

PHENOLIC CONSTITUENTS OF GEORGIA-GROWN BLACKBERRY CULTIVARS:  
FRACTIONATION AND CHARACTERIZATION OF THEIR ANTIOXIDANT, RADICAL-  
SCAVENGING, AND ANTI-INFLAMMATORY CAPACITIES

by

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(Under the direction of RONALD B. PEGG)

ABSTRACT

The aims of this work were to isolate and characterize phenolic constituents of Georgia-grown blackberry (*Rubus* spp.) cultivars (*i.e.*, Navaho, Kiowa, and Ouachita) and to examine their antioxidant and anti-inflammatory capacities. Phenolic compounds were identified and quantified using HPLC and MS techniques. Column chromatography on Amberlite XAD-16 was employed to recover polyphenolic extracts, which were further fractionated on Sephadex LH-20. ESI-MS studies confirmed the presence of cyanidin-3-*O*-glucoside as the predominant anthocyanin in all the cultivars; cyanidin-3-*O*-rutinoside and cyanidin-3-*O*-xyloylrutinoside were also present in the Kiowa cultivar. Ellagitannin isomers; *i.e.*, lambertianin A and lambertianin C were confirmed in all blackberry cultivars with pedunculagin and lambertianin D also being detected in Navaho and Kiowa blackberries, respectively. Total phenolics content (TPC) and total anthocyanins content were determined spectrophotometrically using the classical Folin-Ciocalteu assay and a pH differential method, respectively. Antioxidant capacities were assessed by the FRAP and TEAC assays; values for the preparations ranged from 26.1±2.7 to 178.1±6.9 mmol Fe<sup>2+</sup> equivalents/100g of fraction (d.w.) and 13.6±0.7 to 79.4±0.9 μmol Trolox

equivalents/g of fraction (d.w.), respectively. All fractions collected inhibited the formation of advanced glycation endproducts (AGEs); the % inhibition of AGEs ranged from  $20.7 \pm 1.9$  to  $79.4 \pm 2.2\%$ . Significant positive correlations ( $p < 0.05$ ) were determined for all fractions based on the TPC vs the FRAP and TEAC assays as well as % inhibition of protein glycation. The anti-inflammatory effects of low- and high-molecular-weight phenolic fractions (LMPF and HMPF, respectively) from blackberries were examined by assessing the effects of edema and polymorphonuclear (PMN) leukocyte infiltration 24 h after topical application of an irritant, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), using the mouse ear model. The mouse ears of treatment groups were applied a solution of the LMPF, HMPF, or indomethacin, a non-steroidal anti-inflammatory drug (NSAID), 30 min after TPA application. The NSAID, LMPF, and HMPF (from all three cultivars) significantly ( $p < 0.05$ ) reduced TPA-induced injury when compared to the TPA-positive control group. Myeloperoxidase (MPO) activity, a biomarker for PMN leukocyte infiltration, was also significantly ( $p < 0.05$ ) reduced for treatment groups. Inhibition of both edema and MPO activity indicates marked anti-inflammatory activities arising from the phenolic constituents in the blackberries.

INDEX WORDS: Blackberries, Polyphenolics, Anthocyanins, Hydrolyzable tannins, Sephadex LH-20 chromatography, Antioxidant activity, Anti-inflammatory activity, Myeloperoxidase activity, Advanced glycation endproducts

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## DEDICATION

Mom, Dad  
Anupam and Rahul

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## TABLE OF CONTENTS

	Page
ACKNOWLEDGMENTS.....	v
LIST OF TABLES.....	vii
LIST OF FIGURES.....	x
CHAPTER	
1. INTRODUCTION.....	1
2. LITERATURE REVIEW.....	14
3. CHARACTERIZATION OF PHENOLIC COMPOUNDS IN GEORGIA-GROWN BLACKBERRY CULTIVARS.....	170
4. <i>IN VITRO</i> INHIBITION OF PROTEIN GLYCATION AND ANTIOXIDANT ACTIVITIES BY POLYPHENOLICS EXTRACTED FROM SOUTHEASTERN U.S. RANGE BLACKBERRIES.....	222
5. TOPICAL ANTI-INFLAMMATORY ACTIVITIES OF POLYPHENOLICS EXTRACTED FROM SOUTHEASTERN U.S. RANGE BLACKBERRY CULTIVARS USING THE 12- <i>O</i> -TETRADECANOYLPHORBOL-13-ACETATE (TPA) MODEL OF MOUSE EAR INFLAMMATION .....	271
6. SUMMARY AND CONCLUSION.....	318
INDEX OF ABBREVIATIONS.....	321

## LIST OF TABLES

	Page
Table 2.1: Phenolic classes found in plants.....	36
Table 3.1: Relative yields (%) of CBEs and PPEs isolated from 100-g fresh blackberries of the Navaho, Kiowa, and Ouachita varieties.....	202
Table 3.2: Phenolic compounds and selected phenolic classes identified in Georgia-grown Navaho, Kiowa, and Ouachita blackberries.....	203
Table 3.3: Percent relative yield, total phenolics content, and total anthocyanins content of fractions isolated from Navaho, Kiowa, and Ouachita blackberry cultivars.....	204
Table 3.4: Phenolic compounds and selected phenolic classes identified in blackberry fractions of the Navaho cultivar.....	205
Table 3.5: Phenolic compounds and selected phenolic classes identified in blackberry fractions of the Kiowa cultivar.....	206
Table 3.6: Phenolic compounds and selected phenolic classes identified in blackberry fractions of the Navaho cultivar.....	207
Table 3.7: Interpretation of the MALDI-TOF-MS for hydrolyzable tannins in the acetonitrile-FXN-VII of Navaho blackberries.....	208
Table 4.1: Total phenolics content of ethanolic and acetonitrile fractions isolated from Navaho, Kiowa, and Ouachita cultivars of Georgia-grown blackberries using a Sephadex LH-20 column.....	260

Table 4.2:	Phenolic compounds identified in ethanolic and acetonic fractions isolated from Navaho, Kiowa, and Ouachita cultivars of Georgia-grown blackberries using a Sephadex LH-20 column.....	261
Table 5.1:	Relative yield (%) of CBE, PPE, LMPF, and HMPF from lyophilized Georgia-grown blackberry cultivars (Navaho, Kiowa, and Ouachita).....	308
Table 5.2:	Total phenolics content and antioxidant activity of the LMPF and HMPF from Georgia-grown blackberry cultivars (Navaho, Kiowa, and Ouachita).....	309
Table 5.3:	Experimental design for the TPA-induced mouse ear edema study.....	310

## LIST OF FIGURES

	Page
Figure 2.1: A commercial blackberry-growing operation in South Georgia and parts of the fruit.....	15
Figure 2.2: Biosynthesis of phenolic compounds.....	28
Figure 2.3: The phenolic acid family.....	37
Figure 2.4: Other non-flavonoid phenolics of importance.....	41
Figure 2.5: Basic structure and carbon-numbering system for the diphenylpropane (C <sub>6</sub> – C <sub>3</sub> – C <sub>6</sub> ) nucleus of flavonoids.....	43
Figure 2.6: Chemical structures of selected compounds from the flavonoids' family.....	47
Figure 2.7: Chemical structures of selected compounds from the flavonoids' family.....	51
Figure 2.8: Biosynthesis and hydrolysis of hydrolyzable tannins.....	56
Figure 2.9: Chemical structures of selected hydrolyzable tannins and their monomers.....	59
Figure 2.10: Chemical structures of selected condensed tannins and their monomers.....	67
Figure 2.11: Metabolic pathway of quercetin.....	77
Figure 2.12: UV/VIS absorption spectra of selected phenolic compounds present in blackberry.....	88
Figure 2.13: Pathway of ROS formation, the lipid peroxidation process, and the role of glutathione and non-phenolic antioxidants in the control of oxidative stress.....	96
Figure 2.14: The role of phenolic compounds as antioxidants.....	102

Figure 2.15: The formation of advanced glycation endproducts (AGEs) and selected structures.....	105
Figure 2.16: The effects of AGEs on oxidative stress.....	110
Figure 2.17: Chemical reactions of selected <i>in vitro</i> antioxidant activity assays.....	117
Figure 2.18: Arachidonic acid pathway .....	124
Figure 2.19: Schematic diagram of NF- $\kappa$ B activation.....	128
Figure 2.20: Schematic diagram of NF- $\kappa$ B as an inflammatory regulator.....	130
Figure 3.1: Chemical structures of selected phenolic compounds endogenous to blackberry.....	209
Figure 3.2: Schematic process flow diagram detailing the isolation of ethanolic and acetonitrile fractions from lyophilized blackberries using extraction and column chromatography strategies.....	213
Figure 3.3: Representative HPLC chromatograms of a Navaho blackberry sample with UV/VIS-DAD detection.....	214
Figure 3.4: Representative HPLC chromatogram of FXN-VII from Navaho blackberries with UV-DAD detection at 280 nm.....	217
Figure 3.5: On-line UV/VIS DAD spectra of gallic acid, galloyl esters, ellagitannins, as well as ellagic acid and its derivatives.....	218
Figure 3.6: MALDI-TOF-MS of FXN-VII from Navaho, Kiowa, and Ouachita blackberries.....	219
Figure 4.1: A classical pathway of advanced glycation endproducts (AGEs) formation in the Maillard reaction.....	262
Figure 4.2: Chemical structures of selected AGEs.....	263

Figure 4.3:	Schematic process flow diagram detailing the isolation of ethanolic and acetonetic fractions from lyophilized blackberries using extraction and column chromatography strategies.....	264
Figure 4.4:	Percent inhibition of fructose-mediated bovine serum albumin (BSA) glycation by blackberry fractions.....	265
Figure 4.5:	Ferric reducing antioxidant power (FRAP) values of blackberry fractions.....	266
Figure 4.6:	Trolox equivalent antioxidant capacity (TEAC) values of blackberry fractions..	267
Figure 4.7:	Structural requirements of flavonoids for inhibition of protein glycation and radical-scavenging capacity.....	268
Figure 4.8:	Correlations established between the total phenolics content (TPC), ferric reducing antioxidant power (FRAP), and Trolox equivalent antioxidant capacity (TEAC) values of blackberry fractions from the Navaho, Kiowa, and Ouachita cultivars.....	269
Figure 4.9:	Correlations established between the percent inhibition of fructose-mediated bovine serum albumin (BSA) glycation, total phenolics contents (TPCs), and ferric reducing antioxidant power (FRAP) values of blackberry fractions from the Navaho, Kiowa, and Ouachita cultivars.....	270
Figure 5.1:	Process flow diagram detailing the isolation and separation of LMPF and HMPF from blackberry cultivars.....	311
Figure 5.2:	Relative tannin contents in the LMPF and HMPF of three blackberry cultivars.	312
Figure 5.3:	HPLC chromatogram with diode array detection at $\lambda = 520$ nm of a LMPF from one of the blackberry cultivars examined.....	313
Figure 5.4:	Change in ear thickness (mm) of mice after 4 and 24 h of TPA-induced injury..	314

Figure 5.5:	Change in mass (mg) of 6-mm ear punches after 24 h of TPA-induced injury...	315
Figure 5.6:	Colorimetric assay determination of myeloperoxidase (MPO) activity for biopsies of mouse ears after 24 h of TPA-induced injury.....	316
Figure 5.7:	Correlations established between the percent inhibition of edema, total phenolics contents (TPCs), and ferric reducing antioxidant power (FRAP) values of the LMPFs and HMPFs from blackberries.....	317

# CHAPTER 1

## INTRODUCTION

### 1.1 Blackberry Morphology and Horticulture

The blackberry (Family, Rosaceae; and Genus, *Rubus*) is a small round fruit that grows on flowering shrubs or trailing vines. Each blackberry is an aggregate fruit consisting of a cluster of tiny fruits called drupelets. Species in this genus have a tremendous diversity. They grow either as bushes over 5 m tall, trailing vines, semi-erect or erect canes (Clark *et al.*, 2007). In the process of domestication, excellent genotypes with many improved characteristics have been developed over the years. The three cultivars chosen for this study, Kiowa (an erect thorny variety), Navaho (an erect thornless variety), and Ouachita, (a thornless, very erect cane variety), were released from the University of Arkansas blackberry breeding program. These cultivars were specifically developed for the Southeastern U.S. and selected for cultivation in Georgia because they grow well in warm and humid climates. Since initiation in 1964, the Arkansas breeding program has released 13 cultivars, ten of which have been patented. Some of their major objectives were to develop superior genotypes containing the following traits: improved thornless characteristics, erect canes, fruit firmness, large fruit size, high yields, higher total soluble solids, improved post-harvest handling characteristics, and more recently primocane fruiting (Clark, 1999). Mature blackberries (*Rubus* spp.) were harvested in May 2006 and 2007 for this study from Jacob W. Paulk Farms, Inc., a commercial blackberry grower (Wray, GA).



## 1.2 Blackberries and Human Health

Blackberry juice and ethanolic extracts have been used in folk medicine to treat many ailments like migraines as well as digestive, inflammatory and cardiac disorders (Bhakuni *et al.*, 1997). As a food category, berries are among 50 products ranked highest in antioxidant levels (Halvorsen *et al.*, 2006). Over the past decade, numerous independent investigations have firmly established that the dietary intake of berries has had a positive and profound impact on general human health, performance, and disease prevention (Bagchi *et al.*, 2004; Seeram, 2008).

Biological activities are largely attributed to their polyphenolic constituents (Nijveldt *et al.*, 2001; Bagchi *et al.*, 2004). Blackberries rank highest in berry antioxidant concentrations (Pellegrini *et al.*, 2003; Seeram *et al.*, 2006) primarily due to marked anthocyanin contents, predominantly cyanidin-3-*O*-glucoside (C3G), ellagitannins and ellagic acid derivatives (Moyer and others 2002; Naczki and Shahidi, 2004). Ding *et al.* (2006) demonstrated that C3G, isolated from blackberries, was able to scavenge ultraviolet B-induced  $\bullet\text{OH}$  and  $\text{O}_2^{\bullet-}$  radicals in a cultured JB6 mouse epidermal cell system. This system has been used extensively as an *in vitro* transformation model for the study of inflammatory tumor promotion. Hassimoto *et al.* (2008) reported that consumption of anthocyanin-rich blackberry juice had a pronounced effect at increasing catalase content, an antioxidant enzyme, in human blood plasma.

Inflammation is a physiological and protective response to injury and tissue destruction in the body. Studies have shown that fruit phenolics such as quercetin, ellagic acid, gallic acid, and anthocyanins retard inflammation by various mechanisms. For example, Seeram *et al.* (2001) reported that anthocyanins from cherries and berries (tart cherries, sweet cherries, bilberries, blackberries, blueberries, cranberries, elderberries, and strawberries) possess potent anti-inflammatory effects *in vitro* by inhibiting cyclooxygenase-1 (COX-1) and cyclooxygenase-2

(COX-2) activity, classical markers of inflammation and tumor promotion. Afaq *et al.* (2005) demonstrated that topical applications of anthocyanins and hydrolyzable tannin-rich pomegranate fruit extracts on mice afforded significant inhibition against 12-*O*-tetradecanoylphorbol acetate (TPA)-induced development of skin edema, hyperplasia, as well as epidermal ornithine decarboxylase (ODC) and COX-2 activities. Soon after, Pergola *et al.* (2006) demonstrated the anti-inflammatory activity of a C3G-enriched blackberry extract by suppressing nitric oxide production in J774 murine macrophage cell lines. J774 murine macrophage cell lines are commonly employed in biochemical studies aimed at understanding the physiology of monocyte-macrophages.

Ding *et al.* (2006) postulated chemopreventative and chemotherapeutic activities of C3G in experimental animals. The authors proposed that anthocyanins were interfering with the formation of signal transduction molecules such as activation protein-1 (AP-1), mitogen-activated protein kinase (MAPK), nuclear factor-kappa B (NF- $\kappa$ B), COX-2, and tumor necrosis factor-alpha (TNF- $\alpha$ ). These signal transduction molecules are commonly monitored as biomarkers of inflammation. They are also important targets for modulating inflammation and carcinogenesis. For example, TNF- $\alpha$  inhibitors display a significant effect in retarding inflammatory disease and carcinogenesis (Sturm *et al.*, 2003).

Myeloperoxidase (MPO), a key oxidant-producing enzyme during inflammation, is another important inflammatory biomarker. Inflammatory stimuli, for example, reactive oxygen species (ROS), tissue injury, and infection result in the secretion of MPO in activated neutrophils. MPO catalyzes the reaction of H<sub>2</sub>O<sub>2</sub> with chlorides to produce hypochlorous acid (HOCl), an antimicrobial oxidant. Based on an increasing number of inflammatory-mediated disorders, HOCl has been shown to be a harmful agent (Nauseef, 2001). Kato *et al.* (2003)

demonstrated that phenolics such as epigallocatechin gallate (EGCG), epicatechin gallate (ECG), and curcumin were strong inhibitors of MPO activity thereby, reducing the incidence of inflammation.

There have been many reports on the inhibitory effects of phenolic compounds found in plants relating to the formation of advanced glycation end products (AGEs) (Kim and Kim 2003; Wu and Yen 2005). The formation of AGEs is an important biochemical abnormality that accompanies diabetes. In turn, AGEs induce inflammation by activating RAGEs (to be discussed in detail later). AGEs formation induces free-radical production leading to oxidative stress, which has been implicated in the aging process as well as in the pathogenesis of the complications of diabetes (retinopathy, neuropathy, and nephropathy), atherosclerosis, and Alzheimer's disease (Wu and Yen 2005). To date, there has been limited research regarding fruit phenolics and AGEs inhibition. Babu *et al.* (2006) reported on the antiglycation activity of green tea phenolics in diabetic rats. Farrar *et al.* (2007) demonstrated that ethanolic extracts of muscadine grape skins and seeds could inhibit AGEs formation. Unfortunately, there is no available data on blackberry phenolics and AGEs.

### **1.3 Classes of Phenolic Compounds in Blackberries**

Several classes of phenolic compounds are endogenous to blackberries such as hydroxybenzoic acids, hydroxycinnamic acids, flavonoids, and tannins. Major phenolic compounds include hydrolyzable tannins and anthocyanins, with hydroxycinnamic acids, flavonols, and flavan-3-ols (including proanthocyanidins, PACs) being present in lesser amounts (Cho *et al.*, 2004; Siriwoharn and Wrolstad, 2004; Mertz *et al.*, 2007). The cyanidin-containing compounds are predominantly anthocyanins (Fan-Chiang and Wrolstad, 2005). Ellagitannins are

predominantly hydrolyzable tannins (Hager *et al.*, 2008) and are polymers of hexahydroxydiphenic (HHDP) acid. A majority of the ellagitannin structures that have been characterized in the fruits and leaves of blackberries are polymerized forms of galloyl-bis-HHDP. The oligomeric ellagitannins, so far characterized in blackberries, are the dimers sanguin H-6 and lambertianins A, the trimer lambertianin C, and the tetramer lambertianin D (Hager *et al.*, 2008; Mertz *et al.*, 2007). There is pronounced variation evident among cultivars (Jiao and Wang, 2000; Siriwoharn *et al.*, 2004), which may be influenced by a large number of factors such as agrotechnical practices, climatic conditions, and post-harvest manipulations (*i.e.*, storage, transportation, etc.) (Waterman and Mole, 1994).

#### **1.4 Blackberry Production in the United States**

Blackberry production has increased markedly in the past ten years due to improved cultivars and horticultural practices (Clark, 2005; Strik *et al.*, 2007). Strik *et al.* (2007) estimated that 20,035 ha of blackberries were planted and commercially cultivated worldwide in 2005 producing 140,292 tons of blackberries. This acreage represented, approximately, a 45% increase from 1995, compared to the U.S., which increased its planted area by 28% from 1995 to 2005. Although Serbia accounted for the greatest blackberry acreage, the U.S. yielded the greatest deliverable, commercial product in 2005 (Strik *et al.*, 2007). Sixty-five percent of the blackberries cultivated in the U.S. came from Oregon; California came in second with 283 ha and 2,359 tons of berries while Texas reported 275 ha and 726 tons in 2005. Arkansas had 243 ha, a 60% increase in planted area from 1995, resulting in about 1,400 tons of berry production (Strik *et al.*, 2007). Ninety-five percent of all commercially-harvested blackberries were processed into value-added products such as frozen berries and juices, while the remaining 5%

were marketed fresh. It is notably important that Georgia has less acreage than the states mentioned.

### **1.5 Blackberry Production in Georgia**

In Georgia, blackberries are grown on a moderately large scale with the area of cultivation tripling over the last ten years to 127 ha in 2005 (Strik *et al.*, 2007). Development of new cultivars, bred by and released from the Blackberry Breeding Program at the University of Arkansas, has shown good adaptation to the warm, humid climatic conditions of Georgia and other regions of the southeastern U.S., as well as some fungal resistance to rosette caused by *Cercospora rubi*. This has resulted in greater blackberry production. Blackberries are also commonly referred to as brambles. They usually grow satisfactorily in soils ranging from sand to clay but grow best in loamy sand or clay loam soils (Krewer *et al.*, 2004). Navaho, Kiowa, and Ouachita, released from the University of Arkansas, are the primary cultivars presently grown in Georgia. These three erect-type cultivars are well suited to the warm and humid climate of the Southeastern U.S. and possess improved post-harvest handling characteristics thereby offering great marketing opportunities to the state of Georgia (Strik *et al.*, 2007).

### **1.6 Research Objectives**

It is hypothesized that the antioxidative and anti-inflammatory properties of berry phenolics contribute to chemopreventive or chemoprotective activities in humans after consumption and thereby reduce chronic diseases, for example, cancer, cardiovascular disease, neurodegenerative disease, and diabetes (Dashwood, 2007; Viasoli and Hagen, 2007, Bourre, 2006; Banini *et al.*, 2006; Schroder, 2007). Furthermore, it is postulated that the high antioxidant

concentrations (Pellegrini *et al.*, 2003) in blackberries will have a profound effect on AGEs inhibition. The absence of significant research on blackberry phenolics and their impact on inflammation deems it important to examine the effects of phenolics isolated from blackberries on MPO activity. Examining potential health benefits afforded by the three cultivars grown in the Southeastern U.S. will provide valuable data regarding blackberry phenolics which is currently lacking and improve marketing opportunities in the state of Georgia because commercial production is expected to increase in future years.

### **1.6.1 Characterization of Phenolic Compounds in Georgia-grown Cultivars of Blackberries**

The first objective of the present study involved the isolation and fractionation of phenolic constituents from blackberry cultivars grown in the Southeastern U.S. (*i.e.*, Navaho, Kiowa, and Ouachita) using classical liquid chromatography. Solid phase extraction (SPE) and fractionation techniques were employed to remove non-phenolic compounds (Naczk and Shahidi 2004). Amberlite XAD-16 and Sephadex LH-20 packings were used to fractionate and isolate phenolics from fruit matrices with various organic solvent systems as mobile phases (Naczk and Shahidi 2004; Mazza *et al.*, 2004). Once separated each fraction was lyophilized and stored under refrigerated conditions until use. The total phenolics content (TPC) was determined for each fraction colorimetrically using the classical Folin-Ciocalteu assay (Singleton and Rossi, 1965) and the total anthocyanins content (TACY) of the freeze-dried berries and fractions was estimated by a pH differential method using two buffer systems as described by Giusti and Wrolstad (2001). Phenolic profiles of the individual fractions were characterized by reversed-phase high performance liquid chromatography (RP-HPLC) and using a C<sub>18</sub> (octadecylsilyl-

modified silica) column and an ultraviolet-visible diode array detection (UV/VIS DAD) system. Matrix-assisted laser desorption/ionization-time-of-flight-mass spectrometry (MALDI-TOF-MS) and electrospray ionization mass spectrometry (ESI-MS) measurements were performed for structural identification of hydrolyzable tannins and the anthocyanins in the blackberry fractions, respectively.

### **1.6.2 *In Vitro* Inhibition of Protein Glycation and Antioxidant Activities by Polyphenolic Fractions Extracted from Blackberry Cultivars**

The second objective of the present study was to investigate the *in vitro* antiglycation and antioxidant activities of phenolic constituents isolated from three Georgia-grown cultivars (*i.e.*, Navaho, Kiowa, and Ouachita) using standard assays. For this work, antiglycation activity was performed using a bovine serum albumin (BSA)/fructose system and fluorescence spectroscopy. This assay is based on detecting Maillard reaction products which are formed when fructose reacts with albumin in neutral aqueous solution at 37 °C and produces yellow-brown fluorescent derivatives. Pentosidine and crossline are examples of such reaction products (Wells-Knecht *et al.*, 1996). A reduction in fluorescence is measured as the inhibition of AGEs formation by phenolic compounds.

Antioxidant assays employed in this study included the ferric reducing antioxidant power (FRAP) assay and Trolox equivalent antioxidant capacity (TEAC) assay. The TPC was determined for each fraction colorimetrically using the classical Folin-Ciocalteu assay (Singleton and Rossi, 1965) and correlations with data for the TPC, FRAP, TEAC and antiglycation activity assays were tested.

### **1.6.3 Topical Anti-inflammatory Activities of Polyphenolics Extracted from Georgia-grown Cultivars using the 12-*O*-Tetradecanoylphorbol-13-Acetate (TPA)-Mediated Mouse Ear Model of Topical Inflammation**

The third research objective was to examine the anti-inflammatory activity of blackberry fractions using the TPA-mediated mouse ear model of topical inflammation. High-molecular-weight phenolic fractions (HMPPF) and low-molecular-weight phenolic fractions (LMPPF) were isolated from blackberry cultivars by classical column chromatography techniques. Mouse ear edema was induced by treatment with TPA, a potent inflammatory tumor promoter widely used with an animal model for testing anti-inflammatory activity. TPA applied topically to mouse ears promotes a cascade of events of the inflammatory process such as, vascular permeability, edema, and activation of polymorphonuclear (PMN) cells. Edema was expressed as the increase in ear thickness due to inflammation and measured at 0, 4, and 24 h after TPA administration. Activation of PMN cells by inflammatory stimuli resulted in the secretion of the MPO enzyme. Tissue MPO activity was measured in ear biopsies taken from both ears 24 h after TPA administration according to the method of Suzuki *et al.* (1983) and as modified by De Young *et al.* (1989).



## LITERATURE CITED

- Afaq, F.; Saleem, M.; Krueger, C. G.; Reed, J. D.; Mukhtar, H. Anthocyanin- and hydrolyzable tannin-rich pomegranate fruit extract modulates MAPK and NF- $\kappa$ B pathways and inhibits skin tumorigenesis in CD-1 mice. *Int. J. Cancer* **2005**, *113*, 423–433.
- Babu, P. V. A.; Sabitha, K. E.; Shyamaladevi, C. S. Green tea impedes dyslipidemia, lipid peroxidation, protein glycation and ameliorates Ca<sup>2+</sup>-ATPase and Na<sup>+</sup>/K<sup>+</sup>-ATPase activity in the heart of streptozotocin-diabetic rats. *Chem. Biol. Interact.* **2006**, *162*, 157–164.
- Bagchi, D.; Sen, C. K.; Bagchi, M.; Atalay, M. Anti-angiogenic, antioxidant, and anti-carcinogenic properties of a novel anthocyanin-rich berry extract formula. *Biochem. (Moscow)* **2004**, *69*, 75–80.
- Banini, A. E.; Boyd, L. C.; Allen, J. C.; Allen, H. G.; Sauls, D. L. Muscadine grape products intake, diet and blood constituents of non-diabetic and type 2 diabetic subjects. *Nutrition* **2006**, *22*, 1137–1145.
- Bhakuni, D. S.; Bittner, M.; Marticorena, C.; Silva, M.; Weldt, F.; Hoeneisen, M.; Hartwell, J. L. Screening of Chilean plant for anticancer activity. *J. Nat. Prod. (Lloydia)* **1976**, *39*, 225–243.
- Bourre, J. M. Effects of nutrients (in food) on the structure and function of the nervous system: Update on dietary requirements for brain. Part 1: Micronutrients. *J. Nutr. Health Aging* **2006**, *10*, 377–385.
- Cho, M. J.; Howard, L. R.; Prior, R. L.; Clark, J. R. Flavonoid glycosides and antioxidant capacity of various blackberry, blueberry and red grape genotypes determined by high-performance liquid chromatography/mass spectrometry. *J. Sci. Food Agric.* **2004**, *84*, 1771–1782.
- Clark, J. R. Changing times for eastern United States blackberries. *HortTechnology* **2005**, *15*, 491–494.
- Clark, J. R. The blackberry breeding program at the University of Arkansas: Thirty-plus years of progress and developments for the future. *Acta Horticulture* **1999**, *505*, 73–77.
- Clark, J. R.; Stafne, E. T.; Hall, H. K.; Finn, C. E. Blackberry breeding and genetics. In *Plant Breeding Reviews*. Janick, J., Ed.; Wiley and Sons: Hoboken, NJ, 2007; pp. 19–144.
- Dashwood, R. H. Frontiers in polyphenols and cancer prevention. *J. Nutr.* **2007**, *137*, 267S–269S.

- De Young, L. M.; Kheifets, J. B.; Ballaron, S. J.; Young, J. M. Edema and cell infiltration in the phorbol ester-treated mouse ear are temporally separate and can be differentially modulated by pharmacologic agents. *Agent Actions* **1989**, *26*, 335–341.
- Ding, M.; Feng, R.; Wang, S. Y.; Bowman, L.; Lu, Y.; Qian, Y.; Castranova, V.; Jiang, B.-H.; Shi, X. Cyanidin-3-glucoside, a natural product derived from blackberry, exhibits chemopreventive and chemotherapeutic activity. *J. Biol. Chem.* **2006**, *281*, 17359–17368.
- Fan-Chiang, H.-J.; Wrolstad, R. E. Anthocyanin pigment composition of blackberries. *J. Food Sci.* **2005**, *70*, C198–C202.
- Farrar, J. L.; Hartle, D. K.; Hargrove, J. L.; Greenspan, P. Inhibition of protein glycation by skins and seeds of the muscadine grape. *BioFactors* **2007**, *30*, 193–200.
- Giusti, M. M.; Wrolstad, R. E. Characterization and measurement of anthocyanins by UV-visible spectroscopy. In *Current Protocols in Food Analytical Chemistry*. Wrolstad, R. E.; Schwartz, S. J., Eds.; Wiley and Sons: New York, NY, 2001; pp. F.1.2.1–F.1.2.13.
- Hager, T. J.; Howard, L. R.; Liyanage, R.; Lay, J. O.; Prior, R. L. Ellagitannin composition of blackberry as determined by HPLC-ESI-MS and MALDI-TOF-MS. *J. Agric. Food Chem.* **2008**, *56*, 661–669.
- Halvorsen, B. L.; Carlsen, M. H.; Phillips, K. M.; Bøhn, S. K.; Holte, K.; Jacobs, D. R., Jr.; Blomhoff, R. Content of redox-active compounds (ie, antioxidants) in foods consumed in the United States. *Am. J. Clin. Nutr.* **2006**, *84*, 95–135.
- Hassimotto, N. M. A.; da Silva Pinto, M.; Lajolo, F. M. Antioxidant status in humans after consumption of blackberry (*Rubus fruticosus* L.) juices with and without defatted milk. *J. Agric. Food Chem.* **2008**, *56*, 11727–11733.
- Jiao, H.; Wang, S. Y. Correlation of antioxidant capacities to oxygen radical scavenging enzyme activities in blackberry. *J. Agric. Food Chem.* **2000**, *48*, 5672–5676.
- Kato, Y.; Nagao, A.; Terao, J.; Osawa, T. Inhibition of myeloperoxidase-catalyzed tyrosylation by phenolic antioxidants *in vitro*. *Biosci. Biotechnol. Biochem.* **2003**, *67*, 1136–1139.
- Kim, H. Y.; Kim, K. Protein glycation inhibitory and antioxidative activities of some plant extracts *in vitro*. *J. Agric. Food Chem.* **2003**, *51*, 1586–1591.
- Krewer, G.; Fonseca, M.; Brannen, P.; Horton, D. (December 2004). Home garden raspberries and blackberries; URL: (<http://pubs.caes.uga.edu/caespubs/pubs/PDF/C766.pdf>) (accessed August 2009), Cooperative Extension Service, The University of Georgia, College of Agricultural and Environmental Sciences.
- Mazza, G.; Cacace, J. E.; Kay, C. D. Methods of analysis for anthocyanins in plants and biological fluids. *J. AOAC Int.* **2004**, *87*, 129–145.

- Mertz, C.; Cheynier, V.; Günata, Z.; Brat, P. Analysis of phenolic compounds in two blackberry species (*Rubus glaucus* and *Rubus adenotrichus*) by high-performance liquid chromatography with diode array detection and electrospray ion trap mass spectrometry. *J. Agric. Food Chem.* **2007**, *55*, 8616–8624.
- Moyer, R. A.; Hummer, K. E.; Finn, C. E.; Frie, B.; Wrolstad, R. E. Anthocyanins, phenolics, and antioxidant capacity in diverse small fruits: *vaccinium*, *rubus*, and *ribes*. *J. Agric. Food Chem.* **2002**, *50*, 519–525.
- Naczek, M.; Shahidi, F. Extraction and analysis of phenolics in food. *J. Chromatogr. A* **2004**, *1054*, 95–111.
- Nauseef, W. M. Contributions of myeloperoxidase to proinflammatory events: More than an antimicrobial system. *Int. J. Hematol.* **2001**, *74*, 125–133.
- Nijveldt, R. J.; van Nood, E.; van Hoorn, D. E. C.; Boelens, P. G.; van Norren, K.; van Leuwen, P. A. M. Flavonoids: a review of probable mechanisms of action and potential applications. *Am. J. Clin. Nutr.* **2001**, *74*, 418–425.
- Pellegrini, N.; Serafini, M.; Colombi, B.; del Rio, D.; Salvatore, S.; Bianchi, M.; Brighenti, F. Total antioxidant capacity of plant foods, beverages and oils consumed in Italy assessed by three different in vitro assays. *J. Nutr.* **2003**, *133*, 2812–2819.
- Pergola, C.; Rossi, A.; Dugo, P.; Cuzzocrea, S.; Sautebin, L. Inhibition of nitric oxide biosynthesis by anthocyanin fraction of blackberry extract. *Nitric Oxide* **2006**, *15*, 30–39.
- Schröder, H. Protective mechanisms of the Mediterranean diet in obesity and type 2 diabetes. *J. Nutr. Biochem.* **2007**, *18*, 149–160.
- Seeram, N. P.; Adams, L. S.; Zhang, Y.; Lee, R.; Sand, D.; Scheuller, H. S.; Heber, D. Blackberry, black raspberry, blueberry, cranberry, red raspberry, and strawberry extracts inhibit growth and stimulate apoptosis of human cancer cells in vitro. *J. Agric. Food Chem.* **2006**, *54*, 9329–9339.
- Seeram, N. P.; Momin, R. A.; Nair, M. G.; Bourquin, L. D. Cyclooxygenase inhibitory and antioxidant cyanidin glycosides in cherries and berries. *Phytomedicine* **2001**, *8*, 362–369.
- Singleton, V. L.; Rossi, J. A., Jr. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* **1965**, *16*, 144–158.
- Siriwoharn, T.; Wrolstad, R. E. Polyphenolic composition of Marion and Evergreen blackberries. *J. Food Sci.* **2004**, *69*, FCT233–FCT240.

- Siriwoharn, T.; Wrolstad, R. E.; Finn, C. E.; Pereira, C. B. Influence of cultivar, maturity, and sampling on blackberry (*Rubus* L. hybrids) anthocyanins, polyphenolics, and antioxidant properties. *J. Agric. Food Chem.* **2004**, *52*, 8021–8030.
- Strik, B. C.; Clark, J. R.; Finn, C. E.; Bañados, M. P. Worldwide blackberry production. *HortTechnology* **2007**, *17*, 205–213.
- Sturm, J. W.; Magdeburg, R.; Berger, K.; Petruch, B.; Samel, S.; Bönninghoff, R.; Keese, M.; Hafner M.; Post, S. Influence of TNFA on the formation of liver metastases in a syngenic mouse model. *Int. J. Cancer* **2003**, *107*, 11–21.
- Suzuki, K.; Ota, H.; Sasagawa, S.; Sakatani, T.; Fujikura, T. Assay method for myeloperoxidase in human polymorphonuclear leukocytes. *Anal. Biochem.* **1983**, *132*, 345–352.
- Visioli, F.; Hagen, T. M. Nutritional strategies for healthy cardiovascular aging: Focus on micronutrients. *Pharmacol. Res.* **2007**, *55*, 199–206
- Waterman, P.G.; Mole, S. Why are phenolic compounds so important? In *Analysis of Phenolic Plant Metabolites*. Lawron J. H.; Likens, G. E., Eds.; Wiley and Sons: Hoboken, NJ, 1994; pp. 44–65.
- Wu, C.-H.; Yen, G.-C. Inhibitory effect of naturally occurring flavonoids on the formation of advanced glycation endproducts. *J. Agric. Food Chem.* **2005**, *53*, 3167–3173.

## CHAPTER 2

### LITERATURE REVIEW

#### 2.1 Blackberry: Introduction

‘... and the running blackberry would adorn the parlors of heaven’. A line from the poem “*Leaves of Grass*”, written in the nineteenth century by the famous American poet Walt Whitman, confirms that the blackberry is more than 2,000 years old. Findings from archeological digs in Ireland, Denmark, and England revealed that blackberries were important in the diets of Vikings, and their teas were featured in ancient Roman dispensaries. A tea, made with the whole blackberry plant, was used by Native Americans to treat dysentery, cholera, and upset stomach. Today blackberries are consumed fresh or processed, and are promoted as a rich source of polyphenolics, current compounds of interest because of their anti-inflammatory effects (Hollman, 1996; Bravo, 1998; Sellappan *et al.*, 2002; Wang and Ou, 2002; Mertz *et al.*, 2007).

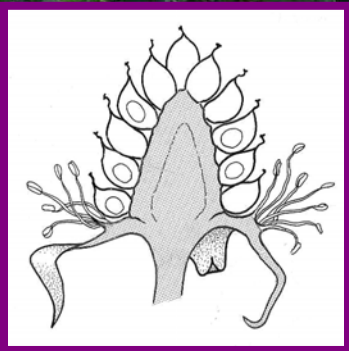
Blackberries (Family, Rosaceae; and Genus, *Rubus*) are small, round, aggregate berries comprising a cluster of tiny fruits called drupelets. Each drupelet contains a single seed (pyrene) and adheres to the central torus or receptacle [**Figure 2.1(A)**]. *Rubus* species have tremendous diversity: they grow as either bushes over 5 m tall, trailing vines, semi-erect or erect canes (Clark *et al.*, 2007). They are represented predominantly in the northern hemisphere, excepting desert regions. In the process of domestication, excellent genotypes with many improved characteristics have been developed.

**Figure 2.1:** A commercial blackberry-growing operation in South Georgia and parts of the fruit: **(A)** Jacob W. Paulk Farms, Inc.; **(B)** blackberry receptacle; and **(C)** aggregate fruit.

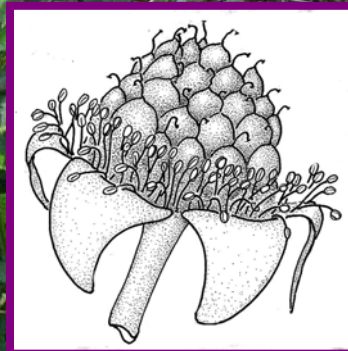
(A)



(B) Receptacle



(C) Aggregate fruit



In the United States, there are two major blackberry breeding programs. which include the University of Arkansas (UA), initiated by Dr. Jim Moore in 1964 and currently directed by Dr. John R. Clark, and the United States Department of Agriculture–Agriculture Research Service (USDA-ARS), Corvallis, OR. The USDA-ARS program is the oldest continuous blackberry breeding program in the world, active since 1928 and directed by Chad E. Finn. Major objectives of the breeding programs have included improvement in plant and fruit characteristics such as erect growth habit, thornlessness, adaptation to climates in the upper south and midwest U.S., compatibility to mechanical harvesting, high disease resistance, high fertility, large fruit size, good flavor, and fruit firmness (Clark, 1999). Under these programs, several new cultivars have been developed including Cherokee (1974), Comanche (1974), Cheyenne (1976), Shawnee (1985), Choctaw (1988), Kiowa (1996), and Ouachita (1989). All Southeastern-grown cultivars have been released from the UA and grow very well in warm and humid climates.

### **2.1.1 Classification**

Blackberries can be classified into three types based on their growth habit: (1) erect, which produces self-supporting canes; (2) semi-erect, where canes are partially erect but require a trellis for support; and (3) trailing, where canes are not erect and require a trellis for support. Thorny and thornless cultivars are inherent to all three types. Trailing and semi-erect types have few root buds and usually produce primocanes from crown buds. Erect blackberries, on the other hand, have many vegetative buds on the roots and readily produce primocanes from both roots and crowns.

Blackberries have perennial root systems with biennial canes (*i.e.*, the canes live two years and then die). The two cane types are primocanes (first-year canes) and floricanes,



(second-year canes). Until 2004, blackberry floricanefruiting had been the basis of all blackberry production; *Prime-Jim* and *Prime-Jan* were released by the UA under the *Prime-Ark* Primocane-Fruiting Blackberry Series as the first primocane-fruited cultivars. These new cultivars were targeted for home garden use and *Prime-Jan* has been recommended for limited commercial trial. Primocane-fruited provides growers with the advantage of two crops per growing season.

### **2.1.2 Plant Habit**

The state of Oregon is the number one producer of blackberries. Most blackberries are grown west of the Cascade Mountains in the Willamette Valley where fertile soils, mild winters and cool summers allow growers to produce high-quality berries with good flavor and color. Blackberries usually grow satisfactorily in soils ranging from sand to clay but grow best in loamy sand or clay loam soils with a pH of 6.0 to 6.5 (Krewer *et al.*, 2004). In general, blackberries are recommended for areas with moderate winter temperatures above -12 °C.

Important components of plant habit include the characteristics of cane growth. The University of Arkansas (UA) Blackberry Breeding Program has stressed erect canes coupled with the incorporation of thornlessness. Fruit ripening times vary substantially and this is an important trait for consideration, especially for breeders who want to expand their blackberry production.

Blackberries are remarkably free of serious disease, insect, and pest problems. Crops are grown in a monoculture with fungicidal applications as standard pest management practice. Insecticides, or acaricides, are applied only for specific problems (Clark and Finn, 2008). In general, new thornless cultivars released from the UA are resistant to double blossom, or rosette

(*Cercospora rubi* [G. Wint.] Plakidas), a serious disease prevalent in the Southeastern U.S. (Buckley *et al.*, 1995). Thorny cultivars have shown some susceptibility for this disease.

### **2.1.3 Fruit Composition**

On average, blackberries contain 1.4% protein, 0.5% lipid, and 5.3% dietary fiber. The total carbohydrate content of the fruit has been reported as 9.6% by the USDA National Nutrient Database for Standard Reference (2005). Moisture and ash content ranged from 88.2 to 94.0% and 0.20 to 0.30%, respectively (Hassimoto *et al.*, 2008). Total sugars and total soluble solids (TSS) averaged 4.88% and varied from 6 to 12 °Brix, respectively (Hassimoto *et al.*, 2008; Mertz *et al.*, 2007; Thomas *et al.*, 2005). Degrees Brix (°Brix) is a measure of the dissolved sugar-to-water mass ratio of a liquid and is determined with a refractometer. A 10 °Brix solution is equal to a 10% (w/v) glucose solution. Fruit flavor is dependent on a balance between sugars (fructose and glucose) and organic acids (Moing *et al.*, 2001). Organic acids are measured as titratable acidity (TA). Navaho, a cultivar examined in this study, has been reported as having the highest TSS/TA ratio (Thomas *et al.*, 2005). Malic acid, the predominant organic acid found in blackberries (Kafkas *et al.*, 2006), and ascorbic acid concentrations ranged from 8 to 10 mg/100 g of fresh fruit (Thomas *et al.*, 2005). Some detailed information on the chemical composition of blackberries like vitamins, minerals, and fatty acids can be found in the USDA National Nutrient Database for Standard Reference. The phenolic compounds of blackberries are discussed later in **Section 2.8**.

## **2.1.4 Cultivars Grown in the Southeastern United States**

### **2.1.4.1 Erect Thornless Cultivars**

Apache, Arapaho, Navaho, and Ouachita are examples of erect thornless cultivars predominately grown in the Southeastern U.S. These were released by the UA's Blackberry Breeding Program. The TSS of these berries range from 8 to 12 °Brix and weights range from 3.5 to 10 g. Apache produces the largest fruit of the three thornless cultivars (*i.e.*, Arapaho, Navaho, and Ouachita).

Navaho is the first fully developed erect thornless cultivar in the world (Moore and Clark, 2000). It has an excellent flavor, fruit firmness, and resistance to rosette disease. Navaho is grown widely in the South and Southwestern U.S. (Moore and Clark, 2000). Prolonged and late ripening are disadvantages associated with this cultivar.

Ouachita, recently released in 2004 from the UA, has many desirable characteristics such as consistently high yields, large fruit sizes, and good post-harvest keeping qualities. The fruit size is larger than that of Arapaho and Navaho with yields comparable to Apache (Clark and Moore, 2005). Ouachita is the largest selling Arkansas variety and the most widely adapted and planted berry (Clark, 2009).

### **2.1.4.2 Erect Thorny Cultivars**

Chicksaw, Choctaw, Kiowa, and Brazos are a few examples of erect, thorny varieties. Most erect, thorny varieties have been released by the UA; however, Brazos was released by the Texas Agricultural Experiment Station in 1959 and has primarily been used for jams, jellies, and baking due to its high acid content (Andersen and Crocker, 2008).

The TSS of these berries varies from 8 to 12 °Brix and weights range from 8 to 14 g. Of these cultivars, Chickasaw produces the highest yields; berries are firm, long, and cylindrical, with an average weight of about 7 to 10 g. Choctaw berries are medium sized (~5 g) and Kiowa produces a large-sized berry with good firmness and flavor.

### **2.1.5 Characteristic Focus of Current Breeders**

The UA blackberry breeding program was established in 1964 and has released 13 cultivars of which ten are patented. Some of the major objectives of this program have been to develop superior genotypes that contain traits described in the following sections.

#### **2.1.5.1 Plant Adaptation and Habit**

Some common goals to improve plant adaptation and habits are as follows: (1) adaptation to reduced-chill environments; (2) expansion of the ripening season; (3) adequate resistance to sunburn, which is seen as the appearance of a few to many white drupelets on the fruit, affecting the fruit quality; and (4) primocane fruiting, which has several advantages including the potential a two crop harvest from the same plant in the same year; *i.e.*, floricanes followed by primocanes, reduction in pruning costs and circumvention of winter injury (Clark and Finn, 2005).

#### **2.1.5.2 Fruit Quality**

Regardless of whether fruit is processed or used fresh, fruit quality includes the following attributes: fruit flavor (sweetness, acidity, astringency, aromatic components, etc.), color, firmness, ease of fruit separation from the plant, and seediness (size and mouthfeel during

consumption). Enhancement of sweetness along with reduced acidity and astringency levels are a top priority in most breeding programs today. Berries with very low acidity may have a “flat” flavor (Hall, 1990). The TSS are generally high in trailing blackberry cultivars; *e.g.*, the Boysenberry cultivar has a TSS of 11 to 13 °Brix compared to the erect, thornless Chester cultivar which has a TSS of 8 °Brix. Navaho and Ouachita cultivars range from 10 to 12 °Brix (Clark, 2005).

### **2.1.5.3 Pest Resistance**

Blackberries tend to be free of serious disease and insect problems. All new cultivars developed by the UA Blackberry Breeding Program must be resistant to double blossom/rosette (*Cercospora rubi* [G. Wint.] Plakidas), a serious disease in Southeastern U.S. blackberries (Ellis *et al.*, 1991). Thorny cultivars show some degree of susceptibility (Buckley *et al.*, 1995) and affect blackberries grown in the Southeastern U.S.

### **2.1.5.4 Post-harvest Quality**

The post-harvest quality of a fruit is determined by the response of the cultivar to storage and/or handling practices from the time the fruit ripens on the plant until it reaches the consumer. Fully black, shiny berries are desirable for the fresh market. Any indication of decay, leakage of juice, mushiness of fruit, or presence of substantial red drupelets limit consumer appeal (Perkins-Veazie and Clark, 2005). Multi-year evaluations have been essential to fully determine the post-harvest potential of new genotypes bred.

#### **2.1.5.5 Thornlessness**

It is expected that higher proportions of new cultivars will be thornless in the future as this is an important trait desired by most blackberry producers; *i.e.*, pick-your-own marketers and mechanically picked berries.

#### **2.1.5.6 Processing Parameters**

Berry firmness is quite important during harvest because berries must be sufficiently firm to allow ripened fruit to easily separate from the plant and to go through a mechanical harvester with little visible damage. Intense color, flavor, high TSS, reduced TA, and the perception of low “seediness” are desirable characteristics of berries by the processing industry (Hall *et al.*, 2002). These qualities must be maintained for berries that are frozen and later thawed, dried, canned, or juiced.

#### **2.1.5.7 Nutraceutical/Antioxidant Levels**

Substantial variation has been reported in the antioxidant capacities among cultivars; *i.e.*, by the oxygen radical absorbance capacity (ORAC<sub>FL</sub>) (Clark *et al.*, 2002). This variation can occur from year-to-year for some genotypes. Cho *et al.* (2004) found differences in the anthocyanin and flavanol contents among cultivars; therefore, advance selective breeding programs ought to measure ORAC<sub>FL</sub> values, anthocyanins contents, or other phenolic compound levels, to breed specifically for increased antioxidant levels.

## 2.2 Phenolic Compounds and Their Location in Fruits

Phenolic compounds are found throughout the plant kingdom and are the most widespread class of metabolites in nature. Phenolic compounds are unevenly distributed in fruits, either at the subcellular level or in the tissues (Macheix *et al.*, 1990). They are present in high concentrations in the epidermis of leaves and in the skin of fruits. As lignin, they form an integral part of cell-wall structures and are the second most abundant organic compound on earth, after cellulose (Strack, 1997).

The simple molecules, soluble phenolic compounds and their derivatives accumulate and are stored in vacuoles (Guern *et al.*, 1987; Monties, 1989; Ibrahim and Barron, 1989). For example in grapes, flavonol glycosides, anthocyanins, and hydroxycinnamic esters accumulate in the vacuoles of subepidermal cells (Moskowitz and Hrazdina, 1981). Accumulation of soluble phenolic compounds is greater in the external tissues of fleshy fruits (epidermal and subepidermal layers) than in the internal tissue (mesocarp and pulp) (Macheix *et al.*, 1990; Wollenweber, 1994). In berries, phenolic compounds are located mostly in the skins. In many fruits (*e.g.*, black currant, grape, apple, and peach) flavonol glycosides are mainly, or even only, located in the outer part of the fruit or in the epicarp (Hawker *et al.*, 1972; Wildanger and Herrmann 1973; Pérez-Ilzarbe *et al.*, 1991; Price *et al.*, 1999). Anthocyanins, on the other hand, may be distributed throughout the fruit (*i.e.*, strawberry and red raspberry) (Macheix *et al.*, 1990) while catechins and tannins are more abundant in the external tissues of fruits than internal ones.

There is little information about the localization of hydroxybenzoic acids in fruits (Macheix *et al.*, 1990). They are either present in the skin or pulp, as in tomatoes and melons, (Schmidlein and Herrmann, 1975) or found only in the skin, as in grapes (Singleton and Trousdale, 1983). According to Daniel *et al.* (1989), 96% of ellagic acid in strawberries is

present in the pulp and 4% in the seeds. In contrast, Maas *et al.* (1991) reported that the ellagic acid content was higher in the seeds, 8.5 to 9 mg/g, than in the pulp, 1.5 to 1.6 mg/g, of ripe strawberries.

### **2.3 Phenolic Compounds and Their Biosynthesis**

Phenolic compounds are secondary metabolites of plants. They are defined as compounds possessing an aromatic ring bearing one or more hydroxy substituents (Harborne, 1967; Mann, 1987). Their chemical structure may range from simple compounds like phenol, with a gram molecular weight of 94 g/mole, to highly polymerized compounds such as PACs or hydrolyzable tannins, which range in gram molecular weights from 500 to greater than 10,000 g/mole (Santos-Buelga and Scalbert, 2000).

In plants, phenolic compounds occur primarily in their mono-glycosylated form (Shahidi and Naczki, 1995; Manach *et al.*, 2004). Glucose is the most commonly encountered sugar. Galactose, rhamnose, xylose, and arabinose are also present but less common while mannose, fructose, glucuronic, and galacturonic acids are rare (Bravo, 1998; Iwashina, 2000). Phenolic compounds may also be conjugated with aliphatic organic acids, amines, lipids, oligosaccharides or other substituents (Carotenuto *et al.*, 1997; Bravo, 1998; Norbaek and Kondo, 1999; Lin *et al.*, 2002). In addition to conjugation, the aromatic ring(s) of phenolic compounds contain varying levels of hydroxylation and methoxylation. More than 8,000 phenolic compounds have been identified in plants due to variations in complexity of structure which is contributed by conjugation, hydroxylation and methoxylation of molecules (Crozier *et al.*, 2006; Wrolstad, 2005).



Phenolic compounds are produced in plants via the shikimate or phenylpropanoid pathways. It is estimated that 100,000 to 200,000 secondary metabolites exist; approximately 20% of the carbon fixed by photosynthesis is channeled into the phenylpropanoid pathway (Pereira *et al.*, 2009). The biosynthesis of phenylpropanoid compounds is activated in response to environmental stresses such as wounding, pathogen infection, and/or UV irradiation.

Plant metabolism begins with photosynthesis, wherein, UV light energy from the sun is absorbed by chlorophyll to synthesize NADPH [nicotinamide adenine dinucleotide phosphate (reduced form)] and ATP (adenosine triphosphate) which act as reducing and activating agents, respectively, in metabolic reactions. In the subsequent 'dark reaction' of photosynthesis, carbon dioxide is reduced to produce four-, five-, six-, and seven-carbon atom-containing carbohydrates, including glucose. An overview of the biosynthesis of phenolic compounds is given in **Figure 2.2(A)**.

The key precursor intermediates in the biosynthesis of phenolic compounds include shikimic acid, a central intermediate in the shikimate pathway and acetyl coenzyme A (acetyl CoA), the starting unit for the acetate pathway. CoA is an important cofactor used by enzymes to activate substrates for subsequent reactions.

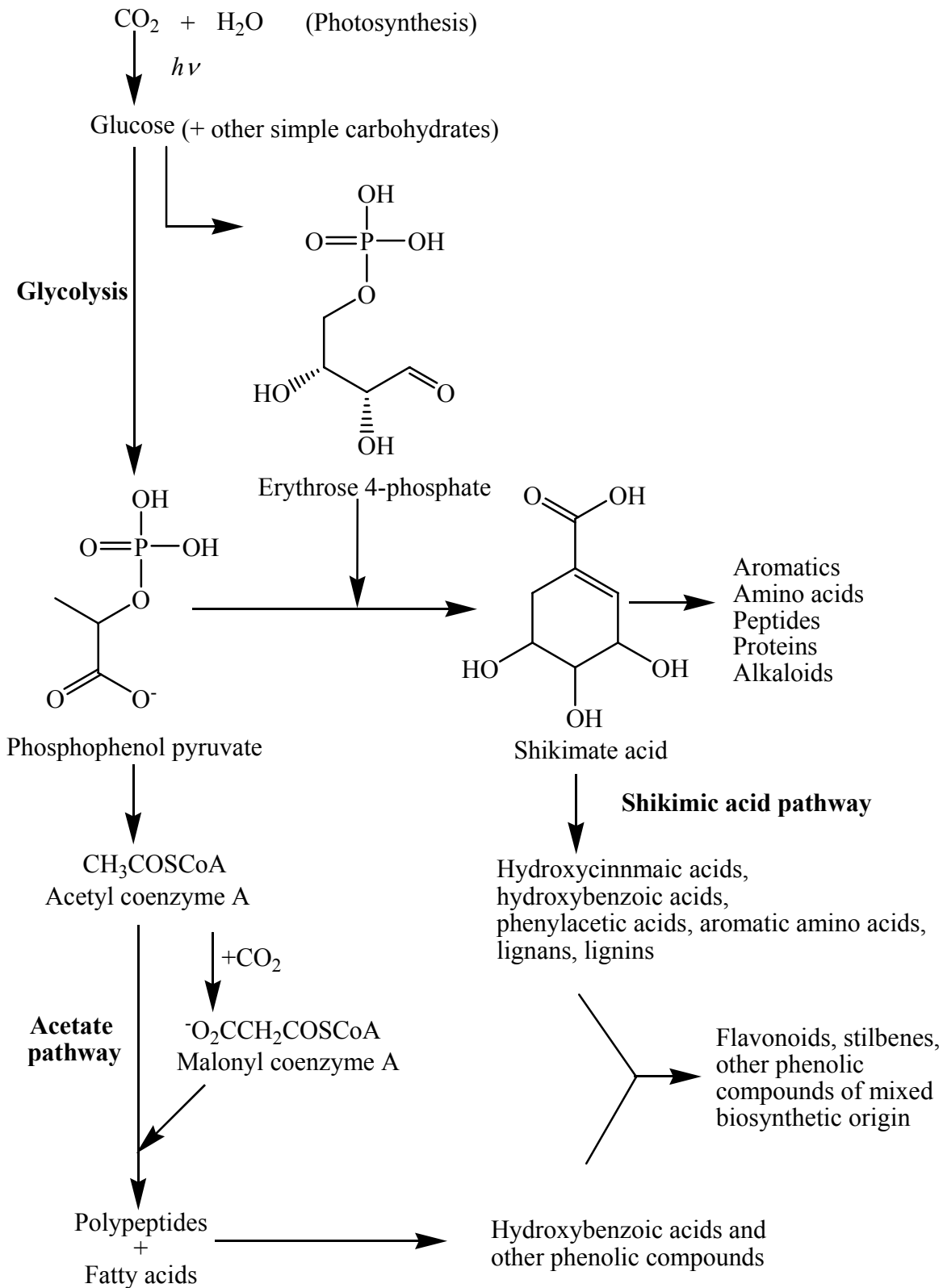
Phenylalanine is a common precursor for most phenolic compounds in higher plants (Dixon and Paiva, 1995; Herrmann *et al.*, 1995). All phenylpropanoids are derived from cinnamic acid, which is formed from phenylalanine by the action of phenylalanine ammonia-lyase (PAL). PAL is the branch point enzyme between primary (Heller *et al.*, 1988) and secondary (phenylpropanoid) metabolism (Heller *et al.*, 1988; Hahlbrock and Scheel, 1989; Dixon *et al.*, 1992).

Several simple phenylpropanoids (with the basic C<sub>6</sub> – C<sub>3</sub> carbon skeleton of phenylalanine) are produced from cinnamate via a series of hydroxylation, methoxylation, and dehydration reactions. These phenylpropanoids include *p*-coumaric, caffeic, ferulic, and sinapic acids, and simple coumarins [Figure 2.2(B)]. The precursors for the synthesis of all flavonoid variants including anthocyanins, are malonyl CoA and *p*-coumaroyl CoA. Chalcone synthase (CHS) catalyzes the stepwise condensation of three acetate units from malonyl CoA with a *p*-coumaroyl CoA to yield tetrahydrochalcone. CHS is the first step in the branch of the pathway that produces flavonoids including isoflavones, flavones, flavonols, and anthocyanins (Crozier *et al.*, 2000). An overview of the shikimic acid pathway is depicted in Figure 2.2(B) (Mann, 1987; Holton and Cornish, 1995; Dewick, 2002).

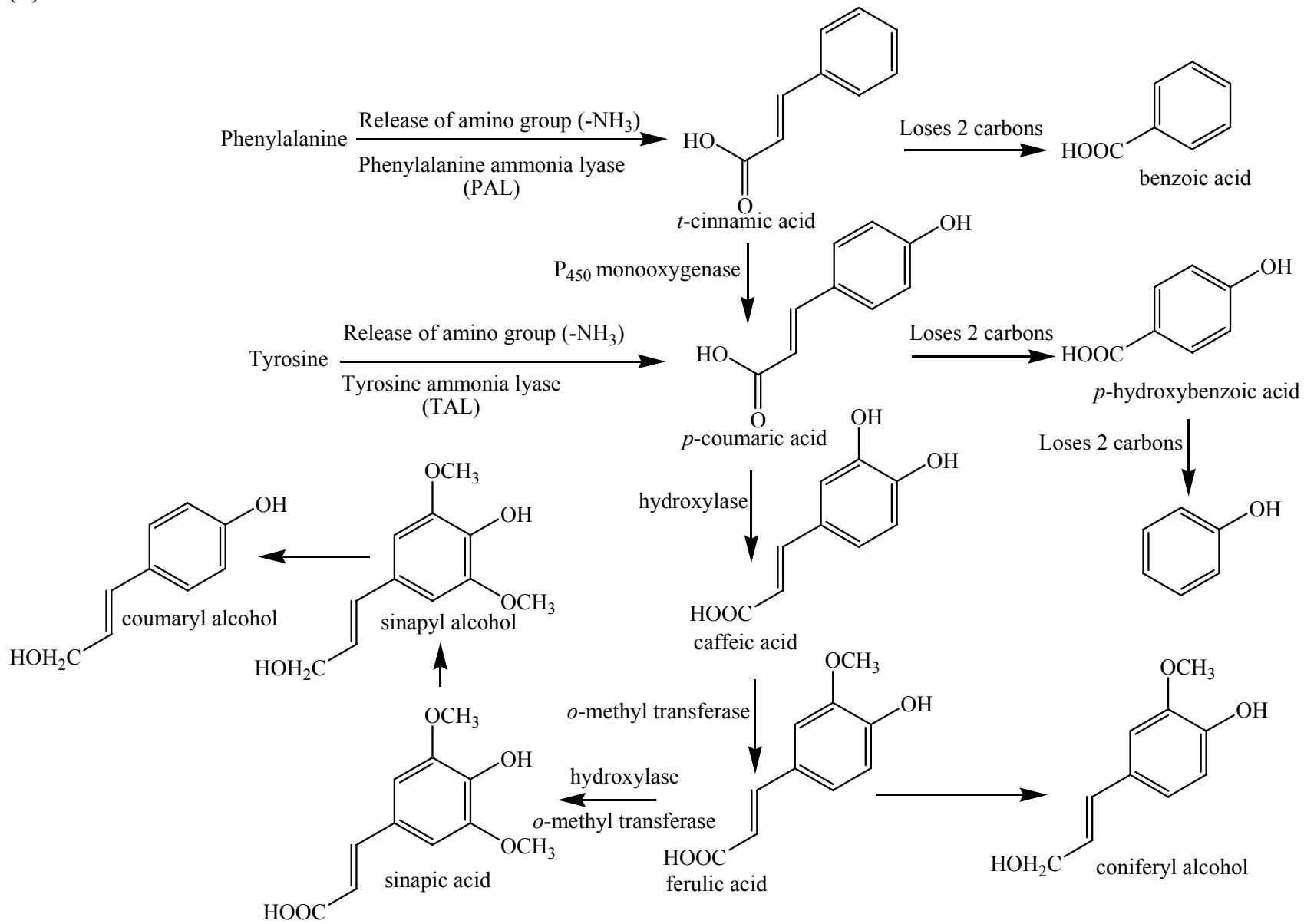
With regard to anthocyanin biosynthesis, flavanone (naringenin) is converted to dihydrokaempferol (DHK) by flavanone 3-hydroxylase (F3H). DHK can subsequently be hydroxylated by flavonoid 3'-hydroxylase (F3'H) to produce dihydroquercetin (DHQ) or by flavonoid 3',5'-hydroxylase (F3'5'H) to produce dihydromyricetin (DHM). At least three enzymes are required for converting the colorless dihydroflavonols (DHK, DHQ, and DHM) to anthocyanins. The first of these enzymatic conversions is the reduction of dihydroflavonols to flavan-3,4-*cis*-diols (leucoanthocyanidins) by dihydroflavonol 4-reductase (DFR). Further oxidation, dehydration, and glycosylation of the different leucoanthocyanidins produce the corresponding brick-red pelargonidin, red cyanidin, and blue delphinidin pigments. Anthocyanidin-3-*O*-glucosides can be modified further in many species by glycosylation, methoxylation, and acylation reactions as illustrated in Figure 2.2(C) (Holton and Cornish, 1995).

**Figure 2.2:** Biosynthesis of phenolic compounds. **(A)** Overview of the biosynthetic pathway for phenolic generation; **(B)** Formation of phenylpropanoids; and **(C)** Formation of anthocyanins (Adapted from Mann, 1987; Dixon and Paiva, 1995; Holton and Cornish, 1995). Abbreviations are as follows: 3GT: flavonoid-3-glucosyltransferase; 5GT: anthocyanin-5-*O*-glucosyltransferase; ANS: anthocyanidin synthase; DFR: dihydroflavonol-4-reductase; DHK: dihydrokaempferol; DHM: dihydromyricetin; DHQ: dihydroquercetin; F3H: flavanones-3-hydroxylase; F3'H: flavonoid-3'-hydroxylase; F3'5'H: flavonoid 3',5'-hydroxylase; FLS: flavonol synthase; Glc: glucose; and Rha: Rhamnose.

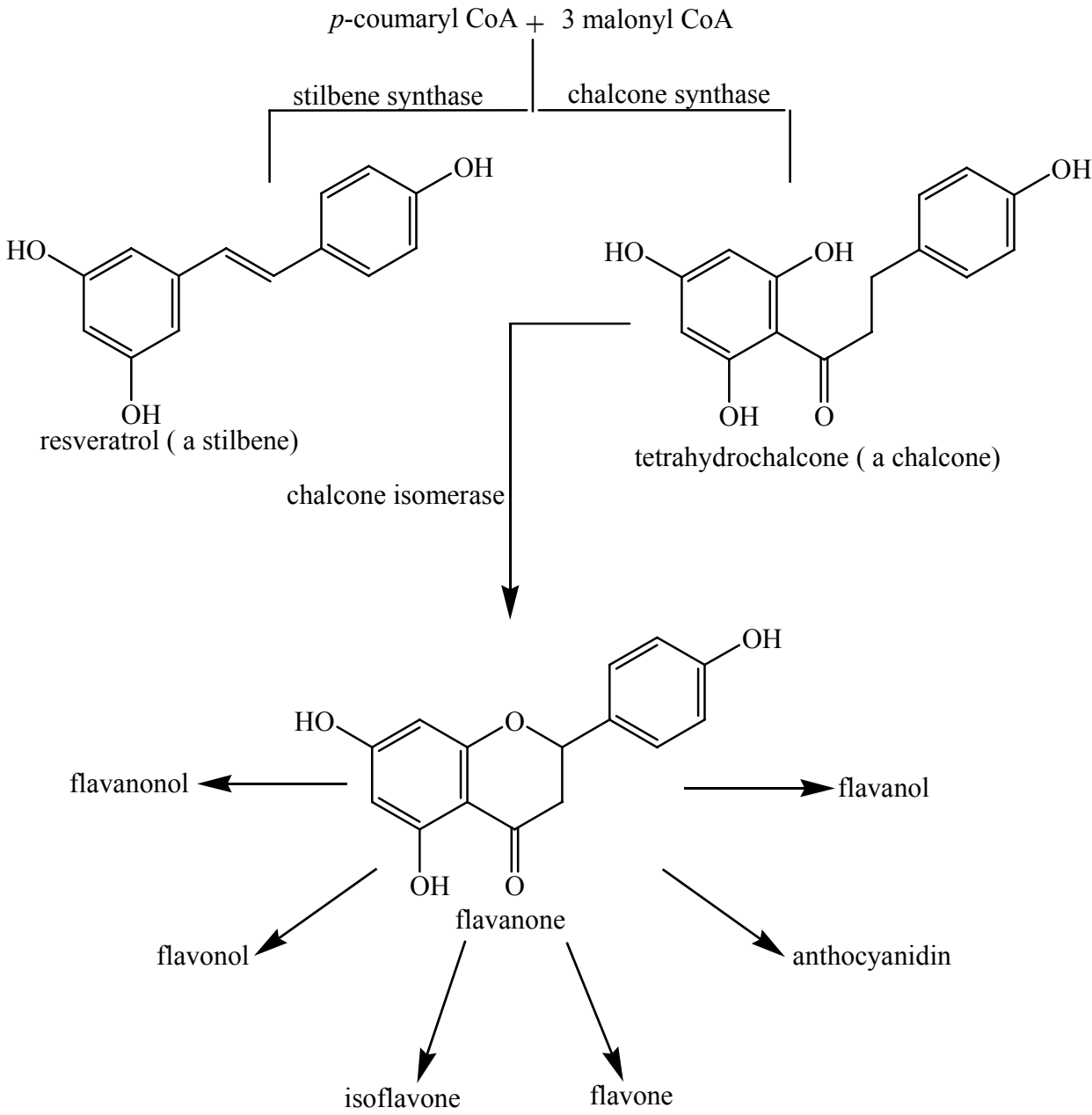
(A)



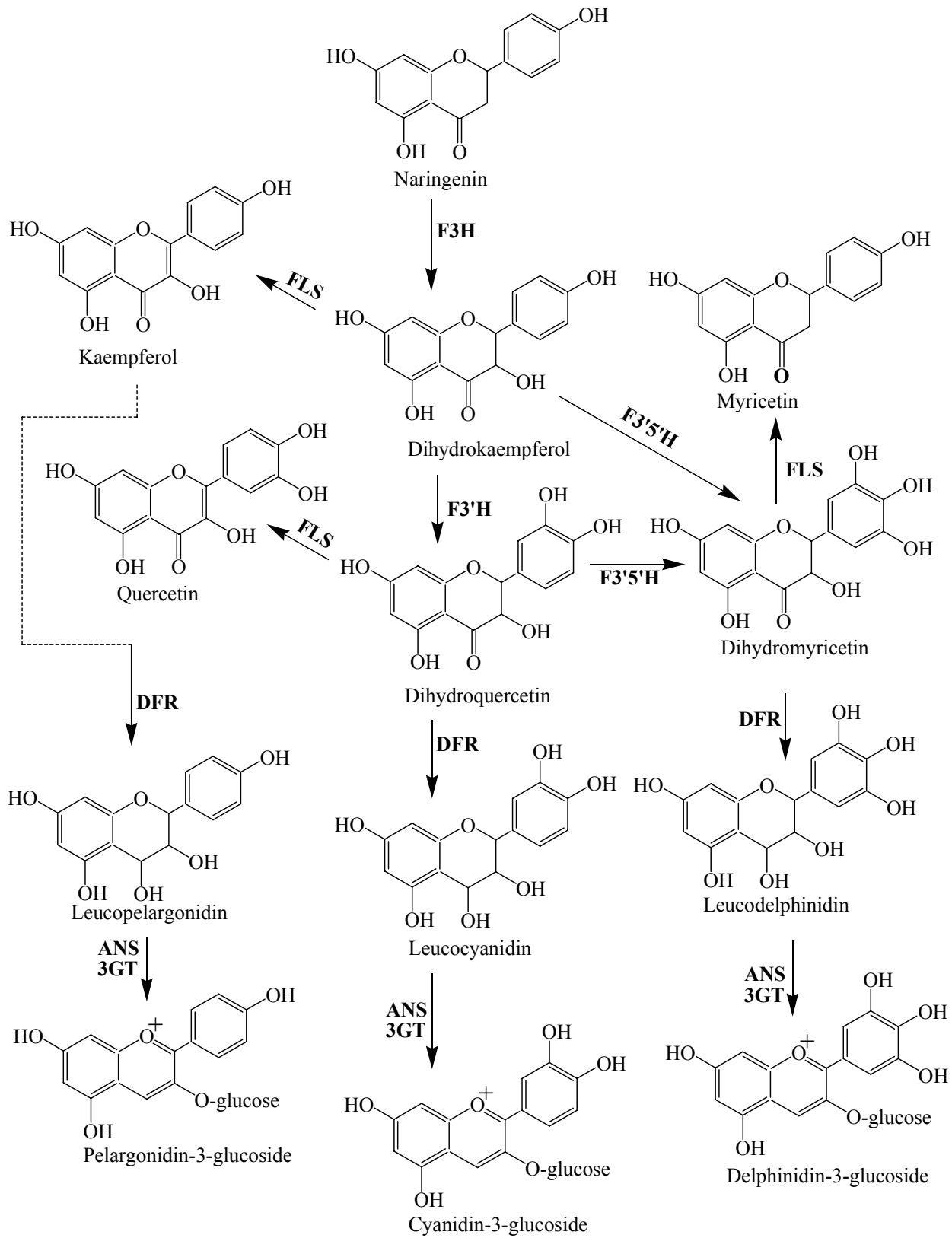
(B)



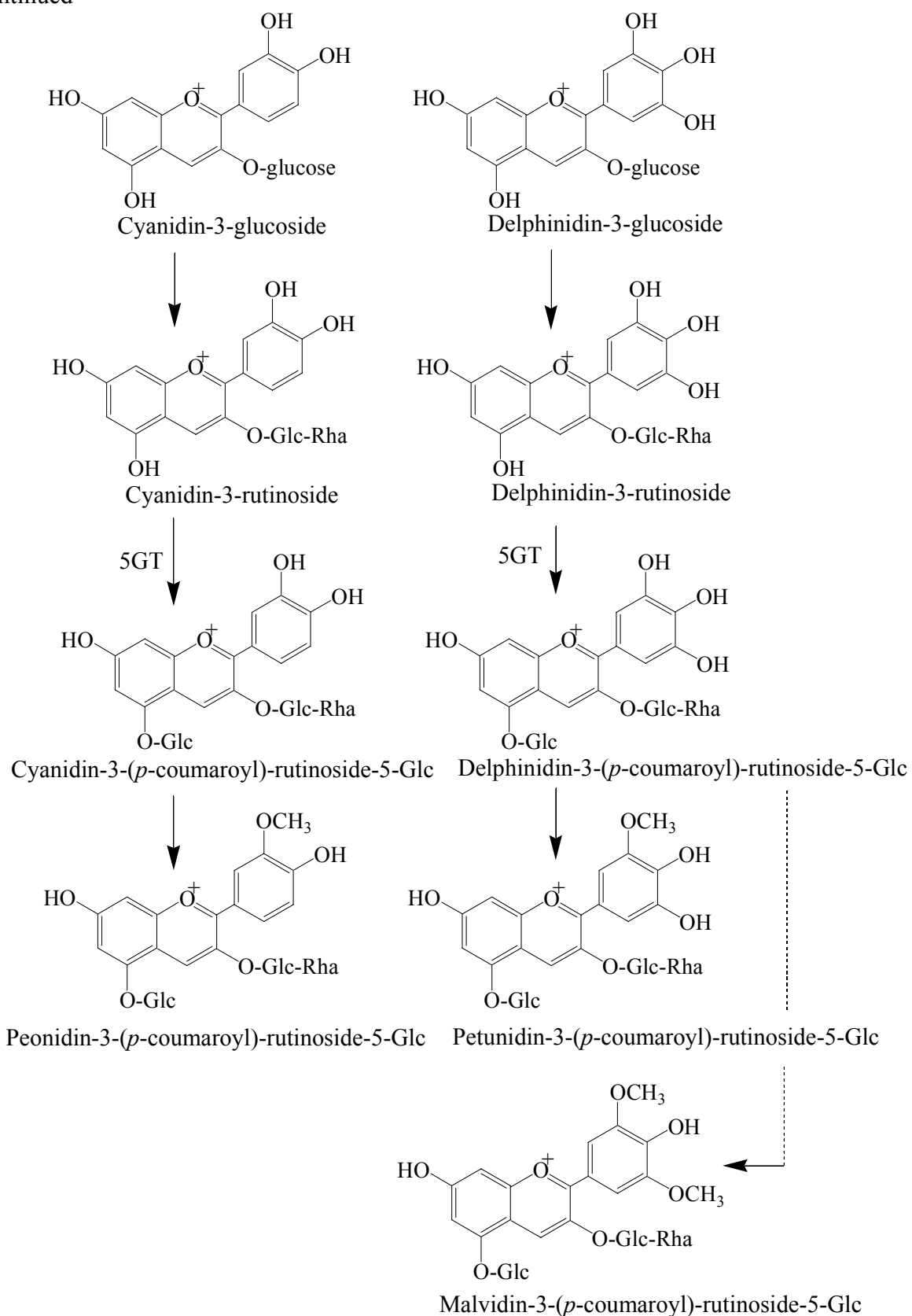
(B) Continued



(C)



(C) Continued





Variations due to hydroxylation, glycosylation, methylation, acylation, and polymerization result in different classes of phenolic compounds. The structures of these phenolics vary from simple molecules such as phenolic acids, to highly polymerized compounds (tannins). Phenolic acids contain at least one phenol group, flavonoids have at least two rings, whereas tannins are polymers of flavonoid units or esters of phenolic acids.

## 2.4 Classification of Phenolic Compounds

Phenolic compounds can be divided into at least 13 different classes depending on their basic carbon skeleton structure (Shahidi and Naczki, 1995; Bravo, 1998; Crozier *et al.*, 2006; Manach *et al.*, 2004) as shown in **Table 2.1**. Distinction between these classes is drawn first on the basis of the number of constitutive carbon atoms, and then by the structure of the basic skeleton. Selected phenolic compounds of importance are discussed in this section.

### 2.4.1 Simple Phenols

Simple phenols ( $C_6$ ), or free phenols, occur rarely in nature. Some examples of simple phenols are arbutin, catechol, hydroquinone, phloroglucinol, and resorcinol [**Figure 2.3(A)**]. These phenols may be substituted with aliphatic side chains. Many such compounds are responsible for allergic reactions in humans, for example, urushiol III is an alkyl catechol and one of the active principles of poison ivy; a resorcinol derivative,  $A'$ -tetrahydrocannabinol is a hallucinogenic principle of marijuana (Harborne *et al.*, 1999).

## 2.4.2 Hydroxybenzoic Acids

Hydroxybenzoic acids are phenolic compounds with a C<sub>6</sub> – C<sub>1</sub> structure (*e.g.*, gallic acid, vanillic acid, syringic acid, *p*-hydroxybenzoic acid) that contain one carboxylic acid functional group [**Figure 2.3(B)**] (Bravo, 1998; Robbins, 2003). Variations in hydroxybenzoic acids occur due to differing patterns of hydroxylation and methoxylation of their aromatic rings.

Hydroxybenzoic acids are present in nearly all plants (Shahidi and Naczk, 1995; Robbins, 2003). In addition to free and conjugated forms with other compounds, gallic acid is the basic phenolic constituent of hydrolyzable tannins (**Section 2.4.8.1**). Aldehydes such as vanillin and *p*-hydroxybenzaldehyde are common flavor compounds derived from the reduction of hydroxybenzoic acids (Shahidi and Naczk, 1995; Bravo, 1998).

## 2.4.3 Phenylpropanoids and Hydroxy-*trans*-cinnamic Acids

Phenolic compounds consisting of the C<sub>6</sub> – C<sub>3</sub> skeleton are collectively known as phenylpropanoids, which implies that they contain a three-carbon side chain attached to a phenol ring [**Figure 2.3(C)**]. They largely comprise hydroxycinnamic acid derivatives, coumarins, phenylpropenes, and lignans (Briellmann *et al.*, 2006). Hydroxycinnamic acids are the most widely distributed phenolic acids in plant tissues (Robbins, 2003) and are rarely encountered in their free state. They are predominantly found as hydroxyacid esters with quinic, shikimic, or tartaric acid (Herrmann, 1989) flavonoids, or with structural components of the plant such as cellulose, lignin, and protein (Clifford, 1999; Scalbert and Williamson, 2000). Some common natural hydroxycinnamic acids are caffeic, ferulic, *p*-coumaric, and sinapic acid.

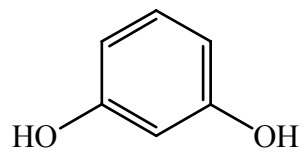
Caffeic acid, and to a lesser extent ferulic acid, are the most prominently occurring phenolic acids in foods of plant origin such as cereals, coffee, fruits, and vegetables (Andreasen

**Table 2.1 Phenolic classes found in plants (Adapted from Harborne, 1980)**

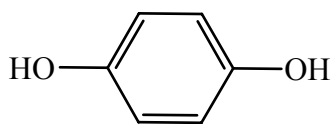
<b>Phenolic Classes</b>	<b>Number of C-Atoms</b>	<b>Basic Carbon Skeleton</b>
Simple phenols, benzoquinones	6	$C_6$
Phenolic acids	7	$C_6 - C_1$
Acetophenone, phenylacetic acid	8	$C_6 - C_2$
Hydroxycinnamic acid, coumarin, isocoumarin	9	$C_6 - C_3$
Naphthoquinone	10	$C_6 - C_4$
Xanthone	13	$C_6 - C_1 - C_6$
Stilbene, anthrachinone	14	$C_6 - C_2 - C_6$
Flavonoids, isoflavonoids	15	$C_6 - C_3 - C_6$
Lignans, neolignans	18	$(C_6 - C_3)_2$
Biflavonoids	30	$(C_6 - C_3 - C_6)_2$
Lignins	$n$	$(C_6 - C_3)_n$
Tannins	$(C_6)_n$	$(C_6 - C_1)_n; (C_6 - C_3 - C_6)_n$

**Figure 2.3:** The phenolic acid family: **(A)** Simple phenols; **(B)** Hydroxybenzoic acids; and **(C)** Hydroxy-*trans*-cinnamic acids.

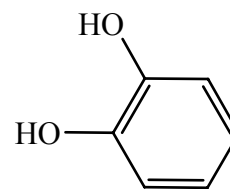
(A)



Resorcinol

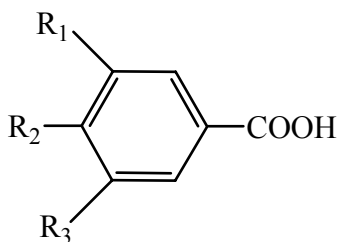


Hydroquinone



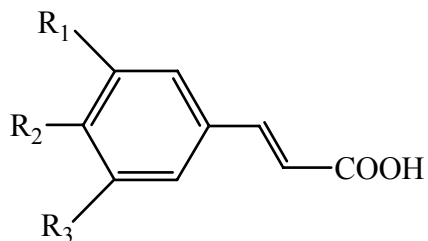
Catechol

(B)



Acid	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>
<i>p</i> -Hydroxybenzoic	H	OH	H
Gallic	OH	OH	OH
Vanillic	OCH <sub>3</sub>	OH	H
Syringic	OCH <sub>3</sub>	OH	OCH <sub>3</sub>

(C)



Acid	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>
<i>p</i> -Coumaric	H	OH	H
Caffeic	OH	OH	H
Ferulic	OCH <sub>3</sub>	OH	H
Sinapic	OCH <sub>3</sub>	OH	OCH <sub>3</sub>

*et al.*, 2000; Scalbert and Williamson, 2000; Robbins, 2003). Chlorogenic acids are a family of esters formed between *trans*-cinnamic acid and quinic acid, which have axial hydroxy moieties at the C-1 and C-3 carbon positions and equatorial hydroxy groups on the C-4 and C-5 carbon positions.

Coumarin is a sweet smelling volatile material that is released from freshly mowed hay (Briellmann *et al.*, 2006). Phenylpropanes are important components of essential oils. For example, anethole and myristicin are the principle components of nutmeg (Archer, 1988) and eugenol is the principle component of the oil in clove (Ntamila and Hassanali, 1976).

#### **2.4.4 Naphthoquinones**

Naphthoquinone (C<sub>6</sub> – C<sub>4</sub>) is an oxidized diphenolic compound having an aromatic ring containing a double ketone functional group [**Figure 2.4(A)**] (Cowan, 1999). The switch between diphenol (hydroquinone) and diketone (quinone) occurs easily through oxidation and reduction reactions (Cowan, 1999). These compounds are usually conjugated with aliphatic isoprenoid units to form phyloquinones (vitamin K<sub>1</sub>): an essential component of the human diet which cannot be synthesized in the human body and serves as a cofactor in blood coagulation.

#### **2.4.5 Xanthones**

The basic structures of xanthones (C<sub>6</sub> – C<sub>1</sub> – C<sub>6</sub>) comprise an important class of oxygenated heterocycles. This includes a pyrylium ring possessing a ketone functional group between two aromatic rings [**Figure 2.4(B)**] (Vieira and Kijjoa, 2005; Gales and Damas, 2005). Mangosteen is a well known source of xanthone,  $\alpha$ -mangostin, and  $\gamma$ -mangostin. Various

biological and medicinal activities (for example, antioxidant, anti-inflammatory, and antiviral) of mangosteen are attributed to the endogenous xanthenes (Pedraza-Chaverri *et al.*, 2008).

#### 2.4.6 Anthraquinones and Stilbenes

Anthraquinones ( $C_6 - C_2 - C_6$ ) possess a diketone linkage between the aromatic moieties. However, anthraquinones are not prominent phenolic compounds in plants [**Figure 2.4(C)**]. Stilbenes also have a  $C_6 - C_2 - C_6$  structure [**Figure 2.4(D)**]. The main dietary source of stilbenes is resveratrol (3,5,4'-trihydroxystilbene) from red wine, which occurs as *cis* and *trans* isomers present in free or glycoside forms (*e.g.*, *trans*-resveratrol and *trans*-resveratrol-3-*O*-glucoside). The protective effects of red wine consumption have been attributed to resveratrol (Crozier *et al.*, 2009).

#### 2.4.7 Flavonoids

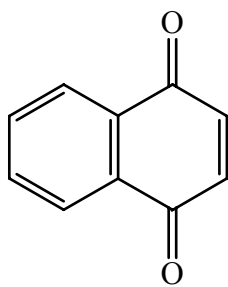
There are an estimated 4,000 known flavonoids (Wrolstad, 2005). This represents the most common and widely distributed group of plant phenolics and accounts for ~60% of dietary phenolic compounds (Harborne and Williams, 2000; Shahidi and Naczki, 2004; Nichenametla *et al.*, 2006). Flavonoids are generally present in the epidermis of leaves and the skin of fruits.

Flavonoids consist of two aromatic rings connected by a three-carbon bridge that usually forms an oxygenated heterocycle. Their common structure is a diphenylpropane ( $C_6 - C_3 - C_6$ ) (Heller *et al.*, 1988; Aruoma *et al.*, 2004; Cai *et al.*, 2006). **Figures 2.5(A)** and **(B)** depict the basic structure and system employed for the carbon numbering of a flavonoid nucleus. Hydroxy groups are usually present at the C-4', C-5, and C-7 positions. The majority of flavonoids exist naturally as glycosides. Sugars and hydroxy groups increase the water solubility of flavonoids,

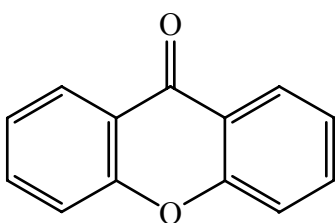
**Figure 2.4:** Other non-flavonoid phenolics of importance. **(A)** Napthoquinones; **(B)** Xanthones; **(C)** Anthraquinones; and **(D)** Stilbenes.



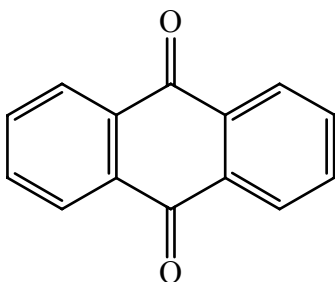
(A)



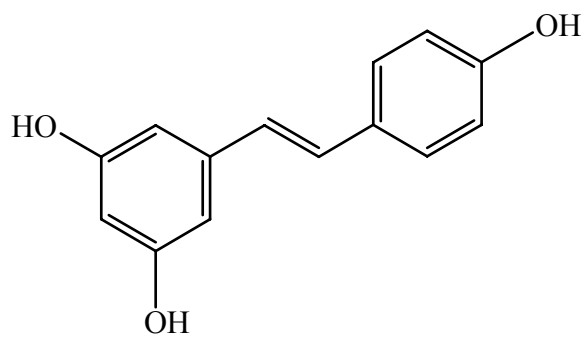
(B)



(C)

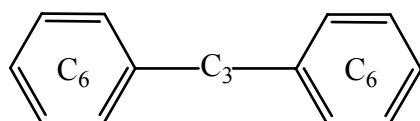


(D)

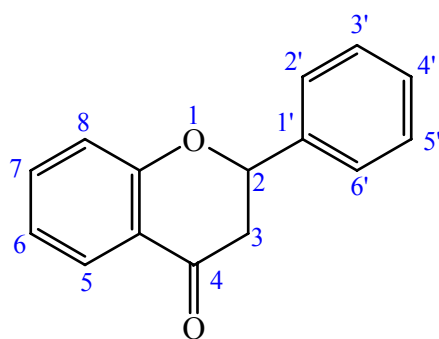


**Figure 2.5:** Basic structure and carbon-numbering system for the diphenylpropane ( $C_6 - C_3 - C_6$ ) nucleus of flavonoids. **(A)** Basic structure; and **(B)** Carbon-numbering system.

(A)



(B)



while methoxy groups and isopentyl unit substituents make flavonoids more lipophilic. Conjugation occurs mostly at the C-3 position of the C-ring, but substitutions can also happen at the C-5, C-7, C-4', C-3', and C-5' positions (Crozier *et al.*, 2009).

Structural variations within the rings subdivide the flavonoids into several families. The main sub-classes of dietary flavonoids are flavanones, flavonols, flavones, flavanols, flavan-3-ols, anthocyanidins, and isoflavones. Minor classes are dihydroflavonols, flavan-3,4-diols, coumarins, chalcones, dihydrochalcones, and aurones.

#### **2.4.7.1 Anthocyanins**

Anthocyanins are the largest group of flavonoids that possess a hydroxylated 2-phenyl benzopyrilium chromophore. Anthocyanins exist in various tissues of higher plants including flowers, fruits, seed coats, leaves, stems, tubers, and roots. They exhibit a wide variety of colors ranging from red to purple and from blue to black (Yoshida *et al.*, 2009). Anthocyanins are water-soluble, glycosylated, and/or acylated flavonoid derivatives. The de-glycosylated, or aglycone, forms are called anthocyanidins and exist as different chemical structures, both colored and uncolored, according to variation in pH (Yoshida *et al.*, 2009). There are six commonly occurring anthocyanidins in higher plants, namely pelargonidin, cyanidin, peonidin, delphinidin, malvidin, and petunidin [**Figures 2.6(A).**]

Cyanidins are the most abundant anthocyanidins while malvidins are the least (Kong *et al.*, 2004). Sugars are present typically at the C-3 position, second at the C-5 position, and very rarely at the C-7 position. These sugars include glucose, galactose, rhamnose, and arabinose, as 3-glycosides or as 3,5-diglycosides (Seeram, 2005). The C-3 glycosylation is a prerequisite for further modifications such as a second glycosylation, acylation, methoxylation, and prenylation

(Springob *et al.*, 2003). Common acylating agents include phenolic acids; *i.e.*, *p*-coumaric, caffeic, ferulic, and sometimes *p*-hydroxybenzoic acids, and a range of aliphatic acids; *i.e.*, acetic, malic, malonic, oxalic, and succinic acid (Seeram, 2005). There are 500 naturally occurring anthocyanins reported due to the diversity of glycosylation and acylation in their basic structure. The flavylum ion structures of commonly found anthocyanidins are shown in **Figure 2.6(A)**.

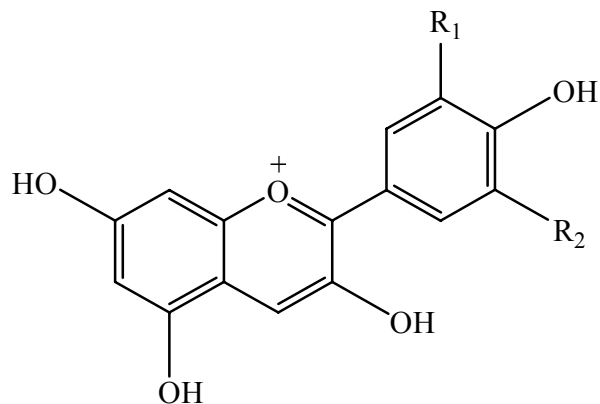
#### 2.4.7.2 Flavanols

Flavanols, also called flavan-3-ols, possess a saturated C<sub>3</sub> element in the heterocyclic C-ring with a hydroxy or galloyl group attached at the C-3 position and are the only class of flavonoids that largely occur in the aglycone form (Seeram, 2005). The most widely distributed members of flavanols in nature are the diastereoisomeric pair (+)-catechin and (-)-epicatechin. Epicatechin has an *ortho*-dihydroxy group in the B-ring at the C-3' and C-4' positions and a hydroxy group at the C-3 position on the C-ring (Yilmaj, 2006). This group also includes epicatechin gallate (ECG), epigallocatechin (EGC), and epigallocatechin-3-gallate (EGCG) [**Figure 2.6(B)**]. ECG differs from epicatechin due to its gallate moiety esterified at the C-3 position of the C-ring. EGC differs from epicatechin due to a hydroxy group at the C-3', C-4', and C-5' positions on the B-ring. However, EGCG has hydroxy groups at the C-3', C-4', and C-5' positions on the B-ring and a gallate moiety esterified at the C-3 position on the C-ring. EGCG is the most abundant catechin in the leaves of green, oolong, and black teas (Yilmaj, 2006).

Monomeric flavanols, hydroxylated at the C-4 position only, or with an additional hydroxy group at the C-3 position of ring C, are known as flavan-4-ols and flavan-3,4-diols,

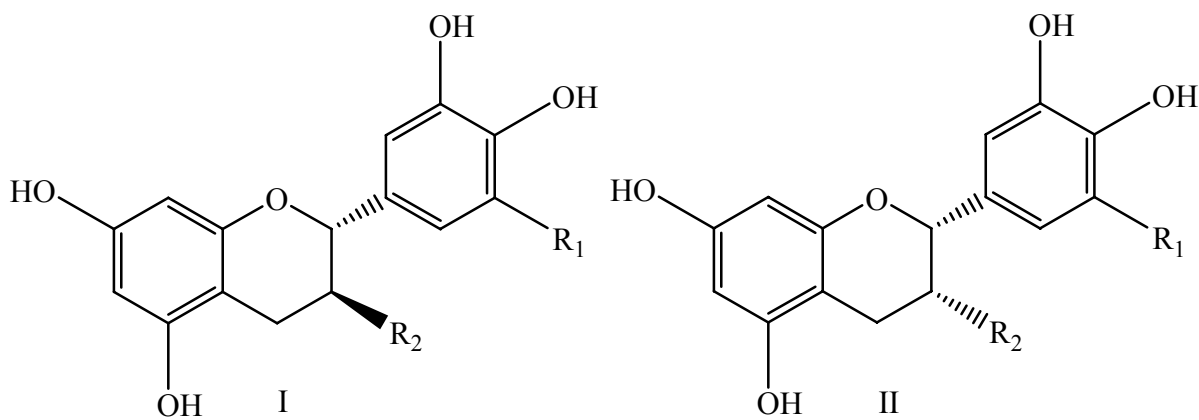
**Figure 2.6:** Chemical structures of selected compounds from the flavonoids' family. **(A)** Anthocyanidins; and **(B)** Flavanols.

(A)



	R <sub>1</sub>	R <sub>2</sub>
Pelargonidin	H	H
Cyanidin	OH	H
Delphinidin	OH	OH
Petunidin	OCH <sub>3</sub>	OH
Peonidin	OH	OCH <sub>3</sub>
Malvidin	OCH <sub>3</sub>	OCH <sub>3</sub>

(B)



Flavanol (I/II)	R <sub>1</sub>	R <sub>2</sub>
(+)-Catechin/(-)-Epicatechin	H	OH
(+)-Catechin gallate/(-)-Epicatechin gallate	H	<i>O</i> -Gallate
(+)-Gallocatechin/(-)-Epigallocatechin	OH	OH
(+)-Gallocatechin gallate/(-)-Galloepicatechin gallate	OH	<i>O</i> -Gallate

respectively. These compounds, also known as leucoanthocyanidins, are converted to anthocyanidins by cleavage of the C-ring hydroxy group upon heating with acid (Ferreira *et al.*, 2006).

### 2.4.7.3 Flavanones

Flavanones are characterized by the absence of C-2-C-3 double bonds and the presence of a chiral center at the C-2 position. In the majority of naturally-occurring flavanones, the C ring is attached to the B ring at the C-2 position in the  $\alpha$ -configuration [**Figure 2.7(A)**]. (Crozier *et al.*, 2006). There are numerous structural variations of flavanones found in plants due to acylation, glycosylation, hydroxylation, methylation, and methoxylation of the A- and B-rings of the carbon skeleton. Glycosylation of flavanones typically occurs at the C-7 position, ring A (Veitch and Grayer, 2008).

Flavanones are abundant in citrus fruits; the most commonly occurring aglycones are hesperedin (tasteless), hesperetin (bitter), and naringenin (bitter), which are present in citrus peel, bitter orange, and grape fruit peel, respectively.

### 2.4.7.4 Flavones

Flavones are similar in structure to flavanones, but they are unsaturated between bonds C-2 and C-3 of the C ring [**Figure 2.7(B)**] (Harborne and Williams, 1976). They may have a wide range of substitutions including hydroxylation, methoxylation, and glycosylation. Most flavones exist as 7-*O*-glycosides. Flavones are not widely distributed in plants as compared to other flavonoids, however, they have been reported in celery, parsley, and some herbs.



Polymethoxylated flavones such as tangeretin and nobiletin are found in citrus species (Crozier *et al.*, 2008).

#### **2.4.7.5 Flavonols**

Flavonols are the most widespread flavonoid in the plant kingdom (Harborne and Williams, 2000). The basic aglycone structure of flavonols differs from that of flavones in that, the C-3 position of the flavonol is hydroxylated/glycosylated. Conjugation occurs at the C-3 position of the C-ring, but substitutions can also occur at the C-5, C-7, C-4', C-3', and C-5' positions (Crozier *et al.*, 2008).

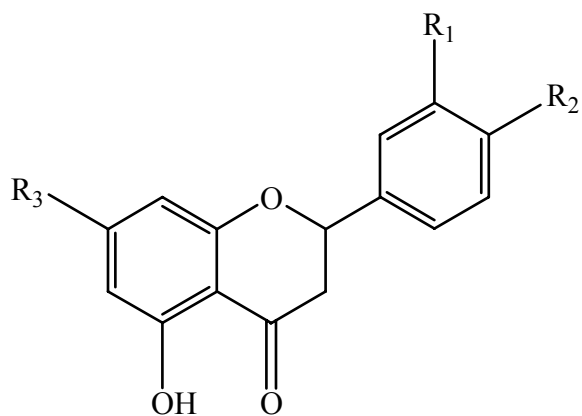
Commonly occurring flavonol aglycones include kaempferol, myricetin, and quercetin. The structures of these compounds are depicted in **Figure 2.7(C)**. Flavonols are usually found as glycoside derivatives where the associated sugar moiety is glucose or rhamnose. Galactose, arabinose, xylose, and glucuronic acids are less common. More than 1,400 glycosylated flavonols have been identified in plants (Harborne and Williams, 2000). Glycosides of quercetin such as rutin, are the most predominant, naturally-occurring flavonols.

#### **2.4.7.6 Isoflavones**

Isoflavones are characterized by the attachment of the B-ring to the C-ring at the C-3 position, rather than C-2 [**Figure 2.7(D)**]. The aglycones – diadzein, genistein, and glycitein – and their respective glycosides (free, malonyl, and acetyl forms) are the major isoflavones that have been characterized in plants. They are found almost exclusively in leguminous plants, with the highest concentrations determined in soybeans (Crozier *et al.*, 2008).

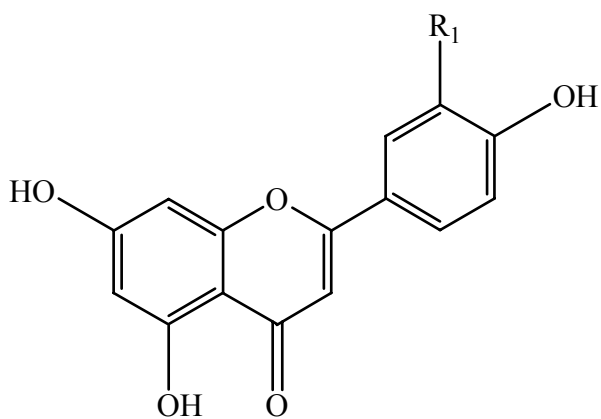
**Figure 2.7:** Chemical structures of selected compounds from the flavonoids' family. **(A)** Flavanones; **(B)** Flavones; **(C)** Flavonols; and **(D)** Isoflavones.

(A)



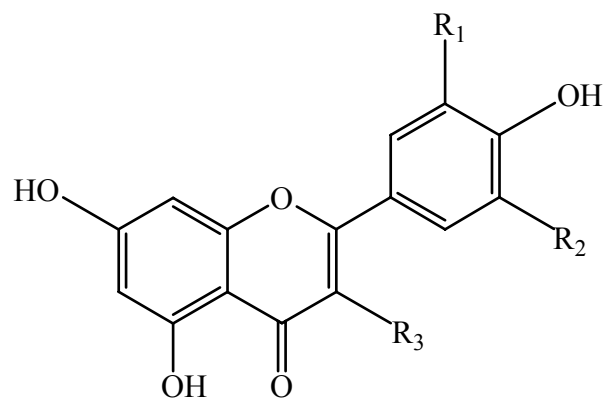
Flavanone	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>
Naringenin	H	OH	OH
Hesperetin	OH	OCH <sub>3</sub>	OH
Hesperedin	OH	OCH <sub>3</sub>	<i>O</i> -Rutinoside

(B)



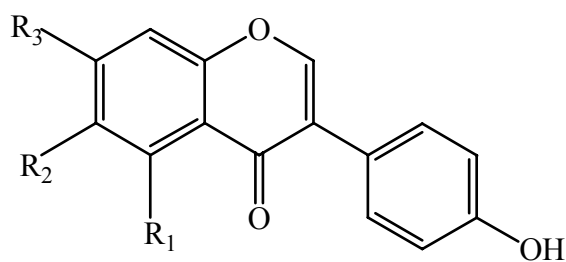
Flavone	R <sub>1</sub>
Apigenin	H
Luteolin	OH

(C)



Flavonol	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>
Kaempferol	H	H	OH
Quercetin	OH	H	OH
Myricetin	OH	OH	OH
Rutin	OH	H	<i>O</i> -Rutinoside

(D)



Isoflavone	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>
Diadzein	H	H	OH
Diadzin	H	H	<i>O</i> -Glucose
Genistein	OH	H	OH
Genistin	OH	H	<i>O</i> -Glucose
Glycitein	H	OCH <sub>3</sub>	OH
Glycitin	H	OCH <sub>3</sub>	<i>O</i> -Glucose

#### **2.4.7.7 Other Minor Flavonoids**

In addition to the aforementioned main subclasses of flavonoids, there are three minor flavonoid subclasses: these include chalcones, dihydrochalcones, and aurones. The unique feature which distinguishes chalcones and dihydrochalcones from other flavonoids is the open chain three-carbon structures linking the A- and B-rings in place of the heterocyclic C-ring. This three-carbon linkage between the aromatic rings is unsaturated in chalcones and saturated in dihydrochalcones.

#### **2.4.8 Tannins**

Tannins are phenolic compounds of intermediate to high molecular weights ranging from 500 to > 30,000 Da (Serrano *et al.*, 2009) and can form insoluble complexes with carbohydrates and proteins through hydrogen bonding of the hydroxyl groups (Reed, 1995). The term “tannin” was first introduced in 1796 referring to compounds used in tanneries to transform animal hides into leather by forming stable tannin-protein complexes with skin collagen (Bravo, 1998; Serrano *et al.*, 2009).

Tannins are classified into two major groups: the hydrolyzable tannins which are polymers of gallic acid and condensed tannins which are composed of flavanol polymers (Manach *et al.*, 2004).

##### **2.4.8.1 Hydrolyzable Tannins**

Hydrolyzable tannins are esters of gallic acid and ellagic acid. They are readily hydrolyzed by acids (or enzymes) to sugars, or related polyhydric alcohols and a phenolic carboxylic acid. Depending on the nature of the phenolic carboxylic acid, the hydrolyzable

tannins are subdivided into gallotannins (GTs) and ellagitannins (ETs). Hydrolysis of GTs yield gallic acid while hydrolysis of ETs yield hexahydroxydiphenic acid (HHDP), which is normally isolated as its stable dilactone, ellagic acid [**Figure 2.8(A)**].

Pentagalloylglucose (1, 2, 3, 4, 6-penta-*O*-galloyl- $\beta$ -D-glucopyranose) is precursor for GTs and ETs (Clifford and Scalbert, 2000). Additional galloyl subunits can attach to the galloyls on the pentagalloylglucose molecule by oxidative bonding to form characteristic 3,4,5,3',4',5' hexahydroxydiphenoyl (HHDP) moieties and larger GT molecules (Mueller-Harvey, 2001; Serrano *et al.*, 2009). Principle steps in the biosynthesis of GTs and ETs are presented in **Figure 2.8(B)**.

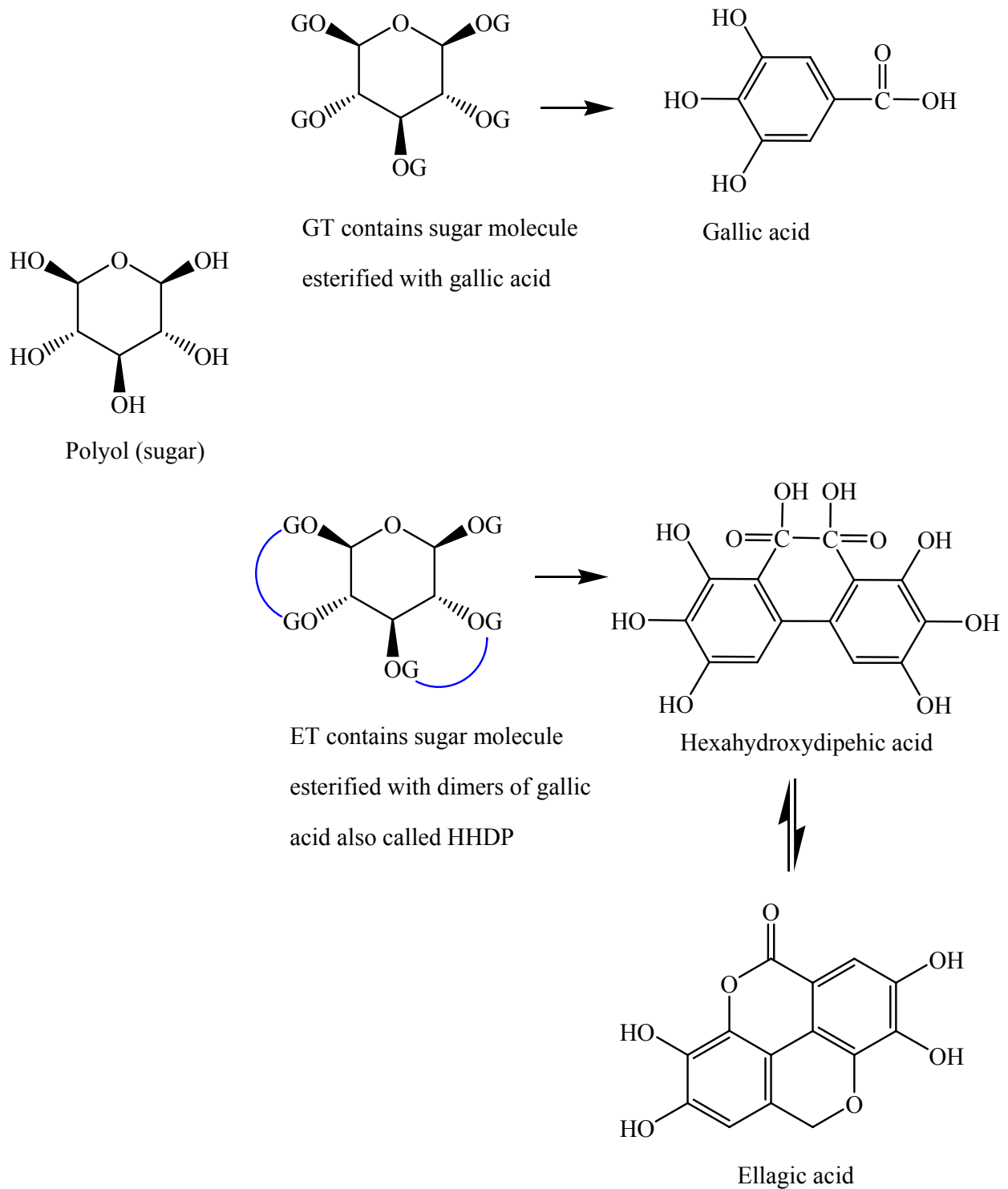
Most ETs are esters mixed with both HHDP and gallic acid (**Figure 2.9**). ET monomers can be further oxidized in plants to form dimers, trimers, and tetramers with molecular weights up to 4,000 Da (Seeram, 2005). Five hundred ETs with diverse structures have been identified. Structural variations among monomers occur due to the metabolic modification of HHDP. In addition, intermolecular C-O oxidative bonding between the HHDP group in ETs and a galloyl group, in another monomer, produces additional structural variation leading to a large number of compounds in oligomeric ET subclasses (Okuda *et al.*, 2009).

#### **2.4.8.2 Proanthocyanidins (PACs)/Condensed Tannins**

Proanthocyanidins (PACs) or condensed tannins are formed by condensation of monomeric units consisting of chains of flavan-3-ol units. The building block for PACs are (+)-catechin and its isomer (-)-epicatechin. Oxidative condensation occurs between the C-4 position of the C-ring and the C-6 or C-8 position of the A-ring on adjacent flavanol units. These PACs

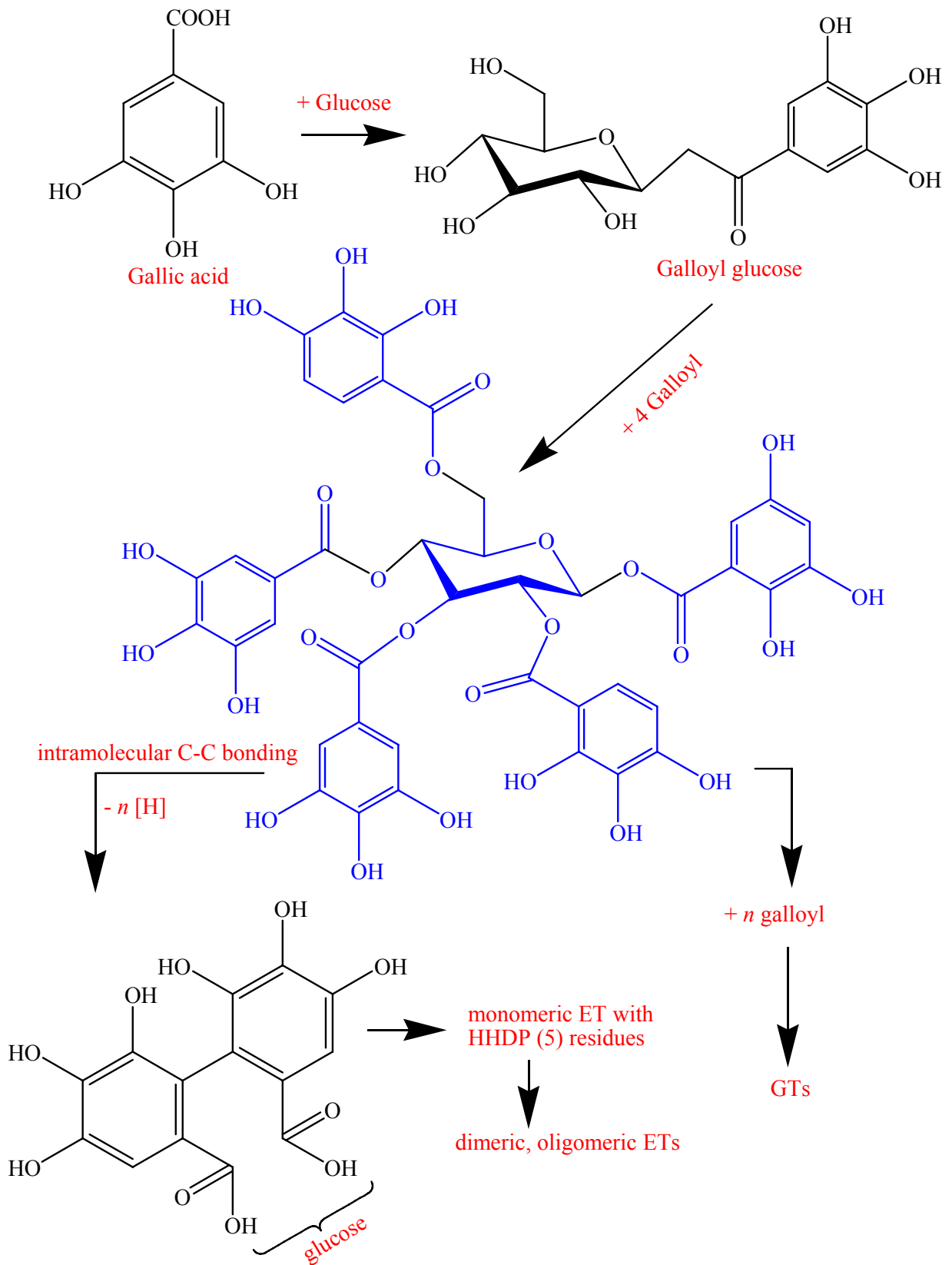
**Figure 2.8:** Biosynthesis and hydrolysis of hydrolyzable tannins. **(A)** Hydrolysis of GTs and ETs; and **(B)** Principal steps in the biosynthesis of GTs and ETs. Abbreviations are as follows: ETs, ellagitannins; G, gallic acid; GTs, Gallotannins; and HHDP: hexahydroxydiphenic acid.

(A)



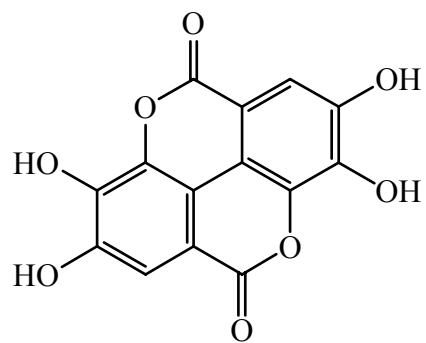


(B)

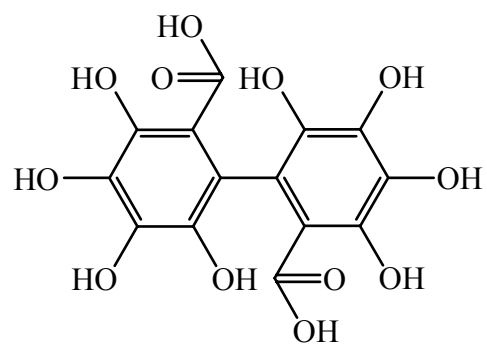


**Figure 2.9:** Chemical structures of selected hydrolyzable tannins and their monomers. **(A)** Ellagic acid; **(B)** Hexahydroxydiphenic acid (HHDP); **(C)** Pentagalloylglucose; **(D)** Pedunculagin, bis-HHDP glucopyranose; **(E)** Ellagitanins, galloyl-HHDP; **(F)** Galloyl-bis-HHDP glucopyranose; **(G)** Dimer, sanguin H-6/lambertianin A; **(H)** Dimer, lambertianin B; **(I)** Trimer, lambertianin C; and **(J)** Tetramer, lambertianin D. (Adapted from Hager *et al.*, 2008; Vrhovsek *et al.*, 2006).

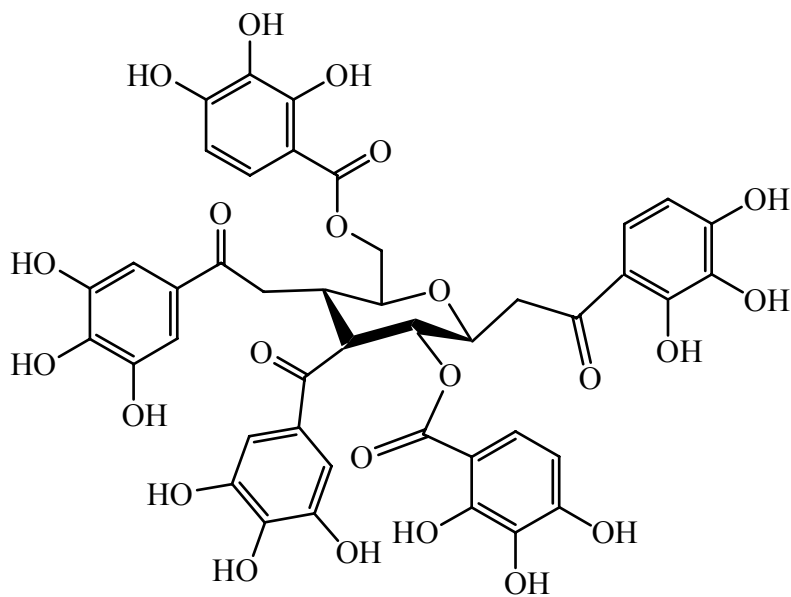
(A)



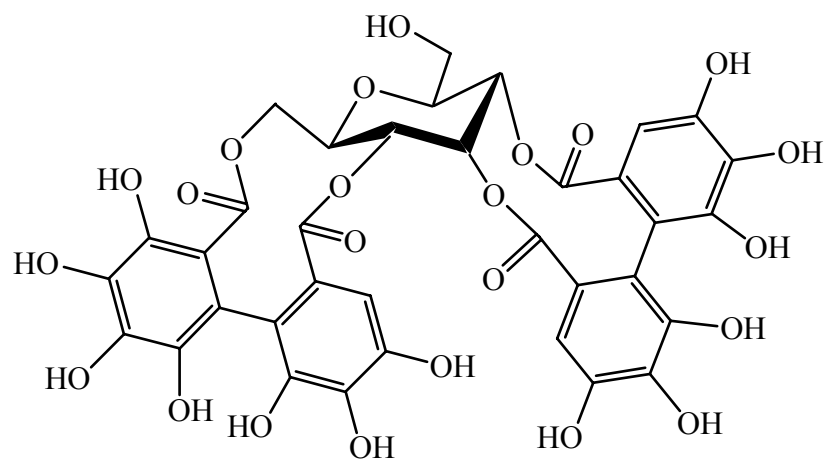
(B)



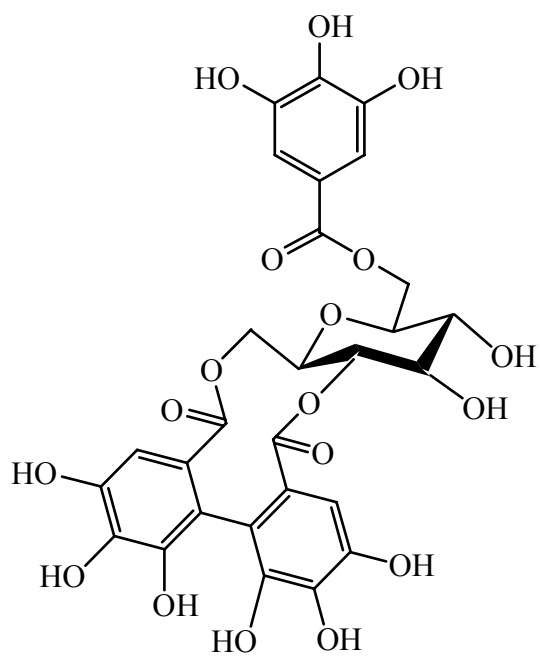
(C)



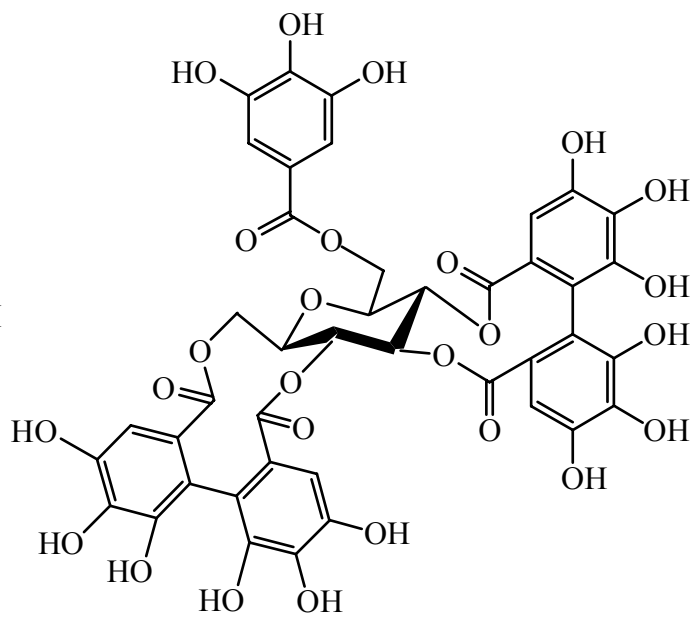
(D)



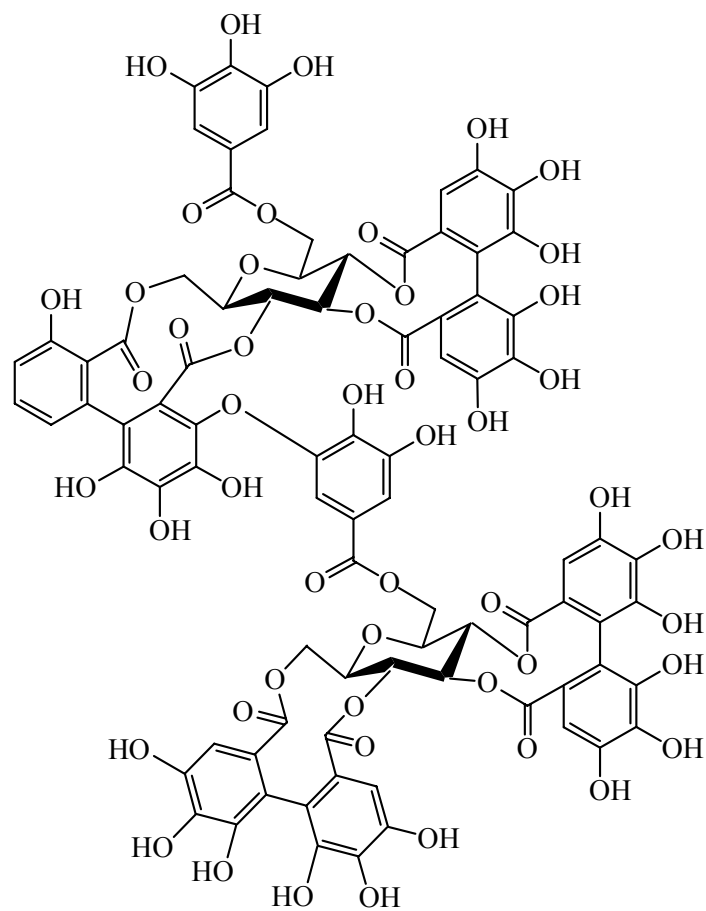
(E)



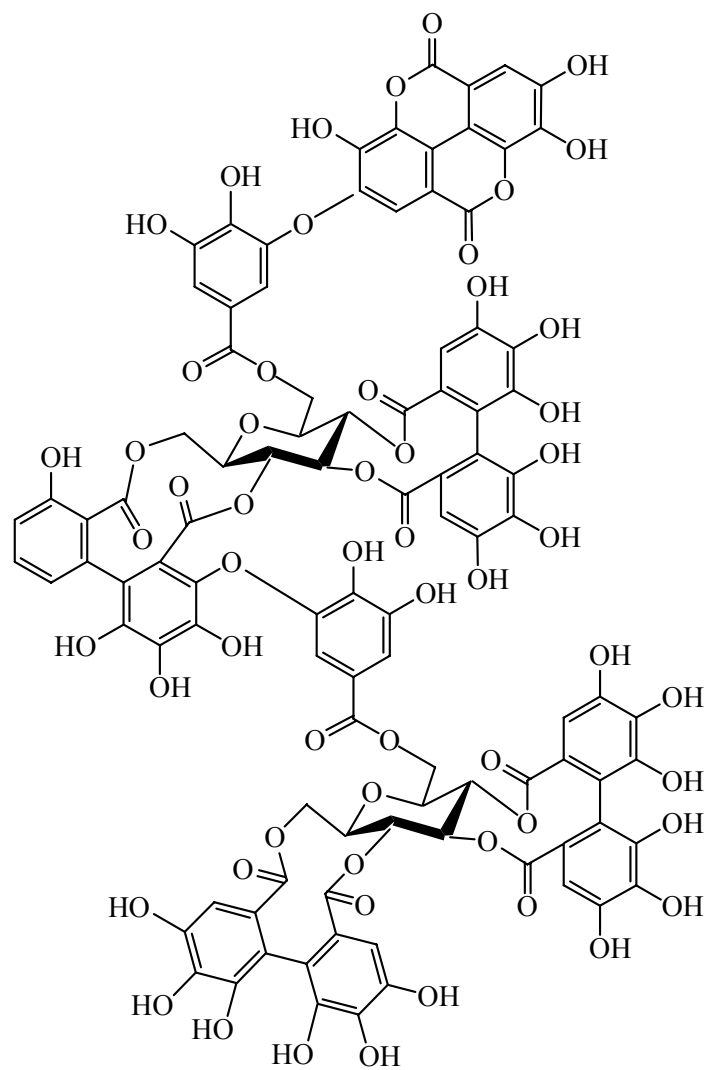
(F)



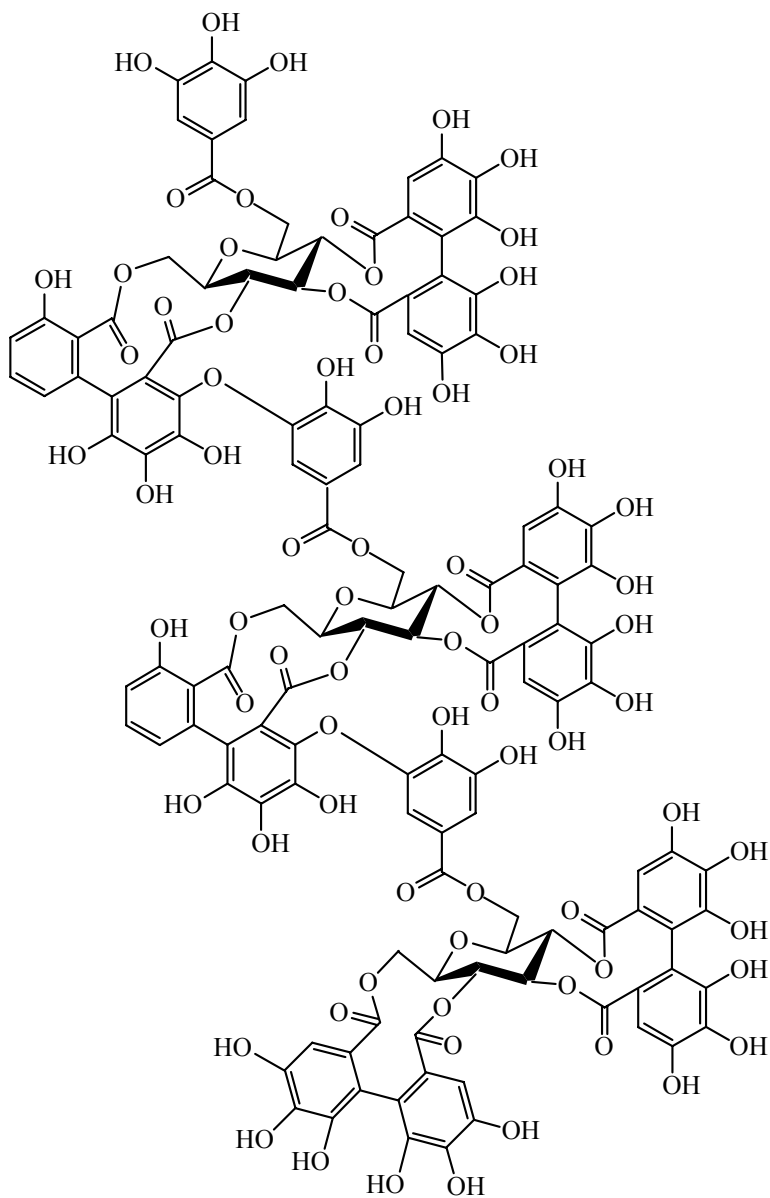
(G)



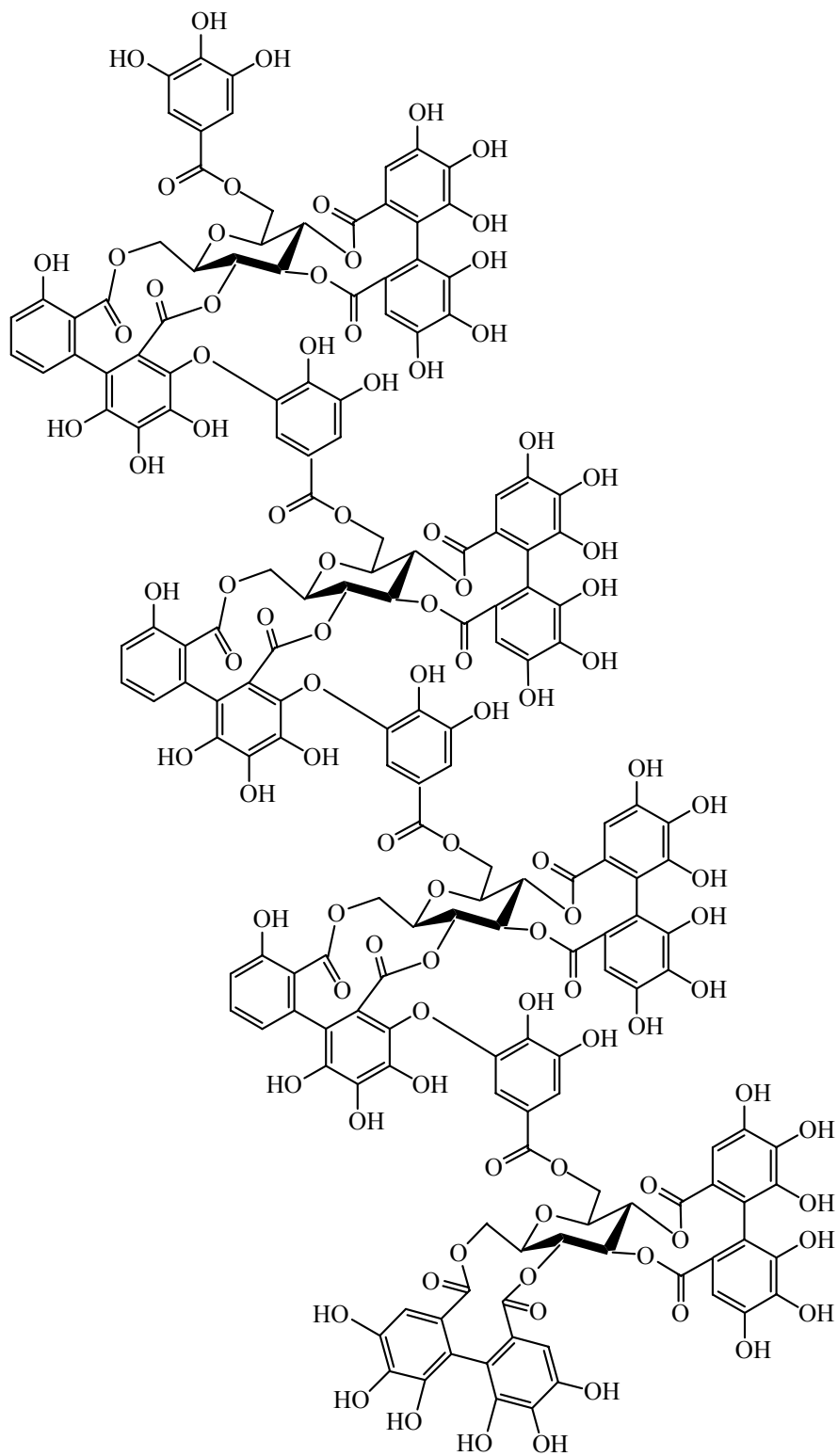
(H)



(I)



(J)





are referred to as B-type PACs. In some plants, an additional C-2→C-7 ether linkage can be found and these PACs are often called A-type PACs (Serrano *et al.*, 2009).

Proanthocyanidins are typically described by their degree of polymerization (DP). Monomers have a DP = 1 while oligomers and polymers have a DP of 2 to 10 and a DP > 10, respectively (Santos-Buelga and Scalbert, 2000; Gu *et al.*, 2003; Gu *et al.*, 2004). The most abundant type of PACs found in plants consists exclusively of epicatechin units called procyanidins. The least common PACs, containing (-)-epiafzelechin and (+)-afzelechin, are epigallocatechin sub-units called propelargonidins and prodelphinidins, respectively (Santos-Buelga and Scalbert, 2000; Gu *et al.*, 2004; USDA, 2004; Crozier *et al.*, 2009).

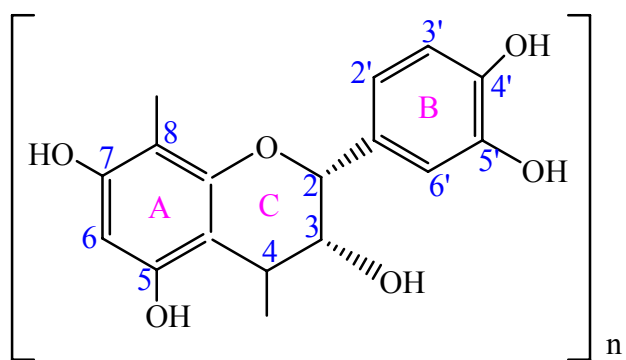
The chemical structures of A- and B-type PACs are depicted in **Figure 2.10**. The A- and B-type linkages are illustrated where monomeric units are primarily linked through single 4→6 or 4→8 carbon carbon bonds.

## 2.5 Role of Phenolic Compounds in Plants

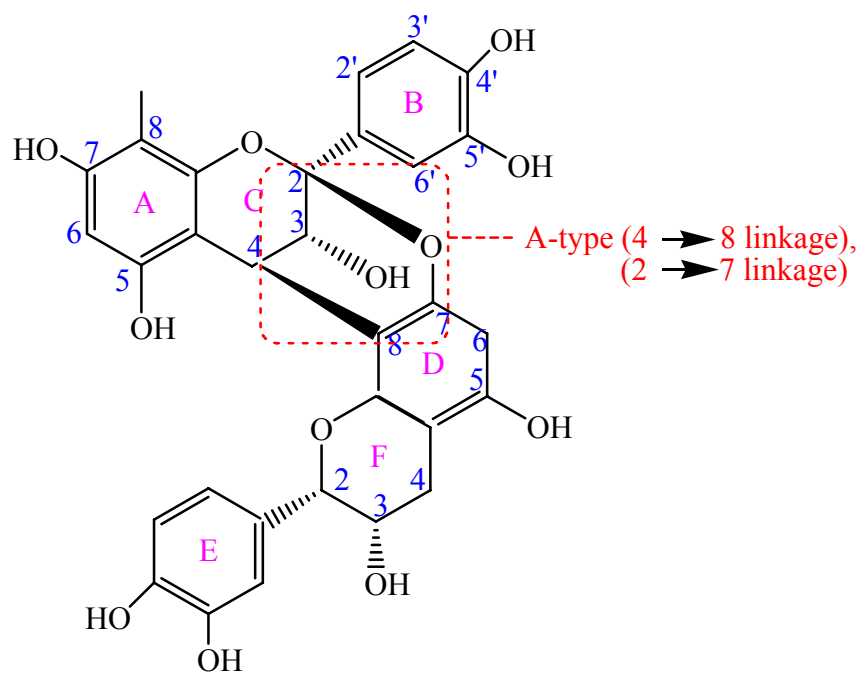
Phenolic compounds are secondary metabolites that play important roles in the defense and physiology of plants. They can be categorized into three broad groups: phytoalexins, phytoanticipins, and signal molecules (Dixon *et al.*, 2002). Phytoalexins help plants fight microbial diseases. For example, kaempferol and quercetin 3-glucoside can inhibit *Verticillium* fungi that cause plant wilt (Picman *et al.*, 1995) and catechin can inhibit the growth of pathogenic fungi (*Aspergillus* species) (Weidenborner *et al.*, 1990). Mechanisms responsible for phenolic toxicity to microorganisms include enzyme inhibition, complexation with extracellular proteins and cell wall disruption (Mason and Wasserman, 1987; Scalbert, 1991; Chabot *et al.*, 1992).

**Figure 2.10:** Chemical structures of selected condensed tannins and their monomers. **(A)** Condensed tannin monomeric unit; **(B)** A-type linkage in which monomeric units are primarily joined through single 2→7 ether bonds, this is in addition to B-type linkages; and **(C)** B-type linkage in which monomeric units are primarily joined through single 4→8 ether bonds.

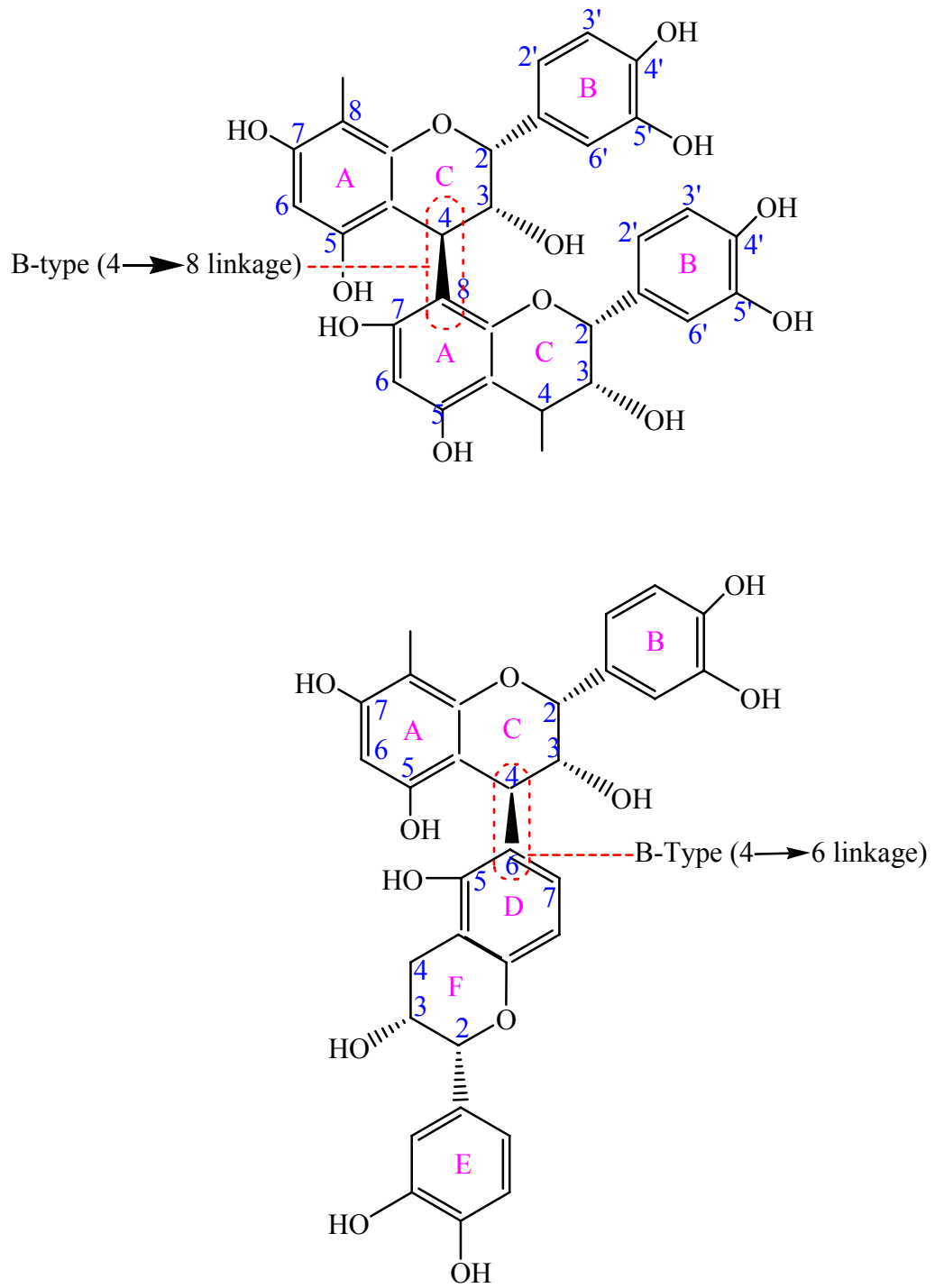
(A)



(B)



(C)



Many phenolic compounds play important roles as signal molecules. For example, dehydrodiconiferyl glucosides are potential modulators of cell division (Woo *et al.*, 1999) and other flavonoid glucosides can regulate auxin transport (Jacob and Rubery, 1988). Salicylic acid is a regulator of local and systemic pathogen-induced defense gene activation, the oxidative burst, and pathogen-induced cell death (Dempsey *et al.*, 1999).

In plants, phenylpropanoids are released in response to wounding of tissue like that resulting from feeding by herbivores and insects. Increased levels of the phenylpropanoids, coumestrol and coumarin, are toxic to potential herbivores causing estrogenic and anticoagulant effects (Smith, 1982). Chlorogenic acid and rutin are toxic and inhibit growth of fruitworm larvae (*Heliothis zea*), feeding on leaves containing these compounds (Isman and Duffey, 1982).

Some of the phenolic compounds are allelopathic; *i.e.*, they are released by plants to inhibit the growth of other plants and thereby reduce competition (Mann, 1987; Bais *et al.*, 2003). For example, phlorizin is a chalcone released from the roots of apple trees to reduce competition from other plants near the tree (Mann, 1987).

One important protective role of flavonoids is to filter harmful UV radiation, preventing damage to the plant (Dixon *et al.*, 2002). Flavonoids, mainly flavones and flavonols, exhibit high absorption in the UV-B region (280-320 nm), thereby protecting plants against UV-B damage and subsequent cell death by protecting DNA from dimerization and breakage (Dixon and Paiva, 1995).

Some flavonols function as attractants for pollen and seed dispersal. For example, kaempferol and its glycosides are induced by both wounding and pollination in petunia stigmas and appear to be required for normal pollen development (Mo *et al.*, 1992; van der Meera *et al.*, 1992; Vogt *et al.*, 1994). Although flavonols are not colored, they absorb strongly in the UV

region and can be seen by insects; kaempferol 3-glucoside, and quercetin 3-glucoside have been reported to attract insects for pollen dispersal in the flowers of Leguminaceae (Harborne and Boardley, 1983; Mann, 1987).

Anthocyanins are the most widespread pigments in the plant kingdom and are responsible for most of the red, blue, purple, and intermediate hues in flowers and fruits (Lev-Yadun *et al.*, 2009; Springob *et al.*, 2003). The color imparted by anthocyanins to flowers and fruits is believed to attract specific species of animals, birds, and insects for pollen and seed dispersal (Harborne, 2001; Lev-Yadun *et al.*, 2009; Springob *et al.*, 2003). Anthocyanins and flavones increase in response to high visible light levels which help to attenuate the amount of light reaching the photosynthetic cells (Beggs *et al.*, 1987).

Nutritional stress also results in increased concentrations of phenylpropanoids. For example, low nitrogen promotes flavonoids, isoflavonoid nod gene inducers and chemoattractants for nitrogen fixing symbionts (Wojtaszek *et al.*, 1993); low iron levels cause increased release of phenolic acids that help in solubilizing metals thereby, facilitating their uptake (Marschner, 1991).

## **2.6 Phenolic Compounds in Food Industry**

### **2.6.1 Sensory Attributes of Phenolic Compounds**

Phenolic compounds may contribute desirable or non-desirable flavors to foods (Scalbert and Williamson, 2000; Shahidi and Naczki, 2004; Kyle and Duthie, 2006). The characteristic flavor of vanilla extract is due to the benzoic acid derivative vanillin (*p*-hydroxybenzaldehyde). Strawberry volatiles contain esters of phenolic acids such as methyl cinnamic and ethylbenzoic acid and several coumarins contribute to lime and mandarin flavors (Shahidi and Naczki, 1995).

Chalcones and dihydrochalcones are sweet in flavor, although the onset of sweetness is reportedly slow and leaves a lingering aftertaste (Dubois *et al.*, 1977). Sinapine is a bitter phenolic acid derivative found in rapeseed which is linked to a fishy taint in eggs (Butler *et al.*, 1982; Shahidi and Naczk, 1992). Free ferulic acid is a precursor of *p*-vinyl guaiacol which is responsible for the objectionable flavor in stored orange juice which is produced by carboxylation of ferulic acid (Naim *et al.*, 1988).

The color of many fruits and fruit products is attributable to phenolic compounds. Anthocyanin pigments that are naturally present in raw fruits and certain vegetables are also responsible for the desirable colors in foods prepared from these plant products (Lev-Yadun *et al.*, 2009).

Apart from sensory properties, phenolic compounds have significant potential to increase the shelf life of food due to their antioxidant and free radical scavenging abilities (Shahidi, 2000). Food manufacturers use food-grade phenolic antioxidants to prevent deterioration of product quality and nutritional value due to oxidation (Shahidi and Naczk, 2004). Synthetic and natural antioxidants are used routinely in foods, especially those containing oils and fats. Common synthetic antioxidants used in foods include butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and *tert*-butylhydroquinone (TBHQ). Natural antioxidants used in food product manufacturing include, ascorbic acid and phenolic compounds extracted from plants (Schuler, 1990; Sakihama *et al.*, 2002; Shahidi and Naczk, 2004) such as tocopherols and rosemary extract (Schuler, 1990).

The sensory attributes typically attributed to phenolic compounds are bitterness and astringency (Shahidi and Naczk, 1995). Astringency is related to the ability of the phenolic compound to precipitate salivary proteins, bringing about a drying sensation over the surface of

the tongue (Bate-Smith, 1973). Tannins are the most astringent phenolic compounds. However less complex phenolic compounds such as catechin and *p*-coumaric acid can also impart astringency (Delcour *et al.*, 1984; Huang and Zayas, 1991). Hydroxycinnamic acid derivatives have been found to contribute bitter flavors in berries such as cranberries (Marwan and Nagel, 1982), while catechin and epicatechin have been reported to contribute the bitterness in red wine (Kallithraka *et al.*, 1997), tea, and cocoa powder (Drewnowski and Gomez-Carneros, 2000). Flavanone glycosides such as hesperidin, naringin, and neohesperidin are major contributors to the bitter taste of citrus fruits (USDA, 1998).

### **2.6.2 Dietary Intake of Phenolic compounds**

The average per capita consumption of phenolic compounds in the U.S. has been estimated to range from 255 mg/day (Vinson *et al.*, 2001) to as high as 1,000 mg/day (Kyle and Duthie, 2006). Anthocyanins, flavonols, flavanols, phenolic acids, and tannins are the major phenolic compounds found in foods (Macheix *et al.*, 1990; Shahidi and Naczki, 1995). In the U.S., the daily intake of anthocyanins has been estimated to be 12.5 mg/day (Prior and Wu, 2006) and 53.6 mg/day for PAC (DP > 2) (Prior and Gu, 2005). The intakes could easily be over 200mg/day if a regular diet of fruit and berries was consumed (Prior and Wu, 2006). In Spanish population intake of hydrolyzable tannins and phenolic acids are estimated at around 1250 mg/day (Calixto *et al.*, 2007) and intake of highly polymerized PACs at ~450 mg/day (Saura-Calixto *et al.*, 2007); literature data is limited concerning ETs and GTs intake (Serrano *et al.*, 2009).

In 2003, due to the growing awareness of the potential importance of flavonoids in diet, the United States Department of Agriculture (USDA) established a database containing the



flavonoid content of foods compiled from bibliographic sources. New foods are added to the database periodically and the information is made available on the USDA website (<http://www.nal.usda.gov/nutrientdata>).

## **2.7 Bioavailability and Metabolism of Phenolic Compounds**

There are many factors that influence the extent and rate of absorption of ingested compounds by the small intestine. These include physiochemical factors such as molecular size, lipophilicity, solubility, and biological factors. These biological factors include gastric and intestinal transit time, lumen pH, membrane permeability, and first pass metabolism (Lin *et al.*, 1999).

Phenolic compounds are usually found conjugated to sugars and organic acids. Sugar attachment increases the water solubility and limits the passive diffusion (Williamson *et al.*, 2000). The first stage of metabolism is deglycosylation/deconjugation; *i.e.*, cleavage of either the glycosyl or glucuronosyl moiety from the phenolic backbone, resulting in aglycone formation (Leese and Semenza, 1973; Day *et al.*, 1998).

Aglycones can be formed in the lumen by membrane-bound lactase phlorhizin-hydrolase (LPH) action and they are absorbed passively through the epithelium (Day *et al.*, 2000; Nemeth *et al.*, 2003). Many phenolic aglycones are hydrophobic and can passively diffuse through biological membranes (Williamson *et al.*, 2000).

Deconjugation is catalyzed by fecal microbial enzymes ( $\alpha$ -rhamnosidase,  $\beta$ -glucosidase, and  $\beta$ -glucuronidase) (Selma *et al.*, 2009). The  $\beta$ -glucosidase present in the epithelial cells of the human small intestine and liver also hydrolyze various phenolic glycosides (Day *et al.*, 1998).

Some phenolics can be transported through the gut epithelium as glycosides by sugar transporters.

Once absorbed, phenolics may be metabolized by phase-I enzymes, responsible for hydroxylation and demethylation, and phase-II enzymes, responsible for the removal of xenobiotics by conjugation of phenolics with glucuronide or sulphate. Conjugation is a common detoxification reaction which increases solubility and molecular mass (Smith, 1973).

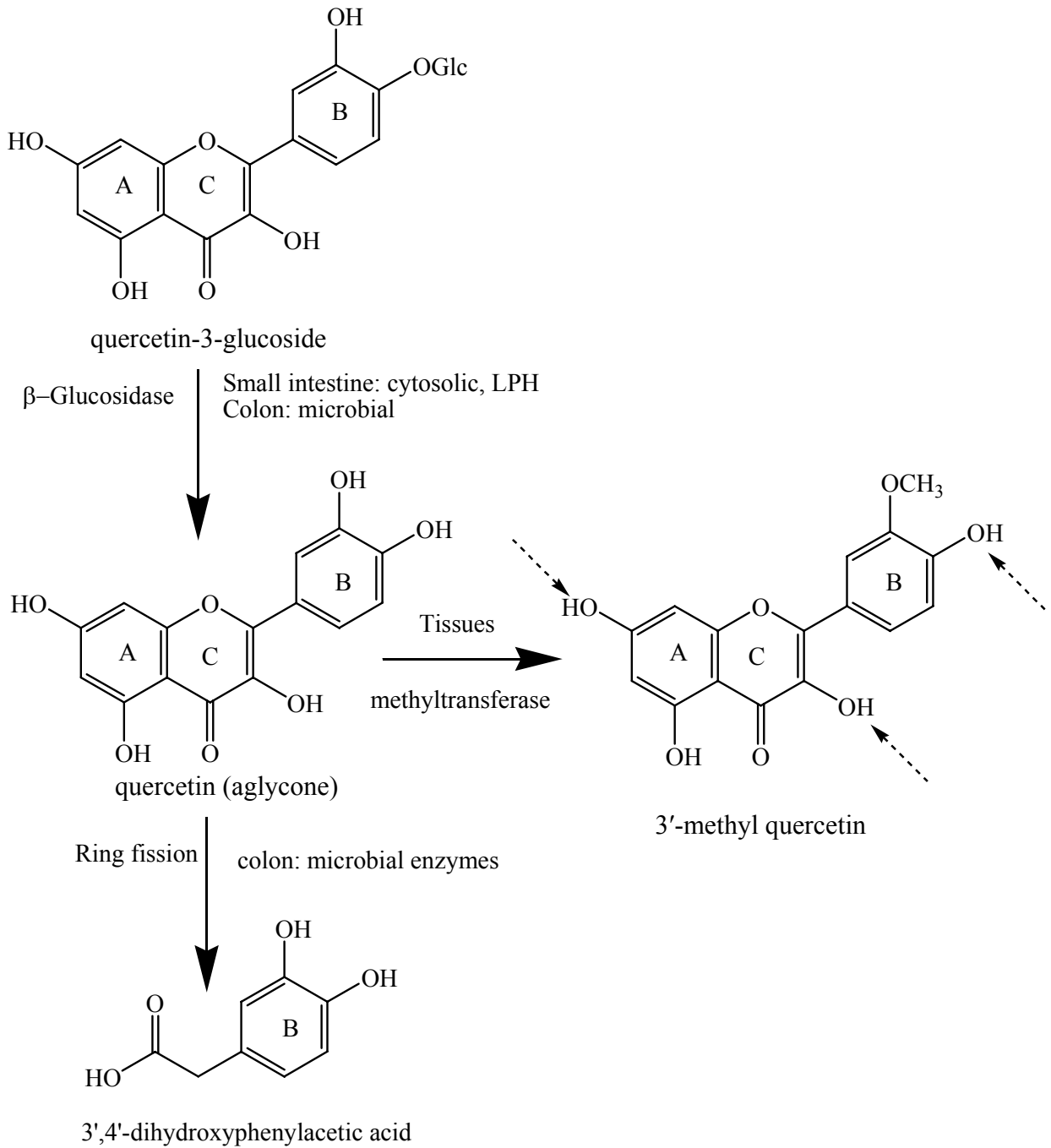
Microbial metabolites are absorbed from the colon after deconjugation and transformation by human cell enzymes into phase II conjugates including methyl ether glucuronides and sulfates metabolized in the liver, resulting in their glucuronidated and sulfated derivatives (Yeh and Yen 2003; Yeh and Yen, 2006; Rechner and Kuhnle, 2002). Through enterohepatic recirculation, conjugated compounds are excreted by the liver as components of bile into the intestine and the deconjugated compounds are regenerated by microbial enzymes before being reabsorbed.

The metabolic pathway of quercetin is summarized in **Figure 2.11** (Williamson *et al.*, 2000).

### **2.7.1 Bioavailability and Metabolism of HMW Phenolic Compounds**

During small intestine digestion, high-molecular-weight PACs can form complexes with protein, starch, and digestive enzymes including pectinase, amylase, lipase, protease and  $\beta$ -galactosidase (Chung *et al.*, 1998), resulting in the formation of less digestible complexes with digestive enzymes. Four types of linkages (*i.e.*, hydrogen bonding, hydrophobic interactions, electrostatic, and covalent bonding) are found in PAC-protein complexes, which are less soluble and less accessible to enzymes (Goel *et al.*, 2005). Phenylacetic, phenylpropionic, and phenylbutyric

**Figure 2.11:** Metabolic pathway of quercetin. Dotted arrows indicate the position of sulfate and/or glucuronide conjugation (Adapted from Williamson *et al.*, 2000). Abbreviations are as follows: Glc, glucose; and LPH, lactase phloridzin hydrolase.



acids are the main metabolites produced from PAC metabolism by gut microflora (Déprez *et al.*, 2000).

Regarding hydrolyzable tannin bioavailability from the small intestine, few studies have evaluated the rate of hydrolysis into monomers (ellagic acid or gallic acid) during enzymatic digestion in the stomach and small intestine. It has been reported that hydrolyzable tannins are degraded to gallic acid, pyrogallol, phloroglucinol, and finally, to acetate and butyrate via the sequential actions of different bacterial enzymes (Whitley *et al.*, 2003). GTs are easily degraded by bacteria, fungi, and yeast, while the galloyl residues of galloyl esters in ETs can be only hydrolyzed by microbes. Tannase (tannin acyl hydrolase, EC 3.1.1.20), produced by a group of microorganisms such as fungi, yeast, and bacteria, is active in galloyl residues of galloyls esters, as well as on hexahydroxydiphenoyl and other ETs. It has both esterase and depsidase activities resulting in hydrolysis of ester and depside bonds in GTs, releasing glucose and gallic acid. Unlike PACs, colonic bacteria have been identified that are capable of metabolizing hydrolyzable tannins. Lactobacilli with tannase activity have been isolated from human feces (Osawa *et al.*, 2000). The presence of lactobacilli with distinct tannase activity suggests that gallic acid from GTs may be available during colonic fermentation.

## **2.8 Phenolic Compounds in Blackberries**

Blackberries are rich sources of anthocyanins and other phenolic compounds. The phenolic content of blackberries (*Rubus* spp.) has been reported to range from 383 to 844 mg gallic acid equivalents/100 g fresh weight (Fukumoto and Mazza, 2000; Moyer *et al.*, 2002; Sellappan *et al.*, 2002; Siriwoharn and Wrolstad, 2004). Anthocyanins are the major phenolic class in this berry, with concentrations reported from 75 to 225 mg cyanidin-3-*O*-glucoside

(C3G)/100 g fresh weight (Siriwoharn and Wrolstad, 2004; Fan-Chiang and Wrolstad, 2005); C3G is the major anthocyanin ranging from 45-95%. There are a number of distinguishable anthocyanins in blackberries and include C3G, cyanidin-3-*O*-arabinoside, cyanidin-3-*O*-rutinoside, cyanidin-3-*O*-xyloside, pelargonodin-3-*O*-glucoside, cyanidin 3-(6'-malonyl)-glucoside, and cyanidin-3-*O*-dioxaloylglucoside (Wu and Prior, 2005). Siriwoharn *et al.*, (2004) reported quercetin glycoside as the primary flavonol. Catechin- and epicatechin-based PACs and ellagic acid derivatives dominant the phenolic constituent in blackberry seeds. The presence of quercetin and kaempferol glycosides have also been reported by previous researchers in blackberries (Wald *et al.*, 1986; Gu *et al.*, 2004; Mertz *et al.*, 2007).

Major phenolic compounds in high molecular weight fractions from berries are hydrolyzable tannins (GTs and ETs) with PACs present in lesser amounts (Seeram, *et al.*, 2006). ETs are a major class of phenolics largely responsible for the astringent and antioxidant properties of blackberries. The *Rubus* ETs are mostly oligomeric. Although ETs are present in all parts of the fruit (seeds, torus, and flesh), they are most abundant in seeds. The primary unit of an ET is the bis-HHDP glucopyranose, commonly known as pedunculagin [**Figure 2.9(E)**]. *Rubus* species oligomeric ETs contain ellagic acid and gallic acid moieties, the sanguisorbolyl linking ester group. When exposed to acids or bases, ester bonds are hydrolyzed and the HHDP spontaneously cyclizes into ellagic acid. The ETs of *Rubus* berries and leaves are a complex mixture containing both monomeric and oligomeric ETs. The monomers consist of ellagic acid glycosides (Zafrilla *et al.*, 2001) and relatively simple ETs such as galloyl-bis-HHDP-glucosides which have been isolated both as  $\alpha$ - and  $\beta$ -glucopyranoside derivatives (Gupta *et al.*, 1982). The oligomeric ETs, so far characterized, are the dimers sanguiin H-6, sanguiin H-10 (lambertianins A), lambertianins B, the trimer lambertianin C, and the tetramer lambertianin D

with a molecular weight of 3,740 g (Mullen *et al.*, 2003). Earlier, Sanguin H-10 was reported only in *Sanguisorba officinalis* (Tanaka, *et al.*, 1985). Such oligomers are formed as a second phase in oxidative metabolism by intermolecular C-O oxidative bonding between the galloyl and the hexahydroxydiphenoyl moieties of two galloyl-bis-HHDP-glucoside units (Gupta, *et al.*, 1982). In the case of the *Rubus* and *Sanguisorba* species, this type of reaction produces the sanguisorbol linking ester group (Gupta *et al.*, 1982; Tanaka *et al.*, 1993; Haslam *et al.*, 1998).

## **2.9 Extraction of Phenolic Compounds**

Phenolic compounds are not uniformly distributed in plant tissue. Insoluble phenolic compounds are mainly deposited in the cell wall as lignin and simple molecules (flavonoids and ferulic esters). Soluble phenolic compounds are stored in the vacuoles of plant cells (Bengoechea *et al.*, 1997; Robbins, 2003) and extraction is the primary step to recover and isolate these compounds, from plant materials, before analysis.

Various factors influence extraction of these compounds from the plant tissue such as the extraction method employed, chemical nature of phenolic compounds, and presence of interfering substances (Naczk and Shahidi, 2004).

The chemical nature of plant phenolics vary from simple to highly polymerized substances that include varying proportions of phenolic acids, phenylpropanoids, anthocyanins, and tannins. They may also exist as conjugates with carbohydrates, proteins, and other non-phenolic components (waxes, fats, terpenes, and chlorophylls) (Robbins, 2003). Therefore, phenolic extracts of plant materials are always a mixture of different classes of phenolics and non-phenolic components (Naczk and Shahidi, 2004).

The first step of extraction is the preparation of slurry by milling/grinding/homogenization of freeze-dried or frozen plant material with a suitable solvent system in a blender or homogenizer (Stilkas, 2007). Ultrasonics have also been applied to facilitate the extraction of phenolic compounds from plant matrices (Vinatoru *et al.*, 1997).

The solubility of phenolic compounds is dependent on the polarity of the solvent used, the degree of phenolic polymerization, the interaction of the phenolics with other food constituents and the formation of insoluble complexes (Naczk and Shahidi, 2004). Commonly used solvents for phenolic compound extractions include aqueous solutions of acetone (50-80% v/v), ethanol (95% v/v), ethyl acetate or methanol (Naczk and Shahidi, 2004; Stalikas, 2007). These solvent systems along with appropriate extraction conditions destroy cell membranes and simultaneously dissolve phenolic compounds (Naczk and Shahidi, 2004). Aqueous methanol solutions are the most commonly employed solvents for extracting phenolic compounds, particularly phenolic acids and flavonoids from fruit and vegetable material (Merken and Beecher, 2000). Phenolic compounds tend to be quite stable in methanol. Tannins are typically extracted with 70% (v/v) aqueous acetone (Naczk and Shahidi, 2006).

The extraction of phenolic compounds from plant material is also influenced by the ratio of solvent-to-sample, for example, Naczk *et al.*, (1992) found that changing the solvent-to-sample ratio from 5:1 to 10:1 increased the extraction of total phenolics from 773 to 805 mg/100 g of canola meal.

In plant material, extraction times also affect the recovery; longer extraction times increase the chance of phenolic oxidation unless reducing agents are added to the solvent system (Naczk and Shahidi, 2004).



Anthocyanins are usually extracted from plant material with an acidified organic solvent, most commonly methanol. Typical acidulants include acetic acid, citric acid, formic acid, hydrochloric acid, and trifluoroacetic acid (Wrolstad, 1993; Durst and Wrolstad, 2005). The concentration of acidic extracts of anthocyanins before purification may cause losses of labile acyl and sugar residues. In order to avoid this detrimental effect, weak organic acids such as formic or acetic acid are used for the extraction (Moore *et al.*, 1982; Antolovich *et al.*, 2000).

After extraction of soluble phenolic compounds, insoluble phenolic compounds remain in the plant matrices. These compounds are bound to insoluble carbohydrates and proteins within the plant matrix and lignins of the cell wall. Saponification, prior to extraction, is employed to cleave the ester linkage to the cell walls (Robins, 2003) using less polar solvents (dichloromethane, chloroform, hexane, benzene). These solvents are suitable for the extraction of nonpolar extraneous compounds (waxes, oils, sterols, chlorophyll) from the plant matrix.

## **2.10 Separation and Purification of Phenolic Compounds**

### **2.10.1 Chromatography**

The development of chemistry in this century, and especially the rapid development in the chemistry of natural products after WWII, is directly connected with the utilization of chromatographic separation methods. The techniques of liquid chromatography, which are now considered to be classical, played important roles in the separation and purification of phenolic compounds (Merken and Beecher, 2000; Robbins, 2003; Shahidi and Naczki, 2004). Techniques employed included paper chromatography (Harborne, 1967), thin-layer chromatography (TLC) (Harborne, 1967; Anderson and Francis, 1985), open-tubular column chromatography

(Anderson, 1988), solid-phase extraction (SPE) (Hong and Wrolstad, 1990), and high performance liquid chromatography (HPLC) (Merken and Beecher, 2000).

#### **2.10.1.1 Classical Column Chromatography**

Classical column chromatography (CCC) has been an important tool in isolating natural products. CCC is a glass tube filled with either a dry or wet stationary phase, whereby, phenolic extracts are typically loaded onto the top of an adsorbent column (the stationary phase) and components are eluted by varying the polarity of the solvent (mobile phase) (Takeoka and Dao, 2002). The stationary phase, or adsorbant packing, is usually finely ground powders or gels. Three packings, widely used for the fractionation and isolation of phenolics are: Amberlite XAD-7 (Anderson, 1988; Kraemer-Schafhalter *et al.*, 1998), Amberlite XAD-16 (Mazza *et al.*, 2004), and Sephadex LH-20 (Amarowicz and Shahidi, 1994; Kantz and Singleton, 1990).

#### **2.10.1.2 Solid-Phase Extraction (SPE)**

Solid-phase extraction is a commonly used sample preparation technique to remove all the non-phenolic substances such as sugars and organic acids. Small, disposable C<sub>18</sub> cartridges are the preferred method for sample cleanup and fractionation of phenolic acids and flavonoids (Kim and Lee, 2005). Typically, sample components (phenolic compounds) to be determined or isolated are retained quantitatively on the SPE cartridge while interfering components (sugars and organic acids) are washed from the cartridge using water as mobile phase. Finally the retained sample is eluted from the cartridge using a suitable solvent system (Takeoka and Dao, 2002).

### **2.10.1.3 Semi-Preparative High Performance Liquid Chromatography (HPLC)**

Further separation and purification of phenolic compounds is carried out on a preparative or semi-preparative HPLC (Amarowicz and Shahidi 1996; Bunzel *et al.*, 2004) column prior to analysis and identification.

### **2.10.1.4 Hydrolysis**

Some sample preparation methods of phenolic extracts include acid, alkaline or enzymatic hydrolysis to cleave acyl and glycosyl moieties from the phenolic compounds (Kraushofer and Sontag, 2002; Vrhovsek *et al.*, 2006). Vigorous acid or alkaline hydrolysis may degrade the most unstable aglycones, while some of the glycosides may not be hydrolyzed completely yielding inconsistent results (Tolonen and Uusitalo, 2004). For example, Hertog *et al.*, (1992) reported that even under optimized hydrolysis conditions, the true phenolic compound content in foods may be underestimated by up to 50%. Hydrolysis simplifies the identification of phenolic compounds and provides important information concerning the total amount of individual aglycones in foods. It does not reflect the authentic structure of phenolic glycosides consumed in the diet.

## **2.11 Analysis of Phenolic Compounds**

### **2.11.1 Spectroscopy**

A number of spectrophotometric methods for phenolic compound quantification in plant materials have been developed. Based on different principles, these assays are used to determine various structural groups present in phenolic compounds (Goldstein and Swain, 1963; Price and Butler, 1977; Slinkard and Singleton, 1977; Porter *et al.*, 1986; Brava and Mateos, 2008). These

include the classical Folin-Ciocalteu method by Singleton and Rossi (1965) for total phenolic content (TPC), pH differential method for anthocyanins (Giusti and Wrolstad, 2001), vanillin method for PACs (Sun *et al.*, 1998), and protein precipitation method (Hagerman and Butler, 1978) for PACs and hydrolyzable tannins.

### **2.11.2 High Performance Liquid Chromatography (HPLC)**

The most widely used technique for the isolation and identification of phenolic compounds is HPLC (Merken and Beecher, 2000; Määttä *et al.*, 2003; Robbins, 2003). After purification of phenolic extracts using various techniques discussed in **Section 2.9**, samples may be loaded onto an analytical HPLC column.

There are numerous reviews published on the application of HPLC methodology for the analysis of phenolic compounds (Merken and Beecher, 2000; Robbins, 2003). HPLC columns are typically reversed phase (RP), ranging from 100 to 300 mm in length and 4.6 mm in diameter. Elution systems are usually binary, with an aqueous acidified polar solvent such as aqueous acetic, formic, or phosphoric acid (solvent A), and a less polar organic solvent such as methanol or acetonitrile (solvent B) (Merken and Beecher, 2000). Thermostatically controlled columns are normally held at ambient or slightly above ambient temperatures and injections generally range from 1 to 100  $\mu\text{L}$  (Merken and Beecher, 2000; Robbins, 2003).

Phenolic acids and flavonoids show characteristic UV range absorbance patterns from 190 to 380 nm (Merken and Beecher, 2000; Robbins, 2003). Anthocyanin pigments have intense absorption in the visible range with maximum absorbance at 510 to 525 nm (Williams and Grayer, 2004). Because of these strong UV/VIS absorption properties, phenolic compounds are most commonly detected with a photodiode array (DAD) detector (Merken and Beecher, 2000).

Structural characteristics of the flavonoids can be determined from the UV/VIS spectra and two absorption bands, referred to as Band I and Band II (Merken and Beecher, 2000; Kim and Lee, 2005). Band I, with a maximum absorption in the range of 300 to 550 nm, arises from the B-ring (Sivam, 2002). Band II, with a maximum absorption range of 240 to 285 nm, is believed to arise from the A-ring of flavonoids.

UV spectra of flavones and flavonols have a Band II peak around 240 to 280 nm and a Band I peak around 300 to 380 nm (Mabry *et al.*, 1970). Due to the fact that there is little or no conjugation between the A- and B-rings, UV spectra of flavanones and isoflavones usually have an intense B and II peak and a small Band I peak (Mabry *et al.*, 1970); similarly, the lack of conjugation in flavanols result in the absence of Band I peaks in the spectra of these compounds.

Anthocyanins show Band II and Band I absorption maxima in the 265 to 275 and 465 to 560 nm regions, respectively (Robards and Antolovich, 1997). Structural properties of the anthocyanins can be obtained from spectral data including the nature of their aglycone (anthocyanidin), the position of attachment of the sugar molecule, and acylation by phenolic acids (Mabry *et al.*, 1970; Brouillard, 1982). The presence of glycosidic substituents can be differentiated by reversed phase HPLC retention characteristics. The nature of carbohydrate substitution has no effect on the anthocyanin absorbance spectrum (Harborne, 1967; Williams and Grayer, 2004).

Phenolic acids with the benzoic acid structure have their maximum absorbance wavelength ( $\lambda_{\text{max}}$ ) in the 200 to 290 nm range. The cinnamic acid derivatives, due to additional conjugation, show an additional broad absorbance band from 270 to 360 nm (Robbins, 2003).

Based on the UV/VIS absorbance spectra and retention times ( $t_r$ ), the phenolic class and flavonoid sub-class can be identified for each chromatographic peak separated and detected by

HPLC-DAD (Merken and Beecher, 2000; Robbins, 2003); Identified compounds are quantified with reference standards. **Figure 2.12(A)** and **(B)** present the absorption spectra of phenolic acids, anthocyanins, and ETs (Määttä *et al.*, 2004).

### 2.11.3 Mass Spectrometry (MS)

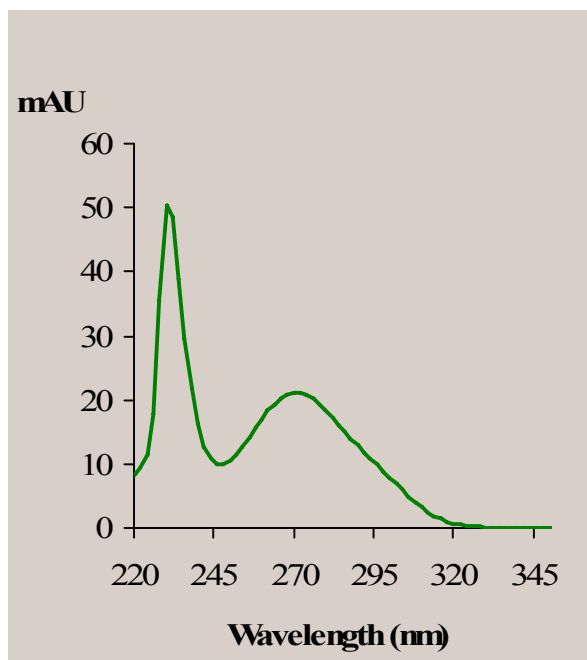
Much research has been devoted to the structural elucidation of flavonoids by mass spectrometry (Stobiecki, 2000; Cuyckens and Claeys, 2004). Several groups have reported flavonoid identification by electron ionization (EI) (Giusti *et al.*, 1999; Cuyckens and Claeys, 2004) or fast atom bombardment (FAB) mass spectrometry (Ma *et al.*, 2001; Takanyama *et al.*, 2005). More recently, electrospray ionization (ESI) (Maatta *et al.*, 2003; Tsao *et al.*, 2003) and matrix-assisted laser desorption ionization (MALDI) (Wang and Sporns, 1999) have been used to analyze flavonoids. Giusti *et al.*, (1999) applied electrospray and tandem mass spectrometry for anthocyanins characterization. Mertz *et al.*, (2007) used ESI-MS for characterization of hydrolyzable tannins in Mexican and South American blackberries. **Figure 2.12** presents absorption spectra of phenolic compounds present in blackberries: (A) gallic acid, (B) galloyl esters, (C) ellagic acid and its derivative, (D) ellagitannins, and (E) cyanidin-3-*O*-glucoside.

## 2.12 Phenolic Compounds and Human Health

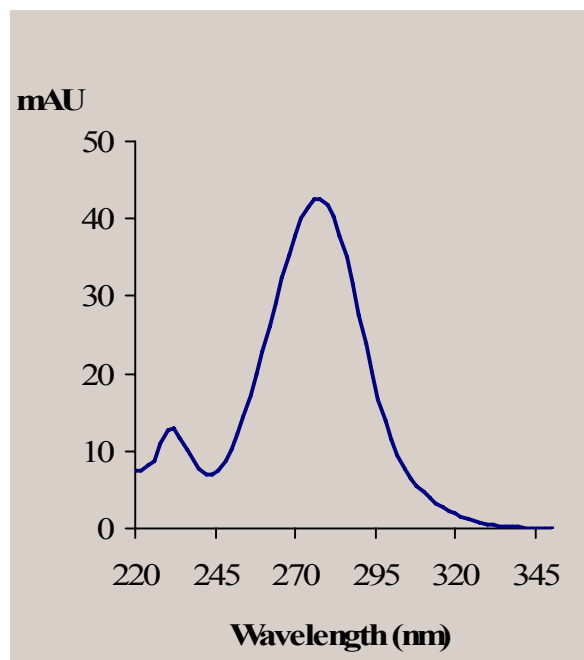
Epidemiological studies have shown that the increased intake of polyphenols is associated with a reduced risk of development of aging and degenerative diseases such as atherosclerosis, cardiovascular diseases, cancer, diabetes, inflammation, and neurodegenerative disorders (Yang *et al.*, 2001; Dashwood, 2007; Rahman, 2008; Davis *et al.*, 2007; Visioli and Hagen, 2007; Banini *et al.*, 2006).

**Figure 2.12:** UV/VIS absorption spectra of selected phenolic compounds present in blackberry. **(A)** Gallic acid; **(B)** Galloyl esters; **(C)** Ellagic acid and its derivative; **(D)** Ellagitannins; and **(F)** Cyanidin-3-*O*-glucoside.

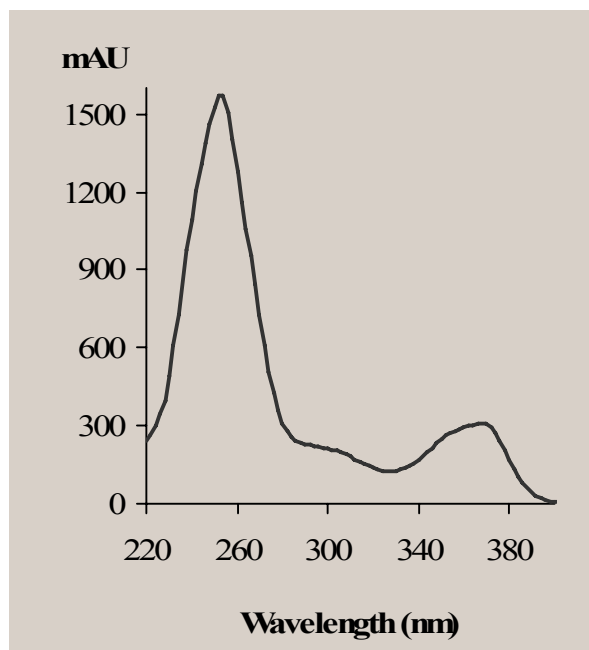
(A)



(B)

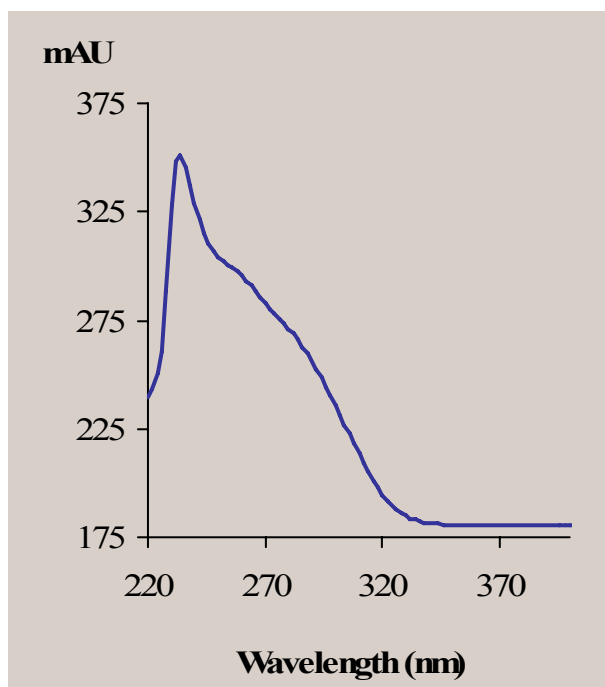


(C)

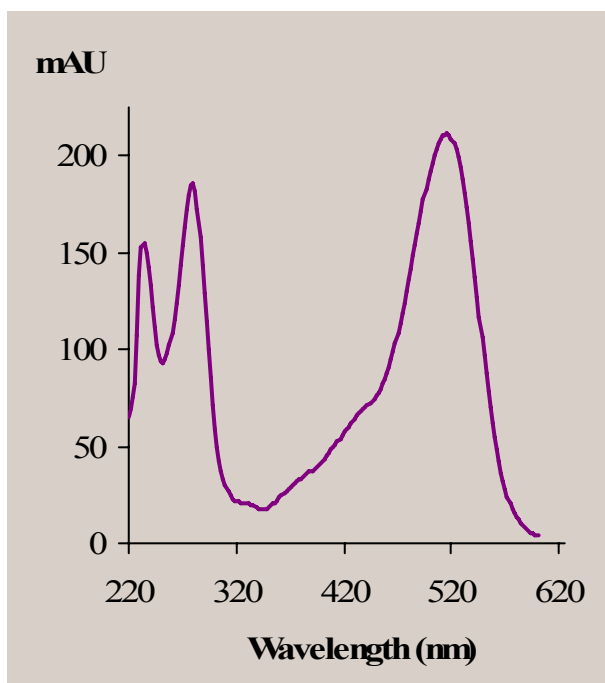




(D)



(E)



Most of these biological actions have been attributed to the antioxidant capacities of phenolic compounds such as direct scavenging of or detoxification of reactive oxygen species (ROS), blocking ROS production, and sequestration of transition-metal ions. ROS formation in the body is discussed in **Section 2.13** and the proposed antioxidant mechanism of phenolic compounds is discussed in **Section 2.14**. Antioxidant capacities of phenolic compounds may also offer indirect protection: (1) by activating endogenous defense systems such as glutathione peroxidase (GSHPx), superoxide dismutase (SOD), catalase (CAT) or glutathione reductase (GR) (Collins, 2005), and (2) by modulating cellular signaling processes such as DNA binding, glutathione biosynthesis, and activation of nuclear factor-kappa B (NF- $\kappa$ B), activator protein-1(AP-1), protein kinase (PK), and mitogen-activated protein kinase (MAPK) proteins [extracellular signal-regulated protein kinase (ERK), c-jun N-terminal kinase (JNK) and P38] (Packer, 2005; Han *et al.*, 2007; Hayes and McLellan, 1999; Masella *et al.*, 2005).

Activation of cytoplasmic PKs and the MAPK play a major role in modulating many transcription factor activities (Karin, 1995). MAPKs are important cellular signaling components that convert various extracellular signals into intracellular responses through serial phosphorylation cascades (Cobb and Goldsmith, 1995). Three structurally related but biochemically and functionally distinct MAPK signal transduction pathways have been identified. They are ERK, JNK, and p38 (Su and Karin, 1996; Kyriakis and Avruch, 1996). The ERKs are activated by mitogens, whereas the JNKs and p38 MAPKs are activated by environmental stress, UV light, osmotic shock, and inflammatory cytokines (Davis, 2000). Activation of JNK and p38 MAPK phosphorylate, and activate c-Jun proteins which in turn lead to the activation of AP-1; a transcription factor (Chang and Karin, 2001) that has been shown to play a critical role in inflammatory responses and tumorigenesis (Hasselblatt *et al.*, 2008).

Several investigators have reported that phenolic compounds, including anthocyanins, can protect the cells from damage via protecting DNA from oxidation (Galvano *et al.*, 2004; Meiers *et al.*, 2001). Curcumin, quercetin, and grape skin procyanidin extract increased several antioxidant enzyme activities such as GSHPx, SOD, CAT, and GRed *in vivo* and *in vitro* assays (Alía *et al.*, 2006; Molina *et al.*, 2003; Shen *et al.*, 2007; Yousef *et al.*, 2009; Valerio *et al.*, 2001; Nishinaka, *et al.*, 2007). These enzymes are critical cellular defenses involved in the detoxification of  $O_2^{\bullet-}$  and  $H_2O_2$ . Paraoxonase-1 is another antioxidative enzyme that functions in preventing the formation of oxidized lipoproteins (Shih *et al.*, 1998). Quercetin decreased lipid peroxidation, upregulated the expression of serum high density lipoprotein (HDL)-associated paraoxonase 1(PON-1) in the HuH7 human hepatoma cell line (Gouedard *et al.*, 2004) and increased intracellular glutathione (GSH) in COS-1 cells (Myhrstad *et al.*, 2002). Lee *et al.*, 2009 suggested that *p*-coumaric acid prevented lipid peroxidation and cell death under oxidative stress due to high levels of glucose and arachidonic acid in bovine aortic endothelial cell lines. Du *et al.*, (2007) confirmed induction of SOD, catalase, and GSH in cultured cardiomyocytes by catechin and PAC B<sub>4</sub> isolated from grape seed extract (GSE). The cardiovascular protective effects of GSE are believed to be ascribed to its antioxidative properties.

Several studies have shown that polyphenols exerted anti-atherosclerosis activities and conferred cardioprotection. Karthikeyan *et al.*, (2007) have reported the beneficial effects of grape skin procyanidin extract in cardioprotection against isoproterenol-induced myocardial injury in rats. Proanthocyanidins could significantly reduce cardiomyocyte apoptosis by inhibiting ischemia/reperfusion-induced activation of JNK-1 and c-Jun in male Sprague Dawley rats (Dasgupta and Milbrandt, 2007). Resveratrol showed decreases in the expression of the vascular cell adhesion molecule-1 (VCAM-1) (Carluccio *et al.*, 2003), cyclooxygenase-2 (COX-

2) (Subbaramaiah *et al.*, 1998), and matrix metalloproteinase-9 (MMP-9) mRNA (Li *et al.*, 2003) through suppression activation of the nuclear factor AP-1 (Subbaramaiah *et al.*, 1998).

Recently, there has been considerable interest in the neuroprotective effects of phenolic antioxidants (Hartman *et al.*, 2006). Blueberries have been linked to improved cognitive performance as a result of decreased oxidative stress in the brain (Papandreou *et al.*, 2009). Curcumin has been shown to disrupt existing plaques and restore distorted neurites in an Alzheimer mouse model (Garcia-Alloza *et al.*, 2007). Resveratrol had an impact on cognitive deficits by activating the phosphorylation of protein kinase C (PKC), secreting transthyretin to prevent amyloid  $\beta$ -peptide (this peptide has consequently been regarded as the principal toxic factor in the neurodegeneration of Alzheimer's disease), aggregation in cultured rat hippocampal cells (Bastianetto *et al.*, 2007), and stimulating adenosine monophosphate (AMP) kinase activity in Neuro2a cells and primary neurons (Dasgupta and Milbrandt, 2007).

Phenolic compounds can modulate diverse biochemical processes involved in carcinogenesis such as antitumor activities by inhibition of cellular proliferation and angiogenesis, blockade of tumor cell cycle progression, and induction of programmed cell death *in vivo* and *in vitro* (Kunnumakkara *et al.*, 2007; Collett *et al.*, 2004). Cellular signaling cascades mediated by NF- $\kappa$ B or AP-1 play an important role in regulating many of the aforementioned biochemical processes (Kundu *et al.*, 2004; Collett *et al.*, 2004). Delphinidin, cyanidin, and petunidin are reported to inhibit TPA-induced AP-1 transcriptional activity and cell transformation in JB6 cell lines (Hou *et al.*, 2004).

Numerous experimental studies have demonstrated an antidiabetic effect of phenolic compounds. For example, Koboyashi *et al.*, (2000) have shown that the green tea polyphenols EGCG and ECG inhibited glucose transport, possibly by sodium-dependent glucose transporter 1

(SGLT1) inhibition, in the small intestines of rabbits (Koboyashi *et al.*, 2000). Song *et al.*, (2002) presented evidence for quercetin-mediated inhibition of the facilitated diffusion glucose transporter 2 (GLUT2) in Chinese hamster ovary cells. Anthocyanins inhibited  $\alpha$ -glucosidase activity and reduced blood glucose levels after starch-rich meals; this is a proven clinical therapy for controlling type II diabetes (McDougall *et al.*, 2005).

Anti-inflammatory activities of phenolic compounds are discussed later in **Section 2.18.5**. Research, in recent years, has shown oxidative and free-radical-mediated reactions in degenerative processes such as aging and chronic diseases (*e.g.*, cancer, coronary heart disease, and neurogenerative disorders such as Alzheimer's disease) (Puido *et al.*, 2000; Masella *et al.*, 2005; Collins, 2005).

### **2.13 Reactive Oxygen Species and Oxidative Stress**

Reactive oxygen species are referred to as free radicals which are formed by a partial reduction of oxygen during normal metabolism or inflammatory responses yielding highly reactive molecules such as superoxide anion radical ( $O_2^{\bullet-}$ ), hydroxyl ( $\bullet OH$ ), hydroperoxyl ( $HOO\bullet$ ), and peroxy ( $ROO\bullet$ ).

Free radicals can be defined as molecules or molecular fragments containing one or more unpaired electrons in atomic or molecular orbitals (Halliwell, 2006). This unpaired electron(s) usually gives a considerable degree of reactivity to the free radical. Radicals derived from oxygen represent the most important class of radical species generated in living systems (Miller *et al.*, 1990). Molecular oxygen (dioxygen) has a unique electronic configuration which makes it a radical. The addition of one electron to dioxygen forms  $O_2^{\bullet-}$  (Miller *et al.*, 1990). The  $O_2^{\bullet-}$ , arising either through metabolic processes or following oxygen "activation" by physical

irradiation, is considered the “primary” ROS, and can further interact with other molecules to generate the “secondary” ROS. This process occurs either directly or indirectly through enzyme- or metal-catalyzed processes. Various pathways of ROS formation are outlined in **Figure 2.13** (Valko *et al.*, 2007).

ROS are involved in various important functions in our body for example, energy production, phagocytosis, cell growth, and intracellular signaling regulation (Masella *et al.*, 2005; Valko *et al.*, 2007). ROS may also be highly damaging, as they can attack biological macromolecules (lipids, protein, and DNA), induce oxidation, cause membrane damage, enzyme inactivation, and DNA damage (Valko *et al.*, 2004; Valko *et al.*, 2007). Thus, there are “two faces” of free radicals in biology in that they serve as signaling and regulatory molecules at physiologic levels, but also function as highly deleterious and cytotoxic oxidants at pathologic levels (Freidovich, 1999).

Similar to ROS, reactive nitrogen species (RNS) are produced during normal cellular metabolism (Valko *et al.*, 2007). Nitric oxide ( $\text{NO}^\bullet$ ) is the most commonly found RNS that acts as an important oxidative biological signaling molecule in a large variety of physiological processes including neurotransmission, blood pressure regulation, defense mechanisms, smooth muscle relaxations, and immune system regulation (Archer, 1993; Alderton *et al.*, 2001; Bergendi *et al.*, 1999). RNS can either be beneficial or harmful to a living system.

**Figure 2.13:** Pathway of ROS formation, the lipid peroxidation process, and the role of glutathione (GSH) and non-phenolic antioxidants (vitamin A, vitamin C, lipoic acid) in the control of oxidative stress. **Reaction 1:** Formation of  $O_2^{\bullet-}$ ; **Reaction 2:** Formation of  $H_2O_2$  by SOD; **Reaction 3:** Scavenging of  $H_2O_2$  by GSHPx; **Reaction 4:** Reduction of GSH; **Reaction 5:** Fenton reaction; **Reaction 6:** Formation of  $L^{\bullet}$ ; **Reaction 7:** Formation of  $LOO^{\bullet}$ ; **Reaction 8:** Formation of LOOH and  $T-O^{\bullet}$ ; **Reaction 9:** Regeneration of vitamin E by vitamin C and the formation of  $Asc^{\bullet}$ ; **Reaction 10:** Regeneration of vitamin E by GSH; **Reaction 11:** Reduction of GSSG and  $Asc^{\bullet}$  by DHLA and the formation of ALA; **Reaction 12:** Regeneration of DHLA; **Reaction 13:** Reduction of LOOH; **Reaction 14:** Reaction of LOOH with metal ions (*e.g.*,  $Fe^{2+}$ ); **Reaction 15:** Cyclization of  $LO^{\bullet}$ ; **Reaction 16:** Formation of 4-hydroxynonenal; **Reaction 17:** Formation of glutathyl adduct; **Reaction 18:** Cyclization of  $LOO^{\bullet}$  to form an intermediate product for MDA; **Reaction 19:** Formation of MDA; **Reaction 20:** MDA reacts with MDA base (Adapted from Valko *et al.*, 2007). Abbreviations are as follows:  $Asc^{\bullet}$ , ascorbate radical;  $AscH^-$ , ascorbate monoanion;  $G_{Red}$ , glutathione reductase; GSH, glutathione; GSHPx, glutathione peroxidase; GSSG, oxidized glutathione; GST, glutathione-*s*-transferase;  $LO^{\bullet}$ , lipid alkoxy radical;  $LOO^{\bullet}$ , lipid peroxy radical;  $L^{\bullet}$ , lipid radical;  $O_2^{\bullet-}$ , superoxide anion radical; SOD, superoxide dismutase; T-O, Vitamin E; and  $T-O^{\bullet}$ , Vitamin E radical.





The cells of the immune system during the onset of inflammatory processes, (Carr *et al.*, 2000) produce  $O_2^{\bullet-}$  and  $NO^{\bullet}$ : These reactive species react together to produce peroxynitrite ( $ONOO^{\bullet}$ ). Peroxynitrite is a potent oxidant that can cause DNA fragmentation and lipid peroxidation (Carr *et al.*, 2000).

The effects of ROS/NOS causing potential biological damage are termed as oxidative stress/ nitrosative stress (Ridnour *et al.*, 2004). This occurs in biological systems when there is overproduction of ROS/NOS and deficiency of enzymatic and non-enzymatic antioxidants (Valko *et al.*, 2007). These ROS contain an unpaired electron, like  $O_2^{\bullet-}$ ,  $^{\bullet}OH$ ,  $HOO^{\bullet}$ ,  $ROO^{\bullet}$ , alkoxy ( $RO^{\bullet}$ ),  $NO^{\bullet}$ , or  $ONOO^{\bullet}$  (Issa *et al.*, 2006). ROS are electrophilic in nature. They attack the nucleophilic centers in cells, which results in initiation of undesirable events such as lipid peroxidation, protein oxidation and DNA damage.

The human body is equipped with effective endogenous antioxidant defenses against ROS, which include, detoxifying enzymes such as SOD, CAT, GSHPx, and GRed. The defense system also includes high molecular-weight antioxidants such as albumin and ferritin, and low-molecular weight ones such as GSH (Harman, 1995).

In normal physiological conditions, the antioxidant defense system maintains the balance between oxidation and oxidative scavenging. When an imbalance between free radical generation and the body defense mechanisms occurs, oxidative stress evolves yielding oxidative damage to cellular and extra cellular macromolecules (*e.g.*, protein, lipids, and nucleic acids) causing tissue injury (Halliwell and Gutteridge, 1989; Halliwell and Aruoma, 1991; Halliwell and Chirico, 1993; Meydani *et al.*, 1995) and affecting immune function (Meydani *et al.*, 1995; Hughes, 1999).

Free radicals are constantly produced in the body by environmental agents such as UV and ionizing radiations. Secondly, from by-products of normal cellular metabolism that include ROS (superoxide anions, hydroxyl radicals, and hydrogen peroxide) derived from oxidative respiration and products of lipid peroxidation (Fang *et al.*, 2002).

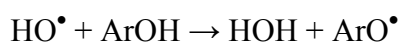
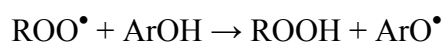
Oxidative stress has been implicated in various pathological conditions involving cardiovascular disease, cancer, neurological disorders, diabetes, ischemia/reperfusion, and aging (Dalle-Donne *et al.*, 2006; Dhalla *et al.*, 2000; Jenner 2003; Sayre *et al.*, 2001). These diseases fall into two groups: (1) diseases characterized by pro-oxidants shifting the thiol/disulphide redox state and impairing glucose tolerance, often referred to as “mitochondrial oxidative stress” causing conditions such as cancer and diabetes mellitus and (2) diseases characterized by “inflammatory oxidative conditions” and enhanced activity of either NADPH oxidase (leading to atherosclerosis and chronic inflammation) or xanthine oxidase-induced formation of ROS (implicated in ischemia and reperfusion injury). The process of aging is largely due to the damaging consequence of free radical action (lipid peroxidation, DNA damage, and protein oxidation) (Harman, 1956). Convincing evidence for the association of oxidative/ nitrosative stress and acute and chronic diseases relies on validated biomarkers of oxidative stress.

#### **2.14 Phenolic Compounds and Antioxidant mechanism**

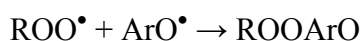
The antioxidant mechanism of phenolic compounds involves scavenging free radicals, chelating transition-metal ions involved in free-radical production, and inhibiting the enzymes participating in free-radical generation (Yang *et al.*, 2001; Aruoma, 2002; Hensley *et al.*, 2004). Free radical scavengers are compounds that are capable of donating electrons or hydrogen atoms to inhibit a free radical reaction (Halliwell *et al.*, 1995).

An antioxidant effect is observed by scavenging free radicals that are involved in slowing or inhibiting the oxidative chain reaction. The free radical scavenging activity of phenolic compounds is generally attributed to their ability to donate a hydrogen atom to reduce ROS radicals (Halliwell *et al.*, 1995). In doing so, the phenolic compounds are converted to oxidized phenoxy radicals (ArO•) that are stable due to resonance-stabilized delocalization of the unpaired electron over the aromatic ring [**Figure 2.14 (A)**] (Pietta, 2000; Aruoma, 2002).

For example, the reduction of peroxy and hydroxyl radicals by phenolic compounds can be represented as follows:



whereby, ArOH represents the phenolic compound and ArO• is the phenoxy radical. Phenoxy radical intermediates are relatively stable therefore, further oxidation reactions are not easily initiated. Non-radical products may also be formed by the coupling of ROS radicals with phenoxy radicals (Halliwell *et al.*, 1995) as follows:



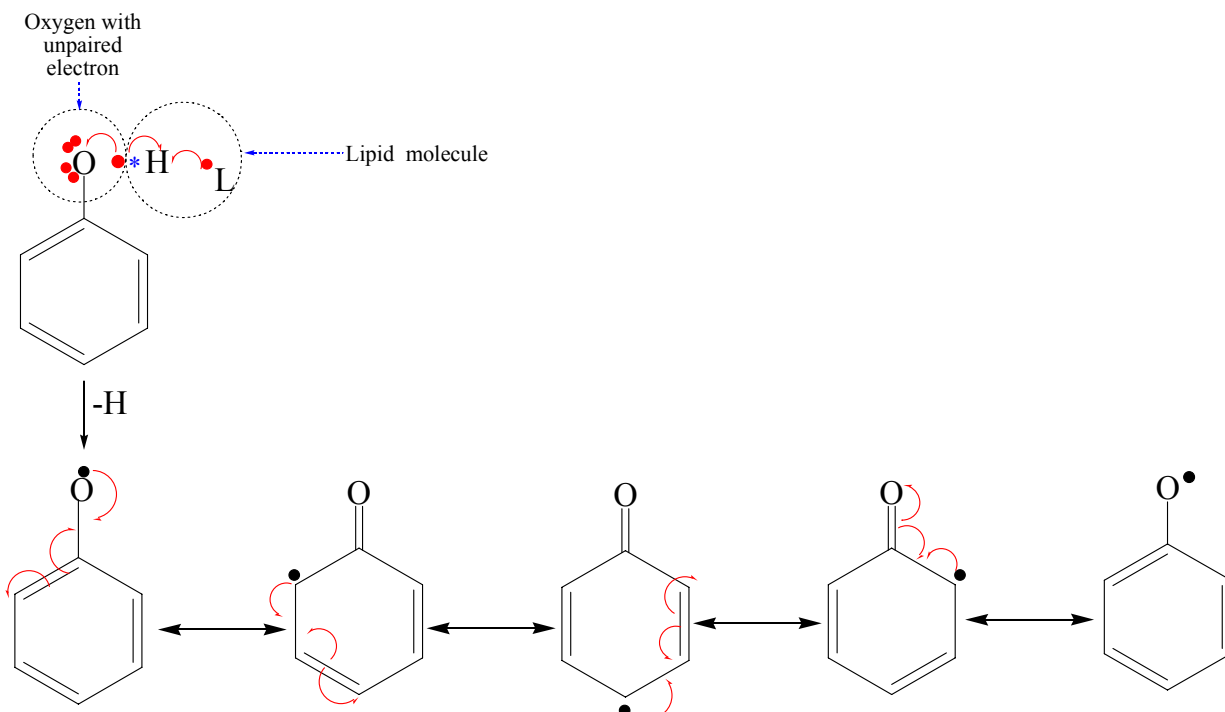
Phenolic compounds may also enhance the antioxidant activity of nonphenolic antioxidants by regenerating the oxidized forms of these compounds. For example, phenolic compounds have been reported to regenerate dehydroascorbic acid to its reduced form (Cossins *et al.*, 1998). Pedrielli and Skibsted (2002) reported synergistic antioxidant interactions between

flavonoids and  $\alpha$ -tocopherol *in vitro*. Their studies included the flavonoids (+)-catechin, (-)-epicatechin, and quercetin. The combination of any one of these flavonoids with  $\alpha$ -tocopherol resulted in longer induction periods and efficient inhibition of oxidation. All experimental results indicated that  $\alpha$ -tocopherol was regenerated by the flavonoid. The suggested mechanism of interaction was by hydrogen atom transfer between the flavonoid and  $\alpha$ -tocopherol. Phenolic compounds may also impart antioxidant properties by functioning as chelators of metal ions that are capable of catalyzing oxidation (Gordon, 1990; Aruoma, 2002). Phenolic acids and flavonoids have been shown to complex with iron (Rice-Evans *et al.*, 1996) and copper ions (Brown *et al.*, 1998) to provide secondary antioxidant effects.

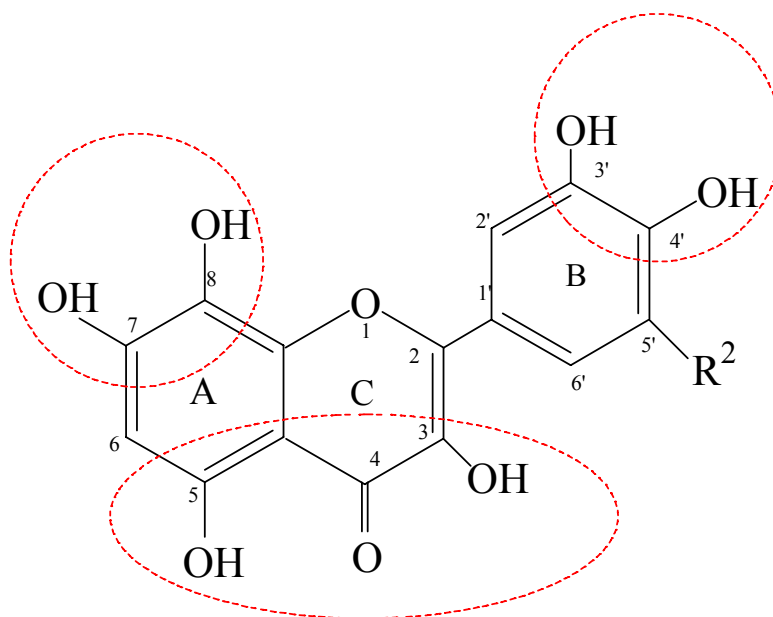
The radical-scavenging activity of flavonoids depends on the molecular structure and the substitution pattern of hydroxyl groups; *i.e.*, the availability of phenolic hydrogens and the possibility of stabilization of the resulting phenoxyl radicals *via* hydrogen bonding or by expanded electron delocalization (Bors *et al.*, 1990; Rice-Evans *et al.*, 1996). Structural requirements considered essential for effective scavenging activity are: (1) the presence of a 3',4'-dihydroxy; *i.e.*, an *O*-dihydroxy group (catechol structure) in the B-ring, possessing electron donating properties and being a radical target, (2) 3-OH moiety of the C-ring (Sichel *et al.*, 1991), (3) C-2=C-3 double bond conjugated with a 4-keto group, which is responsible for electron delocalization from the B-ring, and (4) presence of both 3-OH and 5-OH groups in combination with a 4-carbonyl function and C-2=C-3 double bond (Bors *et al.*, 1990; Rice-Evans *et al.*, 1996; Sichel *et al.*, 1991; Cao *et al.*, 1997; Amić *et al.*, 2003) [Figure 2.14 (B)].

**Figure 2.14:** The role of phenolic compounds as antioxidants. **(A)** Antioxidative mechanism of phenolic compounds; **(B)** Structural criteria that modulate the free-radical scavenging activity of phenolic compounds. Dotted circles indicate the presence of dihydroxy groups in phenolics possessing electron-donating properties.

(A)



(B)



## 2.15 Advanced Glycation Endproducts (AGEs)

AGEs are a complex group of compounds formed via a nonenzymatic reaction between reducing sugars and amine residues on proteins, lipids, or nucleic acids. The major AGEs *in vivo* appear to be formed from highly reactive intermediate carbonyl groups, known as  $\alpha$ -dicarbonyls or oxoaldehydes, including 3-deoxyglucosone, glyoxal, and methylglyoxal (BrownLee, 2001; Thornalley, 1996).

A classical pathway of AGE formation is shown in **Figure 2.15(A)**. Reducing sugars such as glucose react non-enzymatically with amino groups of proteins, lipids, and nucleic acids through a series of reactions and form Schiff bases and Amadori products (Singh *et al.*, 2001). The Amadori products then degrade into  $\alpha$ -dicarbonyl compounds that are more reactive than the parent sugars with respect to their ability to react with amino groups of proteins. These  $\alpha$ -dicarbonyl compounds or  $\alpha$ -ketoaldehydes react with amino groups of proteins to form cross-linked, yellow-brown, fluorescent, insoluble, irreversible compounds, called AGEs (Hayase *et al.*, 1996; Yim *et al.*, 1995; Ahmed 2005).

These compounds include N<sup>E</sup>-carboxymethyllysine (CML), pentosidine, pyrroline, crosslines, glyoxal-lysine dimer (GOLD), and methylglyoxal-lysine dimer (MOLD) (Bierhaus *et al.*, 1998). Pentosidine and CML [**Figure 2.15(B)**] are the best characterized AGEs (Singh *et al.*, 2001). Pentosidine and CML may also serve as biomarkers of oxidative stress resulting from carbohydrate and lipid oxidation reactions (Requena *et al.*, 1996).

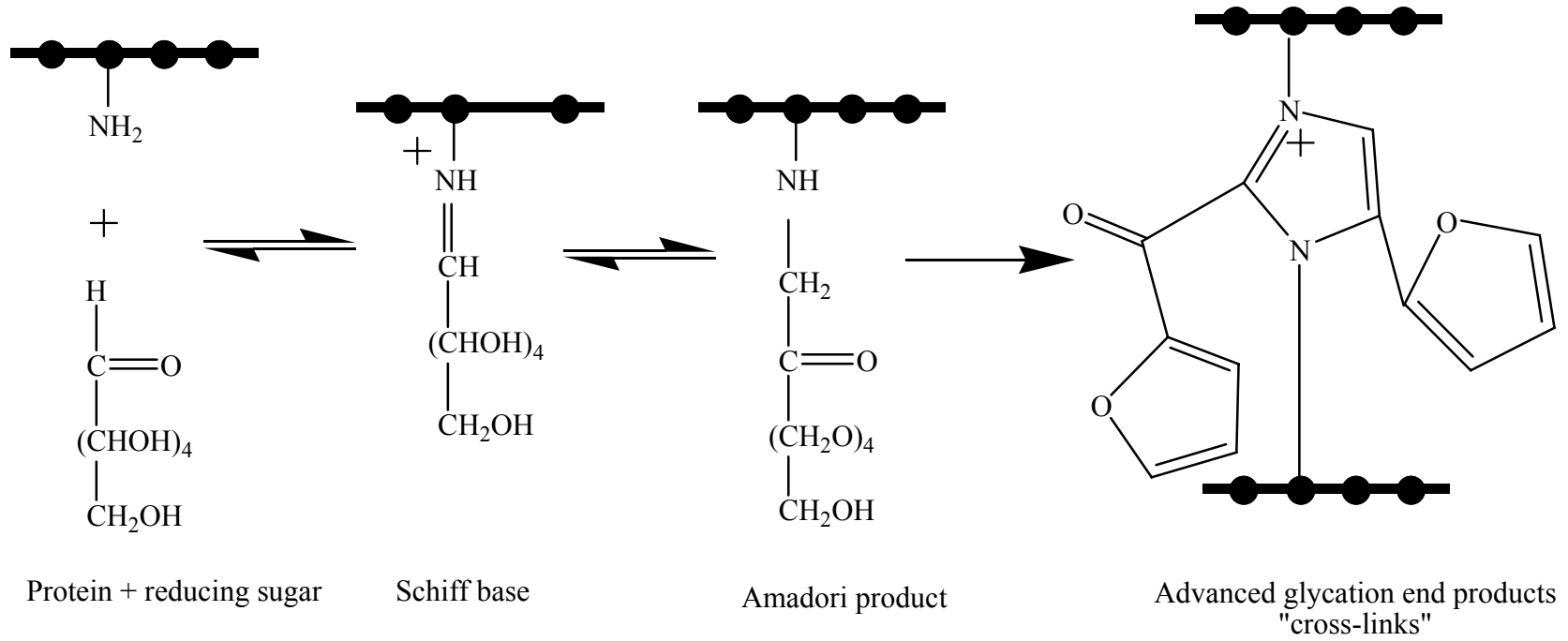
### 2.15.1 AGEs and Health

AGEs have been associated with the presence of increased oxidative damage to tissues (Kim *et al.*, 2003). The accumulation of AGEs in the body leads to structural and functional

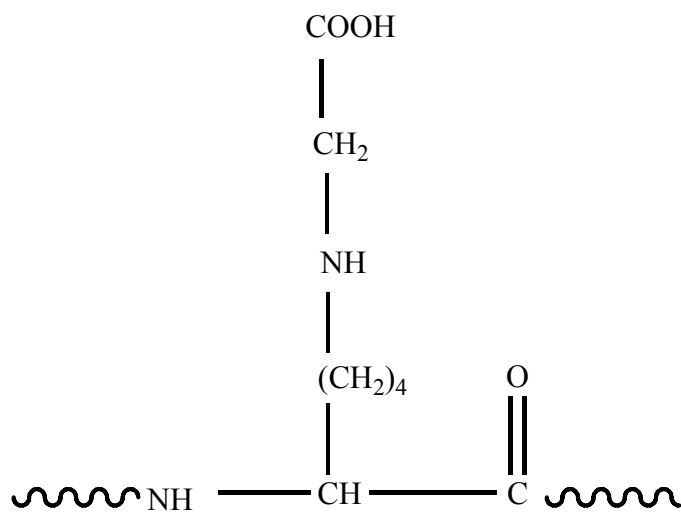
**Figure 2.15:** The formation of advanced glycation endproducts (AGEs) and selected structures. **(A)** A classical AGEs formation pathway involving the Maillard reaction: reducing sugars condense with amino groups of macromolecules to form reversible Schiff base adducts. Intramolecular rearrangements lead to chemically-stabilized Amadori products. These products then give rise to a number of complex reactions (*e.g.*, dehydration, oxidation, cyclization, and scission) leading to the formation of AGEs; and **(B)** Structures of some AGEs. (Adapted from Bierhaus *et al.*, 1998).



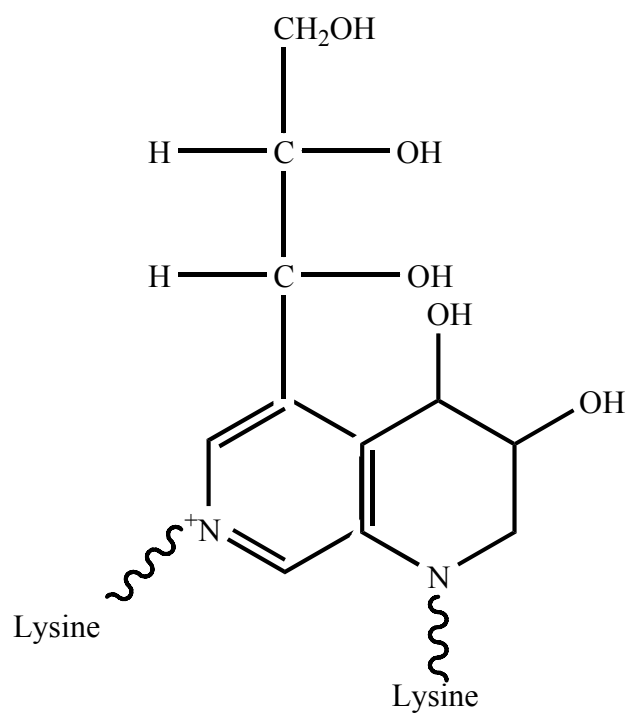
(A)



(B)

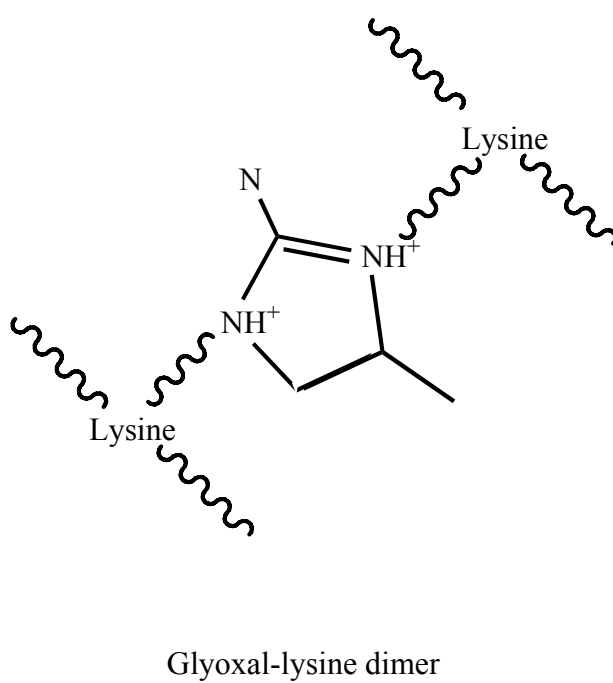
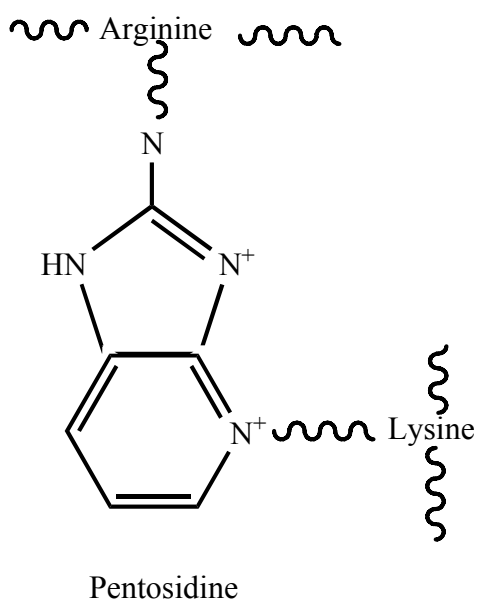


$N^\epsilon$ -(carboxymethyl) lysine



Crossline

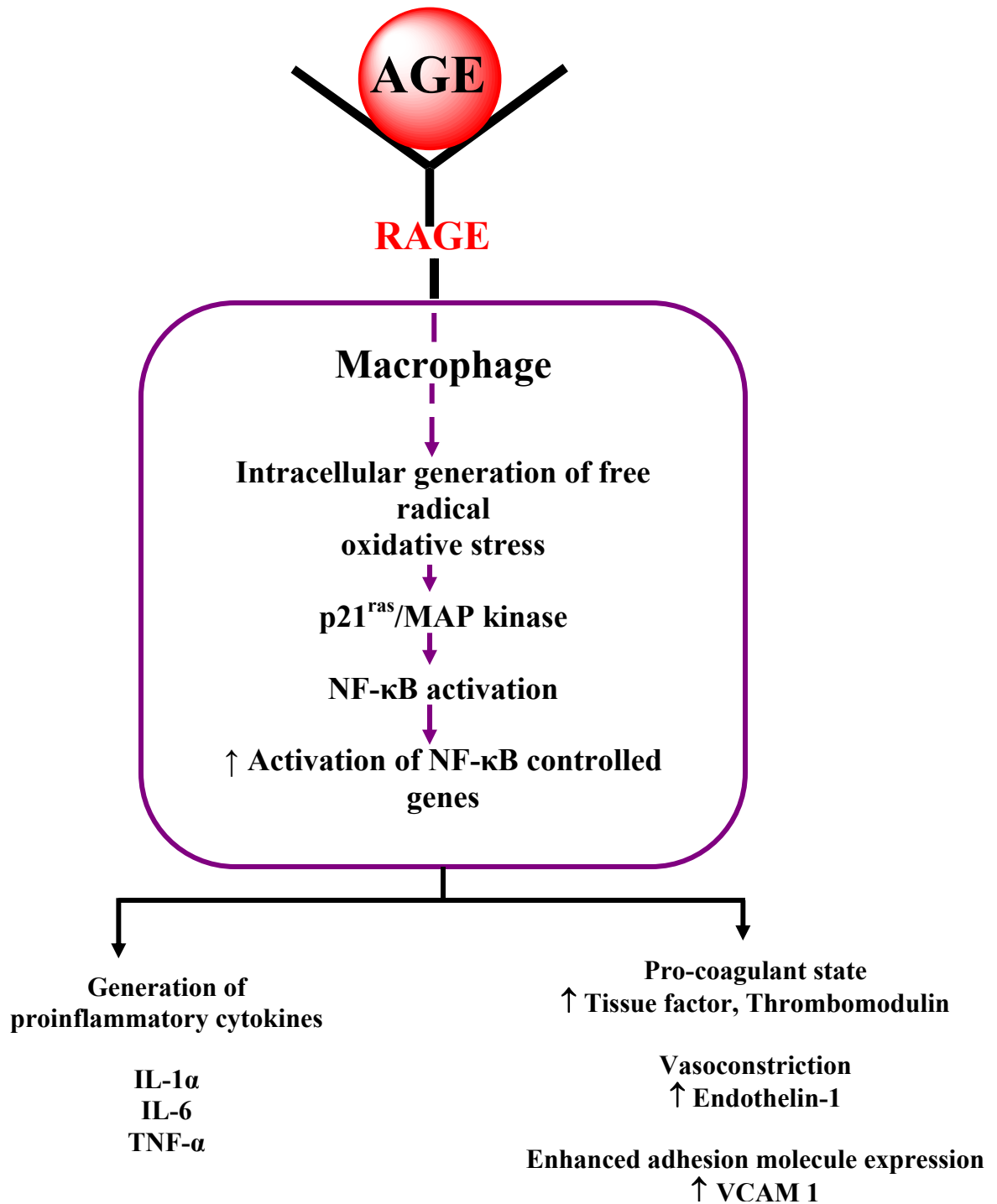
(B) Continued



modifications of tissue proteins. There is emerging evidence that protein glycation is implicated in the aging process, as well as the pathogenesis of the complications due to diabetes (retinopathy, neuropathy, nephropathy, and atherosclerosis) and Alzheimer's disease (Lapolla *et al.*, 2005). AGEs accumulate in most sites associated with diabetic complications including the kidney, retina, and atherosclerotic plaques (Hammes *et al.*, 1999; Makita *et al.*, 1994). Modified proteins, both glycated and oxidized, promote an enhancement of oxidative, pro-inflammatory cascades (Iwashima, 1999).

Several AGE receptors (RAGE), that include macrophage scavenger receptor Types I and Type II, have been identified (Stitt *et al.*, 1997). These AGE receptors are expressed on various cells such as smooth muscle cells, monocytes, macrophages, endothelial cells, etc. Expression of these receptors is increased in diabetic patients (Singh *et al.*, 2001). Binding of AGEs to their receptors (RAGE) (Schmidt *et al.*, 1994) results in depletion of cellular antioxidant defense mechanisms (*e.g.*, glutathione and vitamin C) (Bierhaus *et al.*, 1997) and induces formation of ROS (Sato, 2006). *In vitro* studies have shown that AGE-RAGE binding on macrophages leads to oxidative stress and activation of the transcription factor NF- $\kappa$ B (**Figure 2.16**) (Schmidt *et al.*, 1994). This promotes the expression of NF- $\kappa$ B regulated genes such as the procoagulant tissue factor (Esposito *et al.*, 1989; Bierhaus *et al.*, 1997) and the adhesion molecule VCAM-1 (Schmidt *et al.*, 1995; Vlassara *et al.*, 1995). All of these have been associated with the early stages of atherosclerosis. Therefore, AGEs are not only markers, but also mediators of chronic vascular complications (Bierhaus, 1998)

**Figure 2.16:** The effects of AGEs on oxidative stress (Adapted from Singh *et al.*, 2001). Abbreviations are as follows: AGE, advanced glycation endproducts; IL-6, interleukin-6; IL-1 $\alpha$ , interleukin-1 $\alpha$ ; MAP, mitogen-activated protein; NF- $\kappa$ B, nuclear factor-kappa B; p21<sup>ras</sup>, GTP (guanosine-5'-triphosphate)-binding protein; RAGE, receptor for AGEs; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; and VCAM-1, vascular cell adhesion molecule 1.



### 2.15.2 AGEs and Phenolic Compounds

Oxidative reactions are now known to participate in the process of AGE formation, therefore, antioxidants and/or radical-scavengers may prevent glycation (Fu *et al.*, 1994). There is an abundance of literature examining antioxidants, plant extracts, and specific phenolic compounds inhibiting glycation. Vitamin E, a model antioxidant, inhibits both LDL and albumin glycation (Haidara *et al.*, 2006).

Emerging research in this decade has demonstrated, *in vitro* and *in vivo*, the inhibitory activities of phenolic compounds against protein glycation. For example, flavonoids (such as, quercetin, kaempferol, flavanols, catechin, epicatechin, and procyanidin oligomers) were found to be effective inhibitors of AGE formation (Urios, *et al.*, 2007). Similar results of AGE inhibition were exhibited by phenolics, isolated from *Chrysanthemum* species (Tsuji-Naito, *et al.*, 2009), muscadine grape seed and skin (Farrar, *et al.*, 2007), guava leaves (Wu, *et al.*, 2009) and spices such as cinnamon, (Dearlove, *et al.*, 2008; Saraswat, *et al.*, 2009; Sajithlal, *et al.*, 1998) in a BSA/glucose *in vitro* system.

Several investigators also examined the inhibitory effects of phenolic compounds against AGE formation in animal model studies. For example, studies performed by Sajithlal *et al.*, (1998) revealed that curcumin is a potent inhibitor of AGE formation and crosslinking of collagen, in diabetic rats. Babu *et al.*, (2006), reported that the oral administration of green tea extract impeded the accumulation of aortic collagen, extent of glycation, formation of AGEs and cross-linking of collagen, in diabetic rats. Hanumura and Aoki (2008), reported inhibitory effects of Acerola polyphenols, predominantly PACs and cyanidin-3- $\alpha$ -O-rhamnside, on  $\alpha$ -glucosidase and AGEs production in rats. Therefore, it has been suggested that phenolic

compounds and natural sources, rich in phenolics, might offer a novel source of glycation inhibitory agents.

## 2.16 Determination of Antioxidant Activity

Antioxidants are compounds that, in low concentration, can prevent biomolecules (*e.g.*, protein, nucleic acids, polysaturated lipids, and sugars) from undergoing damage through free-radical mediated reactions (Diplok, 1994). They can inhibit the oxidizing chain in several ways such as direct quenching of ROS, inhibition of enzymes, and chelation of metal ions ( $\text{Fe}^{2+}$ ,  $\text{Cu}^+$ ).

There are two main mechanisms are suggested by which antioxidant components play a protective role. The first one is called hydrogen atom transfer (HAT) whereby, the free radical removes a hydrogen atom from an antioxidant (ArOH) compound and during the process the antioxidant becomes a radical itself. A more stable  $\text{ArO}^\bullet$  radical corresponds to a greater efficiency of the antioxidant ArOH, so that it is unlikely to react with a substrate. The second mechanism is the single-electron transfer (SET) whereby, the antioxidant gives an electron to the free radical becoming, itself, a radical cation species. A radical cation must be sufficiently stable so that it will not react with a substrate (Leopoldini *et al.*, 2004).



Several analytical methods have been developed to measure the efficiency of dietary antioxidants, either as pure compounds or extracts. These methods focus on different mechanisms of the antioxidant defense system such as scavenging of oxygen, hydroxyl radicals,



reduction of lipid peroxy radicals, inhibition of lipid peroxidation, or chelation of metal ions (Puido *et al.*, 2000). Depending upon the reactions, these assays are classified into either HAT or SET. The majority of HAT-based assays apply a competitive reaction scheme, in which the antioxidant and substrate compete for thermally-generated peroxy radicals through the decomposition of azo compounds. These assays include the inhibition of induced low-density lipoprotein autoxidation, ORAC, and total radical trapping antioxidant parameter (TRAP). SET-based assays measure the capacity of an antioxidant in the reduction of an oxidant, which changes color when reduced. The degree of color change is correlated with the samples' antioxidant concentration. SET-based assays include the total phenols assay using Folin-Ciocalteu's phenol reagent, Trolox equivalence antioxidant capacity (TEAC), ferric ion reducing antioxidant power (FRAP), total antioxidant potential assay using a Cu(II) complex as an oxidant, and the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay (Huang *et al.*, 2005). These techniques differ from each other in terms of substrates, probes, reaction conditions, and quantification methods. Selected SET and HAT assays are discussed in this section.

### 2.16.1 Trolox Equivalence Antioxidant Capacity (TEAC)

The TEAC assay measures the antioxidant capacity of a given substance, as compared to the standard, Trolox, a water soluble vitamin E analogue. Briefly in this assay, a radical monocation,  $\text{ABTS}^{\bullet+}$  [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] is generated by oxidation of ABTS by potassium persulfate. The  $\text{ABTS}^{\bullet+}$  is a blue/green chromophore with absorption maxima at 734 nm. In the presence of hydrogen-donating antioxidants such as phenolic compounds  $\text{ABTS}^{\bullet+}$  is reduced to colorless ABTS [**Figure 2.17(A)**] (Huang *et al.*, 2005). The extent of decolorization is measured as the percentage inhibition of the  $\text{ABTS}^{\bullet+}$  and

is determined as a function of the concentration of antioxidant and reaction time. This is calculated relative to the reactivity of Trolox as a standard, under similar conditions (Re *et al.*, 1999).

### **2.16.2 Ferric Reducing Antioxidant Power (FRAP) Assay**

The FRAP assay (Benzie and Strain (1996) is a simple method of determining the reduction of ferric-tripyridyltriazine complex [Fe(III)TPTZ] to its ferrous state in the presence of reducing substances such as phenolic compounds. In this assay colorless [Fe(III)TPTZ] is reduced to its ferrous form which has intense blue color at 593 nm [Figure 2.17(B)]. The change in absorption is directly related to the total reducing power of electron donating substances such as, phenolic compounds in the reaction mixture.

### **2.16.3 Oxygen Radical Absorbance Capacity (ORAC<sub>FL</sub>) Assay**

Cao *et al.*, (1993) developed a method called oxygen radical absorbance capacity (ORAC), which measures antioxidant scavenging activity against the peroxy radical induced by 2,2-azobis(2-amidinopropane) dihydrochloride (AAPH) at 37 °C. In the basic assay, the peroxy radical reacts with a fluorescent probe to form an oxidized nonfluorescent product, which can be quantitated easily by fluorescence. In the presence of antioxidant, the oxidation of fluorescent probe is inhibited and fluorescence is maintained for longer period (Prior *et al.*, 2005).

This assay has the following components: (1) an azo radical initiator, normally AAPH; (2) a molecular probe (fluorescence) for monitoring reaction progress such as, fluorescein (FL; 3',6'-dihydroxyspiro[isobenzofuran-1[3H],9'[9H]-xanthen]-3-one); (3) antioxidant such as

phenolic compounds, and (4) reaction kinetic parameters collected for antioxidant capacity quantitation (Huang *et al.*, 2005).

Briefly, ORAC<sub>FL</sub> assay samples, controls, and standards (five different concentrations of Trolox for the construction of a standard curve) are mixed with a fluorescein solution and incubated at 37 °C before AAPH solution is introduced to initiate the reaction. Fluorescence (FL) intensity [485 nm (ex)/525 nm (em)] is measured every minute for a couple of hours. As the reaction progresses, fluorescein is consumed and the FL decreases. In the presence of an antioxidant, however, the FL decay is inhibited [**Figure 2.17(C)**] (Prior *et al.*, 2005).

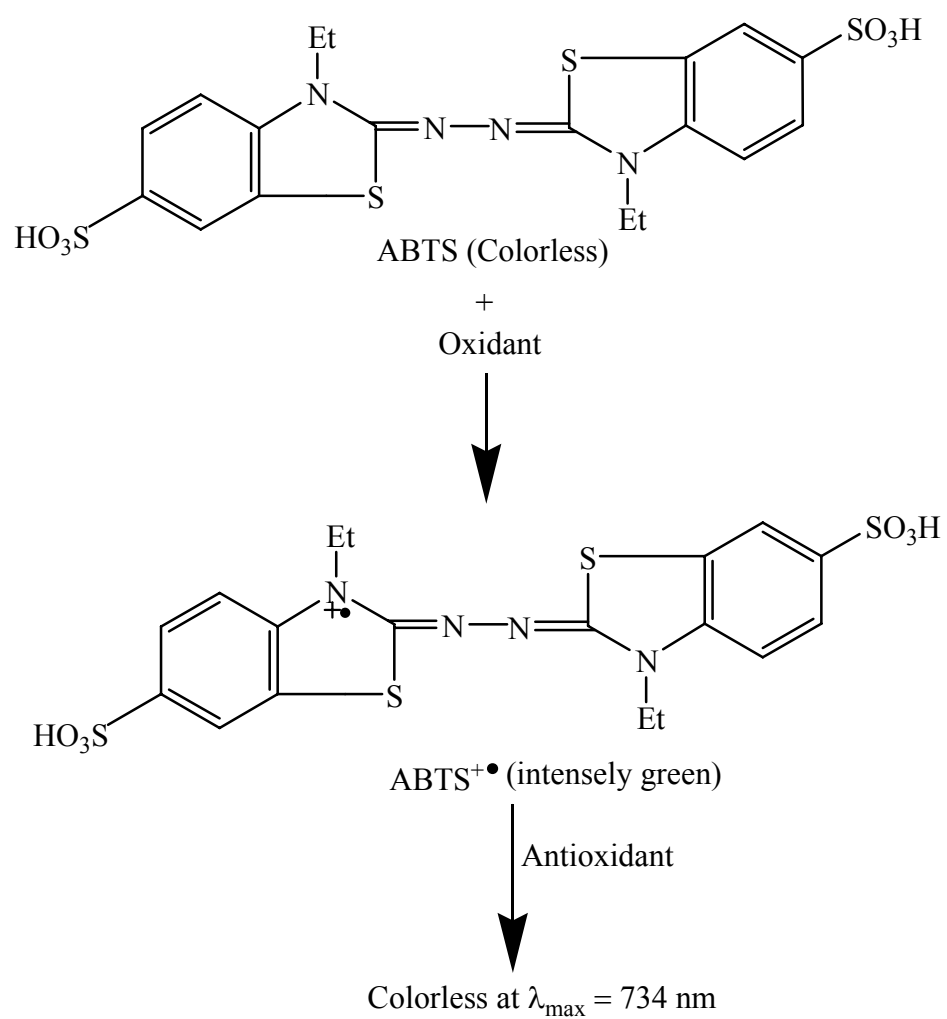
Data reduction from the ORAC<sub>FL</sub> assay is achieved by (1) calculating of the area under the kinetic curve (AUC) and net AUC (AUC<sub>sample</sub> - AUC<sub>blank</sub>), (2) obtaining a standard curve by plotting the concentration of Trolox and the AUC (3) calculating the Trolox equivalents of a sample using the standard curve.

## **2.17 Inflammation**

Inflammation is a protective response intended to eliminate the initial cause of cell injury, as well as necrotic cells and tissues resulting from mechanical injury, chemical toxins, invasion by microorganisms and hypersensitivity (Kumar *et al.*, 2007; Rankin, 2004 ). Several types of cells and molecules play important roles in inflammation. These include blood leukocytes and plasma proteins, cells of vascular walls, and cells of the extracellular matrix (ECM) from the surrounding connective tissue (Kumar *et al.*, 2007; Kundu and Surh, 2008). Inflammation can be acute or chronic (Kumar *et al.*, 2007; Rankin, 2004). In acute inflammation there is an abundance of phagocytic cells (principally neutrophils and macrophages), whereas, in chronic inflammation, lymphocytes and monocytes are predominant (Rankin, 2004). Cardiovascular

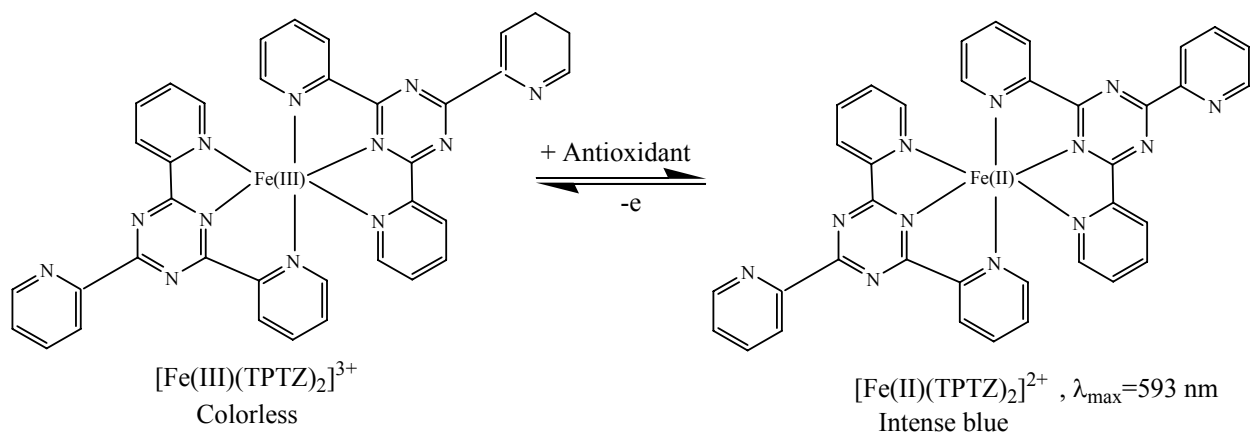
**Figure 2.17:** Chemical reactions of selected *in vitro* antioxidant activity assays. **(A)** TEAC; **(B)** FRAP; and **(C)** ORAC<sub>FL</sub> (Adapted from Huang *et al.*, 2005). Abbreviations are as follows: ABTS, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid); ABTS<sup>•+</sup>, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation; ArO<sup>•</sup>, phenoxyl radical; ArOH, antioxidant; AAPH, 2,2'-azobis-(2-amidinopropane) dihydrochloride; ORAC<sub>FL</sub>, oxygen radical absorbance capacity; ROO<sup>•</sup>, peroxy radical; ROOH, hydroperoxide; FRAP, ferric reducing antioxidant power; TEAC, Trolox equivalent antioxidant capacity; and TPTZ, 2,4,6-tri(2-pyridyl)-s-triazine.

(A)

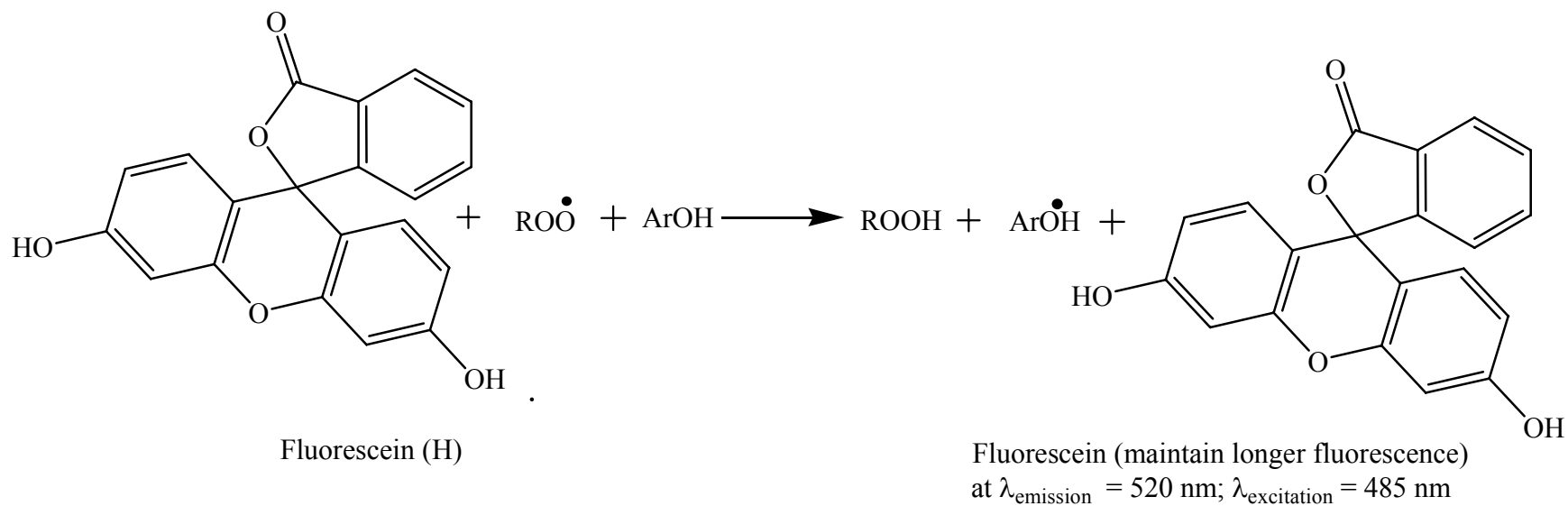
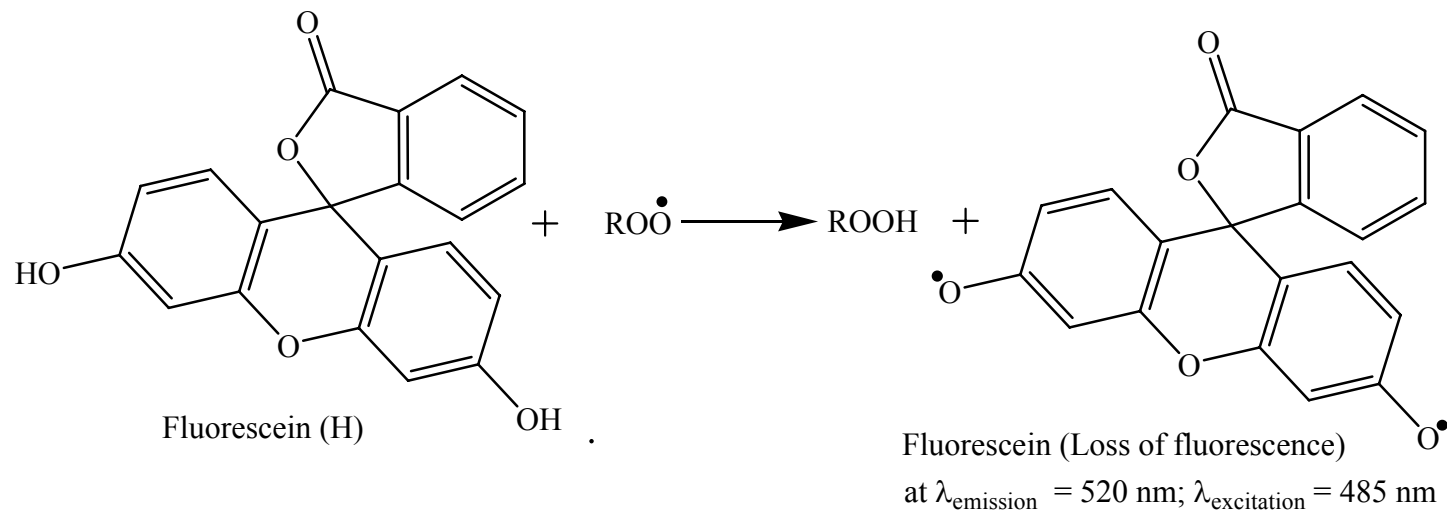


(B)

$\text{Fe}^{3+}\text{-TPTZ} + \text{reducing antioxidant} \rightarrow \text{Fe}^{2+}\text{-TPTZ}$  (intense blue at  $\lambda_{\text{max}} = 593 \text{ nm}$ )



(C)



disease, diabetes, arthritis, cancer, asthma, allergies, irritable bowel syndrome and many others are known to involve chronic inflammation.

Acute inflammation is characterized by fluid and plasma protein exudation and predominantly neutrophilic leukocyte accumulation. External symptoms (cardinal signs) result from vascular changes (vasodilation and increased vascular permeability) and cell recruitment which includes heat, redness (erythema), swelling (edema), pain and sometimes loss of function. Chemical mediators such as histamine, bradykinin, and leukotrienes induce vascular permeability by causing endothelial cell contraction that allows plasma proteins and leukocytes to enter sites of infection or tissue damage. Extravasation of fluid causes edema in tissues. Leukocytes ingest foreign bodies, kill bacteria or other microbes, and eliminate necrotic tissues and foreign substances.

Leukocytic recruitment is a multistep process consisting of loose attachment to rolling on endothelium (mediated by selectins), firm attachment to endothelium (mediated by integrins), and migration through inter-endothelial spaces. Various cytokines [(Tumor necrosis factor-alpha (TNF- $\alpha$ ), interleukin-1 (IL-1)] promote expression of selectin and integrin ligands on endothelial cells. These ligands include ICAM-1 (intercellular adhesion molecule 1) and VCAM-1 (vascular cell adhesion molecule 1). In most forms of acute inflammation, neutrophils predominate in the inflammatory infiltrate during the first 6 to 24 h and are replaced by monocytes in 24 to 48 h (Kumar *et al.*, 2007).

Leukocytic activation results in many processes, such as phagocytosis of particles, production of substances that destroy phagocytosed microbes that remove dead tissues, and the production of mediators [such as prostaglandins and cytokines] that amplify the inflammation reaction. The ingested microbe or foreign substance forms a phagocytic vacuole. The



membrane of the vacuole then fuses with the membrane of a lysosomal granule, thus exposing the ingested particles to the destructive mechanism of leukocytes (Babior, *et al.*, 1973; Kumar *et al.*, 2007).

Phagocytosis stimulates an oxidative burst characterized by a sudden increase in oxygen consumption, glycogen catabolism (glycogenolysis), increased glucose oxidation, and the production of ROS. The generation of oxygen metabolites is due to rapid activation of leukocyte NADPH oxidase, sometimes called the phagocyte oxidase, which oxidizes NADPH and converts oxygen to  $O_2^{\bullet-}$ . The lysosomes of neutrophils contain myeloperoxidase (MPO) enzymes, and in the presence of chloride ions, MPO converts  $H_2O_2$  to  $HOCl^{\bullet}$  (hypochlorous radical), which is a powerful oxidant and antibacterial agent (Rosso *et al.*, 2006).

### **2.17.1 Chemical Mediators of Inflammation**

Chemical mediators are produced either locally by the cells at the site of inflammation, or they may circulate in plasma as inactive precursors and activate with inflammation. Cell-derived mediators are either present in the intracellular granules and are rapidly secreted upon cellular activation (for example, histamine in mast cells), or synthesized *de novo* in response to stimulus (for example, prostaglandins and leukotrienes). Prostaglandins and leukotrienes are synthesized during the metabolism of AA by cyclooxygenase (COX) and lipoxygenase (LOX) enzyme pathways. These mediators promote inflammation by recruiting macrophages, neutrophils, and leukocytes (Moolwaney and Igway, 2005).

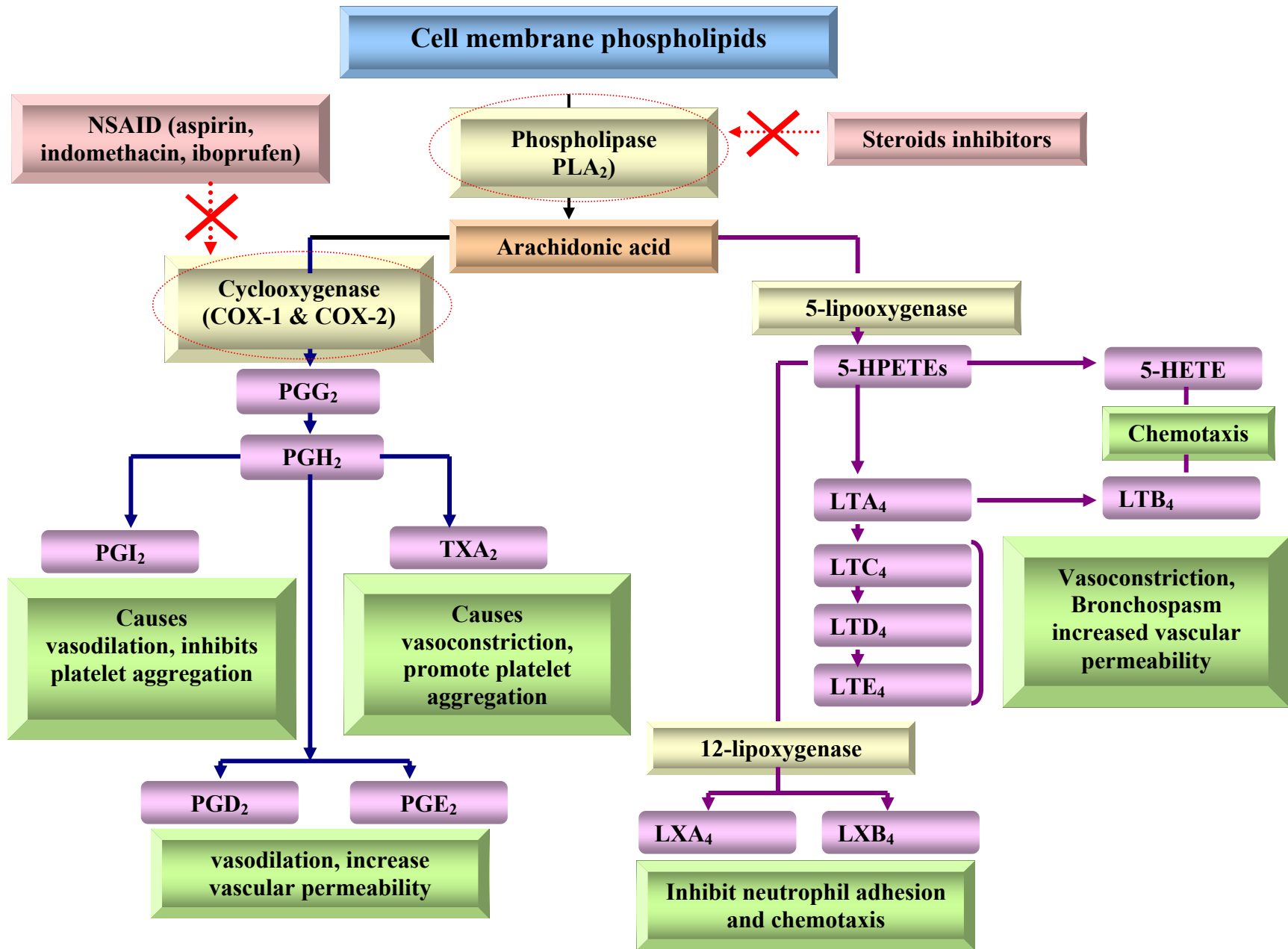
### 2.17.1.1 Arachidonic acid Metabolites

Arachidonic acid (AA) is a 20-carbon fatty acid that is localized in the cellular membrane of phospholipids. Phospholipase A<sub>2</sub> catalyzes the release of AA from membrane phospholipids. The subsequent release of AA from the phospholipid pools allows for AA to be metabolized by either the COX or LOX pathways (**Figure 2.18**).

The COX pathway produces prostaglandins E<sub>2</sub> (PGE<sub>2</sub>), PGD<sub>2</sub>, PGF<sub>2</sub>, PGI<sub>2</sub> (Prostacyclin), and thromboxane A<sub>2</sub> (TXA<sub>2</sub>) (Kumar *et al.*, 2007). PGE<sub>2</sub> can increase free intracellular calcium resulting in keratinocyte differentiation and vascular permeability changes produced by histamine and bradykinin (Lee *et al.*, 2003). PGD<sub>2</sub>, produced in mast cells, a major metabolite of the COX pathway, and a vasodilator, along with PGE<sub>2</sub> and PGF<sub>2</sub> facilitates edema formation. PGE<sub>2</sub> augments pain sensitivity to a variety of other stimuli and interacts with cytokines effecting fever (Rankin, 2004).

5-Lipoxygenase is a predominant enzyme in the AA pathway, present in neutrophils. The 5-hydroperoxy derivative of AA, 5-HPETE (5-hydroperoxyeicosatetraenoic acid) is reduced to 5-HETE (5 hydroxyeicosatetraenoic acid) or converted to a family of leukotrienes. LTB<sub>4</sub> and LTC<sub>4</sub>, produced in neutrophils and some macrophages, are potent chemotactic agents for neutrophils (Lee *et al.*, 2003). LTD<sub>4</sub> and LTE<sub>4</sub> are produced in mast cells and effect vasoconstriction and increased vascular permeability. Prostaglandins and leukotrienes play a large role in inflammation.

**Figure 2.18:** Arachidonic acid pathway (Adapted from Kumar *et al.*, 2007). Abbreviations are as follows: NSAID, non-steroidal anti-inflammatory drug; PLA<sub>2</sub>, Phospholipase A<sub>2</sub>; COX-1 and 2, Cyclooxygenase-1 and 2; PGD<sub>2</sub>, Prostaglandins D<sub>2</sub>; PGE<sub>2</sub>, Prostaglandins E<sub>2</sub>; PGG<sub>2</sub>, Prostaglandins G<sub>2</sub>; PGI<sub>2</sub>, Prostacyclin; 5HPETE, 5-hydroperoxyeicosatetraenoic acid; 5HETE, 5-hydroxyeicosatetraenoic acid; TXA<sub>2</sub>, thromboxane A<sub>2</sub>; LTA<sub>4</sub>, leukotrienes A<sub>4</sub>; LTB<sub>4</sub>, leukotrienes B<sub>4</sub>; LTC<sub>4</sub>, leukotrienes C<sub>4</sub>; LTD<sub>4</sub>, leukotrienes D<sub>4</sub>; LTE<sub>4</sub>, leukotrienes E<sub>4</sub>; LXA<sub>4</sub>, Lipoxin A<sub>4</sub>; LXB<sub>4</sub>, Lipoxin B<sub>4</sub>. Red-dotted circles indicate the inhibition point of action by NSAIDs or steroids. Red crosses indicate the point of action by NSAIDs or steroids.



### **2.17.1.2 Cytokines**

Cytokines play an extremely important role in mediating the process of inflammation. The actions of cytokines may be additive, synergistic, or inhibitory with respect to immune system function (Rankin, 2004). Interleukins (IL) are polypeptide cytokines named for their ability to mediate communication between leukocytes. Two important cytokines are TNF- $\alpha$  and IL-1; they are produced by activated macrophages, mast cells, and endothelial cells. Their main role in inflammation is endothelial activation (Kumar *et al.*, 2007). They stimulate expression of adhesion molecules on endothelial cells resulting in increased leukocytic binding and recruitment. TNF- $\alpha$  also increases activation of neutrophils (Kumar *et al.*, 2007).

Chemoattractant cytokines (chemokines) are a family of small structurally related proteins that act primarily as chemoattractants, for different subsets of leukocytes. Two main functions of chemokines are leukocytic recruitment and activation of leukocytes. Leukocytic activation increases the affinity of leukocyte integrins for their ligands on endothelial cells (Kumar *et al.*, 2007; Rankin, 2004). Synthesis of chemokines is stimulated by TNF- $\alpha$  and interferon gamma (IFN- $\gamma$ ), which are themselves cytokines (Rankin, 2004).

## **2.18 Inflammation and NF- $\kappa$ B**

NF- $\kappa$ B regulates the expression of many genes involved in inflammatory and immune responses (Baldwin, 1996; Ghosh *et al.*, 1998; Pahl, 1999) and cellular growth properties (Barkett and Gilmore, 1999).

NF- $\kappa$ B is a heterodimer, usually consisting of two proteins, a p65 (also called relA) and a p50 subunit, which is found in cytoplasm, bound to I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$ , which prevents entrance of the heterodimer to the nucleus. When these cells are stimulated, specific kinases phosphorylate

I $\kappa$ B causing its release from the heterodimer and its rapid degradation by proteasomes. The release of NF- $\kappa$ B from I $\kappa$ B results in the passage of NF- $\kappa$ B into the nucleus, where it binds to specific sequences in the promoter regions of target genes (Barnes and Karin, 1997; Yamamoto and Gaynor, 2001) (**Figure 2.19**).

Many stimuli activate NF- $\kappa$ B, including cytokines, activators of PKC, phorbol esters, bacterial toxins, free radicals and ultraviolet radiation. All these stimuli act by means of PKs that phosphorylate I $\kappa$ B, thereby rendering NF- $\kappa$ B active (Bell *et al.*, 2003).

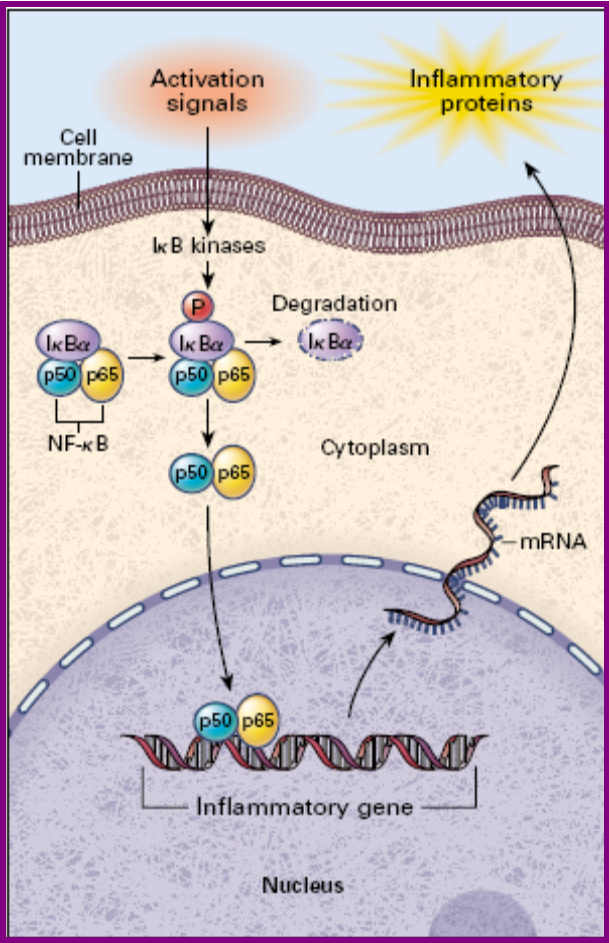
Cytokines production caused by increased gene expression induced by activation of NF- $\kappa$ B is also involved in the activation of NF- $\kappa$ B. IL-1 $\beta$  and TNF- $\alpha$  can directly activate the NF- $\kappa$ B pathway, thus establishing a positive autoregulatory loop that can amplify the inflammatory response and increase the duration of chronic inflammation (**Figure 2.20**) (Barnes and Karin, 1997; Yamamoto and Gaynor, 2001).

NF- $\kappa$ B also stimulates the expression of enzymes whose products contribute to the pathogenesis of the inflammatory process, including the inducible form of nitric oxide synthase, which generates NO and the inducible COX-2, which generates prostanoids (Pahl, 1999).

NF- $\kappa$ B regulates the expression of several genes that encode adhesion molecules such as the intercellular adhesion molecule 1, vascular-cell adhesion molecule 1, E-selectin, interleukin-1 $\beta$ , TNF- $\alpha$ , interleukin-6, granulocyte–macrophage colony-stimulating factor, and many chemotactic cytokines (chemokines) (Barnes and Karin, 1997; Pahl, 1999; Gerondakis *et al.*, 1998).

Thus, activation of NF- $\kappa$ B leads to the induction of multiple genes that regulate immune and inflammatory responses and has been associated with many diseases involving inflammation such as cancer, autoimmune diseases, atherosclerosis, diabetes, asthma, rheumatoid arthritis,

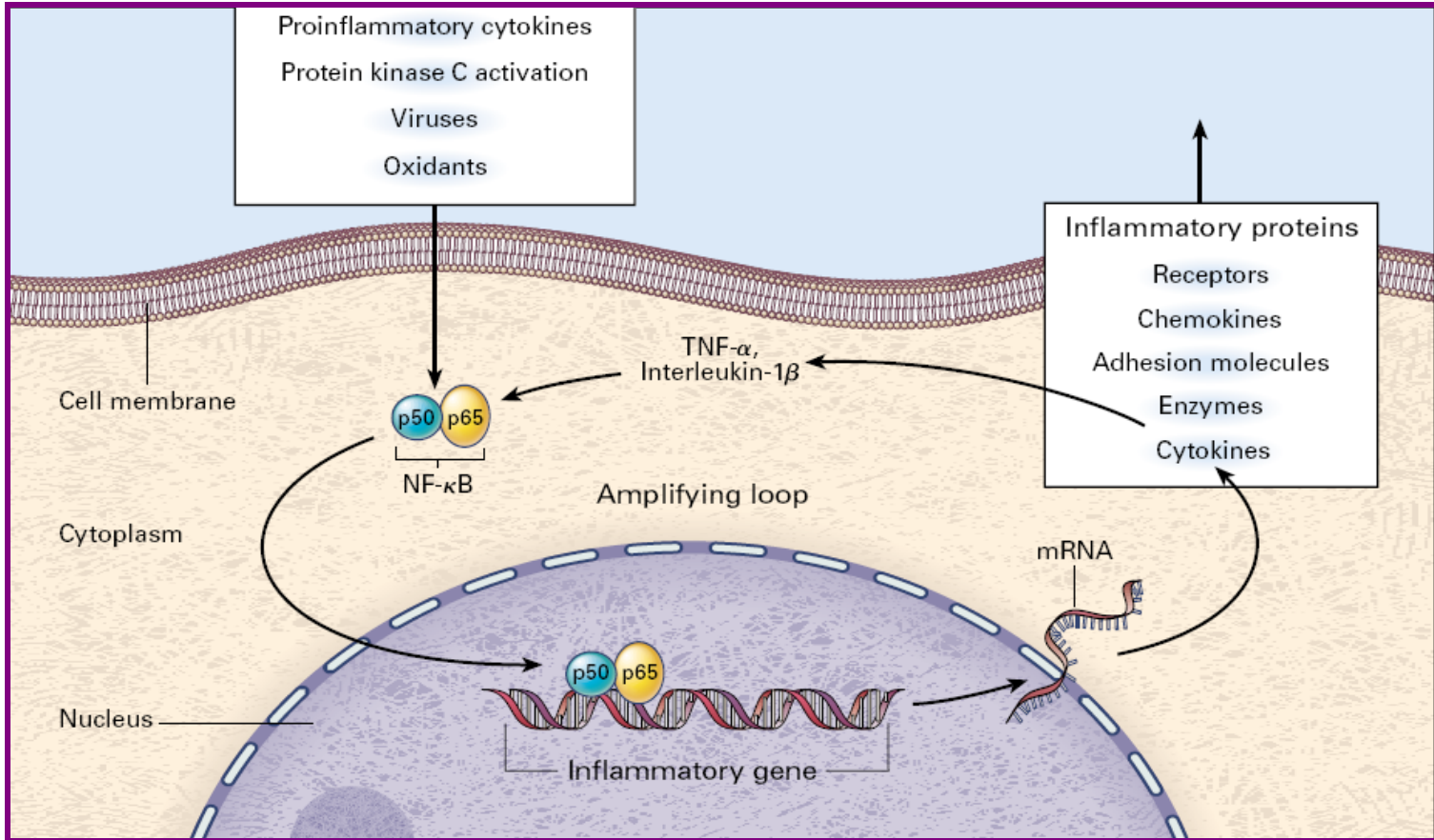
**Figure 2.19:** Schematic diagram of NF- $\kappa$ B activation. Activation of NF- $\kappa$ B involves the phosphorylation and subsequent proteolytic degradation of the inhibitory protein I $\kappa$ B by specific I $\kappa$ B kinases. The free NF- $\kappa$ B (a heterodimer of p50 and p65) then passes into the nucleus where it binds to  $\kappa$ B sites in the promoter regions of genes for inflammatory proteins such as cytokines, enzymes, and adhesion molecules. P denotes protein, and mRNA messenger RNA (Barnes and Karin, 1997 – Permission to reproduce to this image has been secured from the publisher)





**Figure 2.20:** Schematic diagram of NF- $\kappa$ B as an inflammatory regulator. NF- $\kappa$ B can be activated by a variety of inflammatory signals, resulting in the coordinated expression of over 200 genes for several cytokines, enzymes, and adhesion molecules. The cytokines interleukin-1 $\beta$  and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) are both activated and amplified by NF- $\kappa$ B. (Barnes and Karin, 1997 – Permission to reproduce to this image has been secured from the publisher)

## Inflammatory signals



psoriasis, inflammatory bowel disease, and other inflammatory diseases Barnes and Karin, 1997; Yamamoto and Gaynor, 2001; Younes, A. 2003; Wu, J.T. 2005; Gambari, R. 2004; Farabegoli, F. 2005, Kaur, M. 2007; Edwards, M.R. 2006).

## **2.19 Inflammation and 12-*O*-Tetradecanoylphorbol-13-acetate**

Mouse ear edema induced by phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) has been widely used as an animal model for testing anti-inflammatory activity. In this model, TPA stimulates COX-2 expression which increases production of many inflammatory mediators including, PGE<sub>2</sub> and LTB<sub>4</sub>. These mediators are associated with increased ear edema and hyperplasia (Chun *et al.*, 2004).

Prostaglandins and leukotrienes affect initial responses to inflammation by increasing vasodilatation (redness) and vascular permeability (swelling) (Funk, 2001). LTB<sub>4</sub> promotes neutrophil chemotaxis; PGE<sub>2</sub> can increase free intracellular calcium resulting in keratinocytic differentiation and vascular permeability changes produced by histamine and bradykinin (Lee *et al.* 2003); TPA also stimulates keratinocytes to release cytokines, TNF- $\alpha$  and IL-1 $\beta$ , which have been reported to accelerate edema in the mouse ear (Murakawa *et al.*, 2006; Murphy *et al.*, 2000).

The mechanism of action of TPA activity is not well understood, however, it is believed to start with activation of PKC (Cataisoon *et al.*, 2003). PKC has been implicated in events that lead to keratinocyte differentiation, epidermal tumor growth, and cutaneous inflammation (Szaefer *et al.*, 2007).

The PKC pathway is part of a signal transduction system that is dependent upon the turnover of phosphatidylinositol bisphosphate (PIP<sub>2</sub>). PKC is activated by diacylglycerol

(DAG), a second messenger that is formed by the actions of phospholipase C. PKC contains binding sites for both DAG and phorbol esters. Therefore, TPA activates PKC and activated PKC phosphorylates various cellular enzymes and receptors such as the nuclear transcription factor NF- $\kappa$ B (Wang and Smart, 1999; Stahelin, 2004), which plays an important role in inflammation.

## **2.20 Inflammation and Pharmaceutical Agents**

Non-steroidal anti-inflammatory drugs (NSAID), *e.g.*, aspirin, ibuprofen, and indomethacin, are pharmaceutical agents widely used in the treatment of inflammation and pain management. The anti-inflammatory effects of NSAIDs are believed to be the result of inhibiting the formation of prostaglandins by COX, which converts AA, released by membrane phospholipids, into prostaglandins. Two isoforms of COX, COX-1, and COX-2, have been identified. COX-1 is constitutively expressed in many tissues and is necessary for the protection of the stomach lining (Lee *et al.*, 2003; Funk, 2001). Inhibition of COX-1 causes gastrointestinal disturbances ranging from simple discomfort to bleeding ulcers (Yoon and Baek, 2005). The expression of COX-2 is regulated by mitogens, tumor promoters, and growth factors (Herschman, 1996).

Aspirin and other NSAIDs affect non-selective COX inhibition; *i.e.*, they inhibit both COX-I and COX-2. As a consequence they induce gastric ulcerization and kidney failure due to COX-1 inhibition (Furst, 2007).

Newer generations of NSAID drugs are the selective COX-2 enzyme inhibitors called coxibs (*e.g.*, celecoxib and rofecoxib). These agents are used clinically for the management of arthritis and pain. The marketing of new COX-2 inhibitors has emphasized the advantages of not

blocking the constitutive COX-1 pathway; however, infrequent reports of gastrointestinal problems still exist from the use of these medications.

Coxibs decrease gastrointestinal toxicities, but they also suppress the production of prostacyclin, an inhibitor of platelet aggregation. Additionally, the balance of prostanoid synthesis then favors the formation of eicosanoids produced by COX-1 such as pro-thrombotic thromboxanes. These imbalances, due to selective COX-2 inhibition, may explain the increase in cardiovascular complications in many patients taking coxibs. Subsequently, coxibs have been pulled off the market (Funk, 2001; *et al.*, 2005).

Other classes of anti-inflammatory agents such as corticosteroids and disease-modifying anti-rheumatic agents, all produce adverse side effects. One such example is glucocorticoids, which modulate the activity of transcription factors, including NF- $\kappa$ B, AP-1 (Necela and Cidlowski, 2004). The long-term clinical utility of glucocorticoids is limited due to undesirable adverse effects, including suppression of hypothalamus-pituitary-adrenal (HPA) axis, increased serum glucose, induction of osteoporosis and glaucoma, altered electrolyte balance, insomnia, and other behavioral alterations (Zimmermann *et al.*, 2009).

## **2.21 Inflammation and Phenolic Compounds**

A large number of dietary phenolics with known anti-inflammatory activities are consumed in food. These phenolic compounds are capable of inhibiting enzymes such as COX and LOX, NOS) and cytokines like TNF- $\alpha$ , IL-6, IL- $\beta$  and the transcription factor (NF- $\kappa$ B), which are involved in inflammation.

Anthocyanin-rich berry extracts showed considerable inhibitory effects on NO production in LPS/interferon- $\gamma$  (IFN- $\gamma$ ) activated RAW 264.7 macrophages (Wang and Mazza,

2002): NO is known to be an important mediator of acute and chronic inflammation. Delphinidin, an anthocyanin, inhibited NF- $\kappa$ B activation by blocking LPS-induced I $\kappa$ B- $\alpha$  degradation and p65 translocation to the nucleus (Hou *et al.*, 2005); transcription of COX-2 was down-regulated by kaempferol and cyanidin in LPS activated macrophages (O'Leary *et al.*, 2004).

Topical application of anthocyanins and a hydrolyzable tannin-rich pomegranate fruit extract on mouse skin significantly inhibited phosphorylation by MAPKs, activation of NF- $\kappa$ B, and activity of COXs; the later being an important enzyme involved in mediating the inflammatory process (Afaq *et al.* 2005). Similar findings were exhibited by cocoa polyphenols, predominantly flavonol and procyanidins, on mouse skin (Lee *et al.*, 2006). Pre-treatment with a green tea extract [enriched with catechin and epigallocatechin gallate (EGCG)] inhibited COX-2 expression, which was resulting from TPA-induced injury on the mouse skin (Kundu *et al.*, 2003). Tsuda *et al.*, (2002) showed a suppression of MPO activity by C3G in rats after ischemia reperfusion injury.

Karlsen *et al.*, (2007) conducted human trials with anthocyanin supplementation, demonstrating inhibition of NF- $\kappa$ B activation and decreased plasma concentrations of pro-inflammatory chemokines, cytokines, and inflammatory mediators in human subjects. Most of these phenolic compounds are also present in blackberry cultivars.

## **2.22 Blackberries and Health Benefits**

Scientific evidence indicates that berries have diverse biological effects attributed to their phenolic compounds. As a food category, berries are among 50 products that are ranked highest in antioxidant concentrations (Halvorsen *et al.*, 2006), with blackberries being the most effective

antioxidant (Pellegrini *et al.*, 2003; Seeram *et al.*, 2006). Blackberries contain high levels of anthocyanins (predominantly, C3G), ellagitannins, ellagic acid and its derivatives (Moyer *et al.*, 2002).

For centuries, blackberry extracts and juice have been used in folk medicine to treat numerous ailments (Bhakuni *et al.*, 1997). Teas made from whole blackberry plants were used by Native Americans to treat dysentery, cholera, and upset stomach and were also featured in ancient Roman dispensaries.

Several studies over the past decade have shown that antioxidant and anti-inflammatory activities are attributable to blackberry phenolics and may play roles in mechanisms for chemopreventive and chemotherapeutic activities of blackberries. For instance, Hassan and Yousef (2009) demonstrated that treatment of blackberry juice significantly ( $p < 0.05$ ) decreased the levels of 2-thiobarbituric acid reactive substances and NO generated by NaF-induced free radical generation in the liver of male albino rats. 2-Thiobarbituric acid reactive substances were used to measure the extent of lipid peroxidation induced by NaF-induced hepatic damage. Blackberry juice was also effective at restoring the activities of GSH, SOD and CAT (endogenous antioxidant enzymes in the body) in compromised rats.

Anthocyanin extracts from three different blackberry cultivars (*i.e.*, Hull, Chester, and Black-satin), exhibited anticancer activities in human colorectal cancer cell line HT-29, human breast cancer cell line MCF-7 (her2 negative and ER+) and human leukemia cell line HL-60 (Dai *et al.*, 2009). Hassimoto *et al.*, (2008) reported consumption of anthocyanin rich blackberry juice, had a pronounced effect in increasing catalase content, an antioxidant enzyme, in human blood plasma.

Rossi *et al.*, (2003) showed that blackberry extracts, rich in anthocyanins, reduce nitrosative stress and neutrophil infiltration in carrageenan-evoked inflammation, in rats. Blackberry anthocyanins, also inhibited nitrite production in LPS-induced rats (Pergola *et al.*, 2006). Ding *et al.*, (2006) demonstrated that C3G, isolated from blackberries, exhibited inhibitory effects on the activity and expression of AP-1, NF- $\kappa$ B, COX-2, and TNF- $\alpha$ . However, there is limited data on human studies pertaining to absorption of phenolics from blackberries and the impact in human disease.



## LITERATURE CITED

- Afaq, F.; Saleem, M.; Krueger, C. G.; Reed, J. D.; Mukhtar, H. Anthocyanin- and hydrolyzable tannin-rich pomegranate fruit extract modulates MAPK and NF- $\kappa$ B pathways and inhibits skin tumorigenesis in CD-1 mice. *Int. J. Cancer* **2005**, *113*, 423–433.
- Ahmed, N. Advanced glycation endproducts-role in pathology of diabetic complications. *Diabetes Res. Clin. Pract.* **2005**, *67*, 3–21.
- Alderton, W. K.; Cooper, C. E.; Knowles, R. G. Nitric oxide synthases: structure, function and inhibition. *Biochem. J.* **2001**, *357*, 593–615.
- Alía, M.; Ramos, S.; Mateos, R.; Granado-Serrano, A. B.; Bravo, L.; Goya, L. Quercetin protects human hepatoma HepG2 against oxidative stress induced by *tert*-butyl hydroperoxide. *Toxicol. Appl. Pharmacol.* **2006**, *212*, 110–118.
- Amarowicz, R.; Shahidi, F. Application of sephadex LH-20 chromatography for the separation of cyanogenic glycosides and hydrophylic phenolic fraction from flaxseed. *J. Liq. Chrom. Rel. Technol.* **1994**, *17*, 1291–1299.
- Amarowicz, R.; Shahidi, F. A rapid chromatographic method for separation of individual catechins from green tea. *Food Res. Int.* **1996**, *29*, 71–76.
- Amić, D.; Davidović-Amić, D.; Bešlo, D.; Trinajstić, N. Structure-radical scavenging activity relationships of flavonoids. *Croatica Chemica Acta* **2003**, *76*, 55–61.
- Andersen, Ø. M. Semipreparative isolation and structure determination of pelargonidin 3-*O*- $\alpha$ -L-rhamnopyranosyl (1 $\rightarrow$ 2)- $\beta$ -D-glucopyranoside and other anthocyanins from the tree *Dacrydium dacrydioides*. *Acta Chem. Scand.* **1988**, *B42*, 462–468.
- Andersen, Ø. M.; Francis, G. W. Simultaneous analysis of anthocyanins and anthocyanidins on cellulose thin layers. *J. Chromatogr.* **1985**, *318*, 450–454.
- Andreasen, M. F.; Christensen, L. P.; Meyer, A. S.; Hansen, Å. Content of phenolic acids and ferulic acid dehydrodimers in 17 rye (*Secale cereale* L.) varieties. *J. Agric. Food Chem.* **2000**, *48*, 2837–2842.
- Antman, E. M.; DeMets, D.; Loscalzo, J. Cyclooxygenase inhibition and cardiovascular risk. *Circulation* **2005**, *112*, 759–770.
- Antolovich, M.; Prenzler, P.; Robards, K.; Ryan, D. Sample preparation in the determination of phenolic compounds in fruits. *Analyst* **2000**, *125*, 989–1009.
- Archer, S. Measurement of nitric-oxide in biological models. *FASEB J.* **1993**, *7*, 349–360.

- Arts, M. J. T. J.; Haenen, G. R. M. M.; Voss, H.-P.; Bast, A. Antioxidant capacity of reaction products limits the applicability of the Trolox Equivalent Antioxidant Capacity (TEAC) assay. *Food Chem. Toxicol.* **2004**, *42*, 45–49.
- Aruoma, O. I. Methodological considerations for characterizing potential antioxidant actions of bioactive components in plant foods. *Mutat. Res.* **2003**, *523*, 9–20.
- Aruoma, O. I. Free radicals, environmental radiation, and cancer. In *Phytopharmaceuticals in Cancer Chemoprevention*. Bagchi, D.; Preuss, H. G., Eds.; CRC Press: Boca Raton, FL, 2004; pp. 79–87.
- Babior, B. M.; Kipnes, R. S.; Curnutte, J. T. Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent. *J. Clin. Invest.* **1973**, *52*, 741–744.
- Babu, P. V. A.; Sabitha, K. E.; Shyamaladevi, C. S. Green tea impedes dyslipidemia, lipid peroxidation, protein glycation and ameliorates Ca<sup>2+</sup>-ATPase and Na<sup>+</sup>/K<sup>+</sup>-ATPase activity in the heart of streptozotocin-diabetic rats. *Chem. Biol. Interact.* **2006a**, *162*, 157–164.
- Babu, P. V. A.; Sabitha, K. E.; Shyamaladevi, C. S. Therapeutic effect of green tea extract on advanced glycation and cross-linking of collagen in the aorta of streptozotocin diabetic rats. *Clin. Exp. Pharmacol. Physiol.* **2006b**, *33*, 351–357.
- Bais, H. P.; Vepachedu, R.; Gilroy, S.; Callaway, R. M.; Vivanco, J. M. Allelopathy and exotic plant invasion: From molecules and genes to species interactions. *Science* **2003**, *301*, 1377–1380.
- Baldwin, A. S., Jr. The NF- $\kappa$ B and I $\kappa$ B proteins: New discoveries and insights. *Annu. Rev. Immunol.* **1996**, *14*, 649–681.
- Banini, A. E.; Boyd, L. C.; Allen, J. C.; Allen, H. G.; Sauls, D. L. Muscadine grape products intake, diet and blood constituents of non-diabetic and type 2 diabetic subjects. *Nutrition* **2006**, *22*, 1137–1145.
- Barkett, M.; Gilmore, T. D. Control of apoptosis by Rel/NF- $\kappa$ B transcription factors. *Oncogene* **1999**, *18*, 6910–6924.
- Barnes, P. J.; Karin, M. Nuclear factor- $\kappa$ B - A pivotal transcription factor in chronic inflammatory diseases. *New Engl. J. Med.* **1997**, *336*, 1066–1071.
- Bastianetto, S.; Brouillette, J.; Quirion, R. Neuroprotective effects of natural products: Interaction with intracellular kinases, amyloid peptides and a possible role for transthyretin. *Neurochem. Res.* **2007**, *32*, 17720–17725.

- Bate-Smith, E. C. Haemanalysis of tannins: The concept of relative astringency. *Phytochemistry* **1973**, *12*, 907–912.
- Beggs, C. J.; Kuhn, K.; Bocker, R.; Wellmann, E. Phytochrome-induced flavonoid biosynthesis in mustard (*Sinapis alba* L.) cotyledons: Enzymic control and differential regulation of anthocyanin and quercetin formation. *Planta* **1987**, *172*, 121–126.
- Bell, S.; Degitz, K.; Quirling, M.; Jilg, N.; Page, S.; Brand, K. Involvement of NF- $\kappa$ B signalling in skin physiology and disease. *Cell. Signal.* **2002**, *15*, 1–7.
- Bengoechea, M. L.; Sancho, A. I.; Bartolomé, B.; Estrella, I.; Gómez-Cordovés, C.; Hernández, M. T. Phenolic composition of industrially manufactured purées and concentrates from peach and apple fruits. *J. Agric. Food Chem.* **1997**, *45*, 4071–4075.
- Benzie, I. F. F.; Strain, J. J. Ferric reducing/antioxidant power assay: Direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. *Methods Enzymol.* **1999**, *299*, 15–27.
- Bergendi, L.; Beneš, L.; Ďuračková, Z.; Ferenčík, M. Chemistry, physiology and pathology of free radicals. *Life Sci.* **1999**, *65*, 1865–1874.
- Bhakuni, D. S.; Bittner, M.; Marticorena, C.; Silva, M.; Weldt, F.; Hoeneisen, M.; Hartwell, J. L. Screening of Chilean plant for anticancer activity. *J. Nat. Prod. (Lloydia)* **1976**, *39*, 225–243.
- Bierhaus, A.; Hofmann, M. A.; Ziegler, R.; Nawroth, P. P. AGEs and their interaction with AGE-receptors in vascular disease and diabetes mellitus. I. The AGE concept. *Cardiovasc. Res.* **1998**, *37*, 586–600.
- Bierhaus, A.; Illmer, T.; Kasper, M.; Luther, T.; Quehenberger, P.; Tritschler, H.; Wahl, P.; Ziegler, R.; Müller, M.; Nawroth, P. P. Advanced glycation endproduct (AGE)-mediated induction of tissue factor in cultured endothelial cells is dependent on RAGE. *Circulation* **1997**, *96*, 2262–2271.
- Bors, W.; Heller, W.; Michel, C.; Saran, M. Flavonoids as antioxidants: Determination of radical-scavenging efficiencies. *Methods Enzymol.* **1990**, *186*, 343–355
- Bravo, L. Polyphenols: Chemistry, dietary sources, metabolism, and nutritional significance. *Nutr. Rev.* **1998**, *56*, 317–333.
- Bravo, L.; Mateos, R. Analysis of flavonoids in functional foods and nutraceuticals. In *Methods of Analysis for Functional Foods and Nutraceuticals*. Hurst, W. J., Ed.; CRC Press: Boca Raton, FL, 2008; pp. 148–206.

- Brouillard, R. Chemical structure of anthocyanins. In *Anthocyanins as Food Colors*. Markakis, P., Ed.; Academic Press: New York, NY, 1982; pp. 1–39.
- Brown, J. E.; Khodr, H.; Hider, R. C.; Rice-Evans, C. A. Structural dependence of flavonoid interactions with  $\text{Cu}^{2+}$  ions: implications for their antioxidant properties. *Biochem. J.* **1998**, *330*, 1173–1178.
- Brownlee, M. Biochemistry and molecular cell biology of diabetic complications. *Nature* **2001**, *414*, 813–820.
- Buckley, B.; Moore, J. N.; Clark, J. R. Blackberry cultivars differ in susceptibility to rosette disease. *Fruit Var. J.* **1995**, *49*, 235–238.
- Bunzel, M.; Funk, C.; Steinhart, H. Semipreparative isolation of dehydrodiferulic and dehydrotriferulic acids as standard substances from maize bran. *J. Sep. Sci.* **2004**, *27*, 1080–1086.
- Butler, E. J.; Pearson, A. W.; Fenwick, G. R. Problems which limit the use of rapeseed meal as a protein source in poultry diets. *J. Sci. Food Agric.* **1982**, *33*, 866–970.
- Cai, Y.-Z.; Sun, M.; Xing, J.; Luo, Q.; Corke, H. Structure-radical scavenging activity relationships of phenolic compounds from traditional Chinese medicinal plants. *Life Sci.* **2006**, *78*, 2872–2888.
- Cao, G.; Alessio, H. M.; Culter, R. G. Oxygen-radical absorbance capacity assay for antioxidants. *Free Radical Biol. Med.* **1993**, *14*, 303–311.
- Cao, G.; Sofic, E.; Prior, R. L. Antioxidant and prooxidant behavior of flavonoids: Structure-activity relationships. *Free Radical Biol. Med.* **1997**, *22*, 749–760.
- Carluccio, M. A.; Siculella, L.; Ancora, M. A.; Massaro, M.; Scoditti, E.; Storelli, C.; Visioli, F.; Distante, A.; De Caterina, R. Olive oil and red wine antioxidant polyphenols inhibit endothelial activation. Antiatherogenic properties of Mediterranean diet phytochemicals. *Arterioscler. Thromb. Vasc. Biol.* **2003**, *23*, 622–629.
- Carotenuto, A.; Fattorusso, E.; Lanzotti, V.; Magno, S.; De Feo, V.; Cicala, C. The flavonoids of *Allium Neapolitanum*. *Phytochemistry* **1997**, *44*, 949–957.
- Carr, A. C.; McCall, M. R.; Frei, B. Oxidation of LDL by myeloperoxidase and reactive nitrogen species. Reaction pathways and antioxidant protection. *Arterioscl. Thromb. Vasc. Biol.* **2000**, *20*, 1716–1723.

- Cataisson, C.; Joseloff, E.; Murillas, R.; Wang, A.; Atwell, C.; Torgerson, S.; Gerdes, M.; Subleski, J.; Gao, J.-L.; Murphy, P. M.; Wiltrout, R. H.; Vinson, C.; Yuspa, S. H. Activation of cutaneous protein kinase C $\alpha$  induces keratinocyte apoptosis and intraepidermal inflammation by independent signaling pathways. *J. Immunol.* **2003**, *171*, 2703–13.
- Chabot, S.; Bel-Rhliid, R.; Chênevert, R.; Piché, Y. Hyphal growth promotion *in vitro* of the VA mycorrhizal fungus, *Gigaspora margarita* Becker and Hall, by the activity of structurally specific flavonoid compounds under CO<sub>2</sub>-enriched conditions. *New Phytol.* **1992**, *122*, 461–467.
- Chang, L.; Karin, M. Mammalian MAP kinase signalling cascades. *Nature* **2001**, *410*, 37–40.
- Cho, M. J.; Howard, L. R.; Prior, R. L.; Clark, J. R. Flavonoid glycosides and antioxidant capacity of various blackberry, blueberry and red grape genotypes determined by high-performance liquid chromatography/mass spectrometry. *J. Sci. Food Agric.* **2004**, *84*, 1771–1782.
- Chun, K.-S.; Cha, H.-H.; Shin, J.-W.; Na, H.-K.; Park, K.-K.; Chung, W.-Y.; Surh, Y.-J. Nitric oxide induces expression of cyclooxygenase-2 in mouse skin through activation of NF- $\kappa$ B. *Carcinogenesis* **2004**, *25*, 445–454.
- Chung, K.-T.; Wei, C.-I.; Johnson, M. G. Are tannins a double-edged sword in biology and health? *Trends Food Sci. Technol.* **1998**, *9*, 168–175.
- Clark, J. R. Changing times for eastern United States blackberries. *HortTechnology* **2005**, *15*, 491–494.
- Clark, J. R.; Moore, J. N. 'Ouachita' thornless blackberry. *HortScience* **2005**, *40*, 258–260.
- Clark, J. R.; Finn, C. E. New trends in blackberry breeding. *Acta Hort.* **2008**, *777*, 41–47.
- Clark, J. R.; Howard, L.; Talcott, S. Antioxidant activity of blackberry genotypes. *Acta Hort.* **2002**, *585*, 475–480.
- Clark, J. R.; Stafne, E. T.; Hall, H. K.; Finn, C. E. Blackberry breeding and genetics. In *Plant Breeding Reviews*. Janick, J., Ed.; Wiley and Sons: Hoboken, NJ, 2007; pp. 19–144.
- Clifford, M. N. Chlorogenic acids and other cinnamates - nature, occurrence and dietary burden. *J. Sci. Food Agric.* **1999**, *79*, 362–372.
- Clifford, M. N.; Scalbert, A. Ellagitannins - nature, occurrence and dietary burden. *J. Sci. Food Agric.* **2000**, *80*, 1118–1125.
- Cobb, M. H.; Goldsmith, E. J. How MAP kinases are regulated. *J. Biol. Chem.* **1995**, *270*, 14843–14846.

- Collett, G. P.; Campbell, F. C. Curcumin induces c-jun N-terminal kinase-dependent apoptosis in HCT116 human colon cancer cells. *Carcinogenesis* **2004**, *25*, 2183–2189.
- Collins, A. R. Antioxidant intervention as a route to cancer prevention. *Eur. J. Cancer* **2005**, *41*, 1923–1930.
- Cossins, E.; Lee, R.; Packer, L. ESR studies of vitamin C regeneration, order of reactivity of natural source phytochemical preparations. *Biochem. Mol. Biol. Intl.* **1998**, *45*, 583–597.
- Cowan, M. M. Plant products as antimicrobial agents. *Clin. Microbiol. Rev.* **1999**, *12*, 564–582.
- Crozier, A.; Clifford, M. N.; Jaganath, I. B. Phenols, polyphenols and tannins: an overview. In *Plant Secondary Metabolites: Occurrence, Structure and Role in the Human Diet*. Crozier, A.; Clifford, M. N.; Hiroshi, A., Eds.; Blackwell Publishing: Ames, Iowa, 2006; pp. 1–24.
- Crozier, A.; Jaganath, I. B.; Clifford, M. N. Dietary phenolics: chemistry, bioavailability and effects on health. *Nat. Prod. Rep.* **2009**, *26*, 1001–1043.
- Crozier, A.; Burns, J.; Aziz, A. A.; Stewart, A. J.; Rabiasz, H. S.; Jenkins, G. I.; Edwards, C. A.; Lean, M. E. J. Antioxidant flavonols from fruits, vegetables and beverages: measurements and bioavailability. *Biol. Res.* **2000**, *33*, 79–88.
- Cuyckens, F.; Claeys, M. Mass Spectrometry in the structural analysis of flavonoids. *J. Mass Spec.* **2004**, *39*, 1–15.
- Dai, J.; Gupte, A.; Gates, L.; Mumper, R. J. A comprehensive study of anthocyanin-containing extracts from selected blackberry cultivars: Extraction methods, stability, anticancer properties and mechanisms. *Food Chem. Toxicol.* **2009**, *47*, 837–847.
- Dalle-Donne, I.; Rossi, R.; Colombo, R.; Giustarini, D.; Milzani, A. Biomarkers of oxidative damage in human disease. *Clin. Chem.* **2006**, *52*, 601–623.
- Daniel, E. M.; Krubnick, A. S.; Heur, Y.-H.; Blinzler, J. A.; Nims, R. W.; Stoner, G. D. Extraction, stability, and quantitation of ellagic acid in various fruits and nuts. *J. Food Comp. Anal.* **1989**, *2*, 338–349.
- Dasgupta, B.; Milbrandt, J. Resveratrol stimulates AMP kinase activity in neurons. *PNAS* **2007**, *104*, 7217–7222.
- Dashwood, R. H. Frontiers in polyphenols and cancer prevention. *J. Nutr.* **2007**, *137*, 267S–269S.
- Davis, N.; Katz, S.; Wylie-Rosett, J. The effect of diet on endothelial function. *Cardiol. Rev.* **2007**, *15*, 62–66.

- Davis, R. J. Signal transduction by the JNK group of MAP kinases. *Cell* **2000**, *103*, 239–252.
- Day, A. J.; DuPont, M. S.; Ridley, S.; Rhodes, M.; Rhodes, M. J. C.; Morgan, M. R. A.; Williamson, G. Deglycosylation of flavonoid and isoflavonoid glycosides by human small intestine and liver  $\beta$ -glucosidase activity. *FEBS Lett.* **1998**, *436*, 71–75.
- Day, A. J.; Cañada, F. J.; Díaz, J. C.; Kroon, P. A.; Mclauchlan, R.; Faulds, C. B.; Plumb, G. W.; Morgan, M. R. A.; Williamson, G. Dietary flavonoid and isoflavone glycosides are hydrolysed by the lactase site of lactase phlorizin hydrolase. *FEBS Lett.* **2000**, *468*, 166–170.
- Dearlove, R. P.; Greenspan, P.; Hartle, D. K.; Swanson, R. B.; Hargrove, J. L. Inhibition of protein glycation by extracts of culinary herbs and spices. *J. Med. Food* **2008**, *11*, 275–281.
- Delcour, J. A.; Vandenberghe, M. M.; Corten, P. F.; Dondeyne, P. Flavor thresholds of polyphenolics in water. *Am. J. Enol. Vitic.* **1984**, *35*, 134–136.
- Dempsey, D. A.; Shah, J.; Klessig, D. F. Salicylic acid and disease resistance in plants. *Crit. Rev. Plant Sci.* **1999**, *18*, 547–575.
- Déprez, S.; Brezillon, C.; Rabot, S.; Philippe, C.; Mila, I.; Lapiere, C.; Scalbert, A. Polymeric proanthocyanidins are catabolized by human colonic microflora into low-molecular-weight phenolic acids. *J. Nutr.* **2000**, *130*, 2733–2738.
- Dewick, P. M. The shikimate pathway: aromatic amino acids and phenylpropanoids. In *Medicinal Natural Products: A Biosynthetic Approach*. Wiley and Sons: Hoboken, NJ, 2009; pp. 137–164.
- Dhalla, N. S.; Temsah, R. M.; Netticadan, T. Role of oxidative stress in cardiovascular diseases. *J. Hypertens.* **2000**, *18*, 655–673.
- Ding, M.; Feng, R.; Wang, S. Y.; Bowman, L.; Lu, Y.; Qian, Y.; Castranova, V.; Jiang, B.-H.; Shi, X. Cyanidin-3-glucoside, a natural product derived from blackberry, exhibits chemopreventive and chemotherapeutic activity. *J. Biol. Chem.* **2006**, *281*, 17359–17368.
- Diplok, A. T. Antioxidants and free radical scavengers. In *Free Radical Damage and its Control*. Rice-Evans, C. A.; Burdon R. H., Eds.; Elsevier: Amsterdam, The Netherlands, 1994; pp. 113–128.
- Dixon, R. A.; Paiva, N. L. Stress-induced phenylpropanoid metabolism. *Plant Cell* **1995**, *7*, 1085–1097.
- Dixon, R. A.; Achnine, L.; Kota, P.; Liu, C.-J.; Reddy, M. S. S.; Wang, L. The phenylpropanoid pathway and plant defence—a genomics perspective. *Mol. Plant Pathol.* **2002**, *3*, 371–390.

- Dixon, R. A.; Choudhary, A. D.; Dalkin, D.; Edwards, R.; Fahrendorf, T.; Gowri, G.; Harrison, M. J.; Lamb, C. J.; Loake, G. J.; Maxwell, C. A.; Orr, J.; Paiva, N. L. Molecular biology of stress induced phenylpropanoid and isoflavonoid biosynthesis in alfalfa. In *Phenolic Metabolism in Plants*. Stafford, H. A.; Ibrahim, R. K., Eds.; Springer-Verlag: New York, NY, 1992; pp. 91–138.
- Drewnowski, A.; Gomez-Carneros, C. Bitter taste, phytonutrients, and the consumer: a review. *Am. J. Clin. Nutr.* **2000**, *72*, 1424–1435.
- Dubois, G. E.; Crosby, G. A.; Saffron, P. Nonnutritive sweeteners: taste-structure relationships for some new simple dihydrochalcones. *Science* **1977**, *195*, 397–399.
- Durst, R. W.; Wrolstad, R. E. Separation and characterization of anthocyanins by HPLC. In *Handbook of Food Analytical Chemistry*. Wrolstad, R. E.; Acree, T. E.; Decker, E. A.; Penner, M. H.; Reid, D. S.; Schwartz, S. J.; Shoemaker, C. F.; Smith, D. M.; Sporns, P., Eds.; Wiley and Sons: Hoboken, NJ, 2005; pp. 33–45.
- Edwards, M. R.; Keadze, T.; Johnson, M. W.; Johnston, S. L. New treatment regimes for virus-induced exacerbations of asthma. *Pulm. Pharmacol. Ther.* **2006**, *19*, 320–334.
- Ellis, M. A.; Converse, R. H.; Williams, R. N.; Williamson, B. Downy mildew. In *Compendium of Raspberry and Blackberry Diseases and Insects*. Ellis, M. A.; Converse, R. H.; Williams, R. N.; Williamson, B., Eds.; APS Press: St. Paul, MN, 1991; pp. 15–16.
- Esposito, C.; Gerlach, H.; Brett, J.; Stern, D.; Vlassara H. Endothelial receptor-mediated binding of glucose-modified albumin is associated with increased monolayer permeability and modulation of cell surface coagulant properties. *J. Exp. Med.* **1989**, *170*, 1387–1407.
- Fan-Chiang, H.-J.; Wrolstad, R. E. Anthocyanin pigment composition of blackberries. *J. Food Sci.* **2005**, *70*, C198–C202.
- Fang Y.-Z.; Yang S.; Wu, G. Free radicals, antioxidants, and nutrition. *Nutrition* **2002**, *18*, 872–879.
- Farabegoli, F. Green tea in human cancer. *Minerva Biotec.* **2005**, *17*, 163–173.
- Farrar, J. L.; Hartle, D. K.; Hargrove, J. L.; Greenspan, P. Inhibition of protein glycation by skins and seeds of the muscadine grape. *BioFactors* **2007**, *30*, 193–200.
- Ferreira, D.; Slade, D.; Marais, J. P. J. Flavans and Proanthocyanidins. In *Flavonoids: Chemistry, Biochemistry and Applications*. Anderson, Ø. M.; Markham, K. R., Eds.; CRC Press: Boca Raton, FL, 2006; pp. 553 – 616.
- Finn, C. E. Blackberries. In *Temperate Fruit Crop Breeding: Germplasm to Genomics*. Hancock, J. F., Ed.; Springer Science: New York, NY, 2008; pp. 83–114.



- Freidovich, I. Fundamental aspects of reactive oxygen species, or what's the matter with oxygen? *Ann. N.Y. Acad. Sci.* **1999**, *893*, 13–18.
- Fu, M. X.; Wells-Knecht, K. J.; Blackledge, J. A.; Lyons, T. J.; Thorpe, S. R.; Baynes, J. W. Glycation, glycoxidation, and cross-linking of collagen by glucose. Kinetics, mechanisms, and inhibition of late stages of the Maillard reaction. *Diabetes* **1994**, *43*, 676–683.
- Fukumoto, L. R.; Mazza, G. Assessing antioxidant and prooxidants activities of phenolic compounds. *J. Agric. Food Chem.* **2000**, *48*, 3597–3604.
- Funk, C. D. Prostaglandins and leukotrienes: Advances in eicosanoid biology. *Science* **2001**, *294*, 1871–1875.
- Furst, D. E.; Ulrich, R. W. Non-steroidal anti-inflammatory drugs, disease-modifying antirheumatic drugs, nonpioid analgesics and drugs used in gout. In *Basic and Clinical Pharmacology*. Katzung, B. G., Ed. McGraw-Hill: Columbus, OH, 2007; pp. 573–598
- Gales, L.; Damas, A. M. Xanthonones-A structural perspective. *Curr. Med. Chem.* **2005**, *12*, 2499–2515.
- Galvano, F.; La Fauci, L.; Lazzarino, G.; Fogliano, V.; Ritieni, A.; Ciappellano, S.; Battistini, N. C.; Tavazzi, B.; Galvano, G. Cyanidins: metabolism and biological properties. *J. Nutr. Biochem.* **2004**, *15*, 2–11.
- Gambari, R. New trends for the development of novel decoy molecules against nuclear factor kappa-B (NF- $\kappa$ B) transcription factor. *Minerva Biotec.* **2004**, *16*, 145–149.
- Garcia-Alloza, M.; Borrelli, L. A.; Rozkalne, A.; Hyman, B. T.; Bacskai, B. J. Curcumin labels amyloid pathology *in vivo*, disrupts existing plaques, and partially restores distorted neurites in an Alzheimer mouse model. *J. Neurochem.* **2007**, *102*, 1095–1104.
- Gerondakis, S.; Grumont, R.; Rourke, I.; Grossmann, M. The regulation and roles of Rel/NF- $\kappa$ B transcription factors during lymphocyte activation. *Curr. Opin. Immunol.* **1998**, *10*, 353–359.
- Ghosh, S.; May, M. J.; Kopp, E. B. NF- $\kappa$ B and Rel proteins: Evolutionarily conserved mediators of immune responses. *Annu. Rev. Immunol.* **1998**, *16*, 225–260.
- Giusti, M. M.; Wrolstad, R. E. Characterization and measurement of anthocyanins by UV-visible spectroscopy. In *Current Protocols in Food Analytical Chemistry*. Wrolstad, R. E.; Schwartz, S. J., Eds.; Wiley and Sons: New York, NY, 2001; pp. F.1.2.1–F.1.2.13.

- Giusti, M. M.; Rodríguez-Saona, L. E.; Griffin, D.; Wrolstad, R. E. Electrospray and tandem mass spectroscopy as tools for anthocyanin characterization. *J. Agric. Food Chem.* **1999**, *47*, 4657–4664.
- Goel, G.; Puniya, A. K.; Aguilar, C. N.; Singh, K. Interaction of gut microflora with tannins in feeds. *Naturwissenschaften* **2005**, *92*, 497–503.
- Goldstein, J. L.; Swain, T. Changes in tannins in ripening fruits. *Phytochemistry* **1963**, *2*, 371–383.
- Gordon, M. H. The mechanism of antioxidant action *in vitro*. In *Food Antioxidants*. Hudson, B. J. F., Ed.; Elsevier: London, UK, 1990; pp. 1–18.
- Gouédard, C.; Barouki, R.; Morel, Y. Dietary polyphenols increase paraoxonase 1 gene expression by an aryl hydrocarbon receptor-dependent mechanism. *Mol. Cell Biol.* **2004**, *24*, 5209–5222.
- Gu, L.; Kelm, M. A.; Hammerstone, J. F.; Beecher, G.; Holden, J.; Haytowitz, D.; Prior, R. L. Screening of foods containing proanthocyanidins and their structural characterization using LC-MS/MS and thiolytic degradation. *J. Agric. Food Chem.* **2003**, *51*, 7513–7521.
- Gu, L.; Kelm, M. A.; Hammerstone, J. F.; Beecher, G.; Holden, J.; Haytowitz, D.; Gebhardt, S.; Prior, R. L. Concentrations of proanthocyanidins in common foods and estimations of normal consumption. *J. Nutr.* **2004**, *134*, 613–617.
- Guern, J.; Renaudin, J. P.; Brown, S. C. The compartmentation of secondary metabolites in plant cell cultures. In *Cell Culture in Phytochemistry*. Constabel, F.; Vasil, I. K., Eds.; Academic Press: London, UK, 1987; pp. 43–76.
- Gupta, R. K.; Al-Shafi, S. M. K.; Layden, K.; Haslam, E. The metabolism of gallic acid and hexahydroxydiphenic acid in plants. Part 2. Esters of (*S*)-hexahydroxydiphenic acid with D-glucopyranose ( $^4C_1$ ). *J. Chem. Soc. Perkin Trans. I* **1982**, 2525–2534.
- Hager, T. J.; Howard, L. R.; Liyanage, R.; Lay, J. O.; Prior, R. L. Ellagitannin composition of blackberry as determined by HPLC-ESI-MS and MALDI-TOF-MS. *J. Agric. Food Chem.* **2008**, *56*, 661–669.
- Hagerman, A. E.; Butler, L. G. Protein precipitation method for the quantitative determination of tannins. *J. Agric. Food Chem.* **1978**, *26*, 809–812.
- Hahlbrock, K.; Scheel, D. Physiology and molecular biology of phenylpropanoid metabolism. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **1989**, *40*, 347–369.
- Haidara, M. A.; Yassin, H. Z.; Rateb, M.; Ammar, H.; Zorkani, M. A. Role of oxidative stress in development of cardiovascular complications in diabetes mellitus. *Curr. Vasc. Pharmacol.* **2006**, *4*, 215–227.

- Hall, H. K. Blackberry breeding. In *Plant Breeding Reviews*. Janick, J., Ed.; Timber Press: Portland, OR, 1990; pp. 249–312.
- Hall, H. K.; Stephens, M. J.; Stanley, C. J.; Finn, C.; Yorgey, B. Breeding New 'Boysen' and 'Marion' cultivars. *Acta Hort.* **2002**, *585*, 91–95.
- Halliwell, B.; Chirico, S. Lipid peroxidation: its mechanism, measurement, and significance. *Am. J. Clin. Nutr.* **1993**, *57* (suppl.), 715S–725S.
- Halliwell, B.; Aruoma, O. I. DNA damage by oxygen-derived species. Its mechanisms and measurement in mammalian systems. *FEBS Lett.* **1991**, *281*, 9–19.
- Halliwell, B. Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. *Plant Physiol.* **2006**, *141*, 312–322.
- Halliwell, B.; Murcia, M. A.; Chirico, S.; Aruoma, O. I. Free radicals and antioxidants in food and *in vivo*: what they do and how they work. *Crit. Rev. Food Sci. Nutr.* **1995**, *35*, 7–20.
- Halvorsen, B. L.; Carlsen, M. H.; Phillips, K. M.; Bøhn, S. K.; Holte, K.; Jacobs, D. R., Jr.; Blomhoff, R. Content of redox-active compounds (ie, antioxidants) in foods consumed in the United States. *Am. J. Clin. Nutr.* **2006**, *84*, 95–135.
- Hammes, H.-P.; Alt, A.; Niwa, T.; Clausen, J. T.; Bretzel, R. G.; Brownlee, M.; Schleicher, E. D. Differential accumulation of advanced glycation end products in the course of diabetic retinopathy. *Diabetologia* **1999**, *42*, 728–736.
- Han, X.; Shen, T.; Lou, H. Dietary polyphenols and their biological significance. *Int. J. Mol. Sci.* **2007**, *8*, 950–988.
- Hanumura, T.; Aoki, H. Toxicological evaluation of polyphenol extract from acerola (*Malpighia emarginata* DC.) fruit. *J. Food Sci.* **2008**, *73*, T55–T61.
- Harborne, J. B. The anthocyanins pigments. In *Comparative Biochemistry of the Flavonoids*. Harborne, J. B., Ed.; Academic Press: New York, NY, 1996; pp. 1–36.
- Harborne, J. B. Twenty-five years of chemical ecology. *Nat. Prod. Rep.* **2001**, *18*, 361–379.
- Harborne, J. B.; Williams, C. A. Flavone and flavonol glycosides. In *The Flavonoids*. Harborne, J. B.; Mabry, T. J.; Mabry, H., Eds.; Chapman and Hall: London, UK, 1976; pp. 376–443.
- Harborne, J. B.; Boardley, M. Trisubstituted flavonol glycosides in *Coronilla Emerus* flowers. *Phytochemistry* **1983**, *22*, 622–625.
- Harborne, J. B.; Williams, C. A. Advances in flavonoid research since 1992. *Phytochemistry* **2000**, *55*, 481–504.

- Harborne, J. B.; Baxter, H.; Moss G. P. Phenols and phenolic acid. In *Phytochemical Dictionary: A Handbook of Bioactive Compounds from Plant*. Harborne, J. B.; Baxter, H.; Moss G. P., Eds.; Taylor and Francis: Philadelphia, PA, 1999; pp. 496–509.
- Harman, D. Aging: A theory based on free radical and radiation chemistry. *J. Gerontol.* **1956**, *11*, 298–300.
- Harman, D. Role of antioxidant nutrients in aging: Overview. *Age* **1995**, *18*, 51–62.
- Hartman, R. E.; Shah, A.; Fagan, A. M.; Schwetye, K. E.; Parsadanian, M.; Schulman, R. N.; Finn, M. B.; Holtzman, D. M. Pomegranate juice decreases amyloid load and improves behavior in a mouse model of Alzheimer's disease. *Neurobiol. Dis.* **2006**, *24*, 506–515.
- Haslam, E. Polyphenols-structure and biosynthesis. In *Practical Polyphenolics: From Structure to Molecular Recognition and Physiological Action*. Cambridge University Press: Cambridge, UK, 1998; pp. 10–70.
- Hassan, H. A.; Yousf, M. I. Mitigating effects of antioxidant properties of black berry juice on sodium fluoride induced hepatotoxicity and oxidative stress in rats. *Food Chem. Toxicol.* **2009**, *47*, 2332–2337.
- Hasselblatt, P.; Gresh, L.; Kudo, H.; Guinea-Viniegra, J.; Wagner, E. F. The role of the transcription factor AP-1 in colitis-associated and  $\beta$ -catenin-dependent intestinal tumorigenesis in mice. *Oncogene* **2008**, *27*, 6102–6109.
- Hassimotto, N. M. A.; da Silva Pinto, M.; Lajolo, F. M. Antioxidant status in humans after consumption of blackberry (*Rubus fruticosus* L.) juices with and without defatted milk. *J. Agric. Food Chem.* **2008**, *56*, 11727–11733.
- Hassimotto, N. M. A.; da Mota, R. V.; Cordenuns, B. R.; Lajolo, F. M. Physico-chemical characterization and bioactive compounds of blackberry fruits (*Rubus* sp.) grown in Brazil. *Ciênc. Tecnol. Aliment.* **2008**, *28*, 702–708.
- Hawker, J. S.; Buttrose, M. S.; Soeffky, A.; Possigham, J. V. A simple method for demonstrating macroscopically the location of polyphenolic compounds in grape berries. *Vitis* **1972**, *11*, 189–192.
- Hayase, F.; Shibuya, T.; Sato, J.; Yamamoto, M. Effects of oxygen and transition metals on the advanced Maillard reaction of proteins with glucose. *Biosci. Biotechnol. Biochem.* **1996**, *60*, 1820–1825.
- Hayes, J. D.; McLellan, L. I. Glutathione and glutathione-dependent enzymes represent co-ordinately regulated defence against oxidative stress. *Free Radic. Res.* **1999**, *31*, 273–300.

- Heller, W.; Forkman, G. Biosynthesis. In *Flavonoids: Advances in Research since 1980*. Harborne, J. B., Ed.; Chapman and Hall: London, UK, 1988, pp. 389–425.
- Hensley, K.; Mou, S.; Pye, Q. N.; Dixon, R. A.; Summner, L. W.; Floyd, R. A. Chemical versus pharmacological actions of nutraceutical phytochemicals: Antioxidant and anti-inflammatory modalities. *Curr. Top. Nutr. Res.* **2004**, *2*, 13–26.
- Herrmann, K. M. The shikimate pathway: early steps in the biosynthesis of aromatic compounds. *Plant Cell* **1995**, *7*, 907–919.
- Herschman, H. R. Prostaglandin synthase 2. *Biochim. Biophys. Acta* **1996**, *1299*, 125–140.
- Hollman, P. C. H.; Hertog, M. G. L.; Katan, M. B. Analysis and health effects of flavonoids. *Food Chem.* **1996**, *57*, 43–46.
- Holton, T. A.; Cornish, E. C. Genetics and biochemistry of anthocyanins biosynthesis. *Plant Cell* **1995**, *7*, 1071–1083.
- Hong, V.; Wrolstad, R. E. Use of HPLC separation/photodiode array detection for characterization of anthocyanins. *J. Agric. Food Chem.* **1990**, *38*, 708–715.
- Hou, D.-X. Potential mechanisms of cancer chemoprevention by anthocyanins. *Curr. Mol. Med.* **2003**, *3*, 149–159.
- Hou, D.-X.; Yanagita, T.; Uto, T.; Masuzaki, S.; Fujii, M. Anthocyanidins inhibit cyclooxygenase-2 expression in LPS-evoked macrophages: Structure-activity relationship and molecular mechanisms involved. *Biochem. Pharmacol.* **2005**, *70*, 417–425.
- Huang, C. J.; Zayas, J. F. Phenolic acid contributions to taste characteristics of corn germ protein flour products. *J. Food Sci.* **1991**, *56*, 1308–1310.
- Huang, D.; Ou, B.; Prior, R. L. The chemistry behind antioxidant capacity assays. *J. Agric. Food Chem.* **2005**, *53*, 1841–1856.
- Huang, D.; Ou, B.; Hampsch-Woodill, M.; Flanagan, J. A.; Deemer, E. K. Development and validation of oxygen radical absorbance capacity assay for lipophilic antioxidants using randomly methylated  $\beta$ -cyclodextrin as the solubility enhancer. *J. Agric. Food Chem.* **2002**, *50*, 1815–1821.
- Hughes, D. A. Effects of dietary antioxidants on the immune function of middle-aged adults. *Proc. Nutr. Soc.* **1999**, *58*, 79–84.
- Ibrahim, R.; Barron, D. Phenylpropanoids. In *Methods in Plant Biochemistry*. Dey, P. M.; Harborne, J. B., Eds.; Academic Press: London, UK, 1989; pp. 75–111.

- Isman, M. B.; Duffey, S. S. Toxicity of tomato phenolic compounds to the fruitworm, *Heliothis Zea. Ent. Exp. Appl.* **1982**, *31*, 370–376.
- Issa, A. Y.; Volate, S. R.; Wargovich, M. J. The role of phytochemicals in inhibition of cancer and inflammation: New directions and perspectives. *J. Food Comp. Anal.* **2006**, *19*, 405–419.
- Iwashima, Y.; Eto, M.; Horiuchi, S.; Sano, H. Advanced glycation end product-induced peroxisome proliferators-activated receptor  $\gamma$  gene expression in the cultured mesangial cells. *Biochem. Biophys. Res. Commun.* **1999**, *264*, 441–448.
- Iwashina, T. The structure and distribution of flavonoids in plants. *J. Plant. Res.* **2000**, *113*, 287–299.
- Jacobs, M.; Rubery, P. H. Naturally occurring auxin transport regulators. *Science* **1988**, *241*, 346–349.
- Jenner, P. Oxidative stress in Parkinson's disease. *Ann. Neurol.* **2003**, *53* (suppl.), S26–S36.
- Kafkas, E.; Kosar, M.; Türemiş, N.; Başer, K. H. C. Analysis of sugars, organic acids and vitamin C contents of blackberry genotypes from Turkey. *Food Chem.* **2006**, *97*, 732–736.
- Kallithraka, S.; Bakker, J.; Clifford, M. N. Evaluation of bitterness and astringency of (+)-catechin and (-)-catechin in red wine and in model solutions. *J. Sens. Stud.* **1997**, *12*, 25–37.
- Kantz, K.; Singleton, V. L. Isolation and determination of polymeric polyphenols using Sephadex LH-20 and analysis of grape tissue extracts. *Am. J. Enol. Vitic.* **1990**, *41*, 223–228.
- Karin, M. The regulation of AP-1 activity by mitogen-activated protein kinases. *J. Biol. Chem.* **1995**, *270*, 16483–16486.
- Karlsen, A.; Retterstøl, L.; Laake, P.; Paur, I.; Kjølrsrud-Bøhn, S.; Sandvik, L.; Blomhoff, R. Anthocyanins inhibit nuclear factor- $\kappa$ B activation in monocytes and reduce plasma concentrations of pro-inflammatory mediators in healthy adults. *J. Nutr.* **2007**, *137*, 1951–1954.
- Karthikeyan, K.; Bai, B. R. S.; Devaraj, S. N. Grape seed proanthocyanidins ameliorates isoproterenol-induced myocardial injury in rats by stabilizing mitochondrial and lysosomal enzymes: An in vivo study. *Life Sci.* **2007**, *81*, 1615–1621.
- Kaur, M.; Agarwal, R. Transcription factors: Molecular targets for prostate cancer intervention by phytochemicals. *Curr. Cancer Drug Targets* **2007**, *7*, 355–367.

- Kim, D. O.; Lee, C. Y. HPLC separation of polyphenolics. In *Handbook of Food Analytical Chemistry: Pigments, Colorants, Flavors, Texture, and Bioactive Food Components*. Wrolstad, R. E.; Acree, T. E., Eds.; Wiley and Sons: Hoboken, NJ, 2005; pp. 483–498.
- Kim, H. Y.; Kim, K. Protein glycation inhibitory and antioxidative activities of some plant extracts in vitro. *J. Agric. Food Chem.* **2003**, *51*, 1586–1591.
- Kobayashi, Y.; Suzuki, M.; Hideo, S.; Arai, S.; Hara, Y.; Suzuki, K.; Miyamoto, Y.; Shimizu, M. Green tea polyphenols inhibit the sodium-dependent glucose transporter of intestinal epithelial cells by a competitive mechanism. *J. Agric. Food Chem.* **2000**, *48*, 5618–5623.
- Kong, J.-M.; Chia, L.-S.; Goh, N.-K.; Chia, T.-F.; Brouillard, R. Analysis and biological activities of anthocyanins. *Phytochemistry* **2003**, *64*, 923–933.
- Kraemer-Schafhalter, A.; Fuchs, H.; Pfannhauser, W. Solid-phase extraction (SPE)-a comparison of 16 materials for the purification of anthocyanins from *Aronia melanocarpa* var nero. *J. Sci. Food Agric.* **1998**, *78*, 435–440.
- Kraushofer, T.; Sontag, G. Determination of some phenolic compounds in flax seed and nettle roots by HPLC with coulometric electrode array detection. *Eur. Food Res. Technol.* **2002**, *215*, 529–533.
- Krewer, G.; Fonseca, M.; Brannen, P.; Horton, D. (December 2004). Home garden raspberries and blackberries; URL: (<http://pubs.caes.uga.edu/caespubs/pubs/PDF/C766.pdf>) (accessed August 2009), Cooperative Extension Service, The University of Georgia, College of Agricultural and Environmental Sciences.
- Kumar, V.; Abbas, A. K.; Fausto, N.; Mitchell, R. N. Acute and chronic inflammation. In *Robbins Basic Pathology*. Saunders Elsevier: Philadelphia, PA., 2006; pp. 31–58.
- Kundu, J. K.; Surh, Y.-J. Inflammation: Gearing the journey to cancer. *Mutat. Res.* **2008**, *659*, 15–30.
- Kundu, J. K.; Chun, K.-S.; Kim, S. O.; Surh, Y.-J. Resveratrol inhibits phorbol ester-induced cyclooxygenase-2 expression in mouse skin: MAPKs and AP-1 as potential molecular targets. *BioFactors* **2004**, *21*, 33–39.
- Kundu, J. K.; Na, H.-K.; Chun, K.-S.; Kim, Y.-K.; Lee, S. J.; Lee, S. S.; Lee, O.-S.; Sim, Y.-C.; Surh, Y.-J. Inhibition of phorbol ester-Induced COX-2 expression by epigallocatechin gallate in mouse skin and cultured human mammary epithelial cells. *J. Nutr.* **2003**, *133*, 3805S–3810S.
- Kunnumakkara, A. B.; Guha, S.; Krishnan, S.; Diagaradjane, P.; Gelovani, J.; Aggarwal, B. B. Curcumin potentiates antitumor activity of gemcitabine in an orthotopic model of pancreatic cancer through suppression of proliferation, angiogenesis, and inhibition of nuclear factor- $\kappa$ B-regulated gene products. *Cancer Res.* **2007**, *67*, 3853–3861.

- Kyle, J. A. M.; Duthie, G. G. Flavonoids in foods. In *Flavonoids Chemistry, Biochemistry and Applications*. Anderson, Ø. M.; Markham, K. R., Eds.; CRC Press: Boca Raton, FL, 2006; pp. 219–262.
- Kyriakis, J. M.; Avruch, J. Protein kinase cascades activated by stress and inflammatory cytokines. *BioEssays* **1996**, *18*, 567–577.
- Lapolla, A.; Traldi, P.; Fedele, D. Importance of measuring products of non-enzymatic glycation of proteins. *Clin. Biochem.* **2005**, *38*, 103–115.
- Lee, J. L.; Mukhtar, H.; Bickers, D. R.; Kopelovich, L.; Athar, M. Cyclooxygenases in the skin: pharmacological and toxicological implications. *Toxicol. Appl. Pharmacol.* **2003**, *192*, 294–306.
- Lee, K. W.; Kundu, J. K.; Kim, S. O.; Chun, K.S.; Lee, H. J.; Surh, Y.-J. Cocoa polyphenols inhibit phorbol ester-induced superoxide anion formation in cultured HL-60 cells and expression of cyclooxygenase-2 and activation of NF- $\kappa$ B and MAPKs in mouse skin in vivo. *J. Nutr.* **2006**, *136*, 1150–1155.
- Lee, S.-J.; Lee, I.-S.; Mar, W. Inhibition of inducible nitric oxide synthase and cyclooxygenase-2 activity by 1,2,3,4,6-penta-*O*-galloyl- $\beta$ -D-glucose in murine macrophage cells. *Arch. Pharm. Res.* **2003**, *26*, 832–839.
- Leese, H. J.; Semenza, G. On the identity between the small intestinal enzymes phlorizin hydrolase and glycosylceramidase. *J. Biol. Chem.* **1973**, *248*, 8170–8173.
- Leopoldini, M.; Marino, T.; Russo, N.; Toscano, M. Antioxidant properties of phenolic compounds: H-atom versus electron transfer mechanism. *J. Phys. Chem. A* **2004**, *108*, 4916–4922.
- Lev-Yadun, S.; Gould, K. S. Role of anthocyanin in plant defence. In *Anthocyanins*. Gould, K.; Davies, K.; Winefield, C., Eds.; Springer Science: New York, NY, 2009; pp. 21–48.
- Li, Y.-T.; Shen, F.; Liu, B.-H.; Cheng, G.-F. Resveratrol inhibits matrix metalloproteinase-9 transcription in U937 cells. *Acta. Pharmacol. Sin.* **2003**, *24*, 1167–1171.
- Lin, J. H.; Chiba, M.; Baillie, T. A. Is the role of the small intestine in first-pass metabolism over-emphasized? *Pharmacol. Rev.* **1999**, *51*, 135–157.
- Lin, J.-H.; Chiou, Y.-N.; Lin, Y.-L. Phenolic glycosides from *Viscum angulatum*. *J. Nat. Prod.* **2002**, *65*, 638–640.
- Ma, Y.-L.; Cuyckens, F.; van den Heuvel, H.; Claeys, M. Mass spectrometric methods for the characterisation and differentiation of isomeric *O*-diglycosyl flavonoids. *Phytochem. Anal.* **2001**, *12*, 159–165.



- Maas, J. L.; Galletta, G. J.; Stoner, G. D. Ellagic acid, an anticarcinogen in fruits, especially in strawberries: A review. *HortScience* **1991**, *26*, 10–14.
- Määttä, K. R.; Kamal-Eldin, A.; Törrönen, A. R. High performance liquid chromatography (HPLC) analysis of phenolic compounds in berries with diode array and electrospray ionization mass spectrometric (MS) detection: *Ribes* species. *J. Agric. Food Chem.* **2003**, *51*, 6736–6744.
- Määttä-Riihinen, K. R.; Kamal-Eldin, A.; Törrönen, A. R. Identification and quantification of phenolic compounds in berries of *Fragaria* and *Rubus* species (Family Rosaceae). *J. Agric. Food Chem.* **2004**, *52*, 6178–6187.
- Macheix, J. J.; Fleuriet, A.; Billot, J. The main phenolics of fruits. In *Fruit Phenolics*. Macheix, J. J.; Fleuriet, A.; Billot, J., Eds.; CRC Press: Boca Raton, FL, 1990; pp. 1–99.
- Makita, Z.; Bucala, R.; Rayfield, E. J.; Fuh, H.; Manogue, K. R.; Cerami, A.; Vlassara, H.; Friedman, E. A.; Kaufman, A. M.; Korbet, S. M.; Barth, R. H.; Winston, J. A. Reactive glycosylation endproducts in diabetic uraemia and treatment of renal failure. *Lancet* **1994**, *343*, 1519–1522.
- Manach, C.; Scalbert, A.; Morand, C.; Rémésy, C.; Jiménez, L. Polyphenols: food sources and bioavailability. *Am. J. Clin. Nutr.* **2004**, *79*, 727–747.
- Mann, J. Natural products: primary and secondary metabolism. In *Secondary Metabolism*. Oxford University Press: New York, NY, 1987; pp. 1–24.
- Marschner, H. Root-induced changes in the availability of micronutrients in the rhizosphere. In *Plant Roots, the Hidden Half*. Waisel, Y.; Eshel, Y.; Kafkafi, U., Eds.; Marcel Dekker: New York, NY, 1991; pp. 503–528.
- Marwan, A. G.; Nagel, C. W. Separation and purification of hydroxycinnamic acid derivatives in cranberries. *J. Food Sci.* **1982**, *47*, 585–588.
- Masella, R.; Di Benedetto, R.; Vari, R.; Filesi, C.; Giovannini, C. Novel mechanisms of natural antioxidant compounds in biological systems: involvement of glutathione and glutathione-related enzymes. *J. Nutr. Biochem.* **2005**, *16*, 577–586.
- Mason, T. L.; Wasserman, B. P. Inactivation of red beet  $\beta$ -glucan synthase by native and oxidized phenolic compounds. *Phytochemistry* **1987**, *26*, 2197–2202.
- Mazza, G.; Cacace, J. E.; Kay, C. D. Methods of analysis for anthocyanins in plants and biological fluids. *J. AOAC Int.* **2004**, *87*, 129–145.

- McDougall, G. J.; Shpiro, F.; Dobson, P.; Smith, P.; Blake, A.; Stewart, D. Different polyphenolic components of soft fruits inhibits  $\alpha$ -amylase and  $\alpha$ -glucosidase. *J. Agric. Food Chem.* **2005**, *53*, 2760–2766.
- Medeiros, R.; Otuki, M. F.; Avellar, M. C. W.; Calixto, J. B. Mechanisms underlying the inhibitory actions of the pentacyclic triterpene  $\alpha$ -amyryn in the mouse skin inflammation induced by phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate. *Eur. J. Pharmacol.* **2007**, *559*, 227–235.
- Meiers, S.; Kemény, M.; Weyand, U.; Gastpar, R.; von Angerer, E.; Marko, D. The anthocyanidins cyanidin, and delphinidin are potent inhibitors of the epidermal growth-factor receptor. *J. Agric. Food Chem.* **2001**, *49*, 958–962.
- Merken, H. M.; Beecher, G. R. Measurement of food flavonoids by high-performance liquid chromatography: A review. *J. Agric. Food Chem.* **2000**, *48*, 577–599.
- Mertz, C.; Cheynier, V.; Günata, Z.; Brat, P. Analysis of phenolic compounds in two blackberry species (*Rubus glaucus* and *Rubus adenotrichus*) by high-performance liquid chromatography with diode array detection and electrospray ion trap mass spectrometry. *J. Agric. Food Chem.* **2007**, *55*, 8616–8624.
- Meydani, S. N.; Wu, D.; Santos, M. S.; Hayek, M. G. Antioxidants and immune response in aged persons: overview of present evidence. *Am. J. Clin. Nutr.* **1995**, *62* (suppl.), 1462S–1476S.
- Middleton, E., Jr.; Kandaswami, C.; Theoharides, T. C. The effects of plant flavonoids on mammalian cells: Implications for inflammation, heart disease, and cancer. *Pharmacol. Rev.* **2000**, *52*, 673–751.
- Miller, D. M.; Buettner, G. R.; Aust, S. D. Transition metals as catalysts of "autoxidation" reactions. *Free Radic. Biol. Med.* **1990**, *8*, 95–108.
- Mittal, A.; Elmets, C. A.; Katiyar, S. K. Dietary feeding of proanthocyanidins from grape seeds prevents photocarcinogenesis in SKH-1 hairless mice: relationship to decreased fat and lipid peroxidation. *Carcinogenesis* **2003**, *24*, 1379–1388.
- Miyata, T.; van Ypersele de Strihou, C.; Kurokawa, K.; Baynes, J. W. Alterations in nonenzymatic biochemistry in uremia: Origin and significance of "carbonyl stress" in long-term uremic complications. *Kidney Int.* **1999**, *55*, 389–399.
- Mo, Y.; Nagel, C.; Taylor, L. P. Biochemical complementation of chalcone synthase mutants defines a role for flavonols in functional pollen. *PNAS* **1992**, *89*, 7213–7217.
- Moing, A.; Renaud, C.; Gaudillère, M.; Raymond, P.; Roudeillac, P.; Denoyes-Rothan, B. Biochemical changes during fruit development of four strawberry cultivars. *J. Amer. Soc. Hort. Sci.* **2001**, *126*, 394–403.

- Molina, M. F.; Sanchez-Reus, I.; Iglesias, I.; Benedi, J. Quercetin, a flavonoid antioxidant, prevents and protects against ethanol-induced oxidative stress in mouse liver. *Biol. Pharm. Bull.* **2003**, *26*, 1398-1402
- Monties, B. Lignins. In *Methods in Plant Biochemistry*. Dey, P. M.; Harborne, J. B., Eds.; Academic Press: London, UK, 1989; pp. 113–157.
- Montpied, P.; de Bock, F.; Rondouin, G.; Niel, G.; Briant, L.; Courseau, A. S.; Lerner-Natoli, M.; Bockaert, J. Caffeic acid phenethyl ester (CAPE) prevents inflammatory stress in organotypic hippocampal slice cultures. *Mol. Brain. Res.* **2003**, *115*, 111–120.
- Moolwaney, A. S.; Igwe, O. J. Regulation of the cyclooxygenase-2 system by interleukin-1  $\beta$  through mitogen-activated protein kinase signaling pathways: A comparative study of human neuroglioma and neuroblastoma cells. *Mol. Brain. Res.* **2005**, *137*, 202–212.
- Moore, A. B.; Francis, F. J.; Clydesdale, F. M. Changes in the chromatographic profile of anthocyanins of red onion during extraction. *J. Food Prot.* **1982**, *45*, 738–743.
- Moskowitz, A. H.; Hrazdina, G. Vacuolar contents of fruit subepidermal cells from *Vitis* species. *Plant Physiol.* **1981**, *68*, 686–692.
- Moyer, R. A.; Hummer, K. E.; Finn, C. E.; Frie, B.; Wrolstad, R. E. Anthocyanins, phenolics, and antioxidant capacity in diverse small fruits: *vaccinium*, *rubus*, and *ribes*. *J. Agric. Food Chem.* **2002**, *50*, 519–525.
- Mueller, M. M. Inflammation in epithelial skin tumours: Old stories and new ideas. *Eur. J. Cancer* **2006**, *42*, 735–744.
- Mueller-Harvey, I. Analysis of hydrolysable tannins. *Anim. Feed Sci. Technol.* **2001**, *91*, 3–20.
- Mullen, W.; Yokota, T.; Lean, M. E. J.; Crozier, A. Analysis of ellagitannins and conjugates of ellagic acid and quercetin in raspberry fruits by LC-MS<sup>n</sup>. *Phytochemistry* **2003**, *64*, 617–624.
- Murakawa, M.; Yamaoka, K.; Tanaka, Y.; Fakuda, Y. Involvement of tumor necrosis factor (TNF)- $\alpha$  in phorbol ester-12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced skin edema in mice. *Biochem. Pharmacol.* **2006**, *71*, 1331–1336.
- Murphy, J.-E.; Robert, C.; Kupper, T. S. Interleukin-1 and cutaneous inflammation: A crucial link between innate and acquired immunity. *J. Invest. Dermatol.* **2000**, *114*, 602–608.
- Myhrstad, M. C.; Carlsen, H.; Nordström, O.; Blomhoff, R.; Moskaug, J. Ø. Flavonoids increase the intracellular glutathione level by transactivation of the  $\gamma$ -glutamylcysteine synthetase catalytical subunit promoter. *Free Radic. Biol. Med.* **2002**, *32*, 386–393.

- Naczki, M.; Shahidi, F. Extraction and analysis of phenolics in food. *J. Chromatogr. A* **2004**, *1054*, 95–111.
- Naczki, M.; Shahidi, F.; Sullivan, A. Recovery of rapeseed tannins by various solvent systems. *Food Chem.* **1992**, *45*, 51–54.
- Naim, M.; Striem, B. J.; Kanner, J.; Peleg, H. Potential of ferulic acid as a precursor to off-flavors in stored orange juice. *J. Food Sci.* **1988**, *53*, 500–504.
- Nauseef, W. M. Contributions of myeloperoxidase to proinflammatory events: More than an antimicrobial system. *Int. J. Hematol.* **2001**, *74*, 125–133.
- Necela, B. M.; Cidlowski, J. A. Mechanisms of glucocorticoid receptor action in noninflammatory and inflammatory cells. *Proc. Am. Thorac. Soc.* **2004**, *1*, 239–246.
- Németh, K.; Plumb, G. W.; Berrin, J.-G.; Juge, R.; Naim, H. Y.; Williamson, G.; Swallow, D. M.; Kroon, P. A. Deglycosylation by small intestinal epithelial cell  $\beta$ -glucosidases is a critical step in the absorption and metabolism of dietary flavonoid glycosides in humans. *Eur. J. Nutr.* **2003**, *42*, 29–42.
- Nichenametla, S. N.; Taruscio, T. G.; Barney, D. L.; Exon, J. H. A review of the effects and mechanisms of polyphenolics in cancer. *Crit. Rev. Food Sci. Nutr.* **2006**, *46*, 161–183.
- Nielsen, S. E.; Breinholt, V.; Justesen, U.; Cornett, C.; Dragsted, L. O. *In vitro* biotransformation of flavonoids by rat liver microsomes. *Xenobiotica* **1998**, *28*, 389–401.
- Nijveldt, R. J.; van Nood, E.; van Hoorn, D. E. C.; Boelens, P. G.; van Norren, K.; van Leuwen, P. A. M. Flavonoids: a review of probable mechanisms of action and potential applications. *Am. J. Clin. Nutr.* **2001**, *74*, 418–425.
- Nilsson, J.; Pillai, D.; Önning, G.; Persson, C.; Nilsson, Å.; Åkesson, B. Comparison of the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and ferric reducing antioxidant power (FRAP) methods to assess the total antioxidant capacity in extracts of fruit and vegetables. *Mol. Nutr. Food Res.* **2005**, *49*, 239–246.
- Nishinaka, T.; Ichijo, Y.; Ito, M.; Kimura, M.; Katsuyama, M.; Iwata, K.; Miura, T.; Terada, T.; Yabe-Nishimura, C. Curcumin activates human glutathione S-transferase P1 expression through antioxidant response element. *Toxicol. Lett.* **2007**, *170*, 238–247.
- Noda, Y.; Kaneyuki, T.; Mori, A.; Packer, L. Antioxidant activities of pomegranate fruit extract and its anthocyanidins: Delphinidin, cyanidin, and pelargonidin. *J. Agric. Food Chem.* **2002**, *50*, 166–171.
- Nohara, Y.; Usui, T.; Kinoshita, T.; Watanabe, M. Generation of superoxide anions during the reaction of guanidine compounds with methylglyoxal. *Chem. Pharm. Bull* **2002**, *50*, 179–184.

- Nørbæk, R.; Kondo, T. Flavonol glycosides from flowers of *Crocus speciosus* and *C. antalyensis*. *Phytochemistry* **1999**, *51*, 1113–1119.
- Ntamila, M. S.; Hassanali, A. Isolation of oil of clove and separation of eugenol and acetyle eugenol. *J. Chem. Educ.* **1976**, *53*, 263–267.
- Odetti, P. R.; Borgoglio, A.; De Pascale, A.; Rolandi, R.; Adezati, L. Prevention of diabetes-increased aging effect on rat collagen-linked fluorescence by *aminoguanidine* and *rutin*. *Diabetes* **1990**, *39*, 796–801.
- Okuda, T.; Yosuda, T.; Hatano, T.; Ito, H. Biosynthesis of ellagitannins. In *Chemistry and Biology of Ellagitannins: An Underestimated Class of Bioactives Plant Polyphenols*. Quideau, S., Ed.; World Scientific Publishing: Hackensack, NJ, 2009; pp. 94–118.
- O’Leary, K. A.; de Pascual-Tereasa, S.; Needs, P. W.; Bao, Y.-P.; O’Brien, N. M.; Williamson, G. Effect of flavonoids and vitamin E on cyclooxygenase-2 (COX-2) transcription. *Mutat. Res.* **2004**, *551*, 245–254.
- Osawa, R.; Kuroiso, K.; Goto, S.; Shimizu, A. Isolation of tannin-degrading lactobacilli from humans and fermented foods. *Appl. Environ. Microbiol.* **2000**, *66*, 3093–3097.
- Oya, T.; Osawa, T.; Kawakishi, S. Spice constituents scavenging free radicals and inhibiting pentosidine formation in a model system. *Biosci. Biotechnol. Biochem.* **1997**, *61*, 263–266.
- Packer, L. Oxidants and antioxidants: mechanism of action and regulation of gene expression by bioflavonoids. In *Oxidative Stress, Inflammation, and Health*. Surh, Y.; Packer, L., Eds.; CRC Press: Boca Raton, FL, 2005; pp. 1–20.
- Pahl, H. L. Activators and target genes of Rel/NF- $\kappa$ B transcription factors. *Oncogene* **1999**, *18*, 6853–6866.
- Papandreou, M. A.; Dimakopoulou, A.; Linardaki, Z. I.; Cordopatis, P.; Klimis-Zacas, D.; Margarity, M.; Lamari, F. N. Effect of a polyphenol-rich wild blueberry extract on cognitive performance of mice, brain antioxidant markers and acetylcholinesterase activity. *Behav. Brain Res.* **2009**, *198*, 352–358.
- Pedraza-Chaverri, J.; Cárdenas-Rodríguez, N.; Orozco-Ibarra, M.; Pérez-Rojas, J. M. Medicinal properties of mangosteen (*Garcinia mangostana*). *Food Chem. Toxicol.* **2008**, *46*, 3227–3239.
- Pedrielli, P.; Skibsted, L. H. Antioxidant synergy and regeneration effect of quercetin, (-)-epicatechin, and (+)-catechin on  $\alpha$ -tocopherol in homogenous solutions of peroxidating methyl linoleate. *J. Agric. Food Chem.* **2002**, *50*, 7138–7144.

- Pellegrini, N.; Serafini, M.; Colombi, B.; del Rio, D.; Salvatore, S.; Bianchi, M.; Brighenti, F. Total antioxidant capacity of plant foods, beverages and oils consumed in Italy assessed by three different in vitro assays. *J. Nutr.* **2003**, *133*, 2812–2819.
- Pergola, C.; Rossi, A.; Dugo, P.; Cuzzocrea, S.; Sautebin, L. Inhibition of nitric oxide biosynthesis by anthocyanin fraction of blackberry extract. *Nitric Oxide* **2006**, *15*, 30–39.
- Pereira, D. M.; Valentão, P.; Pereira, J. A.; Andrade, P. B. Phenolics: From chemistry to biology. *Molecules* **2009**, *14*, 2202–2211.
- Pérez-Illarbe, J.; Hernández, T.; Estrella, I. Phenolic compounds in apples: varietal differences. *Z. Lebensm. Unters. Forsch.* **1991**, *192*, 551–554.
- Perkins-Veazie, P.; Clark, J. R. Blackberry research in Arkansas and Oklahoma. *Proc. N. Amer. Bramble Growers Assn. Ann. Mtg.* **2005**, 39–42.
- Picman, A. K.; Schneider, E. F.; Picman, J. Effect of flavonoids on mycelial growth of *Verticillium albo-atrum*. *Biochem. Sys. Ecol.* **1995**, *23*, 683–693.
- Pietta, P.-G. Flavonoids as antioxidants. *J. Nat. Prod.* **2000**, *63*, 1035–1042.
- Porter, L. J.; Hrtstich, L. N.; Chan, B. G. The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. *Phytochemistry* **1986**, *25*, 223–230.
- Price, C. L.; Knight, S. C. Advanced glycation: A novel outlook on atherosclerosis. *Curr. Pharm. Des.* **2007**, *13*, 3681–3687.
- Price, K. R.; Prosser, T.; Richetin, A. M. F.; Rhodes, M. J. C. A comparison of the flavonol content and composition in dessert, cooking and cider-making apples; distribution within the fruit and effect of juicing. *Food Chem.* **1999**, *66*, 489–494.
- Price, M. L.; Butler, L. G. Rapid visual estimation and spectrophotometric determination of tannin content of sorghum grain. *J. Agric. Food Chem.* **1977**, *25*, 1268–1273.
- Prior, R. L.; Gu, L. Occurrence and biological significance of proanthocyanidins in the American diet. *Phytochemistry* **2005**, *66*, 2264–2280.
- Prior, R. L.; Wu, X. Anthocyanins: structural characteristics that result in unique metabolic patterns and biological activities. *Free Radic. Res.* **2006**, *40*, 1014–1028.
- Prior, R. L.; Wu, X.; Schaich, K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J. Agric. Food Chem.* **2005**, *53*, 4290–4302.

- Puido, R.; Bravo, L.; Saura-Calixto, F. Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. *J. Agric. Food Chem.* **2000**, *48*, 339–3402.
- Rahman, I. Dietary polyphenols mediated regulation of oxidative stress and chromatin remodeling in inflammation. *Nutr. Rev.* **2008**, *66* (suppl.), S42–S45.
- Rankin, J. A. Biological mediators of acute inflammation. *AACN Clinical Issues* **2004**, *15*, 3–17.
- Rechner, A. R.; Kuhnle, G.; Bremner, P.; Hubbard, G. P.; Moore, K. P.; Rice-Evans, C. A. The metabolic fate of dietary polyphenols in humans. *Free Radic. Biol. Med.* **2002**, *33*, 220–235.
- Reed, J. D. Nutritional toxicology of tannins and related polyphenols in forage legumes. *J. Anim. Sci.* **1995**, *73*, 1516–1528.
- Requena, J. R.; Fu, M.-X.; Ahmed, M. U.; Jenkins, A. J.; Lyons, T. J.; Thorpe, S. R. Lipoxidation products as biomarkers of oxidation damage to proteins during lipid peroxidation reactions. *Nephrol. Dial. Transplant.* **1996**, *11* (suppl.), 48–53.
- Rice-Evans, C. A.; Miller, N. J.; Pagana, G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radic. Biol. Med.* **1996**, *20*, 933–956.
- Ridnour, L. A.; Thomas, D. D.; Mancardi, D.; Espey, M. G.; Miranda, K. M.; Paolocci, N.; Feelisch, M.; Fukuto, J.; Wink, D. A. The chemistry of nitrosative stress induced by nitric oxide and reactive nitrogen oxide species. Putting perspective on stressful biological situations. *Biol. Chem.* **2004**, *385*, 1–10.
- Robards, K.; Li, X.; Antolovich, M.; Boyd, S. Characterisation of citrus by chromatographic analysis of flavonoids. *J. Sci. Food Agric.* **1997**, *75*, 87–101.
- Robbins, R. J. Phenolic acids in foods: An overview of analytical methodology. *J. Agric. Food Chem.* **2003**, *51*, 2866–2887.
- Rommel, A.; Wrolstad, R. E. Ellagic acid content of red raspberry juice as influenced by cultivar, processing, and environmental factors. *J. Agric. Food Chem.* **1993**, *41*, 1951–1960.
- Rossi, A.; Serraino, I.; Dugo, P.; Di Paola, R.; Mondello, L.; Genovese, T.; Morabito, D.; Dugo, G.; Sautebin, L.; Caputi, A. P.; Cuzzocrea, S. Protective effects of anthocyanins from blackberry in a rat model of acute lung inflammation. *Free Radic. Res.* **2003**, *37*, 891–900.

- Rosso, R.; Vieira, T. O.; Leal, P. C.; Nunes, R. J.; Yunes, R. A.; Creczynski-Pasa, T. Relationship between the lipophilicity of gallic acid *n*-alkyl esters' derivatives and both myeloperoxidase activity and HOCl scavenging. *Bioorg. Med. Chem.* **2006**, *14*, 6409–6413.
- Rouanet, J.-M.; Décordé, K.; del Rio, D.; Auger, C.; Borges, G.; Cristol, J.-P.; Lean, M. E. J.; Crozier, A. Berry juices, teas, antioxidants and the prevention of atherosclerosis in hamsters. *Food Chem.* **2010**, *118*, 266–271.
- Sajithlal, G. B.; Chithra, P.; Chandrakasan, G. Effect of curcumin on the advanced glycation and cross-linking of collagen in diabetic rats. *Biochem. Pharmacol.* **1998**, *56*, 1607–1614.
- Sakihama, Y.; Cohen, M. F.; Grace, S. C.; Yamasaka, H. Plant phenolic antioxidant and prooxidant activities: Phenolics-induced oxidative damage mediated by metals in plants. *Toxicology* **2002**, *177*, 67–80.
- Santos-Buelga, C.; Scalbert, A. Proanthocyanidins and tannin-like compounds - nature, occurrence, dietary intake and effects on nutrition and health. *J. Sci. Food Agric.* **2000**, *80*, 1094–1117.
- Saraswat, M.; Reddy, P. Y.; Muthenna, P.; Reddy, G. B. Prevention of non-enzymic glycation of protein by dietary agents: prospects for alleviating diabetic complications. *Bri. J. Nutri.* **2009**, *101*, 1714–1721.
- Sato T.; Iwaki M.; Shimogaito N.; Wu X.; Yamagishi S.; Takeuchi M. TAGE (toxic AGEs) theory in diabetic complications. *Curr. Mol. Med.* **2006**, *6*, 351–358.
- Saura-Calixto, F.; Serrano, J.; Goñi, I. Intake and bioaccessibility of total polyphenols in a whole diet. *Food Chem.* **2007**, *101*, 492–501.
- Sayre, L. M.; Smith, M. A.; Perry, G. Chemistry and biochemistry of oxidative stress in neurodegenerative disease. *Curr. Med. Chem.* **2001**, *8*, 721–738
- Scalbert, A. Antimicrobial properties of tannins. *Phytochemistry* **1991**, *30*, 3875–3883.
- Scalbert, A.; Williamson, G. Dietary intake and bioavailability of polyphenols. *J. Nutr.* **2000**, *130*, 2073S–2085S.
- Schmidt, A. M.; Hori, O.; Brett, J.; Yan, S. D.; Wautier, J. L.; Stern, D. Cellular receptors for advanced glycation end products. Implications for induction of oxidant stress and cellular dysfunction in the pathogenesis of vascular lesions. *Arterioscler. Thromb. Vasc. Biol.* **1994**, *14*, 1521–1528.
- Schmidt, A. M.; Hori, O.; Chen, J. X.; Li, J. F.; Crandall, J.; Zhang, J.; Cao, R.; Yan, S. D.; Brett, J.; Stern, D. Advanced glycation endproducts interacting with their endothelial receptor induce expression of vascular cell adhesion molecule-1(VCAM-1) in cultured human endothelial cells and in mice. *J. Clin. Invest.* **1995**, *96*, 1395–1403.



- Schmidtlein, H.; Herrmann, K. Quantitative analysis of phenolic acids by thin-layer chromatography. *J. Chromatogr.* **1975**, *115*, 123–128.
- Schuler, P. Natural antioxidants exploited commercially. In *Food Antioxidants*. Hudson, B. J. F., Ed.; Elsevier Applied Science: New York, NY, 1990; pp. 99–170.
- Seeram, N. P. Berries. In *Nutritional Oncology*. Heber, D.; Blackburn, G.; Go, V. L. W.; Milner, J., Eds.; Academic Press: London, UK, 2006; pp. 615–625
- Seeram, N. P.; Adams, L. S.; Zhang, Y.; Lee, R.; Sand, D.; Scheuller, H. S.; Heber, D. Blackberry, black raspberry, blueberry, cranberry, red raspberry, and strawberry extracts inhibit growth and stimulate apoptosis of human cancer cells in vitro. *J. Agric. Food Chem.* **2006**, *54*, 9329–9339.
- Sellapan, S.; Akoh, C. C.; Krewer, G. Phenolic compounds and antioxidant capacity of Georgia-grown blueberries and blackberries. *J. Agric. Food Chem.* **2002**, *50*, 2432–2438.
- Selma, M. V.; Espín, J. C.; Tomás-Barberán, F. A. Interaction between phenolics and gut microbiota: Role in human health. *J. Agric. Food Chem.* **2009**, *57*, 6485–6501.
- Serrano, J.; Puupponen-Pimi, R.; Dauer, A.; Aura, A.-M.; Saura-Calixto, F. Tannins: Current knowledge of food sources, intake, bioavailability and biological effects. *Mol. Nutr. Food Res.* **2009**, *53*, S310–S329.
- Shahidi, F. Antioxidants in food and food antioxidants. *Nahrung* **2000**, *44*, 158–163.
- Shahidi, F.; Naczki, M. An overview of the phenolics of canola and rapeseed: Chemical, sensory and nutritional significance. *J. Am. Oil Chem. Soc.* **1992**, *69*, 917–924.
- Shahidi, F.; Naczki, M. Biosynthesis, classification and nomenclature of phenolics in food and nutraceuticals. In *Food Phenolics: Sources, Chemistry, Effects, Applications*. Technomic Publishing: Lancaster, PA, 1995; pp. 1–16.
- Shen, S.-Q.; Zhang, Y.; Xiang, J.-J.; Xiong, C.-L. Protective effect of curcumin against liver warm ischemia/reperfusion injury in rat model is associated with regulation of heat shock protein and antioxidant enzymes. *World J. Gastroenterol.* **2007**, *13*, 1953–1961.
- Sichel, G.; Corsaro, C.; Scalia, M.; Di Bilio, A. J.; Bonomo, R. P. In vitro scavenger activity of some flavonoids and melanins against O<sub>2</sub>. *Free Radic. Biol. Med.* **1991**, *11*, 1–8.
- Singh, R.; Barden, A.; Mori, T.; Beilin, L. Advance glycation end-products: a review. *Diabetologia* **2001**, *44*, 129–146.
- Singleton, V. L.; Rossi, J. A., Jr. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* **1965**, *16*, 144–158.

- Singleton, V. L.; Trousdale, E. White wine phenolics: Varietal and processing differences as shown by HPLC. *Am. J. Enol. Vitic.* **1983**, *34*, 27–34.
- Siriwoharn, T.; Wrolstad, R. E. Polyphenolic composition of Marion and Evergreen blackberries. *J. Food Sci.* **2004**, *69*, FCT233–FCT240.
- Siriwoharn, T.; Wrolstad, R. E.; Durst, W. Identification of ellagic acid in blackberry juice sediment. *J. Food Sci.* **2005**, *70*, C189–C197.
- Sivam, G. Analysis of flavonoids. In *Method of analysis for functional foods and Nutraceuticals*. Hurst, W. J., Ed.; CRC Press: Boca Raton, FL, 2002; pp. 363–384.
- Slinkard, K.; Singleton, V. L. Total phenol analysis: Automation and comparison with manual methods. *Am. J. Enol. Vitic.* **1977**, *28*, 49–55.
- Smith, D. A. Toxicity of phytoalexins. In *Phytoalexins*. Bailey, J. A.; Mansfield, J. W., Eds.; Wiley and Sons: Hoboken, NJ, 1982; pp. 218–252.
- Smith, R. L. Species variations in biliary secretion. In *The Excretory Function of Bile*. Wiley and Sons: Hoboken, NJ, 1973; pp. 76–93.
- Song, J.; Kwon, O.; Chen, S.; Daruwala, R.; Eck, P.; Park, J. B.; Levine, M. Flavonoid inhibition of sodium-dependent vitamin C transporter 1 (SVCT1) and glucose transporter isoform 2 (GLUT2), intestinal transporters for vitamin C and glucose. *J. Biol. Chem.* **2002**, *277*, 15252–15260.
- Springob, K.; Nakajima, J.; Yamazaki, M.; Saito, K. Recent advances in the biosynthesis and accumulation of anthocyanins. *Nat. Prod. Rep.* **2003**, *20*, 288–303.
- Stahelin, R. V.; Digman, M. A.; Medkova, M.; Ananthanarayana, B.; Rafter, J. D.; Melowic, H. R.; Cho, W. Mechanism of diacylglycerol-induced membrane targeting and activation of protein kinase C $\delta$ . *J. Biol. Chem.* **2004**, *279*, 29501–29512.
- Stalikas, C. D. Extraction, separation, and detection methods for phenolic acids and flavonoids. *J. Sep. Sci.* **2007**, *30*, 3268–3295.
- Stitt, A. W.; Li, Y. M.; Gardiner, T. A.; Bucala, R.; Archer, D. B.; Vlassara, H. Advanced glycation end products (AGEs) co-localize with AGE receptors in the retinal vasculature of diabetic and of AGE-infused rats. *Am. J. Pathol.* **1997**, *150*, 523–531.
- Stobiecki, M. Application of mass spectrometry for identification and structural studies of flavonoid glycosides. *Phytochemistry* **2000**, *54*, 237–256.
- Strack, D. Phenolic metabolism. In *Plant Biochemistry*. Dey, P.M.; Harborne, J. B., Eds.; Academic Press: London, UK, 1997; pp. 387–416.

- Strik, B. C.; Clark, J. R.; Finn, C. E.; Bañados, M. P. Worldwide blackberry production. *HortTechnology* **2007**, *17*, 205–213.
- Su, B.; Karin, M. Mitogen-activated protein kinase cascades and regulation of gene expression. *Curr. Opin. Immunol.* **1996**, *8*, 402–411.
- Subbaramaiah, K.; Bulic, P.; Lin, Y. L.; Dannenberg, A. J.; Pasco, D. S. Development and use of a gene promoter-based screen to identify novel inhibitors of cyclooxygenase-2 transcription. *J. Biomol. Screen.* **2001**, *6*, 101–110.
- Subbaramaiah, K.; Chung, W. J.; Michaluart, P.; Telang, N.; Tanabe, T.; Inoue, H.; Jang, M.; Pezzuto, J. M.; Dannenberg, A. J. Resveratrol inhibits cyclooxygenase-2 transcription and activity in phorbol ester-treated human mammary epithelial cells. *J. Biol. Chem.* **1998**, *273*, 21875–21882.
- Sun, A. Y.; Wang, Q.; Simonyi, A.; Sun, G. Y. Botanical phenolics and brain health. *NeuroMol. Med.* **2008**, *10*, 259–274.
- Sun, B.; Ricardo-da-Silva, J. M.; Spranger, I. Critical factors of vanillin assay for catechins and proanthocyanidins. *J. Agric. Food Chem.* **1998**, *46*, 4267–4274.
- Szaefer, H.; Kaczmarek, J.; Rybczyńska, M.; Baer-Dubowska, W. The effect of plant phenols on the expression and activity of phorbol ester-induced PKC in mouse epidermis. *Toxicology* **2007**, *230*, 1–10.
- Takanyama, M.; Fukai, T.; Ichikawa, K.; Nomura, T. Identification of prenylated flavonoids using fast-atom bombardment mass spectrometry. *Rapid Commun. Mass Spectrom.* **2005**, *5*, 67–69.
- Takeoka, G.; Dao, L. Anthocyanins. In *Methods of Analysis for Functional Foods and Nutraceuticals*. Hurst, W. J., Ed.; CRC Press: Boca Raton, FL, 2002; pp. 219–241.
- Tanaka, T.; Nonaka, G. I.; Nishioka, I. Tannins and related compounds. Part 28. Revision of the structures of Sanguiins H-6, H-2, and H-3, and isolation and characterization of Sanguiin H-11, novel tetrameric hydrolyzable tannin, and seven related tannins, from *Sanguisorba officinalis*. *J. Chem. Res. (suppl.)* **1985**, *6*, 176–177.
- Thomas, R. H.; Woods, F. M.; Dozier, W. A.; Ebel, R. C.; Nesbitt, M.; Wilkins, B.; Himelrick, D. G. Cultivar variation in physicochemical and antioxidant activity of Alabama-grown blackberries. *Small Fruits Rev.* **2005**, *4*, 57–71.
- Thornalley, P. J. Pharmacology of methylglyoxal: Formation, modification of proteins and nucleic acids, and enzymatic detoxification—A role in pathogenesis and antiproliferative chemotherapy. *Gen. Pharmac.* **1996**, *27*, 565–573.

- Tolonen, A.; Uusitalo, J. Fast screening method for the analysis of total flavonoid content in plants and foodstuffs by high-performance liquid chromatography/electrospray ionization time-of-flight mass spectrometry with polarity switching. *Rapid Commun. Mass Spectrom.* **2004**, *18*, 3113–3122.
- Tsao, R.; Yang, R. Optimization of a new mobile phase to know the complex and real polyphenolic composition: towards a total phenolic index using high-performance liquid chromatography. *J. Chromatogr. A* **2003**, *1018*, 29–40.
- Tsuda, T.; Horio, F.; Kato, Y.; Osawa, T. Cyanidin 3-*O*-beta-D-glucoside attenuates the hepatic ischemia-reperfusion injury through a decrease in the neutrophil chemoattractant production in rats. *J. Nutr. Sci. Vitaminol. (Tokyo)* **2002**, *48*, 134–141.
- Tsuji-Naito, K.; Saeki, H.; Hamano, M. Inhibitory effects of *Chrysanthemum* species extract on formation of advanced glycation end products. *Food Chem.* **2009**, *116*, 854–859.
- Urios, P.; Grigorova-Borsos, A. M.; Sternberg, M. Flavonoids inhibit the formation of the cross-linking AGE pentosidine in collagen incubated with glucose, according to their structure. *Eur. J. Nutr.* **2007**, *46*, 139–146.
- USDA Survey of Phenolic Compounds Produced in Citrus. Technical Bulletin Number 1856. U.S. Department of Agriculture. Peoria, IL, 1998.
- USDA Database for the Proanthocyanidin Content of Selected Foods. U.S. Department of Agriculture, Human Nutrition Research Center, Nutrient Data Laboratory, Beltsville, MD, 2004.
- USDA Composition of Foods Raw, Processed, Prepared. USDA National Nutrient Database for Standard Reference, Release 18. U.S. Department of Agriculture, Agricultural Research Service, Beltsville, MD, 2005.
- Valerio, L. G.; Kepa, J. K.; Pickwell, G. V.; Quattrochi, L. C. Induction of human NAD(P)H:quinone oxidoreductase (NQO1) gene expression by the flavonol quercetin. *Toxicol. Lett.* **2001**, *119*, 49–57.
- Valko, M.; Izakovic, M.; Mazur, M.; Rhodes, C. J.; Telser, J. Role of oxygen radicals in DNA damage and cancer incidence. *Mol. Cell. Biochem.* **2004**, *266*, 37–56.
- Valko, M.; Leibfritz, D.; Moncol, J.; Cronin, M. T.; Mazur, M.; Telser, J. Free radicals and antioxidants in normal physiological functions and human disease. *Int. J. Biochem. Cell Biol.* **2007**, *39*, 44–84.
- van der Meer, I. M.; Stam, M. E.; van Tunen, A. J.; Mol, J. N. M.; Stuitje, A. R. Antisense inhibition of flavonoid biosynthesis in petunia anthers results in male sterility. *Plant Cell* **1992**, *4*, 253–262.

- Veitch, N. C.; Grayer, R. J. Flavonoids and their glycosides, including anthocyanins. *Nat. Prod. Rep.* **2008**, *25*, 555–611.
- Vieira, L. M. M.; Kijjoa, A. Naturally Occuring Xanthones. *Curr. Med. Chem.* **2005**, *12*, 2413–2446.
- Vinatoru, M.; Toma, M.; Radu, O.; Filip, P. I.; Lazurca, D.; Mason, T. J. The use of ultrasound for the extraction of bioactive principals from plantmaterials. *Ultrason. Sonochem.* **1997**, *4*, 135–139.
- Vinson, J. A.; Su, X.; Zubik, L.; Bose, P. Phenol antioxidant quantity and quality in foods: Fruits. *J. Agric. Food Chem.* **2001**, *49*, 5315–5321.
- Visioli, F.; Hagen, T. M. Nutritional strategies for healthy cardiovascular aging: Focus on micronutrients. *Pharmacol. Res.* **2007**, *55*, 199–206
- Vlassara, H.; Fuh, H.; Donnelly, T.; Cybulsky, M. Advanced glycation endproducts promote adhesion molecule (VCAM-1, ICAM-1) expression and atheroma formation in normal rabbits. *Mol. Med.* **1995**, *1*, 447–456.
- Vogt, T.; Pollak, P.; Tarlyn, N.; Taylor, L. P. Pollination- or wound-induced kaempferol accumulation in petunia stigmas enhances seed production. *Plant Cell* **1994**, *6*, 11–23.
- Vrhovsek, U.; Palchetti, A.; Reniero, F.; Guillou, C.; Masuero, D.; Mattivi, F. Concentration and mean degree of polymerization of *Rubus* ellagitannins evaluated by optimized acid methanolysis. *J. Agric. Food Chem.* **2006**, *54*, 4469–4475.
- Wald, B.; Galensa, R.; Herrmann, K.; Grotjahn, L.; Wray, V. Quercetin 3-O-[6''-(3-hydroxy-3-methylglutaroyl)- $\beta$ -galactoside] from blackberries. *Phytochemistry* **1986**, *25*, 2904–2905.
- Wang, H. Q.; Smart, R. C. Overexpression of protein kinase C- $\alpha$  in the epidermis of transgenic mice results in striking alterations in phorbol ester-induced inflammation and COX-2, MIP-2 and TNF- $\alpha$  expression but not tumor promotion. *J. Cell Sci.* **1999**, *112*, 3497–3506.
- Wang, H.; Cao, G.; Prior, R. L. Oxygen radical absorbing capacity of anthocyanins. *J. Agric. Food Chem.* **1997**, *45*, 304–309.
- Wang, H. Q.; Kim, M. P.; Tiano, H. F.; Lanqenbach, R.; Smart, R. C. Protein kinase C- $\alpha$  coordinately regulates cytosolic phospholipase A<sub>2</sub> activity and the expression of cyclooxygenase-2 through different mechanisms in mouse keratinocytes. *Mol. Pharmacol.* **2001**, *59*, 860–866.
- Wang, J.; Sporns, P. Analysis of anthocyanins in red wine and fruit juice using MALDI-MS. *J. Agric. Food Chem.* **1999**, *47*, 2009–2015.

- Wang, J.; Mazza, G. Inhibitory effects of anthocyanins and other phenolic compounds on nitric oxide production in LPS/IFN- $\gamma$ -activated RAW 264.7 macrophages. *J. Agric. Food Chem.* **2002**, *50*, 850–857.
- Weidenbörner, M.; Hindorf, H.; Jha, H. C.; Tsotsonos, P. Antifungal activity of flavonoids against storage fungi of the genus *Aspergillus*. *Phytochemistry* **1990**, *29*, 1103–1105.
- Whitley, A. C.; Stoner, G. D.; Darby, M. V.; Walle, T. Intestinal epithelial cell accumulation of the cancer preventive polyphenol ellagic acid - extensive binding to protein and DNA. *Biochem. Pharmacol.* **2003**, *66*, 907–915.
- Wildanger, W.; Herrmann, K. The phenolics of fruits. II. The flavonols of fruits. *Z. Lebensm. Unters. Forsch.* **1973**, *151*, 103–108.
- Williams, C. A.; Grayer, R. J. Anthocyanins and other flavonoids. *Nat. Prod. Rep.* **2004**, *21*, 539–573.
- Williamson, G.; Day, A. J.; Plumb, G. W.; Couteau, D. Human metabolic pathways of dietary flavonoids and cinnamates. *Biochem. Soc. Trans.* **2000**, *28*, 16–22.
- Wojtaszek, P.; Stobiecki, M.; Gulewicz, K. Role of nitrogen and plant growth regulators in the exudation and accumulation of isoflavonoids by roots of intact white lupine (*Lupinus albus* L.) plants. *J. Plant Physiol.* **1993**, *142*, 689–694.
- Wollenweber, E. Flavones and flavonols. In *The Flavonoids: Advances in Research Since 1986*. Harborne, J. B., Ed.; Chapman and Hall: Cambridge, UK, 1994; pp. 259–335.
- Won, J.-H.; Park, S.-Y.; Nam, S.-G.; Park, H.-J.; Choi, J.-W.; Lee, K.-T. Inhibition of lipopolysaccharide-induced expression of inducible nitric oxide and cyclooxygenase-2 by chiisanoside *via* suppression of nuclear factor- $\kappa$ B activation in RAW 264.7 macrophage cells. *Biol. Pharm. Bull.* **2005**, *28*, 1919–1924.
- Woo, H.-H.; Orbach, M. J.; Hirsch, A. M.; Hawes, M. C. Meristem-localized inducible expression of a UDP-glycosyltransferase gene is essential for growth and development in pea and alfalfa. *Plant Cell* **1999**, *11*, 2303–2315.
- Wrolstad, R. E. Pigments and colorants. In *Handbook of Food Analytical Chemistry: Pigments, Colorants, Flavors, Texture, and Bioactive Food Components*. Wrolstad, R. E.; Acree, T. E.; Decker, E. A.; Penner, M. H.; Reid, D. S.; Schwartz, S. J.; Shoemaker, C. F.; Smith, D. and Sporns, P. Eds.; John Wiley and Sons: Hoboken, NJ, 2005a; pp. 1–70.
- Wrolstad, R. E. Bioactive Food Components. In *Handbook of Food Analytical Chemistry: Pigments, Colorants, Flavors, Texture, and Bioactive Food Components*. Wrolstad, R. E.; Acree, T. E.; Decker, E. A.; Penner, M. H.; Reid, D. S.; Schwartz, S. J.; Shoemaker, C. F.; Smith, D.; Sporns, P., Eds.; Wiley and Sons: Hoboken, NJ, 2005b; pp. 459.

- Wu, C.-H.; Yen, G.-C. Inhibitory effect of naturally occurring flavonoids on the formation of advanced glycation endproducts. *J. Agric. Food Chem.* **2005**, *53*, 3167–3173.
- Wu, J. T.; Kral, J. G. The NF- $\kappa$ B/I $\kappa$ B signaling system: A molecular target in breast cancer therapy. *J. Surg. Res.* **2005**, *123*, 158–169.
- Wu, J.-W.; Hsieh, C.-L.; Wang, H.-Y.; Chen, H.-Y. Inhibitory effects of guava (*Psidium guajava* L.) leaf extracts and its active compounds on the glycation process of protein. *Food Chem.* **2009**, *113*, 78–84.
- Yamamoto, Y.; Gaynor, R. B. Therapeutic potential of inhibition of the NF- $\kappa$ B pathway in the treatment of inflammation and cancer. *J. Clin. Invest.* **2001**, *107*, 135–142.
- Yang, C. S.; Landau, J. M.; Huang, M.-T.; Newmark, H. L. Inhibition of carcinogenesis by dietary polyphenolic compounds. *Annu. Rev. Nutr.* **2001**, *21*, 381–406.
- Yeh, C.-T.; Yen, G.-C. Effects of phenolic acids on human phenolsulfotransferase in relation to their antioxidant activity. *J. Agric. Food Chem.* **2003**, *51*, 1474–1479.
- Yeh, C.-T.; Yen, G.-C. Induction of hepatic antioxidant enzymes by phenolic acids in rats is accompanied by increased levels of multidrug resistance-associated protein 3 mRNA expression. *J. Nutr.* **2006**, *136*, 11–15.
- Yilmaj, Y. Novel uses of catechins in foods. *Trends Food Sci. Technol.* **2006**, *17*, 64–71.
- Yim, H.-S.; Kang, S.-O.; Hah, Y.-C.; Chock, P. B.; Yim, M. B. Free radicals generated during the glycation reaction of amino acids by methylglyoxal. A model study of protein-cross-linked free radicals. *J. Biol. Chem.* **1995**, *270*, 28228–28233.
- Yoon, J.-H.; Baek, S. J. Molecular targets of dietary polyphenols with anti-inflammatory properties. *Yonsei Med. J.* **2005**, *46*, 585–596.
- Yoshida, K.; Mori, M.; Kondo, T. Blue flower color development by anthocyanins: from chemical structure to cell physiology. *Nat. Prod. Rep.* **2009**, *26*, 884–915.
- Younes, A.; Garg, A.; Aggarwal, B. B. Nuclear transcription factor-kappaB in Hodgkin's disease. *Leukemia Lymphoma* **2003**, *44*, 929–935.
- Yousef, M. I.; Saad, A. A.; El-Shennawy, L. K. Protective effect of grape seed proanthocyanidin extract against oxidative stress induced by cisplatin in rats. *Food Chem. Toxicol.* **2009**, *47*, 1176–1183.
- Zafrilla, P.; Federico, F.; Tomás-Barberán, F. A. Effect of processing and storage on the antioxidant ellagic acid derivatives and flavonoids of red raspberry (*Rubus idaeus*) jams. *J. Agric. Food Chem.* **2001**, *49*, 3651–3655.

Zimmermann, G. R.; Avery, W.; Finelli, A. L.; Farwell, M.; Fraser, C. C.; Borisy, A. A. Selective amplification of glucocorticoid anti-inflammatory activity through synergistic multi-target action of a combination drug. *Arthritis Res. Ther.* **2009**, *11*, 1–14.



## CHAPTER 3

### CHARACTERIZATION OF PHENOLIC COMPOUNDS IN GEORGIA-GROWN BLACKBERRY CULTIVARS

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## ABSTRACT

The phenolic composition of Georgia-grown blackberries was determined and involved various extractions, chromatographies, and mass spectral analyses of isolated fractions. Mean total phenolics contents were  $458.9 \pm 1.92$ ,  $408.6 \pm 0.6$ , and  $410.6 \pm 3.4$  mg GAE/100-g berries, f.w. for the Navaho, Kiowa, and Ouachita cultivars, respectively. The total anthocyanin contents were determined to be  $139.7 \pm 2.4$ ,  $150.0 \pm 2.0$ , and  $143.3 \pm 1.1$  mg C3G equivalents/100-g fresh berries for the Navaho, Kiowa, and Ouachita cultivars, respectively. Blackberry phenolics from each cultivar were separated into seven fractions (FXN). Cyanidins as well as ellagitannins, ellagic acid and its derivatives were the major phenolic classes present in most fractions. Electrospray ionization-mass spectrometry (ESI-MS) studies confirmed the presence of cyanidin-3-*O*-glucoside as dominant anthocyanin in all cultivars. The chemical composition of hydrolyzable tannins comprising FXN-VII was examined by matrix-assisted laser desorption/ionization-time of flight-mass spectrometer (MALDI-TOF-MS). The presence of ellagitannin isomers; *i.e.*, degalloylated sanguin H-6/lambertianin A, sanguine H-6/lambertianin A and lambertianin C, were confirmed in all three blackberry varieties. Lambertianin D, the largest ellagitannin, was found only in the Kiowa cultivar whereas pedunculagin (bis-HHDP-glucose) was noted only in Navaho cultivar.

## INTRODUCTION

Over the past decade, there have been increasing numbers of reports indicating that berries are associated with a reduced risk of aging and degenerative diseases such as atherosclerosis (1), cardiovascular diseases (2), cancer (3-5), diabetes (6), inflammation (7-8) and neurodegenerative disorders (9). Most of these biological actions have been attributed to the intrinsic antioxidant capacities of phytochemicals present in the berries (10-14). In terms of antioxidant concentrations amongst berries (*i.e.*, blackberries, blueberries, raspberries, and strawberries), blackberries have ranked highest (15).

Several *in vitro* and *in vivo* studies have reported antioxidant and anti-inflammatory activities afforded by blackberries or their constituents (16-21). A recent human trial with blackberry juice conducted by Hassimoto et al (22) showed that an increase in catalase activity (an endogenous enzyme responsible for the antioxidant defense mechanism in the human body) resulted. These beneficial health effects are associated with the marked concentrations of phenolic compounds in blackberries, predominantly the anthocyanin pigments and hydrolyzable tannins (23-26).

Phenolic compounds include several classes such as hydroxybenzoic acids, hydroxycinnamic acids, flavonoids (*i.e.*, flavones, flavanones, flavanols, flavonols, anthocyanins), and oligomeric forms such as hydrolyzable and condensed tannins/proanthocyanidins. Anthocyanin pigments are the major phenolic compounds present in blackberries. Cyanidin-based anthocyanins and anthocyanidins are well characterized and range from 45 to 90% of the total anthocyanins

present (23). Hydroxycinnamic acids, flavonols, flavan-3-ols, and proanthocyanidins are present in lower amounts (25, 27-30).

Hydrolyzable tannins are the second major class of phenolics endogenous to blackberries (24), and include ellagitannins (ETs) and ellagic acid derivatives. Ellagitannins are esters of hexahydroxydiphenic (HHDP) acid with the monomeric unit of ETs being bis-HHDP glucopyranose, commonly known as pedunculagin, or its galloylated form called galloyl-bis-HHDP glucopyranose (*i.e.*, a specific isomeric form) (31-32). Most ET structures that have been detected in the fruits and leaves of blackberries are oligomeric ETs; that is, polymerized forms of galloyl-bis-HHDP. So far, the oligomeric ETs characterized in blackberries include the dimers sanguin H-6, sanguin H-10/lambertianins A, the trimer lambertianin C, and the tetramer lambertianin D (24-25). The dimer sanguin H-10 has been associated with astringency noted in herbal remedies prepared from the leaves and fruits of blackberries as well as raspberries (33). The chemical structures of selected blackberry phenolic compounds are given in **Figure 3.1**.

The blackberry (Family, Rosaceae; and Genus, *Rubus*) is a small round fruit that grows on flowering shrubs or trailing vines. Each blackberry is an aggregate fruit consisting of a cluster of tiny fruits called drupelets. In 1964 the Blackberry Breeding Program at the University of Arkansas (UA) blackberry was initiated, and over the years has patented and released several new cultivars of blackberries with improved traits. These include thornless characteristics, erect canes, fruit firmness, large fruit size, high yields, higher total soluble solids, improved post-harvest handling, primocane fruiting (34), and most importantly resistance to winter season damage and low temperatures. The majority of the U.S. blackberry crop is grown in the

Willamette Valley west of the Cascade Mountains in Oregon, and is dominated by the trailing type which has long, pliable canes. Navaho, Kiowa, and Ouachita are erect cane cultivars that have been released from UA's Blackberry Breeding Program and have shown good adaptation to the warm, humid climatic conditions of Georgia and other regions of the southeastern U.S., as well as some fungal resistance to rosette caused by *Cercospora rubi*. These cultivars are different from the 'Marion,' 'Oregon Thornless Evergreen,' and 'Boysenberry' varieties which dominant blackberry production areas in the Pacific Northwest. The commercial Navaho, Kiowa, and Ouachita cultivars possess good post-harvest handling characteristics and provide important marketing opportunities to the growing small-fruit industry in the state of Georgia (35).

The objectives of this study were to characterize the polyphenolics from three present-day blackberry cultivars (*i.e.*, Navaho, Kiowa, and Ouachita) grown in Georgia. Targeted extraction and column chromatography strategies were designed to isolate phenolic compound classes, to further fractionate these, and to partially characterize them. Reversed-phase HPLC was employed to separate compounds of collected fractions, to assist in tentatively identifying phenolics based on their spectral characteristics and reference to commercial standards, and to quantify. Electrospray ionization mass spectrometry (ESI-MS) and matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS) were utilized to elucidate the structural composition of the low-molecular-weight (mainly anthocyanins) and high-molecular-weight (mainly hydrolyzable tannins) phenolic constituents in the blackberry fractions.

## MATERIALS AND METHODS

**Chemicals.** Phenolic compounds including (+)-catechin, (-)-epicatechin, quercetin, and a series of phenolic acids comprising gallic, ellagic, vanillic, caffeic, *p*-coumaric, *trans*-cinnamic, protocatechuic, syringic, chlorogenic, and *p*-hydroxybenzoic acids were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). A pure standard of kuromanin chloride (*i.e.*, cyanidin-3-*O*-glucoside chloride) was acquired from Indofine Chemical Co. (Hillsborough, NJ). An anthocyanidins kit (with pelargonidin chloride, cyanidin chloride, delphinidin chloride, & malvidin chloride), a cyanidin derivatives kit (with cyanidin-3-*O*-rutinoside chloride, cyanidin-3,5-di-*O*-glucoside chloride, cyanidin-3-*O*-galactoside chloride, & cyanidin-3-*O*-glucoside chloride), and cyanidin-3-*O*-rhamnoside chloride were procured from Extrasynthese (Genay, France). Chemicals for extraction and analytical assays included Folin-Ciocalteu's phenol reagent, sodium carbonate, sodium hydroxide, potassium chloride, as well as 2,5-dihydroxybenzoic acid, and were obtained from Sigma-Aldrich. The HPLC-grade solvents acetonitrile, water, and methanol were purchased from Fisher Scientific Co. (Suwanee, GA) as were ACS-grade acetone, 95% ethanol (v/v), glacial acetic acid, trifluoroacetic acid (TFA), and hydrochloric acid.

**Collection of Samples.** Ripe blackberries (*Rubus* spp.) were collected at Jacob W. Paulk Farms, Inc. (Wray, GA) in May 2006 and 2007. The three blackberry cultivars collected over two crops years were as follows: Navaho, an erect thornless variety; Kiowa, an erect thorny variety; and Ouachita, a very erect cane and thornless variety. All three cultivars, which grow particularly well in the hot, humid conditions of the southeastern U.S., were patented by and released from

the University of Arkansas. These cultivars are different from those in the dominant blackberry production areas of the Pacific Northwest. Hand-picked blackberries were transported to the Department of Food Science & Technology, UGA, in Athens, GA. The berries were sorted, cleaned, and frozen in polyethylene pouches at  $-40\text{ }^{\circ}\text{C}$ . Representative samples from each cultivar were lyophilized using a UNITOP 600L VirTis™ freeze dryer (The VirTis Company, Inc., Gardiner, NY), transferred to polyethylene pouches, and then stored at  $-40\text{ }^{\circ}\text{C}$  until analyzed.

**Moisture Content Determination of Fresh Blackberries.** The moisture content of fresh berries was determined by drying samples placed in aluminum dishes at  $105\text{ }^{\circ}\text{C}$  using a 5-ft<sup>3</sup> Isotemp® standard lab oven (Fisher Scientific) until a constant mass was reached.

**Preparation of Crude Blackberry Extracts (CBEs).** Freeze-dried whole blackberry samples (*i.e.*, containing fruit receptacles, skins and seeds) from each cultivar were ground in a commercial coffee mill (KitchenAid, St Joseph, MI). Fifteen grams of blackberry powder were mixed with 150 mL of 70% (v/v) acidified acetone (containing 0.1% [v/v] HCl) and blended using a PT-3100 Polytron™ homogenizer (Brinkmann Instruments, Westbury, NY) at 15,000 rpm for 10 min. The slurry was then filtered by gravity through fluted P8 filter paper (Fisher Scientific). This extraction process was repeated 2× as described above. All filtrates were pooled and acetone was evaporated with a Büchi Rotavapor R-210 using a V-700 vacuum pump connected to a V-850 vacuum controller (Büchi Corporation, New Castle, DE) at  $40\text{ }^{\circ}\text{C}$ . Sample extractions of each cultivar were performed in triplicate.

**Preparation of Polyphenolic Extracts (PPEs).** Ten milliliters of each CBE (containing 13% solids) were applied to the top of a chromatographic column (30 mm *i.d.* × 340 mm *e.l.*, Kontes, Vineland, NJ) packed with Amberlite XAD-16 [(bead size: 20-60 mesh), Sigma-Aldrich] and washed with ~300 mL of deionized water to remove sugars and organic acids. After the first 100 mL, the pH of the eluent was checked with pH paper test strips every 20 mL until a neutral pH was reached. The polyphenolic extract (PPE) was then eluted from the column with anhydrous methanol (~300 mL) as the mobile phase. Methanol was evaporated using the Büchi Rotavapor at 40 °C. The PPE was lyophilized using a FreeZone<sup>®</sup> 2.5-L bench-top freeze dryer (Labconco Corporation, Kansas City, MO) to ensure all traces of moisture were removed and then stored in amber-glass bottles in a 4 °C refrigerator.

**Fractionation of the PPE.** For each cultivar, 200 mg of lyophilized PPE were dissolved in 10 mL of 95% (v/v) ethanol, sonicated to facilitate dissolution, and then applied to the top of a chromatographic column (30 mm *i.d.* × 360 mm *e.l.*, Kontes, Vineland, NJ) packed with Sephadex LH-20 [(bead size: 25-100 µm), Sigma-Aldrich]. Fractions were eluted with 95% (v/v) ethanol at a flow rate of 0.6 mL/min. Nine-milliliter fractions were collected in 13 × 100-mm borosilicate glass culture tubes with a fraction collector (Model SC-100, Beckman Coulter Inc., Fullerton, CA). Using UV/VIS absorbance readings at 280, 360, and 520 nm from an Agilent 8453 diode array spectrophotometer (Agilent Technologies, Inc., Wilmington, DE) as a guide, eluent was pooled into six major fractions (*i.e.*, FXN-I thru FXN-VI). In total, 500 mL of ethanol (95% [v/v]) were employed. The system was changed over to 50% (v/v) acetone, and ~300 mL were required to elute FXN-VII, comprising mostly high-molecular-weight phenolics (*i.e.*, hydrolyzable and condensed tannins) from the Sephadex LH-20 column. Organic solvents



were evaporated off from the seven major fractions collected using the Büchi Rotavapor at 40 °C. Fractions were then lyophilized using a FreeZone<sup>®</sup> 2.5-L bench-top freeze dryer (Labconco Corporation, Kansas City, MO) to ensure all traces of moisture were removed and then stored in amber-glass bottles at 4 °C in a refrigerator. A flow diagram presented in **Figure 3.2** illustrates all steps of the process.

**Total Phenolics Content (TPC) Assay.** The TPC was determined for lyophilized berries and the blackberry fractions colorimetrically using the classical Folin-Ciocalteu assay (36). This assay is based on the reduction of a heteropolyphosphotungstate-molybdate complex by phenolic compounds under alkaline conditions yielding a blue color. Briefly, 0.5 mL of a methanolic solution (5 µg/mL) of each fraction (*i.e.*, FXN-I thru FXN-VII) was pipetted into a test tube followed by the addition of 8.0 mL of deionized water, 0.5 mL of 2 N Folin-Ciocalteu's phenol reagent, and 1.0 mL of a saturated Na<sub>2</sub>CO<sub>3</sub> solution. The contents were vortexed for 15 s. After an incubation period of 60 min at room temperature to allow for optimal color development of the samples, absorbance readings were taken at  $\lambda = 750$  nm with the Agilent spectrophotometer. Quantification was based on a standard curve generated with gallic acid. The TPCs were determined from the standard curve, and results were expressed as mg gallic acid equivalents (GAE)/100-mg fraction (d.w.) or 100-g fruit (f.w.).

**Total Monomeric Anthocyanins (TACY) Content.** The total anthocyanin (TACY) contents of lyophilized blackberries and their fractions were determined by the pH differential method described by Giusti and Wrolstad (37). The method is based on a reversible color change of monomeric anthocyanin pigments with an alteration in the pH: the colored oxonium-ion form exists at pH 1.0, and the colorless hemiketal form predominates at pH 4.5. The difference in

absorbance of the pigments at  $\lambda = 510$  nm is proportional to the anthocyanin concentration with results being expressed as cyanidin-3-*O*-glucoside (C3G) equivalents. Degraded anthocyanins in the polymeric form are resistant to color change regardless of pH and therefore are not included in the measurements since they absorb at both pH 4.5 and 1.0. Two buffer systems are employed in the assay and consist of (i) a 0.025 M potassium chloride buffer, pH 1.0, and (ii) a 0.4 M sodium acetate buffer, pH 4.5. Samples were diluted in pH 1.0 and pH 4.5 buffers, but in the concentration range of 25  $\mu\text{g/mL}$  to 100  $\mu\text{g/mL}$ . After an incubation period of 60 min at room temperature to allow for optimal color development of the samples, absorbance readings were taken at  $\lambda = 510$  and 700 nm with the Agilent spectrophotometer. The pH differential absorbance was determined as follows:

$$A = (A_{510\text{nm}} - A_{700\text{nm}}) @ \text{pH } 1.0 - (A_{510\text{nm}} - A_{700\text{nm}}) @ \text{pH } 4.5$$

The TACY value was calculated using the following equation:

$$\text{TACY (mg C3G eq./L)} = (A \times \text{MW} \times \text{DF} \times 1000) / (\epsilon \times \ell)$$

where  $A$  = absorbance; MW = molecular weight (449.2 g/mol); DF = dilution factor; and  $\epsilon$  = molar extinction coefficient (26,900 L  $\text{cm}^{-1}$   $\text{mol}^{-1}$ ). The TACY value was expressed as mg C3G equivalents/100-mg fraction (d.w.) or 100-g fruit (f.w.).

**HPLC Analysis.** An Agilent 1200 Series HPLC system consisting of a quaternary pump with degasser, autosampler, thermostatted column compartment, UV/VIS diode array detection (DAD) with standard flow cell, and 3D ChemStation software (Agilent Technologies) was used for the chromatography. A reversed-phase Luna C<sub>18</sub>(2) column (4.6  $\times$  250 mm, 5  $\mu\text{m}$ ; Phenomenex, Torrance, CA) was utilized. A gradient elution consisting of mobile phase A (H<sub>2</sub>O:CH<sub>3</sub>CN: CH<sub>3</sub>COOH; 93:5:2, v/v/v) and mobile phase B (H<sub>2</sub>O:CH<sub>3</sub>CN:CH<sub>3</sub>COOH;

58:40:2, v/v/v) from 0% to 100% B over a 50-min period at a flow rate of 1 mL/min was employed. Before subsequent injections, the system was re-equilibrated for 10 min using 100% A giving a total run time of 60 min. The injection volume of each fraction (0.5 mg/mL methanol) was 20  $\mu$ L. Detection wavelengths employed were 255 nm (ellagic acid and ellagic acid derivatives), 280 nm (phenolic acids, catechin, epicatechin), 320 nm (phenolic acids), 360 nm (flavonols), and 520 nm (anthocyanins). Tentative identification of separated components was made by matching UV/VIS spectra and retention time mapping with authentic standards. For quantification, calibration curves were constructed for each standard to confirm linearity based on UV/VIS signal as well as for the determination of response factors.

**Electrospray Ionization Mass Spectrometry (ESI-MS) of Anthocyanins.** Mass spectra of blackberry fractions were obtained using an API I-Plus PE SCIEX mass spectrometer (PerkinElmer SCIEX Instruments, Concord, ON, Canada). Each sample ( $\sim$ 1  $\mu$ g/10  $\mu$ L) was dissolved in anhydrous methanol and injected into the mass spectrometer at a flow rate of 0.2 mL/h. The ion-spray voltage was set to 4 kV with the  $Q_0$  rod voltage at 60 V. The source temperature was held at 50  $^{\circ}$ C, and the desolvation gas temperature was maintained at 50  $^{\circ}$ C at a flow rate of 0.8 L/min.

**Matrix-assisted Laser Desorption/Ionization-Time of Flight-Mass Spectrometry (MALDI-TOF-MS).** Samples (1  $\mu$ L of an  $\sim$ 1  $\mu$ g/10  $\mu$ L solution) were mixed with 4  $\mu$ L of a matrix comprising 2,5-dihydroxybenzoic acid at a concentration of 10 mg/mL highly purified water:acetonitrile:TFA (50:50:0.1, v/v/v). Then, 1  $\mu$ L of the resultant solution was spotted onto a flat, stainless-steel MALDI plate and solvents allowed to vaporize in air. Measurements were

performed using a Bruker Daltonics Autoflex™ MALDI-TOF mass spectrometer (Bruker Corporation, Billerica, MA). Ions, including quasimolecular  $[M+H]^+$  and  $[M+Na]^+$  ions, generated by the laser desorption process were introduced into the flight tube with a delay time of 100 ns in the linear positive-ion mode. All mass spectra were collected by averaging the signals of at least 3000 laser shots over the  $m/z$  range of 839 to 6080 using an external standard.

**Hydrolyzable Tannin Composition of FXN-VII.** Assignment of MALDI-TOF mass signals to a particular hydrolyzable tannin structure (*e.g.*, ellagitannin and pedunculagin) was achieved by determining the theoretical or calculated monoisotopic mass according to the following equation:

$$[M+Na]^+ = 634.47 \times \textit{Ellagitannin} + 784.56 \times \textit{Pedunculagin} + 936.66 \times \textit{G-HHDP} + 169.10 \times \textit{Gallate} - 2.02 \times \textit{linkage} + 22.99$$

where ellagitannins (galloyl-HHDP glucopyranose), pedunculagin (bis-HHDP glucopyranose), and G-HHDP (galloyl-bis-HHDP glucopyranose) are numbers of units contained in the hydrolyzable tannin molecule, with ester linkages between the units.

**Statistical Analysis.** System software (SAS, Version 9.1) was used for statistical analysis of data. All observations were expressed as the mean  $\pm$  standard deviation ( $n = 3$ ). Statistical evaluations used *t*-tests and one-way analysis of variance (ANOVA) with cultivar as the variable factor and post-hoc tests for significance of differences by Duncan's multiple comparison test. Statistical significance was considered at  $p < 0.05$ .

## RESULTS AND DISCUSSION

After shipment to the Department of Food Science & Technology at UGA, fresh blackberries from different lots of each of the three cultivars were immediately lyophilized. The moisture contents in the Navaho, Kiowa, and Ouachita cultivars were found to be  $88.14 \pm 2.23$ ,  $90.72 \pm 0.53$ , and  $89.18 \pm 1.3\%$ , respectively.

The solubility of phenolic compounds is governed by a number of factors including the type of solvent (polarity) used, the degree of phenolic polymerization, as well as possible interactions with other food constituents leading to the formation of insoluble complexes. In other words, there is no uniform or completely satisfactory procedure that will guarantee 100% extraction of the phenolics, or even a specific class of phenolic substances, from plant material. Typical solvents employed, either individually or in combination, to extract phenolic constituents include methanol, ethanol, acetone, water, ethyl acetate and, to a lesser extent, propanol and *N,N*-dimethylformamide (38). In this work, a PPE was prepared using a two-step extraction/chromatography approach: the first involved extracting phenolics from lyophilized blackberry samples with 70% (v/v) acidified acetone according to Naczka and Shahidi (39). The resultant CBE was then partially purified using Amberlite XAD-16 resin to remove sugars and nonvolatile organic acids (40). The two sugars generally found in blackberries are glucose and fructose, while phosphoric, malic, citric/isocitric, and quinic acids comprise typical non-volatile organic acids. The Amberlite resin was then washed with water until the pH of the eluent reached 7.0. Following this, the PPE was eluted from the column with anhydrous methanol. The extraction yields for the CBEs are given in **Table 3.1**.

**TPC and TACY Contents in Lyophilized Blackberries.** The Navaho, Kiowa, and Ouachita blackberries had average TPCs of  $459 \pm 1.9$ ,  $409 \pm 0.6$ , and  $411 \pm 3.4$  mg GAE/100-g fresh berries, respectively. Although a significant difference ( $p < 0.05$ ) existed in the TPC values for Navaho with those of the other two cultivars, no difference ( $p > 0.05$ ) was found between Kiowa and Ouachita. Our data showed a higher TPC for Navaho blackberries than the  $304 \pm 6.2$  mg GAE/100-g fresh frozen fruit reported by Moyer and others (41), but a similar value of  $418 \pm 25.8$  mg GAE/100-g fresh fruit given for the Kiowa cultivar by Sellappan et al (42). These TPC values are approximately half of those from Siriwoharn et al. (26), who reported a range of 682 to 1056 mg GAE/100-g fresh fruit with a mean of  $900 \pm 157$  for cultivars like Marion, Waldo, Evergreen, Chester, and Silvan which grow in Oregon. Wide variations in TPC values of blackberries have been reported by several researchers. For instance, Pantelidis and other (43) reported  $2008 \pm 99$  mg GAE/100 g, d.w. in the same cultivar that Wang and Lin (44) reported  $2070 \pm 80$  mg GAE/100 g, f.w., but the locations for sample collection of the cultivar were different.

The TACY values were determined to be  $139.7 \pm 2.4$ ,  $150.0 \pm 2.0$ , and  $143.3 \pm 1.1$  mg C3G equivalents/100-g fresh berries for the Navaho, Kiowa, and Ouachita cultivars, respectively. Furthermore, the TACY values did not differ significantly ( $p > 0.05$ ) amongst the cultivars. These values are similar to those reported by Fan-Chiang and others (23) for Navaho (130 mg C3G eq./100 g, f.w.) and Kiowa (154 mg C3G eq./100 g, f.w.). A value of 126 mg C3G eq./100g, f.w. for Navaho was reported by Moyer and others (41), who also gave a range of 80 to 220 mg C3G eq./100 g f.w. for 27 other hybrids. Our values were somewhat lower than those reported by Cho and others (27) for Navaho (182.3 mg C3G eq./100 g, f.w.) and Kiowa (188.6

mg C3G eq./100 g, f.w.). No data exists in the literature for comparative purposes with the Ouachita cultivar.

**Phenolic Compounds in Lyophilized Blackberries.** The conjugate forms of phenolic compounds were quantified using response factors for their representative standards near the characteristic wavelengths of maximum absorption; that is, 255 nm for ellagic acid and its derivatives; 280 nm for hydroxybenzoic acids and flavan-3-ols, 320 nm for hydroxy-*trans*-cinnamic acids; 360 nm for flavonols and their glycosides; and 520 nm for anthocyanins.

Selected phenolic classes were examined and their mean contents in the three blackberry cultivars are given **Table 3.2**. All values are expressed in mg respective phenolic/100-g fruit, f.w. Representative HPLC chromatograms depicting the separated compounds for Navaho blackberry samples with UV-DAD detection at 280 & 320, and 360 nm as well as VIS-DAD detection at 520 nm are given in **Figure 3.3(A), (B), and (C)**, respectively. The peaks in the HPLC chromatograms were tentatively classified into free and conjugated forms of hydroxy-*trans*-cinnamic acids, hydroxybenzoic acids (*e.g.*, gallic acid), ellagic acid, ellagitannins, flavonols (*e.g.*, quercetin), flavan-3-ols, anthocyanidins, and anthocyanins by comparison of their UV- or VIS-spectra with those of available aglycone standards (*i.e.*, *p*-coumaric acid, caffeic acid, gallic acid, ellagic acid, quercetin, myricetin, kaempferol, (+)-catechin, (-)-epicatechin, pelargonidin, cyanidin, delphinidin, and malvidin) and glycoside standards (cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside, cyanidin-3,5-di-*O*-glucoside, cyanidin-3-*O*-galactoside, and cyanidin-3-*O*-rhamnoside). A minor peak was detected without a UV spectrum typical of the standards available: it remained unidentified. Whenever possible, relative retention times of

identified peaks and elution orders/patterns of RP-18 HPLC chromatograms from literature were critically evaluated and used to further support the tentative identification of phenolic compounds isolated in this work.

In agreement with previous reports, cyanidins and ellagitannins are the major phenolic classes that characterize plants of the *Rubus* species (25, 45). In blackberries, anthocyanin pigments and anthocyanidins (reaction products from acid hydrolysis of anthocyanins, which are often used as an analytical marker for anthocyanin content; see Sellappan et al [42]) were the predominant phenolics present in the samples tested, and this is consistent with literature findings (18, 23). Cyanidin-3-*O*-glucoside was the dominant anthocyanin; it ranged from  $98.8 \pm 3.28$  to  $114.2 \pm 4.01$  mg G3G/100-g fruit (f.w.). Similar values were reported in *Rubus adenotrichus* [125.8 mg G3G/100-g fruit, f.w.] and *Rubus glaucus* [62.7 mg G3G/100-g fruit, f.w.] (25). Our data is somewhat lower than values reported by Cho and others (27) for the Navaho (145.9 mg G3G/100-g fruit, f.w.) and Kiowa (158.3 mg G3G/100-g fruit, f.w.) cultivars. Higher contents were also noted in Oklahoma-grown Navaho (113.7 mg G3G/100-g fruit, f.w.) and Kiowa (141.1 mg G3G/100-g fruit, f.w.) (23). Siriwoharn and others (26), on the other hand, reported lower concentrations for C3G in Oregon blackberries (*i.e.*, 69.8 to 93.9 mg G3G/100-g fruit, f.w.). No literature data exists concerning the Ouachita cultivar. In Kiowa blackberries, cyanidin-3-*O*-rutinoside was detected [see Peak 2 in **Figure 3.3(C)**] and corresponds with literature data (46) and mass spectra results for Kiowa blackberry fractions described later in the Discussion.

Ellagitannins and ellagic acid (both free and conjugated forms) are reported as ellagic acid and ranged from  $41.1 \pm 0.98$  to  $71.5 \pm 0.14$  mg ellagic acid eq./100-g fruit, f.w. The total ellagic acid



content was significantly ( $p < 0.05$ ) higher in the Navaho and Kiowa cultivars than Ouachita. When comparing our findings for Georgia-grown cultivars to other works, Siriwoharn and others (26) reported lower values for ellagitannins and ellagic acid derivatives; that is, only 9.8 to 27.9 mg ellagic acid eq./100-g fruit, f.w. in Oregon-grown blackberry cultivars like Marion, Evergreen, and Waldo. On the other hand, Mertz and others (25) reported higher values of 194.1 mg ellagic acid eq./100-g fruit, f.w. in *Rubus adenotrichus* and 503.8 mg ellagic acid eq./100-g fruit, f.w. in *Rubus glaucus*.

*p*-Hydroxybenzoic acid, *p*-hydroxy-*trans*-cinnamic acid, and the flavan-3-ols (+)-catechin and (-)-epicatechin were minor phenolic compounds detected in the three cultivars investigated. Free and conjugated forms of gallic acid were measured as gallic acid and ranged from  $2.9 \pm 0.01$  to  $5.8 \pm 0.09$  mg GAE/100-g fruit, f.w. The Navaho cultivar contained the greatest amount of gallic acid, followed by Kiowa and then Ouachita. Mertz and others (25) reported a range of 0.99 to 5.49 mg GAE/100-g fruit, f.w. in berry plants from the *Rubus* species. A value within this range was given by Sellappan and others (42) for Kiowa (*i.e.*, 4.12 mg GAE/100-g fruit, f.w.). Hydroxy-*trans*-cinnamic acids such as *trans*-cinnamic and *p*-coumaric acids were detected in the Navaho cultivar; *p*-coumaric acid was also found in Ouachita blackberries. Similar findings have been reported by Sellappan *et al* (42) and Mertz (25) for Kiowa and other *Rubus* species, respectively. Flavan-3-ols were found in all cultivars, but epicatechin was detected only in the Ouachita variety. Quercetin, a flavonol, was detected in both Navaho and Ouachita, and only a trace of caffeic acid was found in the Ouachita variety. No myricetin was detected in any of the blackberry preparations examined in this work, but its presence in blackberry has been reported (42).

Clearly, berry composition is affected by intrinsic factors such as choice of cultivar and genetics, as well as by numerous extrinsic factors such as maturity of the berry, UV-light exposure, climatic conditions, and agronomic practices including method of harvesting as well as post-harvest manipulation (47). Even small differences in the degree of ripeness or sample preparation techniques (*e.g.* grinding, extraction, and isolation) when it comes time for analysis will contribute to variations in the profile of phenolics and the levels determined in the fruit (26).

**Phenolic Compounds Determined in the Blackberry Fractions.** Column chromatography has long been an important tool to isolate natural products. Crude extracts are often subjected to classical column chromatography before further separations by HPLC. In this work, the PPE was loaded onto the top of a lipophilic Sephadex column, and compounds were eluted by varying the polarity of the mobile phase. In most cases, FXN-I and -II absorbed UV radiation at  $\lambda = 280$  and 320 nm, thereby indicating the presence of phenolic acids and flavonoids, while FXN-III thru -V absorbed UV and VIS radiation at  $\lambda = 360$  and 520 nm, respectively, thus indicating the presence of flavonols and anthocyanins. Based on the mass balance, the majority of flavonoids in the blackberry fractions eluted in FXN-III, -IV, and -V. The yields at each stage of extraction and separation of the blackberry fractions are given in **Figure 3.2**.

The relative content (%), TPC, and TACY values for the fractions from each cultivar are summarized in **Table 3.3**. TPC values ranged from  $6.9 \pm 0.07$  to  $77.8 \pm 0.49$  mg GAE/100-mg fraction and the TACY values ranged from  $0.1 \pm 0.01$  to  $45.1 \pm 0.49$  mg C3G eq./100-mg fraction. The largest TACY content was determined for FXN-III of Kiowa berries,  $45.1 \pm 0.5$

mg C3G eq./100-mg fraction, while the lowest was found in FXN-VII of Kiowa,  $0.1 \pm 0.01$  mg C3G eq./100-mg fraction. It is difficult to compare our findings with those reported in the literature, as most researchers have not removed sugars, non-volatile organic acids, and other water-soluble compounds like vitamin C which can interfere with reaction involving Folin-Ciocalteu's phenol reagent (48).

Selected phenolic classes were examined and their mean contents in each of the fractions from the Navaho, Kiowa, and Ouachita cultivars are presented in **Table 3.4**, **3.5**, and **3.6**, respectively. FXN-III, -IV and -V contained the highest concentrations of phenolics and these were predominantly the anthocyanin, cyanidin-3-*O*-glucoside, whose level ranged from a low  $8.12 \pm 0.46$  mg C3G eq./100-mg fraction in Navaho FXN-V to a high of  $67.74 \pm 0.46$  mg C3G eq./100-mg fraction in Ouachita FXN-IV. As well, significant quantities of FXN-VII were recovered, but this acetonic fraction was not characterized by anthocyanins, rather by ellagic acid, its derivatives, and ellagitannins. For the three blackberry varieties, the quantity of ellagic acid and its derivatives ranged from  $21.1 \pm 0.23$  to  $32.2 \pm 0.01$  mg ellagic acid eq./100-mg fraction. A representative HPLC chromatogram of the compounds separated from FXN-VII is depicted in **Figure 3.4**. Free ellagic acid was identified by comparison of its retention time and absorption spectrum to that of a commercial standard. Also based on UV spectra, peaks 2 thru 7 were assigned as being ellagic acid and ellagitannin compounds (**Figure 3.5**), but no further data was acquired for further identification from the HPLC analysis. No catechins or epicatechins were detected in this fraction, and this may be attributable to very small quantities of condensed tannins present in blackberry fruit (30).

**Mass Spectra.** HPLC coupled to mass spectrometry (HPLC-MS) is a powerful tool that can provide useful structural information and allow for tentative compound identification when standard reference compounds are not available and peaks have similar absorption spectra and retention times (44,49). An attempt was made to analyze the fractions rich in anthocyanins by ESI-MS to help with identifying the number and types of sugar moieties attached to cyanidin residues, as well as to detect for the presence of other anthocyanins. Compounds in FXN-VII were further identified by MALDI-TOF-MS with comparisons to theoretical molecular weights from literature data.

**ESI-MS of Anthocyanins.** Full scan ESI-MS data revealed the presence of three molecular cations ( $M^+$ ) at an  $m/z$  of 449, 595, and 727. Precursor-ion scans indicated that the three molecular cations were precursors of cyanidin (with a  $M^+$  at  $m/z$  of 287), and indicative of cyanidin-based anthocyanins. Cyanidin-3-*O*-glucoside, the dominant compound in the blackberry fractions FXN-III thru -V, exhibited an  $M^+$  at  $m/z$  of 449, and also yielded a fragment ion at  $m/z$  of 287 corresponding to cyanidin (*i.e.*,  $M-162$ )<sup>+</sup>. This characteristic mass spectral pattern was observed in all three of the cultivars examined. ESI-MS analysis of the Kiowa cultivar showed an  $M^+$  at  $m/z$  of 595 and an  $(M-308)^+$  fragment (possibly resulting from cleavage of a rutosyl moiety); cyanidin-3-*O*-rutinoside has been reported in blackberries cultivars by other investigators (23, 27). Furthermore, the ESI-MS revealed the presence of a possible molecular ion at an  $m/z$  of 727 and a fragment ion of  $(M-297)^+$ , which could denote loss of xylosyl and glucosyl groups, and suggest the presence of cyanidin-3-*O*-xylosylrutinoside in blackberries. No prior data is available indicating the presence of cyanidin-3-*O*-xylosylrutinoside in blackberry cultivars. Cho and others (27) did report, however, the detection

of cyanidin-3-*O*-xyloside, cyanidin-3-malonylglucoside, and cyanidin-3-dioxalylglucoside in Kiowa and Navaho cultivars as well as the dominant cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutilinoside.

**MALDI-TOF-MS.** MALDI is a soft ionization technique and enables the interpretation of mass-ion signals corresponding to differences in molecular weights attributed to the degree of hydroxylation and the presence of galloylation. Sephadex LH-20 chromatography of the PPE into low- and high-molecular-weight fractions was expected to markedly improve the sensitivity of the MALDI-TOF-MS analysis towards FXN-VII, by removing many of the lower molecular compounds which would reach the mass detector first and lead to its saturation. Yet, it's important to remember, that most of the ellagic acid endogenous to blackberries, eluted from the Sephadex column only when the mobile phase was change over to 50% (v/v) acetone: water.

The MALDI-TOF mass spectra of FXN-VII from Navaho, Kiowa, and Ouachita cultivars are shown in **Figure 3.6(A), (B), and (C)**, respectively. The MALDI-TOF-MS spectra verified the presence of several large molecular mass compounds ranging from an  $m/z$  of 807.1 to an  $m/z$  of 3762.9. The smallest peak detected had an  $m/z$  of 807.1. This was ascribed to bis-HHDP glucopyranose and is the primary unit for an ellagitannin (ET), also commonly referred to as pedunculagin. This peak was present only in Navaho cultivar. The largest peak was detected at an  $m/z$  of 3762.9 and attributed to lambertianin D, a tetramer of galloyl-bis-HHDP glucopyranose. This compound was only found in the Kiowa cultivar. Each unit of galloyl-bis-HHDP glucopyranose has pedunculagin esterified with a galloyl group. The peak detected at an  $m/z$  of 1127.3 is believed to be the  $\text{Na}^+$  adduct of galloyl-bis-HHDP glucopyranose with a gallate

moiety attached to it and a  $M^+$  at an  $m/z$  of 1106.68. The cation with an  $m/z$  of 1741.5 is thought to be the  $Na^+$  adduct of degalloylated sanguin H-6/lambertianin A, with a molecular mass of 1719.2. Compounds detected at an  $m/z$  of 1893.6 and 2827.8 are the  $Na^+$  adduct signals for the sanguine H-6/lambertianin A and lambertianin C, respectively; these were found in FXN-VII of all three Georgia grown cultivars. The compound with a molecular-ion signal at an  $m/z$  of 1591.5 is believed to be the  $Na^+$  adduct of the ellagitannins sanguin H-10 (*i.e.*, the same as sanguin H-6 but with the one less HHDP moiety). The signal detected at an  $m/z$  of 2061 was ascribed as being the  $Na^+$  adduct of lambertianin A with an additional gallic acid moiety attached. The signal at an  $m/z$  of 2525 may be the  $Na^+$  adduct of lambertianin C with the one less HHDP moiety, and a molecular mass of 2828.8. Finally, the compound with an  $m/z$  of 974.46 could not be assigned. A compound with a similar molecular weight was described as an ellagitannin by Hager and others (24). Using ESI-MS, these authors were able to fragment the large molecule and revealed signals at an  $m/z$  of 783 and 631 which are characteristic of ellagitannins. A summary of the assignments based on MALDI-TOF-MS for ellagitannin constituents found in FXN-VII is given in **Table 3.7**.

This study showed that the acetonic fractions (FXN-VII) of all three blackberry cultivars contain a distribution of ellagitannins. A number of these can be separated by chromatographic means, but isolated compounds require further evaluation of their stability and chemical structure by 1-D ( $^1H$  and  $^{13}C\{^1H\}$ ) and 2-D (HMBC and HMQC) NMR techniques and further MS/MS techniques. The ethanolic fractions mainly contain cyanidin-3-*O*-glucoside, ellagic acid, gallic acid, catechin and traces of *trans*-cinnamic and *p*-coumaric acid. Therefore, the blackberry holds

a significant potential as a healthy fruit and a suitable raw material in the development of next-generation functional foods.

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## LITERATURE CITED

- 1) Rouanet, J.-M.; Décordé, K.; del Rio, D.; Auger, C.; Borges, G.; Cristol, J.-P.; Lean, M. E. J.; Crozier, A. Berry juices, teas, antioxidants and the prevention of atherosclerosis in hamsters. *Food Chem.* **2010**, *118*, 266–271.
- 2) Bagchi, D.; Sen, C. K.; Ray, S. D.; Das, D. K.; Bagchi, M.; Preuss, H. G.; Vinson, J. A. Molecular mechanisms of cardioprotection by a novel grape seed proanthocyanidin extract. *Mutat. Res.* **2003**, *523–524*, 87–97.
- 3) Stoner, G. D.; Wang, L.-S.; Zikri, N.; Chen, T.; Hecht, S. S.; Huang, C.; Sardo, C.; Lechner, J. F. Cancer prevention with freeze-dried berries and berry components. *Semin. Cancer Biol.* **2007**, *17*, 403–410.
- 4) Bagchi, D.; Sen, C. K.; Bagchi, M.; Atalay, M. Anti-angiogenic, antioxidant, and anti-carcinogenic properties of a novel anthocyanin-rich berry extract formula. *Biochem. (Mosc.)* **2004**, *69*, 75–80.
- 5) Boivin, D.; Blanchette, M.; Barrette, S.; Moghrabi, A.; Béliveau, R. Inhibition of cancer cell proliferation and suppression of TNF-induced activation of NFkappaB by edible berry juice. *Anticancer Res.* **2007**, *27*, 937–948.
- 6) Cheplick, S.; Kwon, Y.-I.; Bhowmik, P.; Shetty, K. Phenolic-linked variation in strawberry cultivars for potential dietary management of hyperglycemia and related complications of hypertension. *Biores. Technol.* **2010**, *101*, 404–413.
- 7) Duncan, F. J.; Martin, J. R.; Wulff, B. C.; Stoner, G. D.; Tober, K. L.; Oberyszyn, T. M.; Kusewitt, D. F.; VanBuskirk, A. M. Topical treatment with black raspberry extract reduces cutaneous UVB-induced carcinogenesis and inflammation. *Cancer Prev. Res.* **2009**, *2*, 665–672.



- 8) Shukitt-Hale, B.; Lau, F. C.; Carey, A. N.; Galli, R. L.; Spangler, E. L.; Ingram, D. K.; Joseph, J. A. Blueberry polyphenols attenuate kainic acid-induced decrements in cognition and alter inflammatory gene expression in rat hippocampus. *Nutr. Neurosci.* **2008**, *11*, 172–182.
- 9) Halliwell, B. Role of free radicals in the neurodegenerative diseases: therapeutic implications for antioxidant treatment. *Drugs Aging* **2001**, *18*, 685–716.
- 10) Zafra-Stone, S.; Yasmin, T.; Bagchi, M.; Chatterjee, A.; Vinson, J.A.; Bagchi, D. Berry anthocyanins as novel antioxidants in human health and disease prevention. *Mol. Nutr. Food Res.* **2007**, *51*, 675–683.
- 11) Liu, M.; Li, X. Q.; Weber, C.; Lee, C. Y.; Brown, J.; Liu, R. H. Antioxidant and antiproliferative activities of raspberries. *J. Agric. Food Chem.* **2002**, *50*, 2926–2930.
- 12) Nijveldt, R. J.; van Nood, E.; van Hoorn, D. E. C.; Boelens, P. G.; van Norren, K.; van Leeuwen, P. A. M. Flavonoids: a review of probable mechanisms of action and potential applications. *Am. J. Clin. Nutr.* **2001**, *74*, 418–425.
- 13) Fernandez-Pancho, M. S.; Villano, D.; Troncoso, A. M.; Garcia-Parrilla, M. C. Antioxidant activity of phenolic compounds: from in vitro results to in vivo evidence. *Crit. Rev. Food Sci. Nutr.* **2008**, *48*, 649–671.
- 14) Seeram, N. P. Berry fruits for cancer prevention: current status and future prospects. *J. Agric. Food Chem.* **2008**, *56*, 630–635.
- 15) Pellegrini, N.; Serafini, M.; Colombi, B.; del Rio, D.; Salvatore, S.; Bianchi, M.; Brighenti, F. Total antioxidant capacity of plant foods, beverages and oils consumed in Italy assessed by three different in vitro assays. *J. Nutr.* **2003**, *133*, 2812–2819.

- 16) Pergola, C.; Rossi, A.; Dugo, P.; Cuzzocrea, S.; Sautebin, L. Inhibition of nitric oxide biosynthesis by anthocyanin fraction of blackberry extract. *Nitric Oxide* **2006**, *15*, 30–39.
- 17) Seeram, N. P.; Adams, L. S.; Zhang, Y.; Lee, R.; Sand, D.; Scheuller, H. S.; Heber, D. Blackberry, black raspberry, blueberry, cranberry, red raspberry, and strawberry extracts inhibit growth and stimulate apoptosis of human cancer cells in vitro. *J. Agric. Food Chem.* **2006**, *54*, 9329–9339.
- 18) Dai, J.; Gupte, A.; Gates, L.; Mumper, R. J. A comprehensive study of anthocyanin-containing extracts from selected blackberry cultivars: Extraction methods, stability, anticancer properties and mechanisms. *Food Chem. Toxicol.* **2009**, *47*, 837–847.
- 19) Ding, M.; Feng, R.; Wang, S. Y.; Bowman, L.; Lu, Y.; Qian, Y.; Castranova, V.; Jiang, B.-H.; Shi, X. Cyanidin-3-glucoside, a natural product derived from blackberry, exhibits chemopreventive and chemotherapeutic activity. *J. Biol. Chem.* **2006**, *281*, 17359–17368.
- 20) Rossi, A.; Serraino, I.; Dugo, P.; Di Paola, R.; Mondello, L.; Genovese, T.; Morabito, D.; Dugo, G.; Sautebin, L.; Caputi, A. P.; Cuzzocrea, S. Protective effects of anthocyanins from blackberry in a rat model of acute lung inflammation. *Free Radic. Res.* **2003**, *37*, 891–900.
- 21) Shukitt-Hale, B.; Cheng, V.; Joseph, J. A. Effects of blackberries on motor and cognitive function in aged rats. *Nutr. Neurosci.* **2009**, *12*, 135–140.
- 22) Hassimotto, N. M. A.; da Silva Pinto, M.; Lajolo, F. M. Antioxidant status in humans after consumption of blackberry (*Rubus fruticosus* L.) juices with and without defatted milk. *J. Agric. Food Chem.* **2008**, *56*, 11727–11733.
- 23) Fan-Chiang, H.-J.; Wrolstad, R. E. Anthocyanin pigment composition of blackberries. *J. Food Sci.* **2005**, *70*, C198–C202.

- 24) Hager, T. J.; Howard, L. R.; Liyanage, R.; Lay, J. O.; Prior, R. L. Ellagitannin composition of blackberry as determined by HPLC-ESI-MS and MALDI-TOF-MS. *J. Agric. Food Chem.* **2008**, *56*, 661–669.
- 25) Mertz, C.; Cheynier, V.; Günata, Z.; Brat, P. Analysis of Phenolic compounds in two blackberry species (*Rubus glaucus* and *Rubus adenotrichus*) by high-performance liquid chromatography with diode array detection and electrospray ion trap mass spectrometry. *J. Agric. Food Chem.* **2007**, *55*, 8616–8624.
- 26) Siriwoharn, T.; Wrolstad, R. E.; Finn, C. E.; Pereira, C. B. Influence of cultivar, maturity, and sampling on blackberry (*Rubus L. hybrids*) anthocyanins, polyphenolics, and antioxidant properties. *J. Agric. Food Chem.* **2004**, *52*, 8021–8030.
- 27) Cho, J. M.; Howard, L. R.; Prior, R. L.; Clark, J. R. Flavonoid glycosides and antioxidant capacity of various blackberry, blueberry and red grape genotypes determined by high-performance liquid chromatography/mass spectrometry. *J. Sci. Food Agric.* **2004**, *84*, 1771–1782.
- 28) Siriwoharn, T.; Wrolstad, R. E. Polyphenolic composition of Marion and Evergreen blackberries. *J. Food Sci.* **2004**, *69*, FCT233–FCT240.
- 29) Wald, B.; Galensa, R.; Herrmann, K.; Grotjahn, L.; Wray, V. Quercetin 3-*O*-[6''-(3-hydroxy-3-methylglutaroyl)- $\beta$ -galactoside] from blackberries. *Phytochemistry* **1986**, *25*, 2904–2905.
- 30) Xu, Y.; Zhang, Y.; Chen, M.; Tu, P. Fatty acids, tocopherols and proanthocyanidins in bramble seeds. *Food Chem.* **2006**, *99*, 586–590.

- 31) Rommel, A.; Wrolstad, R. E. Ellagic acid content of red raspberry juice as influenced by cultivar, processing, and environmental factors. *J. Agric. Food Chem.* **1993**, *41*, 1951–1960.
- 32) Vrhovsek, U.; Palchetti, A.; Reniero, F.; Guillou, C.; Masuero, D.; Mattivi, F. Concentration and mean degree of polymerization of *Rubus* ellagitannins evaluated by optimized acid methanolysis. *J. Agric. Food Chem.* **2006**, *54*, 4469–4475.
- 33) Gupta, R. K.; Al-Shafi, S. M. K.; Layden, K.; Haslam, E. The metabolism of gallic acid and hexahydroxydiphenic acid in plants. Part 2. Esters of (*S*)-hexahydroxydiphenic acid with D-glucopyranose (<sup>4</sup>C<sub>1</sub>). *J. Chem. Soc. Perkin Trans. 1* **1982**, 2525–2534.
- 34) Clark, J. R.; Stafne, E. T.; Hall, H. K.; Finn, C. E. Blackberry breeding and genetics. In *Plant Breeding Reviews*; Janick, J., Ed.; John Wiley and Sons: Hoboken, NJ, **2007**; *29*, 19–144.
- 35) Strik, B. C.; Clark, J. R.; Finn, C. E.; Bañados, M. P. Worldwide blackberry production. *Hortech. n.* **2007**, *17*, 205–213.
- 36) Singleton, V. L.; Rossi, J. A., Jr. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* **1965**, *16*, 144–158.
- 37) Giusti, M. M.; Wrolstad, R. E. Characterization and measurement of anthocyanins by UV-visible spectroscopy. In *Current Protocols in Food Analytical Chemistry*; Wrolstad, R. E., Ed.; John Wiley and Sons: New York, NY, 2001; F.1.2.1–F.1.2.13.
- 38) Antolovich, M.; Prenzler, P.; Robards, K.; Ryan, D. Sample preparation in the determination of phenolic compounds in fruits. *Analyst* **2000**, *125*, 989–1009.
- 39) Naczk, M.; Shahidi, F. Extraction and analysis of phenolics in food. *J. Chromatogr. A* **2004**, *1054*, 95–111.

- 40) Mazza, G.; Cacace, J. E.; Kay, C. D. Methods of analysis for anthocyanins in plants and biological fluids. *J. AOAC Int.* **2004**, *87*, 129–145.
- 41) Moyer, R. A.; Hummer, K. E.; Finn, C. E.; Frie, B.; Wrolstad, R. E. Anthocyanins, phenolics, and antioxidant capacity in diverse small fruits: vaccinium, rubus, and ribes. *J. Agric. Food Chem.* **2002**, *50*, 519–525.
- 42) Sellappan, S.; Akoh, C. C.; Krewer, G. Phenolic compounds and antioxidant capacity of Georgia-grown blueberries and blackberries. *J. Agric. Food Chem.* **2002**, *50*, 2432–2438.
- 43) Pantelidis, G. E.; Vasilakakis, M.; Manganaris, G. A.; Diamantidis, G. Antioxidant capacity, phenol, anthocyanin and ascorbic acid contents in raspberries, blackberries, red currants, gooseberries and Cornelian cherries. *Food Chem.* **2007**, *102*, 777–783.
- 44) Wang, S. Y.; Lin, H. S. Antioxidant activity in fruit and leaves of blackberry, raspberry and strawberry varies with cultivar and developmental stage. *J. Agric. Food Chem.* **2000**, *48*, 140–146.
- 45) Määttä-Riihinen, K. R.; Kamal-Eldin, A.; Törrönen, A. R. Identification and quantification of phenolic compounds in berries of *Fragaria* and *Rubus* Species (Family Rosaceae). *J. Agric. Food Chem.* **2004**, *52*, 6178–6187.
- 46) Wu, X.; Prior, R. L. Systematic identification and characterization of anthocyanins by HPLC-ESI-MS/MS in common foods in the United States: fruits and berries. *J. Agric. Food Chem.* **2005**, *53*, 2589–2599.
- 47) Parr, A. J.; Bolwell, G. P. Phenols in the plant and in man. The potential for possible nutritional enhancement of the diet by modifying the phenols content or profile. *J. Sci. Food Agric.* **2000**, *80*, 985–1012.

- 48) Georgé, S.; Brat, P.; Alter, P.; Amiot, M. J. Rapid determination of polyphenols and vitamin C in plant-derived products. *J. Agric. Food Chem.* **2005**, *53*, 1370–1373.
- 49) Haddock, E. A.; Gupta, R. K.; Al-Shafi, S. M. K.; Layden, K.; Haslam, E.; Magnolato, D. The metabolism of gallic acid and hexahydroxydiphenic acid in plants: Biogenetic and molecular taxonomic considerations. *Phytochem.* **1982**, *21*, 1049–1062.

## FIGURE CAPTIONS

**Figure 3.1** Benzoic acid derivatives: **(A)** gallic acid,  $R_1=OH$ ,  $R_2=OH$ ,  $R_3=OH$ ; **(B)** *trans*-cinnamic acid derivatives: *p*-Coumaric acid,  $R_1=H$ ,  $R_2=OH$ ; *trans*-cinnamic acid,  $R_1=H$ ,  $R_2=H$ ; **(C)** flavonols: quercetin,  $R_3=OH$ ,  $R_4=OH$ ,  $R_5=H$ ,  $R_3=OH$ ; myricetin,  $R_3=OH$ ,  $R_4=OH$ ,  $R_5=OH$ ,  $R_3=OH$ ; **(D)** anthocyanidins/anthocyanins: cyanidin,  $R_3=OH$ ,  $R_4=OH$ ,  $R_5=H$ ,  $R_3=OH$ ,  $R_5=OH$ ; **(E)** flavan-3-ols: (+)-catechin or (-)-epicatechin; **(F)** ellagic acid; **(G)** hexahydroxydiphenic acid (HHDP); **(H)** galloyl group; **(I)** ellagitannins: galloyl-HHDP; **(J)** pedunculagin: bis-HHDP glucopyranose; **(K)** galloyl-bis-HHDP glucopyranose; **(L)** sanguisorboyl linking ester group [Ref 10]; **(M)** dimer: sanguin H-6/lambertianin; **(N)** dimer: lambertianin B; **(O)** trimer: lambertianin C; and **(P)** tetramer: lambertianin D. (Adapted from [Ref 24 and 32]).

**Figure 3.2** Schematic process flow diagram detailing the isolation of ethanolic and acetic fractions from lyophilized blackberries using extraction and column chromatography strategies. Abbreviations: CBE, crude blackberry extract; PPE, polyphenolic extract; FXN I – VII, fractions I thru VII.

**Figure 3.3(A)** Representative HPLC chromatogram of a Navaho blackberry sample with UV-DAD detection at 280 and 320 nm. Peaks 1, 2, and 3 – galloyl esters; Peak 4 – (+)-catechin; Peaks 5, 6, and 7 – ellagitannins, ellagic acid, and its derivatives.

**Figure 3.3(B)** Representative HPLC chromatogram of a Navaho blackberry sample with UV-DAD detection at 360 nm. Peak 1 – quercetin.

**Figure 3.3(C)** Representative HPLC chromatogram of a Navaho blackberry sample with VIS-DAD detection at 520 nm. Peak 1 – cyanidin-3-*O*-glucoside; and Peak 2 – Cyanidin-3-*O*-rutinoside.

**Figure 3.4** Representative HPLC chromatogram of FXN-VII from Navaho blackberries with UV-DAD detection at 280 nm. Peak 1 – gallic acid; Peaks 2, 3, 4, and 5 – ellagitannins; and Peaks 6 & 7 – ellagic acid and its derivatives.

**Figure 3.5** On-line UV/VIS DAD spectra of (A) gallic acid; (B) galloyl esters; (C) ellagitannins; as well as (D) ellagic acid and its derivatives.

**Figure 3.6** MALDI-TOF-MS of FXN-VII from the blackberry cultivars (A) Navaho, (B) Kiowa, and (C) Ouachita.



**Table 3.1.** Relative yields (%) of CBEs and PPEs isolated from 100-g fresh blackberries of the Navaho, Kiowa, and Ouachita varieties.

Blackberry Cultivars	Moisture content (%)	CBE (%)	PPE (%)
Navaho	88.14 ± 2.23	1.85 ± 0.18	1.27 ± 0.03
Kiowa	90.72 ± 0.53	1.25 ± 0.09	0.80 ± 0.11
Ouachita	89.18 ± 1.30	1.36 ± 0.26	0.90 ± 0.30

Values are means of triplicate analyses ± standard deviation.

Abbreviations: CBE: crude blackberry extract; and PPE: polyphenolic extract.

**Table 3.2.** Phenolic compounds and selected phenolic classes identified in Georgia-grown Navaho, Kiowa, and Ouachita blackberries.<sup>a</sup>

Phenolic compounds	Navaho	Kiowa	Ouachita
gallic acid	5.8 ± 0.09	4.6 ± 0.04	2.9 ± 0.01
<i>trans</i> -cinnamic acid	0.23 ± 0.01	ND	ND
<i>p</i> -coumaric acid	0.20 ± 0.03	ND	0.1 ± 0.00
(+)-catechin	22.7 ± 0.13	17.8 ± 0.40	28.5 ± 1.4
(-)-epicatechin	ND	ND	1.3 ± 0.10
ellagic acid & ellagitannins	71.5 ± 0.14	63.4 ± 0.13	41.1 ± 0.98
quercetin	0.48 ± 0.03	ND	0.43 ± 0.01
cyanidins	101 ± 4.37	98.8 ± 3.28	114 ± 4.01

<sup>a</sup>Values are means of triplicate analyses ± standard deviation. Findings are reported as mg respective phenolic/100-g fruit, f.w. Abbreviations: ND, not detected; f.w., fresh weight.

**Table 3.3.** Percent relative yield, total phenolics content, and total anthocyanins content of fractions isolated from Navaho, Kiowa, and Ouachita blackberry cultivars.<sup>a</sup>

Fraction <sup>b</sup>	<i>Navaho</i>			<i>Kiowa</i>			<i>Ouachita</i>		
	RC <sup>c</sup>	TPC <sup>d</sup>	TACY <sup>e</sup>	RC <sup>c</sup>	TPC <sup>d</sup>	TACY <sup>e</sup>	RC <sup>c</sup>	TPC <sup>d</sup>	TACY <sup>e</sup>
FXN-I	5.1 ± 1.2	11.3 ± 0.96 q	3.2 ± 0.14 x	7.6 ± 1.2	28.2 ± 0.71 p	0.9 ± 0.02 y	6.2 ± 1.3	6.9 ± 0.07 r	0.4 ± 0.08 z
FXN-II	9.8 ± 1.1	16.9 ± 0.12 q	1.9 ± 0.13 z	8.8 ± 0.9	65.7 ± 0.99 p	18.9 ± 1.3 x	10.1 ± 2.6	17.4 ± 0.29 q	14.4 ± 0.95 y
FXN-III	16.4 ± 1.3	77.8 ± 0.49 p	30.7 ± 0.83 y	10.1 ± 1.9	62.1 ± 0.74 r	45.1 ± 0.49 x	11.9 ± 1.0	75.0 ± 0.33 q	22.3 ± 0.86 z
FXN-IV	13.0 ± 1.2	51.3 ± 0.44 r	20.2 ± 0.91 z	13.7 ± 1.9	61.1 ± 0.77 q	42.6 ± 0.86 x	15.1 ± 1.8	69.2 ± 0.13 p	32.4 ± 0.29 y
FXN-V	9.0 ± 1.6	62.7 ± 0.49 q	11.4 ± 0.70 y	6.6 ± 1.2	66.0 ± 0.35 p	9.2 ± 0.91 y	4.2 ± 1.8	46.3 ± 0.57 r	16.8 ± 0.99 x
FXN-VI	6.1 ± 1.5	35.6 ± 0.23 q	5.6 ± 0.49 x	7.9 ± 1.0	68.9 ± 0.64 p	6.0 ± 0.85 x	11.0 ± 2.2	22.7 ± 0.98 r	2.7 ± 0.92 y
FXN-VII	30.4 ± 1.2	75.4 ± 0.34 p	1.0 ± 0.14 x	27.8 ± 1.4	48.6 ± 0.85 r	0.1 ± 0.01 z	27.8 ± 1.8	57.6 ± 0.95 q	0.6 ± 0.13 y

<sup>a</sup>Values are means of triplicate analyses ± standard deviation. When one-way ANOVA was significant, differences in TPC and TACY values of fractions amongst cultivars were determined using Duncan's multiple comparison test ( $p < 0.05$ ). Means with the same letter (represented by pqu & xyz for TPC and TACY values, respectively) in a row are not significantly ( $p > 0.05$ ) different. Abbreviations: RC, relative content; TPC, total phenolics content; TACY, total anthocyanins content; FXN, fraction; GAE, gallic acid equivalents; C3G, cyanidin-3-*O*-glucoside; d.w., dry weight.

<sup>b</sup>FXN-I thru -VI were eluted from the Sephadex LH-20 column with 95% (v/v) as the mobile phase and FXN-VII was eluted with 50% (v/v) acetone:water.

<sup>c</sup>RC values are percentages based on average recoveries of each fraction from 200-mg of PPEs being loaded on a Sephadex LH-20 column for each cultivar. Mass balances indicated ~13.8% loss of the PPEs after column chromatography.

<sup>d</sup>TPC values are expressed as mg GAE/100-mg fraction, d.w.

<sup>e</sup>TACY values are expressed as mg C3G equivalents/100-mg fraction, d.w.

**Table 3.4.** Phenolic compounds and selected phenolic classes identified in blackberry fractions of the Navaho cultivar.<sup>a</sup>

Compound	FXN-I <sup>b</sup>	FXN-II	FXN-III	FXN-IV	FXN-V	FXN-VI	FXN-VII <sup>c</sup>
gallic acid and its derivatives (GTs)	trace	0.43 ± 0.01	ND	ND	ND	ND	3.13 ± 0.02
<i>trans</i> -cinnamic acid	ND	0.44 ± 0.07	ND	ND	ND	ND	ND
<i>p</i> -coumaric acid	trace	0.40 ± 0.01	ND	ND	ND	ND	ND
(+)-catechin	ND	1.25 ± 0.07	ND	7.04 ± 0.06	ND	ND	ND
(-)-epicatechin	ND	0.87 ± 0.01	ND	ND	ND	ND	ND
ellagic acid & its derivatives (ETs)	trace	0.62 ± 0.02	1.05 ± 0.01	1.62 ± 0.04	2.23 ± 0.08	2.43 ± 0.44	25.33 ± 0.01
quercetin & its glycosides	ND	ND	3.17 ± 0.04	0.88 ± 0.09	0.77 ± 0.05	0.97 ± 0.01	ND
cyanidins	ND	ND	28.82 ± 1.11	22.81 ± 0.17	8.12 ± 0.46	7.22 ± 0.08	ND

<sup>a</sup>Values are means of triplicate analyses ± standard deviation and are expressed as mg phenolic standard/100-mg fraction, d.w. Abbreviations: FXN, fraction; GTs, gallotannins; ETs, ellagitannins; ND, not detected; d.w., dry weight.

<sup>b</sup>FXN-I thru -VI were eluted from the Sephadex LH-20 column with 95% (v/v) as the mobile phase and FXN-VII was eluted with 50% (v/v) acetone:water.

<sup>c</sup>Although FXN-VII is rich in ellagi- and gallotannin constituents, gallic acid and ellagic acid (to a greater extent) preferentially come of the Sephadex LH-20 column with the more polar 50% (v/v) acetone:water solvent system.

**Table 3.5.** Phenolic compounds and selected phenolic classes identified in blackberry fractions of the Kiowa cultivar.<sup>a</sup>

Compound	FXN-I <sup>b</sup>	FXN-II	FXN-III	FXN-IV	FXN-V	FXN-VI	FXN-VII <sup>c</sup>
gallic acid and its derivatives (GTs)	4.14 ± 0.29	4.80 ± 0.21	ND	ND	ND	ND	3.44 ± 0.05
<i>trans</i> -cinnamic acid	trace	0.49 ± 0.08	0.65 ± 0.05	ND	ND	ND	ND
<i>p</i> -coumaric acid	ND	ND	ND	ND	ND	ND	ND
(+)-catechin	ND	ND	ND	ND	ND	ND	ND
(-)-epicatechin	ND	ND	ND	ND	ND	ND	ND
ellagic acid & its derivatives (ETs)	0.32 ± 0.02	0.98 ± 0.01	0.95 ± 0.01	0.63 ± 0.01	2.79 ± 0.01	5.43 ± 0.02	32.24 ± 0.10
quercetin & its glycosides	ND	ND	ND	ND	ND	ND	ND
cyanidins	ND	1.75 ± 0.01	40.26 ± 0.41	47.72 ± 0.18	8.91 ± 0.05	trace	ND

<sup>a</sup>Values are means of triplicate analyses ± standard deviation and are expressed as mg phenolic standard/100-mg fraction, d.w. Abbreviations: FXN, fraction; GTs, gallotannins; ETs, ellagitannins; ND, not detected; d.w., dry weight.

<sup>b</sup>FXN-I thru -VI were eluted from the Sephadex LH-20 column with 95% (v/v) as the mobile phase and FXN-VII was eluted with 50% (v/v) acetone:water.

<sup>c</sup>Although FXN-VII is rich in ellagi- and gallotannin constituents, gallic acid and ellagic acid (to a greater extent) preferentially come of the Sephadex LH-20 column with the more polar 50% (v/v) acetone:water solvent system.

**Table 3.6.** Phenolic compounds and selected phenolic classes identified in blackberry fractions of the Ouachita cultivar.<sup>a</sup>

Compound	FXN-I <sup>b</sup>	FXN-II	FXN-III	FXN-IV	FXN-V	FXN-VI	FXN-VII <sup>c</sup>
gallic acid and its derivatives (GTs)	0.70 ± 0.02	trace	0.95 ± 0.02	0.55 ± 0.02	ND	ND	1.14 ± 0.08
<i>trans</i> -cinnamic acid	ND	0.76 ± 0.01	ND	ND	ND	ND	ND
<i>p</i> -coumaric acid	ND	ND	0.27 ± 0.03	ND	ND	ND	ND
(+)-catechin	1.33 ± 0.12	4.92 ± 0.03	ND	ND	ND	ND	ND
(-)-epicatechin	ND	ND	ND	ND	ND	ND	ND
ellagic acid & its derivatives (ETs)	1.27 ± 0.01	2.01 ± 0.12	0.12 ± 0.01	0.12 ± 0.01	0.45 ± 0.01	3.10 ± 0.01	21.06 ± 0.23
quercetin & its glycosides	ND	ND	2.50 ± 0.21	10.20 ± 0.50	ND	ND	ND
cyanidins	ND	1.15 ± 0.01	24.40 ± 0.74	67.74 ± 0.46	12.11 ± 0.83	1.53 ± 0.13	ND

<sup>a</sup>Values are means of triplicate analyses ± standard deviation and are expressed as mg phenolic standard/100-mg fraction, d.w. Abbreviations: FXN, fraction; GTs, gallotannins; ETs, ellagitannins; ND, not detected; d.w., dry weight.

<sup>b</sup>FXN-I thru -VI were eluted from the Sephadex LH-20 column with 95% (v/v) as the mobile phase and FXN-VII was eluted with 50% (v/v) acetone:water.

<sup>c</sup>Although FXN-VII is rich in ellagi- and gallotannin constituents, gallic acid and ellagic acid (to a greater extent) preferentially come of the Sephadex LH-20 column with the more polar 50% (v/v) acetone:water solvent system.

**Table 3.7.** Interpretation of the MALDI-TOF-MS for hydrolyzable tannins in the acetonic FXN-VII of Navaho blackberries.<sup>a</sup>

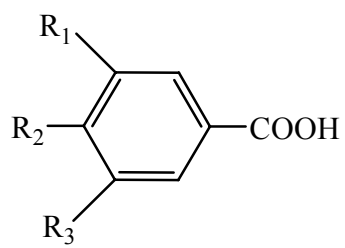
Number of units	Linkages	Cal.	Navaho	Kiowa	Ouachita
		[M+Na] <sup>+</sup>	[M+Na] <sup>+</sup>	[M+Na] <sup>+</sup>	[M+Na] <sup>+</sup>
A 1, Pedunculagin (bis-HHDP glucopyranose)		807.46	807.1	nd	nd
B 1, unidentified		974.89	975.1	975	975.1
C 1, galloyl-bis-HHDP glucopyranose, 1-gallate	1	1126.6	1127.1	1127.1	1127.1
D 1, galloyl-bis-HHDP glucopyranose, 1,1'- galloyl -HHDP glucopyranose	1	1592.1	1591.5	1591.1	1591.3
E 1, galloyl-bis-HHDP glucopyranose, 1-bis-HHDP glucopyranose	1	1742.2	1741.5	1741.1	1741.3
F 2, galloyl-bis-HHDP glucopyranose	1	1894.3	1893.6	1893.1	1893.3
G 2, galloyl-bis-HHDP glucopyranose, 1 gallate	2	2061.3	nd	2061.1	2061.2
H 2, galloyl-bis-HHDP glucose, 1,1'- galloyl -HHDP glucopyranose	2	2526.7	nd	2525	nd
I 3, galloyl-bis-HHDP glucopyranose	2	2828.9	2827.8	2827	2827.2
J 4, galloyl-bis-HHDP glucopyranose	3	3746.6	nd	nd	3746.6

<sup>a</sup>FNX-VII eluted from the Sephadex LH-20 column with 50% (v/v) acetone:water.

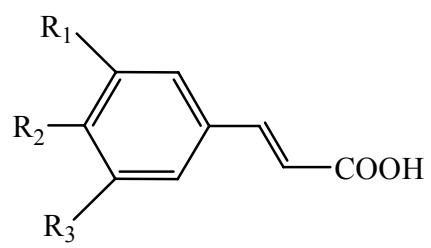
Abbreviations: MALDI-TOF-MS, matrix-assisted laser desorption/ionization-time of flight-mass spectrometer; FXN, fraction; Cal., calculated; [M+Na]<sup>+</sup>, sodium cation adduct of the hydrolyzable tannin's molecular ion; nd, not determined.

**Figure 3.1**

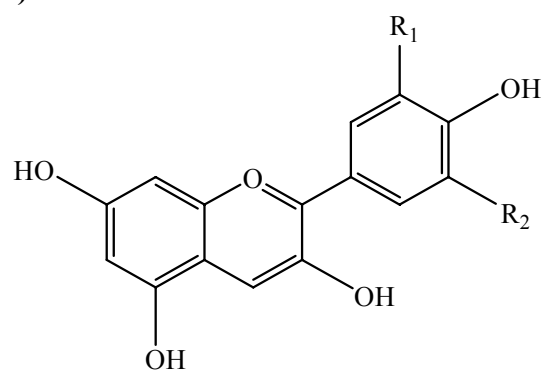
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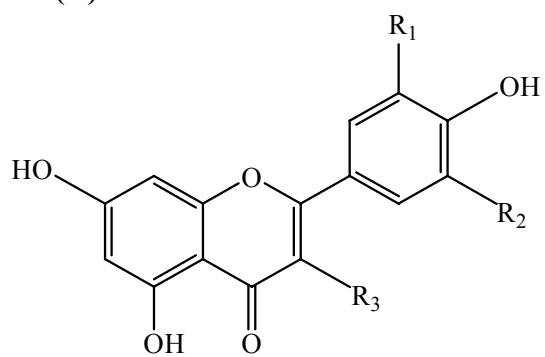
**(B)**



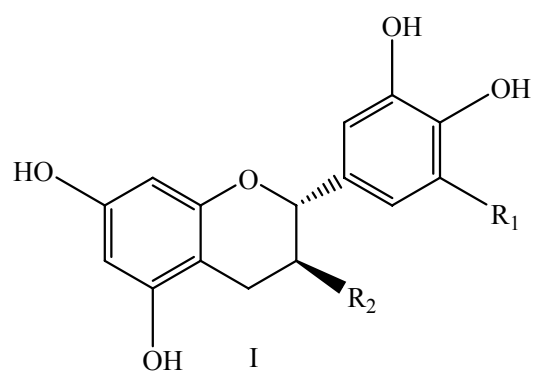
**(C)**



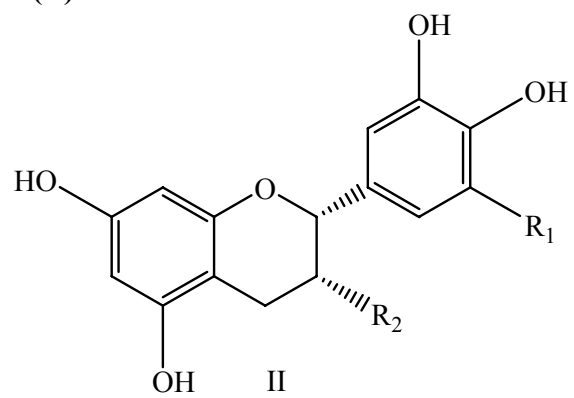
**(D)**



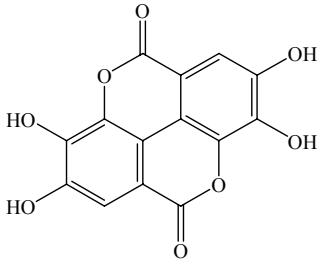
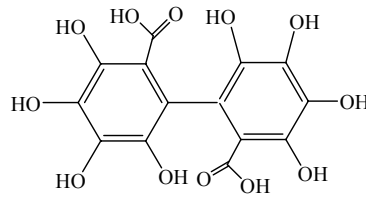
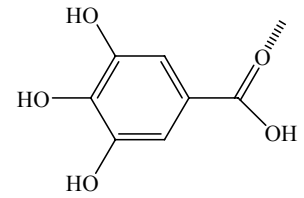
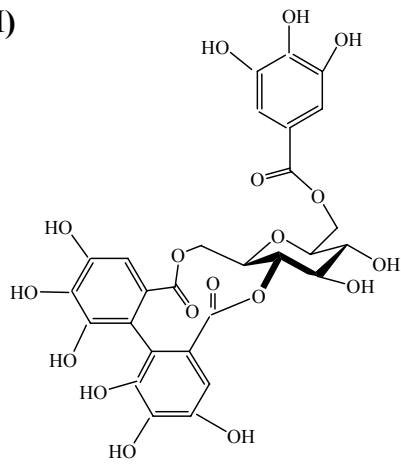
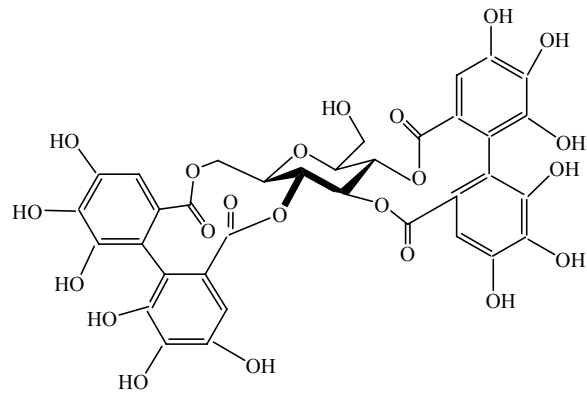
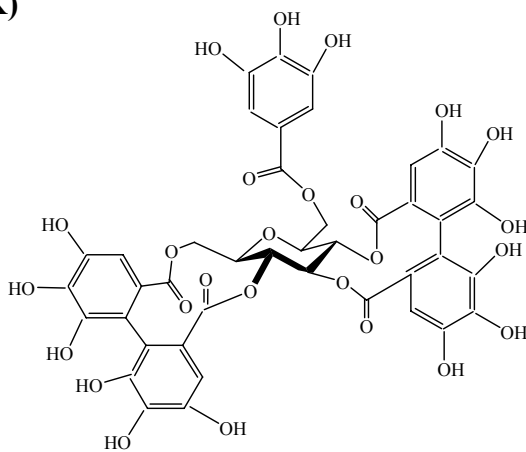
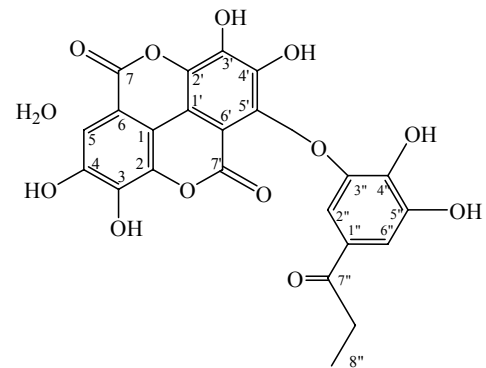
**(E)-I**



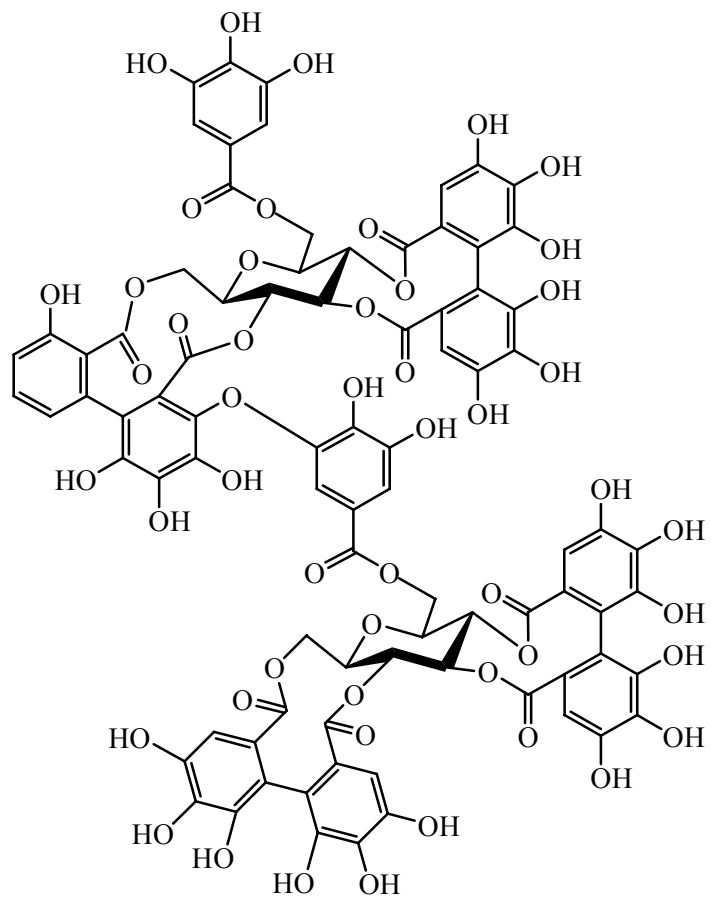
**(E)-II**



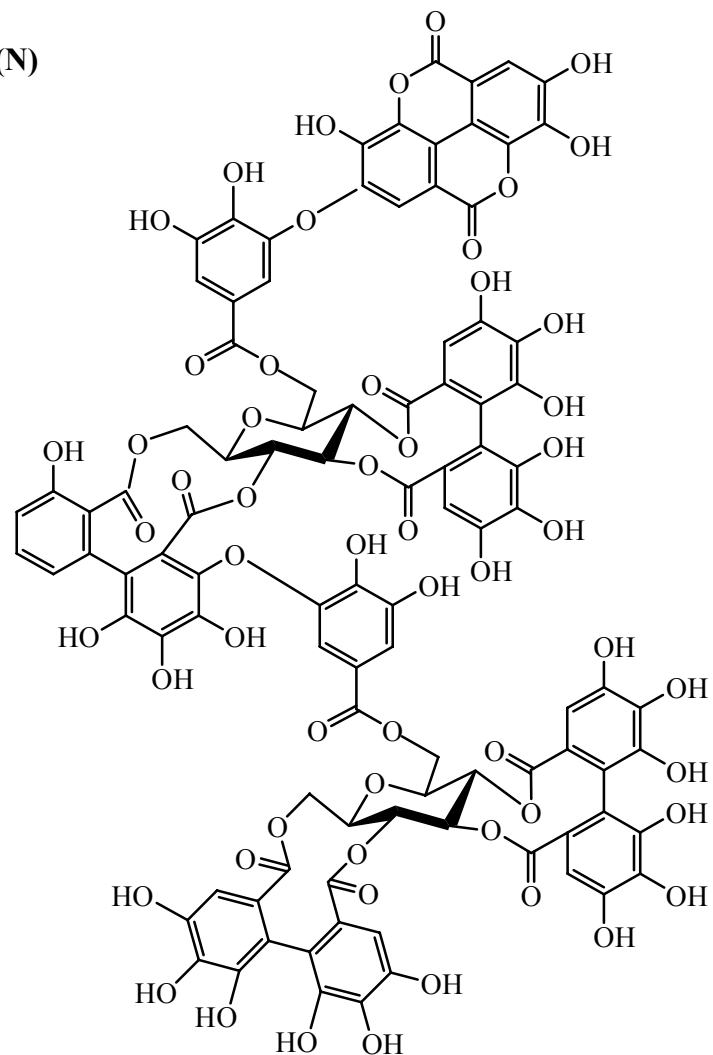


**(F)****(G)****(H)****(I)****(J)****(K)****(L)**

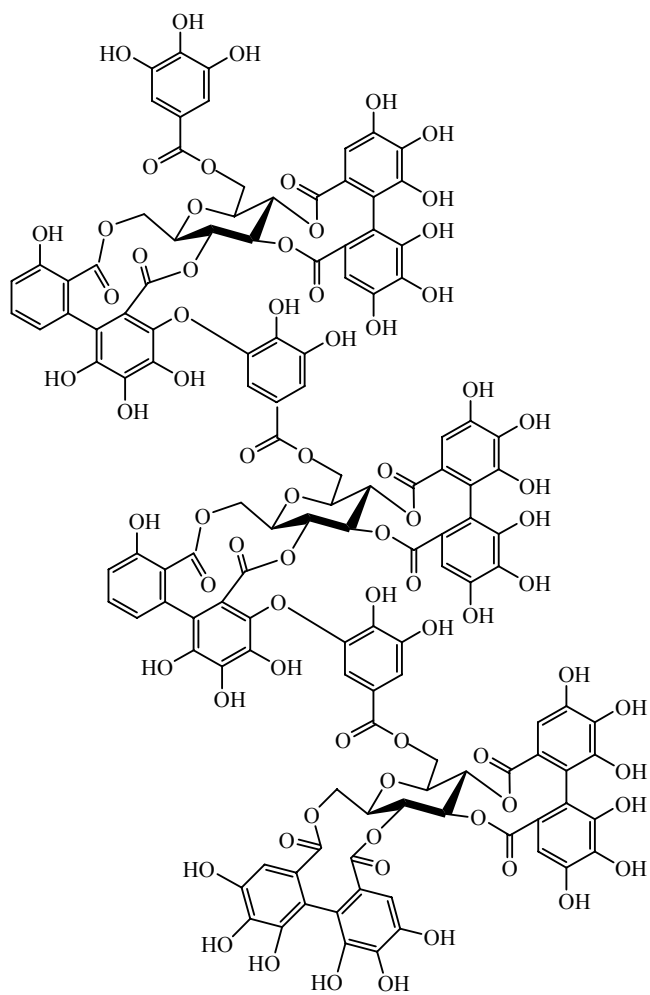
(M)



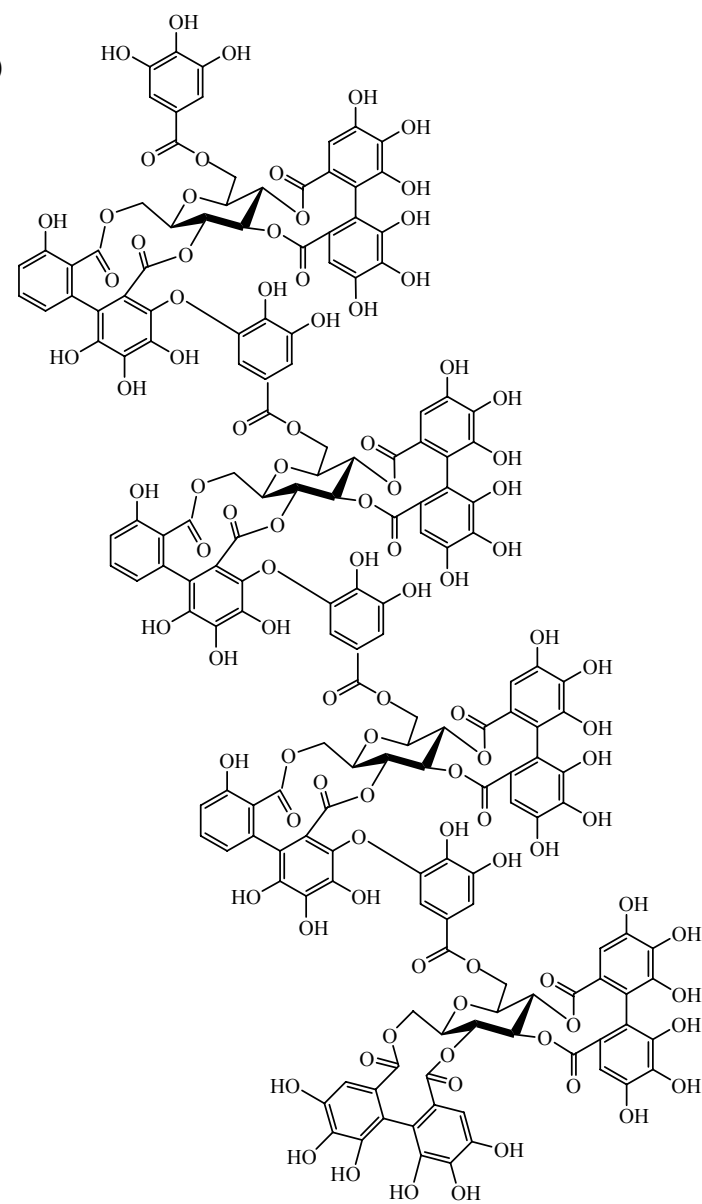
(N)



(O)



(P)



**Figure 3.2**  
**Extraction**

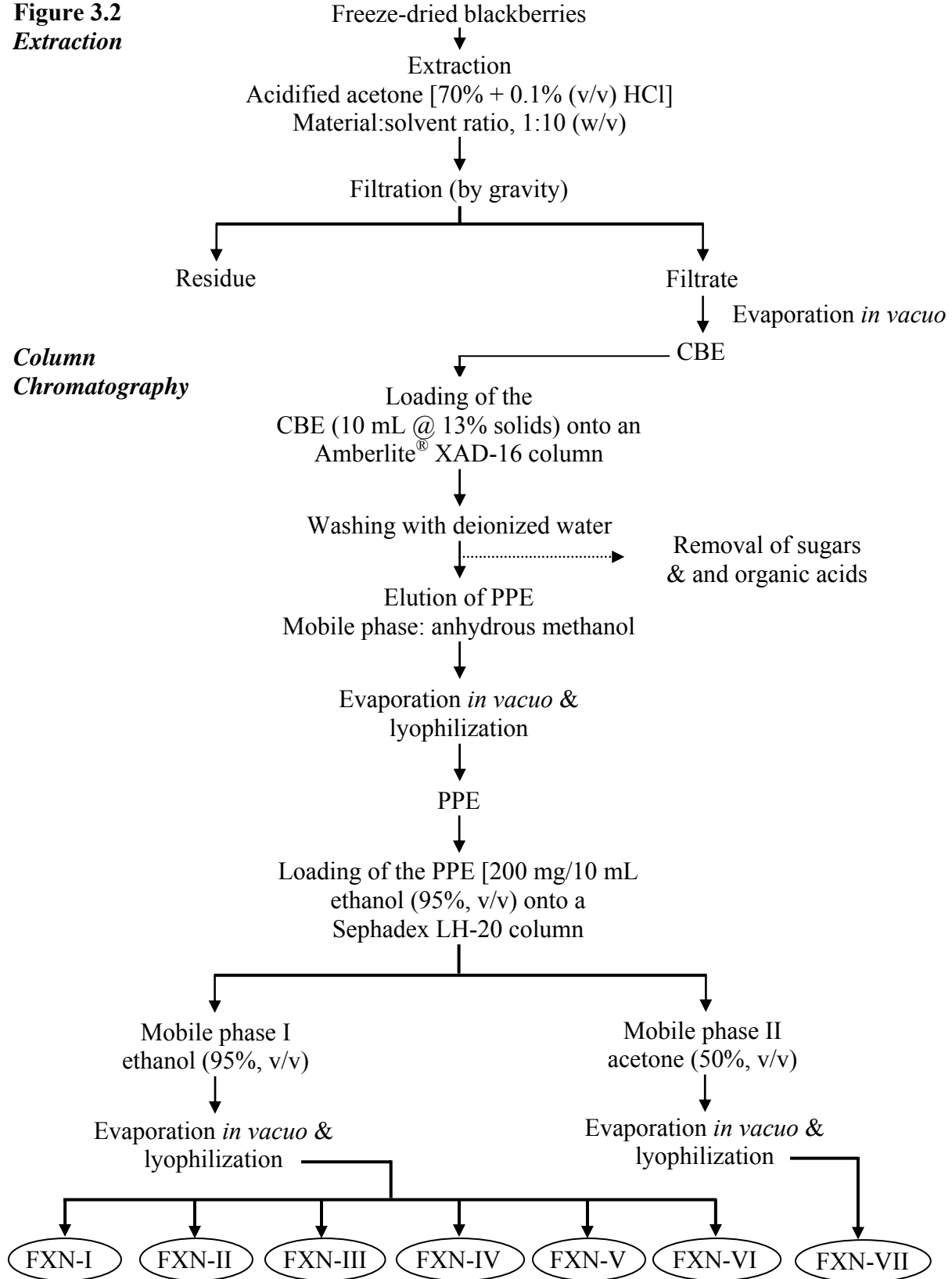


Figure 3.3(A)

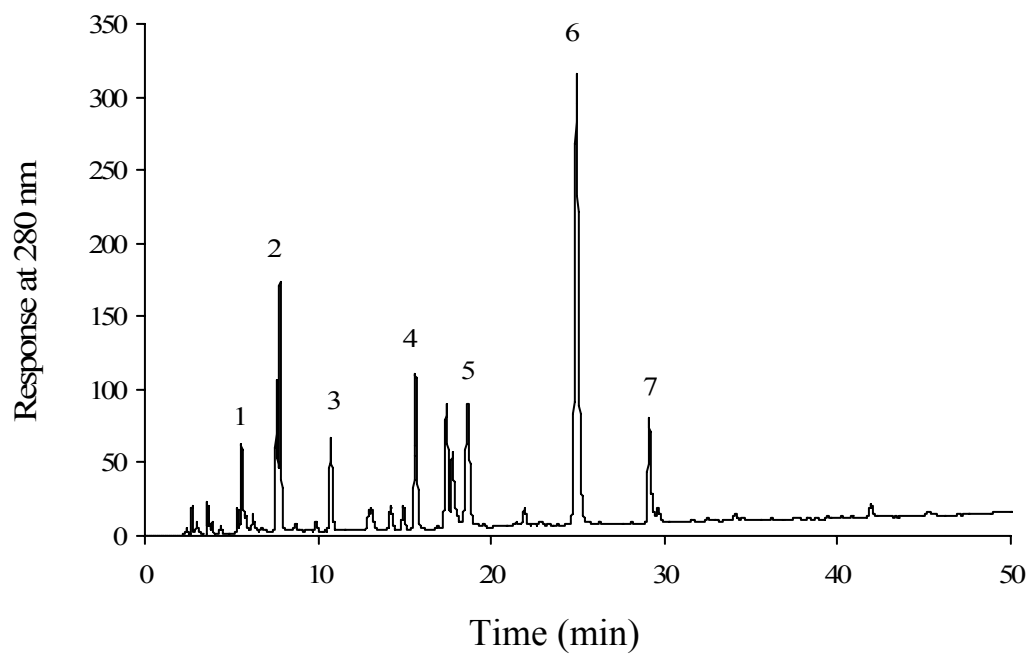


Figure 3.3(B)

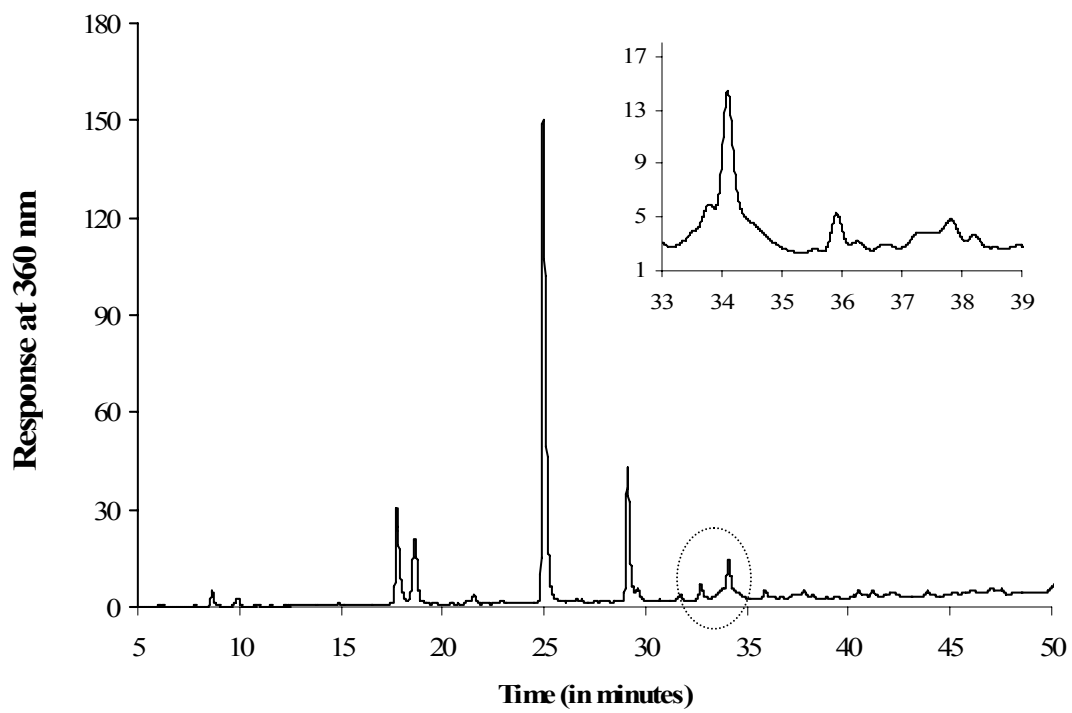


Figure 3.3(C)

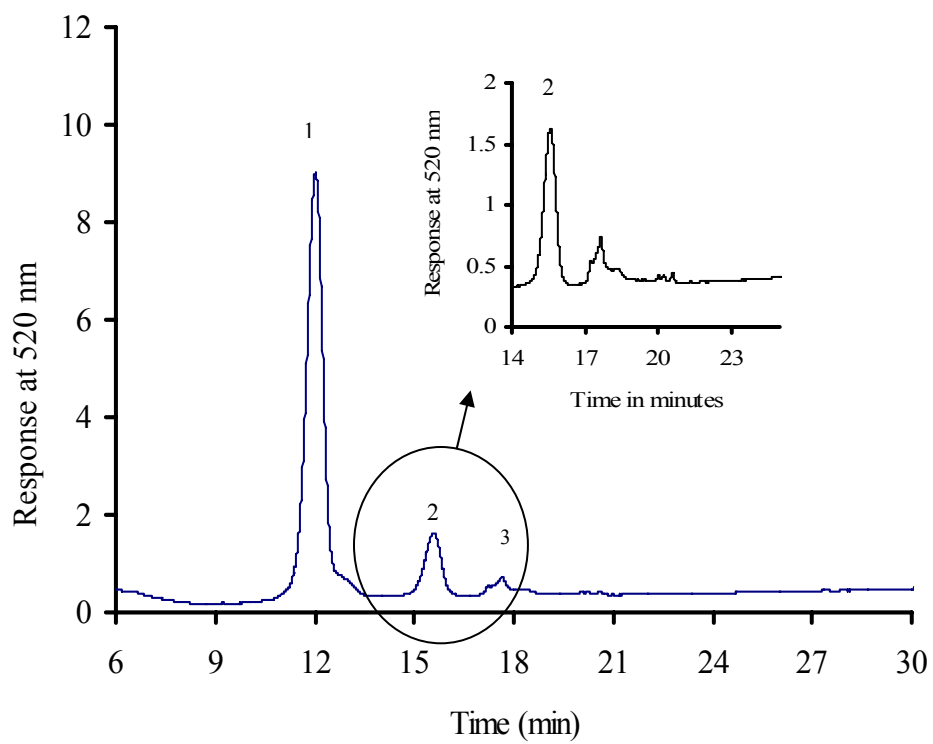
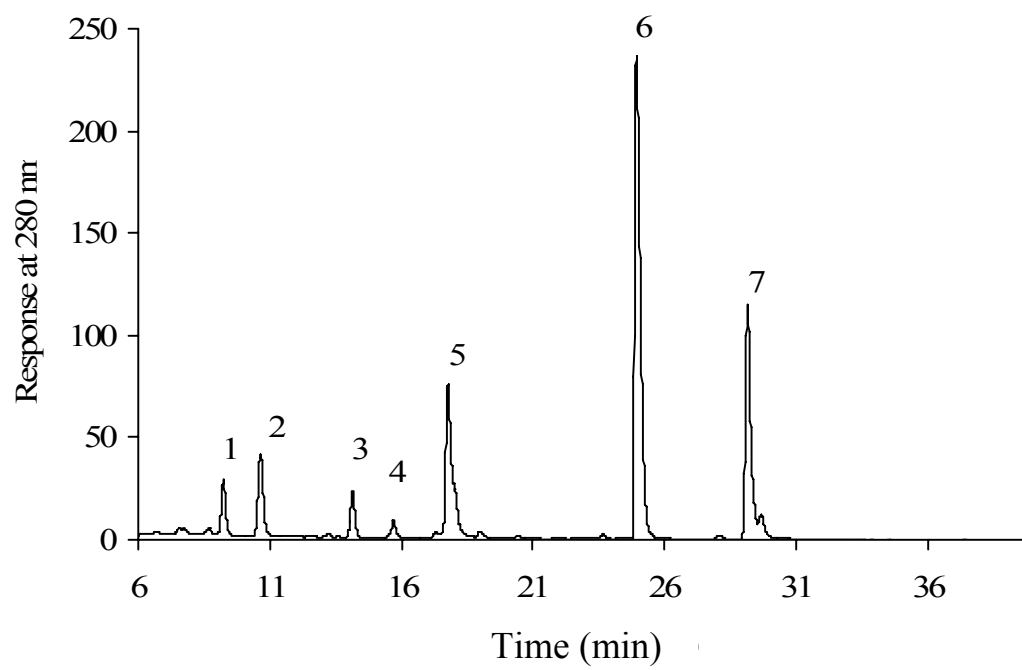


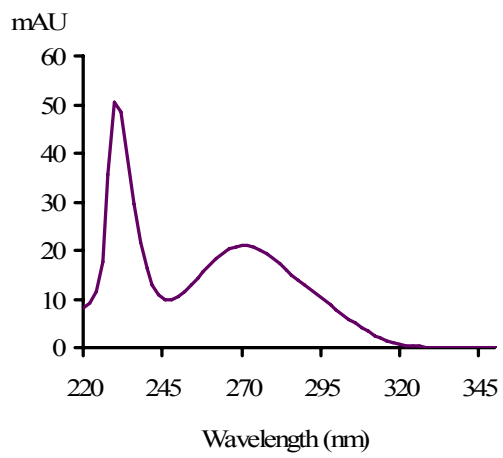
Figure 3.4



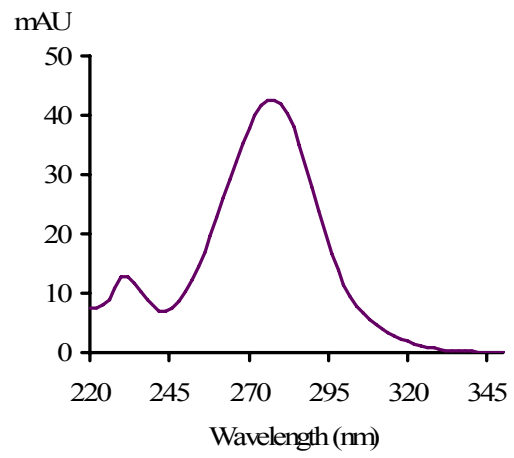


**Figure 3.5**

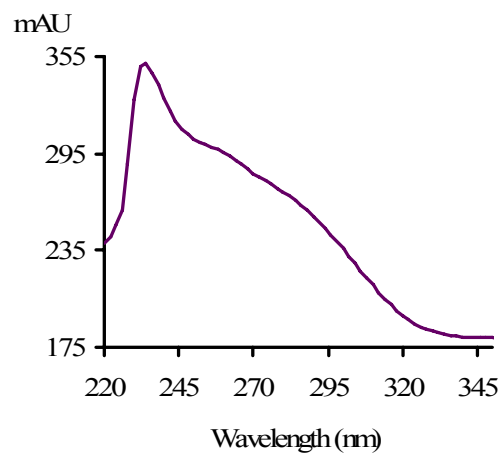
**(A)**



**(B)**



**(C)**



**(D)**

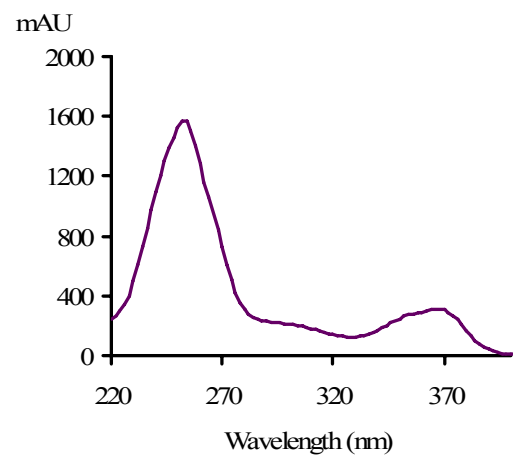


Figure 3.6(A)

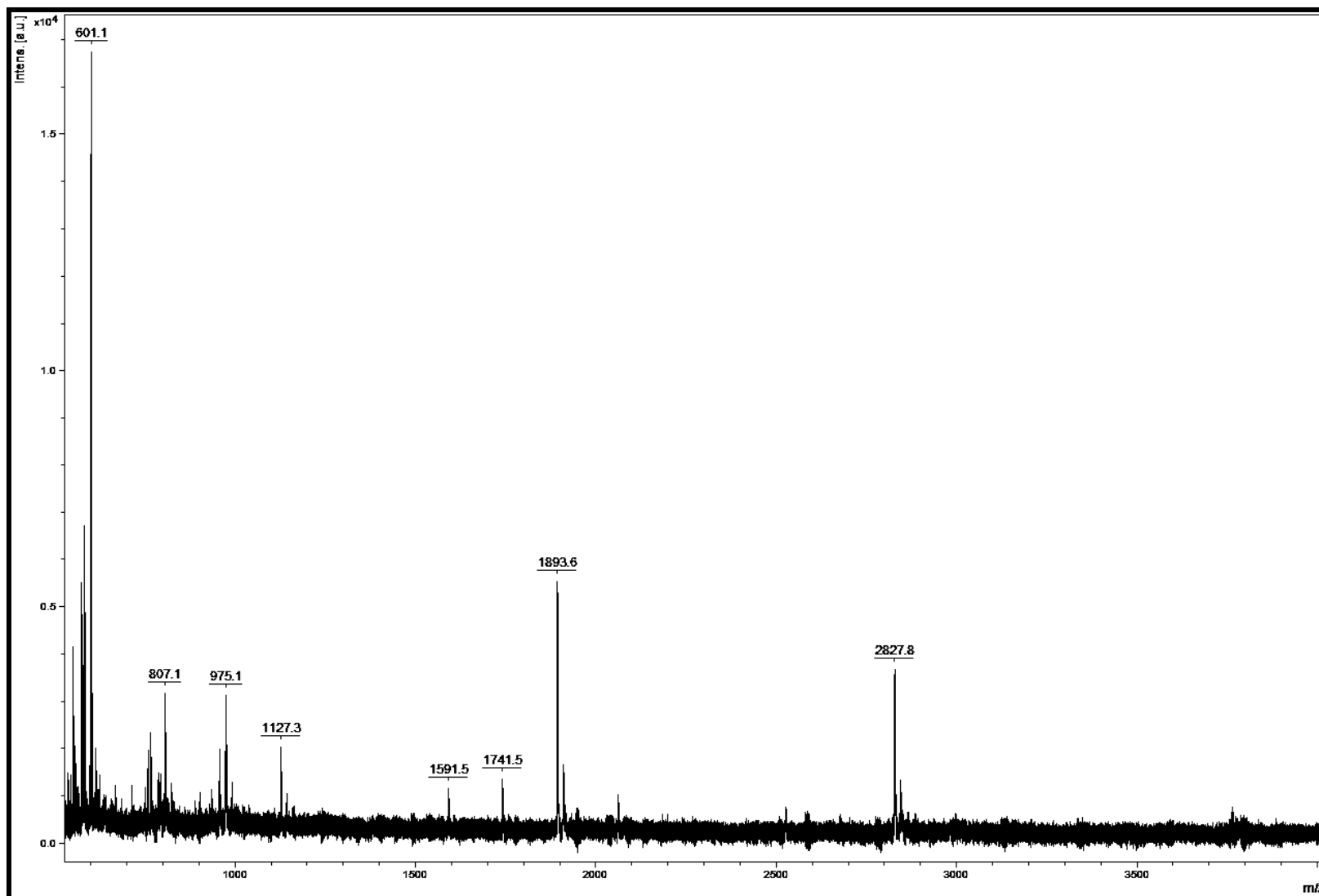


Figure 3.6(B)

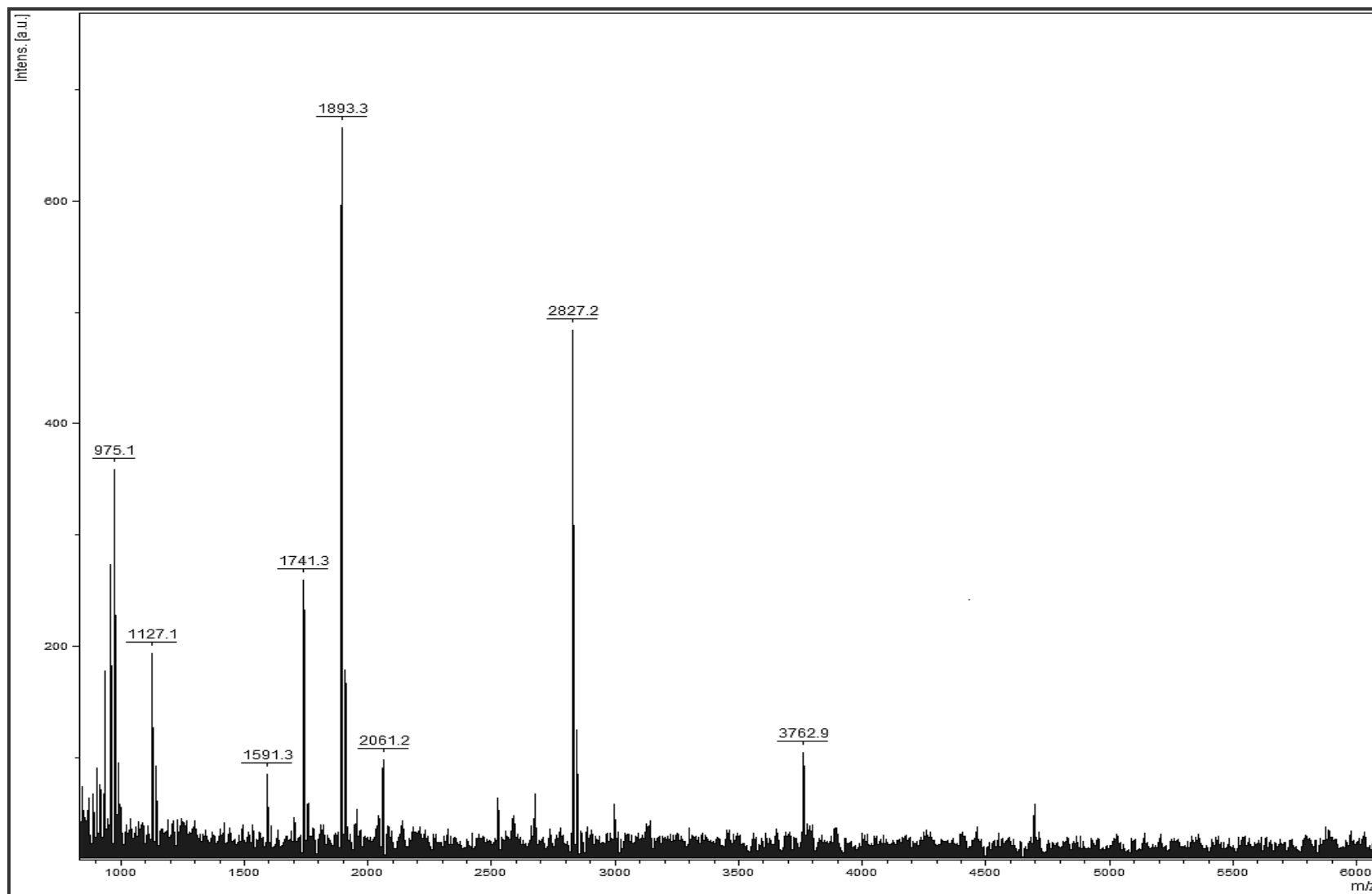
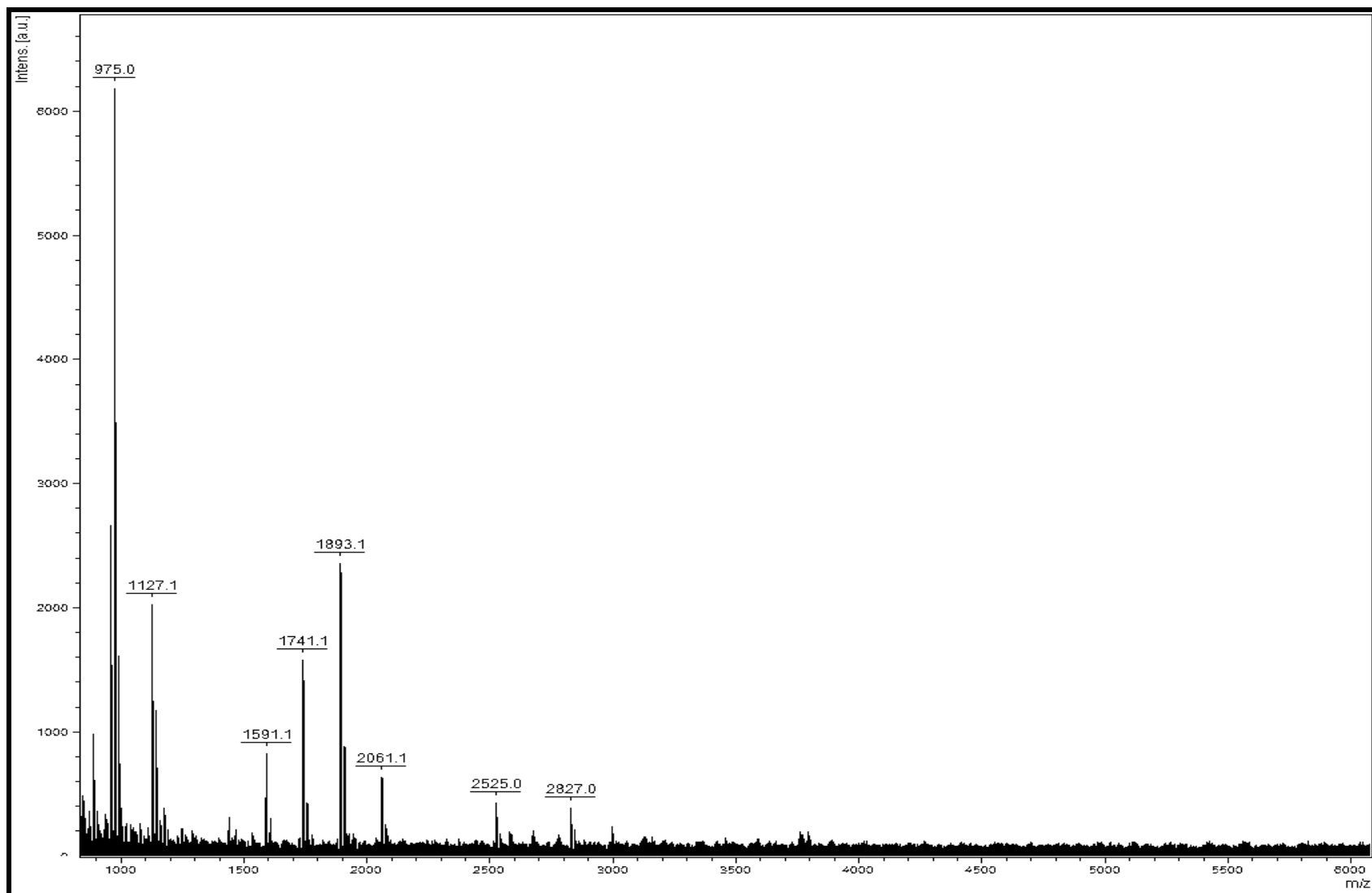


Figure 3.6(C)



## CHAPTER 4

### *IN VITRO* INHIBITION OF PROTEIN GLYCATION AND ANTIOXIDANT ACTIVITIES BY POLYPHENOLICS EXTRACTED FROM SOUTHEASTERN U.S. RANGE BLACKBERRIES

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## ABSTRACT

Blackberries (*Rubus* spp.) are a rich source of phenolic compounds known to possess substantial antioxidant and anti-inflammatory properties. This study examined the effect of ethanol extracts from three blackberry varieties, bred to grow in the warm, humid, climatic conditions of the southeastern U.S., at inhibiting protein glycation *in vitro* using a model system of bovine serum albumin (BSA) and fructose. Blackberry fractions, isolated by targeted chromatographic means from Navaho, Kiowa, and Ouachita cultivars, effectively inhibited the formation of advanced glycation endproducts (AGEs); the inhibition of BSA glycation ranged from 20.7±1.9 to 79.3±2.2%. The fractions contained marked antioxidative activities as assessed by the ferric reducing antioxidant power (FRAP) and Trolox equivalent antioxidant capacity (TEAC) assays; values ranged from 0.88±0.05 to 6.0±0.05 mmol Fe<sup>2+</sup> equivalents/100-mg fraction and 7.7±1.1 to 79.4±0.9 μmol Trolox equivalents/g fraction, respectively. The glycation inhibitory activity was significantly ( $p < 0.05$ ) correlated by linear regression with the total phenolics content (TPC) and antioxidative potency data;  $r^2$  of 0.815 and 0.856 were established with the TPC and FRAP assays, respectively. The positive antiglycation and antioxidative activities demonstrated in this study suggest that certain blackberry constituents may affect critical biological processes and provide a role in preventing glycation-related diabetic complications. Furthermore, these results distinguish blackberries from other edible berries and provide a nutraceutical rationale for greater human consumption and their use in functional foods.

## INTRODUCTION

Reducing sugars in foods such as glucose and fructose can react non-enzymatically with protein and lipid molecules. This process can occur exogenously or endogenously with the production of advanced glycation endproducts (AGEs) via a complex series of chemical reactions (1). Reducing sugars and proteins undergo the Maillard reaction, which involves Amadori rearrangements and the production of Schiff bases. Maillard reaction products are sometimes intermediates or endproducts from the initial glycation reaction. The term has been used inconsistently throughout the literature; so, for the purposes of this work, glycation will refer to as any random interaction between a sugar and a biomolecule (*e.g.*, protein, lipid, nucleic acid) that impairs its function, whereas glycosylation will refer to enzyme-controlled/targeted addition of sugars to proteins or lipid molecules.

The human gastrointestinal tract can absorb AGEs formed in foods during processing operations. At one time, it was believed that AGEs from exogenous glycations were negligible contributors to inflammation and disease states, but recent studies suggest otherwise. Covalent cross-linking of AGEs with tissue proteins can lead to structural and functional modifications of these proteins (2). Cross-linking of proteins is a pathophysiological process and is associated in complications of pre-diabetes, metabolic syndrome, diabetes (retinopathy, neuropathy, and nephropathy) and various age-related diseases such as atherosclerosis, rheumatoid arthritis, and Alzheimer's diseases (1,3-6).

The classical pathway of AGE formation is shown **Figure 4.1** (7). Glycation is initiated by the reaction of the carbonyl group at C-1 with a free amino group of protein to form a Schiff base. These Schiff bases undergo rearrangement and generate early, reversible Amadori reaction products. Subsequently, the Amadori products undergo rearrangement involving further Maillard reactions through a series of complex reactions (*e.g.*, dehydration, oxidation, cyclization, and scission) leading to the generation of “intermediate products” known as  $\alpha$ -dicarbonyls or  $\alpha$ -oxoaldehydes (*e.g.*, 3-deoxyglucosone [3-DG] and methylglyoxal [MGO]) (8). 3-DG is formed by non-oxidative rearrangement and hydrolysis of Amadori products (9), whereas MGO is formed by glycolysis (10) and oxidative decomposition of polyunsaturated fatty acids (11). Accumulation of these reactive dicarbonyl precursors is termed as carbonyl stress (12). The dicarbonyls can react with amino groups of proteins to form cross-linked, yellow-brown, fluorescent and non-fluorescent, insoluble, and irreversible AGEs. Typical glycoxidized AGEs include N<sup>ε</sup>-carboxymethyllysine (CML), pentosidine, pyrraline, crossline, glyoxal-lysine dimer (GOLD), and methylglyoxal-lysine dimer (MGOLD) (8). **Figure 4.2** shows the structures of **(A)** CML (non-fluorescent), **(B)** pentosidine (fluorescent), **(C)** crossline, and **(D)** GOLD; these are well-characterized AGEs (7) which also serve as biomarkers of oxidative stress resulting from carbohydrate and lipid oxidations (13).

Several receptors for AGEs (RAGEs) have been identified on various cells including smooth muscle cells, monocytes, macrophages, endothelial cells, and microglia (14). Binding of AGEs to these receptors results in a depletion of the body’s endogenous antioxidant defense mechanism (*e.g.*, glutathione and Vitamin C) and can lead to the generation of reactive oxygen species (ROS) (15) with subsequent redox activation of NF- $\kappa$ B and the increased production and release



of pro-inflammatory cytokines, growth factors, and adhesion molecules (16-17). *In vitro* studies have shown that AGE-RAGE binding on macrophages will promote the expression of NF- $\kappa$ B-regulated genes such as the procoagulant tissue factor (15, 18) and the adhesion molecule VCAM-1 (19, 20). These biomarkers of inflammation have been associated with the early stages of atherosclerosis.

Because oxidative reactions are known to participate in the formation of AGEs, exogenous sources of antioxidants in our diet may reduce the extent of glycation (21). Phenolic antioxidant compounds are associated with a reduced risk of aging and degenerative diseases such as atherosclerosis, cardiovascular diseases, cancer, diabetes, inflammation and neurodegenerative disorders (22-26). Many of these biological actions have been attributed to their intrinsic antioxidant capacities. These include direct scavenging of ROS, detoxification of ROS, or blocking of ROS production (27) and the indirect protection by modulating cellular signaling processes such as NF- $\kappa$ B activation, glutathione biosynthesis, and protein kinase activation (28-30). Recent studies are documenting the *in vitro* and *in vivo* inhibitory activities of phenolic compounds against protein glycation. Flavonoids like quercetin, kaempferol, catechin, epicatechin, and procyanidin oligomers inhibit AGE formation (31). Specific examples of mixed phenolic extracts which inhibit AGE formation in glucose- or fructose-BSA model systems include those isolated from chrysanthemum species (32), muscadine grape seeds and skins (33), guava leaves (34), and culinary spices such as cinnamon (35, 36). Several researchers have also examined the inhibitory effects of phenolics against AGE formation *in vivo* using animal model studies. For example, Sajithlal et al. (37) reported that curcumin was a potent inhibitor of AGE formation and reduced the cross-linking of AGEs with collagen in diabetic rats. Babu et al. (38)

found that an oral administration of green tea extract to diabetic rats impeded the accumulation of aortic collagen, extent of glycation formation of AGEs, and their cross-linking with collagen. Hanumura and Aoki (39) also reported that the anthocyanidins, cyanidin-3- $\alpha$ -*O*-rhamnoside and pelargonidin-3- $\alpha$ -*O*-rhamnoside as well as quercetin-3- $\alpha$ -*O*-rhamnoside, from acerola (*Malpighia emarginata* DC.) fruit strongly inhibited AGE formation. It has therefore been suggested that phenolic compounds in general, and natural products rich in phenolics may offer a novel source of glycation inhibitory agents.

Berries are among fifty food products that are ranked highest in antioxidant levels (40). Over the past decade, numerous independent investigations have established that the dietary intake of berries has a positive and profound impact on human health, performance, and disease prevention (41-42). The biological activities of berries are largely attributed to their polyphenolic constituents (41, 43). Blackberries ranked highest in berry antioxidant concentrations (42, 44), due to the presence of marked levels of cyanidin-3-*O*-glucoside (C3G) as well as ellagitannins and ellagic acid derivatives (45-46). Ding et al. (28) demonstrated that C3G, isolated from blackberries, was able to scavenge ultraviolet B-induced  $\bullet$ OH and  $O_2^{\bullet-}$  radicals in a cultured JB6 mouse epidermal cell system. Hassimoto et al. (47) reported that the consumption of anthocyanin-rich blackberry juice had a pronounced effect at increasing catalase (an endogenous antioxidant enzyme) levels in human blood plasma.

The inhibition of protein glycation by phenolics isolated from blackberries has not been reported. We therefore investigated the hypothesis that ethanol extracts of blackberry fractions, isolated by targeted chromatographic means, from selected cultivars will inhibit protein glycation. An *in*

*vitro* fructose–bovine serum albumin (BSA) model system and fluorescence spectroscopy were employed to test this hypothesis. Commercial blackberry cultivars (*i.e.*, Navaho, Kiowa, and Ouachita) grown in Georgia were chosen for the study. These cultivars were bred from the Blackberry Breeding Program at the University of Arkansas, and have shown adaptation to the warm, humid climatic conditions of Georgia and other regions of the southeastern U.S., as well as some fungal resistance to rosette caused by *Cercospora rubi*. In addition to determining total phenolics contents (TPCs), antioxidant capacities of the blackberry fractions were assessed by the ferric reducing antioxidant power (FRAP) and Trolox equivalent antioxidant capacity (TEAC) assays. Attempts were then made to establish correlations between the findings from the TPC and antioxidant capacity assays with those of the antiglycation model system experiment.

## **MATERIALS AND METHODS**

**Chemicals.** Phenolic compounds including (+)-catechin, (-)-epicatechin, myricetin, quercetin, and a series of phenolic acids comprising gallic, ellagic, vanillic, caffeic, *p*-coumaric, *trans*-cinnamic, protocatechuic, syringic, chlorogenic, and *p*-hydroxybenzoic acids were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). A pure standard of kuromanin chloride (*i.e.*, cyanidin-3-*O*-glucoside chloride) was acquired from Indofine Chemical Co. (Hillsborough, NJ). Chemicals for analytical and antioxidant assays included 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), potassium persulfate, Trolox, Folin-Ciocalteu's phenol reagent, sodium carbonate, 2,4,6-tri(2-pyridyl)-*s*-triazine (TPTZ), ferric chloride hexahydrate, ferrous sulfate heptahydrate, sodium acetate, acetic acid, bovine serum albumin (Fraction V), D-(-)-fructose,

sodium azide, Chelex<sup>®</sup> 100 (50-100 mesh), monobasic potassium phosphate and dibasic potassium phosphate, all were obtained from Sigma-Aldrich. The HPLC-grade solvents acetonitrile, water, and methanol were purchased from Fisher Scientific Co. (Suwanee, GA) as were, ACS-grade acetone, 95% ethanol (v/v), glacial acetic acid, and hydrochloric acid.

**Collection of Samples.** Ripe blackberries (*Rubus* spp.) were collected at Jacob W. Paulk Farms, Inc. (Wray, GA) in May 2006 and 2007. The three blackberry cultivars collected over two crops years were as follows: Navaho, an erect thornless variety; Kiowa, an erect thorny variety; and Ouachita, a very erect cane and thornless variety. All three cultivars, which grow particularly well in the hot, humid conditions of the southeastern U.S., were patented by and released from the University of Arkansas. These cultivars are different from those in the dominant blackberry production areas of the northwest U.S. Hand-picked blackberries were transported to the Department of Food Science & Technology, UGA, in Athens, GA. The berries were sorted, cleaned, and frozen in polyethylene pouches at  $-40\text{ }^{\circ}\text{C}$ . Representative samples from each cultivar were lyophilized using a UNITOP 600L VirTis<sup>™</sup> freeze dryer (The VirTis Company, Inc., Gardiner, NY), transferred to polyethylene pouches, and then stored at  $-40\text{ }^{\circ}\text{C}$  until analyzed.

**Preparation of Crude Blackberry Extracts (CBEs).** Freeze-dried whole blackberry samples (*i.e.*, containing fruit receptacles, skins and seeds) from each cultivar were ground in a commercial coffee mill (KitchenAid, St Joseph, MI). Fifteen grams of blackberry powder were mixed with 150 mL of 70% (v/v) acidified acetone (containing 0.1% [v/v] HCl) and blended using a PT-3100 Polytron<sup>™</sup> homogenizer (Brinkmann Instruments, Westbury, NY) at 15,000

rpm for 10 min. The slurry was then filtered by gravity through fluted P8 filter paper (Fisher Scientific). This extraction process was repeated 2× as described above. All filtrates were pooled and acetone was evaporated with a Büchi Rotavapor R-210 using a V-700 vacuum pump connected to a V-850 vacuum controller (Büchi Corporation, New Castle, DE) at 40 °C. Sample extractions of each cultivar were performed in triplicate.

**Preparation of Polyphenolic Extracts (PPEs).** Ten milliliters of each CBE (containing 13% solids) were applied to the top of a chromatographic column (30 mm *i.d.* × 340 mm *e.l.*, Kontes, Vineland, NJ) packed with Amberlite XAD-16 [(bead size: 20-60 mesh), Sigma-Aldrich] and washed with ~300 mL of deionized water to remove sugars and organic acids. After the first 100 mL, the pH of the eluent was checked with pH paper test strips every 20 mL until a neutral pH was reached. The polyphenolic extract (PPE) was then eluted from the column with anhydrous methanol (~300 mL) as the mobile phase. Methanol was evaporated using the Büchi Rotavapor at 40 °C. The PPE was lyophilized using a FreeZone<sup>®</sup> 2.5-L bench-top freeze dryer (Labconco Corporation, Kansas City, MO) to ensure all traces of moisture were removed and then stored in amber-glass bottles in a 4 °C refrigerator.

**Fractionation of the PPE.** For each cultivar, 200 mg of lyophilized PPE were dissolved in 10 mL of 95% (v/v) ethanol, sonicated to facilitate dissolution, and then applied to the top of a chromatographic column (30 mm *i.d.* × 360 mm *e.l.*, Kontes, Vineland, NJ) packed with Sephadex LH-20 [(bead size: 25-100 µm), Sigma-Aldrich]. Fractions were eluted with 95% (v/v) ethanol at a flow rate of 0.6 mL/min. Nine-milliliter fractions were collected in 13 × 100-mm borosilicate glass culture tubes with a fraction collector (Model SC-100, Beckman Coulter

Inc., Fullerton, CA). Using UV/VIS absorbance readings at 280, 360, and 520 nm from an Agilent 8453 diode array spectrophotometer (Agilent Technologies, Inc., Wilmington, DE) as a guide, eluent was pooled into six major fractions (*i.e.*, FXN-I thru FXN-VI). In total, 500 mL of ethanol (95% [v/v]) were employed. The system was changed over to 50% (v/v) acetone, and ~300 mL were required to elute FXN-VII, comprising mostly high-molecular-weight phenolics (*i.e.*, hydrolyzable and condensed tannins) from the Sephadex LH-20 column. Organic solvents were evaporated off from the seven major fractions collected using the Büchi Rotavapor at 40 °C. Fractions were then lyophilized using a FreeZone<sup>®</sup> 2.5-L bench-top freeze dryer (Labconco Corporation, Kansas City, MO) to ensure all traces of moisture were removed and then stored in amber-glass bottles at 4 °C in a refrigerator.

**HPLC Analysis.** An Agilent 1200 Series HPLC system consisting of a quaternary pump with degasser, autosampler, thermostatted column compartment, UV/VIS diode array detection (DAD) with standard flow cell, and 3D ChemStation software (Agilent Technologies) was used for the chromatography. A reversed-phase Luna C<sub>18</sub>(2) column (4.6 × 250 mm, 5 µm; Phenomenex, Torrance, CA) was used. A gradient elution consisting of mobile phase A (H<sub>2</sub>O:CH<sub>3</sub>CN: CH<sub>3</sub>COOH; 93:5:2, v/v/v) and mobile phase B (H<sub>2</sub>O:CH<sub>3</sub>CN:CH<sub>3</sub>COOH; 58:40:2, v/v/v) from 0% to 100% B over a 50-min period at a flowrate of 1 mL/min was employed. Before subsequent injections, the system was re-equilibrated for 10 min using 100% A giving a total run time of 60 min. The injection volume of each fraction (0.5 mg/mL methanol) was 20 µL. Detection wavelengths employed were 255 nm (ellagic acid and ellagic acid derivatives), 280 nm (phenolic acids, catechin, epicatechin), 320 nm (phenolic acids), 360

nm (flavonols), and 520 nm (anthocyanins). Tentative identification of separated components was made by matching UV/VIS spectra and retention time mapping with authentic standards.

**Total Phenolics Content (TPC) Assay.** The TPC was determined for the blackberry fractions colorimetrically using the classical Folin-Ciocalteu assay (48). This assay is based on the reduction of a heteropolyphosphotungstate-molybdate complex by phenolic compounds under alkaline conditions yielding a blue color. Briefly, 0.5 mL of a methanolic solution (5 µg/mL) of each fraction (*i.e.*, FXN-I thru FXN-VII) was pipetted into a test tube followed by the addition of 8.0 mL of deionized water, 0.5 mL of 2 N Folin-Ciocalteu's phenol reagent, and 1.0 mL of a saturated Na<sub>2</sub>CO<sub>3</sub> solution. The contents were vortexed for 15 s. After an incubation period of 60 min at room temperature to allow for optimal color development of the samples, absorbance readings were taken at  $\lambda = 750$  nm with the Agilent spectrophotometer. Quantification was based on a standard curve generated with gallic acid as described by Singleton and Rossi (48). The TPCs were determined from the standard curve, and results were expressed as mg gallic acid equivalents (GAE)/100-mg respective fraction.

**Albumin Glycation.** The fluorescence assay to determine the glycation of albumin was performed according to the method described by McPherson et al. (49). This assay is based on the production of Maillard reaction products when fructose reacts with amino moieties of protein in neutral aqueous solution at 37 °C; yellow-brown fluorescent colored derivatives such as pentosidine and crossline are generated (8). A reduction in the fluorescence of reaction products due to intervention by phenolic compounds is reported as the % inhibition in AGE formation. Briefly bovine serum albumin (BSA, 10 mg/mL) was incubated with D-(-)-fructose (250 mM) in

potassium phosphate buffer [200 mM; pH 7.4; 0.02% (w/v) sodium azide] in a 5% (v/v) CO<sub>2</sub> Isotemp<sup>®</sup> incubator (Fisher Scientific) at 37 °C for 72 h. Prior to use, the buffer had been treated with Chelex<sup>®</sup> 100, a styrene-divinylbenzene resin containing iminodiacetic acid groups for chelating transition-metal ions. Sixty microliters of each fraction solution [2.5 mg/mL ethanol, 50% (v/v)] were added to 3 mL of the incubation mixture. Fluorescence intensity was measured at an excitation/emission wavelength pair of  $\lambda = 370/440$  nm using a PerkinElmer LS 55 Luminescence Spectrometer (PerkinElmer Inc., Waltham, MA) with slit widths set at 3 nm. Observed intensities were corrected for the intrinsic fluorescence of the blackberry fractions. The percent inhibition of protein glycation was expressed using the following formula:

$$\% \text{ Inhibition} = \left( 1 - \frac{\text{Sample reading} - \text{BSA reading alone (control)}}{\text{Maximum glycation} - \text{BSA reading alone (control)}} \right) \times 100$$

**(Ferric Reducing Antioxidant Power) Assay.** The antioxidant capacity of each fraction was determined according to the procedure described by Pulido et al. (50). Briefly, the FRAP reagent was prepared fresh each day by adding 2.5 mL of a 10 mM TPTZ [2,4,6-tri(2-pyridyl)-s-triazine] solution in 40 mM HCl, 2.5 mL of 20 mM FeCl<sub>3</sub>•6H<sub>2</sub>O, and 25 mL of acetate buffer (300 mM, pH 3.6). The FRAP reagent was kept warm at 37 °C in a 5-L Isotemp<sup>®</sup> Digital-Control Water Bath (Model 205, Fisher Scientific) until further use. Eighteen hundred microliters of the FRAP reagent and 180  $\mu$ L of 37 °C deionized water were pipetted into borosilicate glass tubes, positioned in the 37 °C water bath. To respective test tubes, 60  $\mu$ L of each sample solution [*i.e.*, 50  $\mu$ g/mL of 50% (v/v) ethanol] were added. The contents were mixed well by pumping the solution through the pipette tip twice. Using a micropipette, 250- $\mu$ L aliquots were transferred to



a prewarmed COSTAR<sup>®</sup> 96-well clear, non-sterile, non-treated microtiter assay plate and inserted into a FLUOstar Omega microplate reader (BMG LABTECH Inc., Durham, NC) set at 37 °C. Absorbance readings were recorded by bottom scanning every 20 s over 30 min at  $\lambda = 595$  nm. Aqueous solutions of known Fe<sup>2+</sup> concentrations in the range of 0.1 to 2.5 mM FeSO<sub>4</sub>•7H<sub>2</sub>O were employed to construct a calibration curve. All analyses were replicated a minimum of three times and FRAP values were expressed as mmol Fe<sup>2+</sup> equivalents/100-mg fraction.

**Trolox Equivalent Antioxidant Capacity (TEAC) Assay.** The TEAC assay evaluates the capacity of a sample to scavenge 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ABTS<sup>•+</sup> radicals (51-52). In short, a 7 mM aqueous ABTS solution was mixed with 2.45 mM potassium persulfate and incubated in the dark for 12 to 16 h. The resultant ABTS<sup>•+</sup> solution (blue-green) was gravity filtered through P8 filter paper. The ABTS<sup>•+</sup> stock was diluted with 95% (v/v) ethanol until an absorbance of  $0.70 \pm 0.02$  at  $\lambda = 734$  nm was reached using the Agilent spectrophotometer; the ABTS<sup>•+</sup> working solution was held at 30 °C in the 5-L Isotemp<sup>®</sup> Digital-Control Water Bath (Fisher Scientific). Trolox standards were prepared at concentrations ranging from 0.2 to 2 mM for the development of a standard curve. A 10- $\mu$ L aliquot of sample or standard was combined with 1 mL of the ABTS<sup>•+</sup> working solution in a glass cuvette held at 30 °C in the spectrophotometer using an Agilent 89090A Peltier temperature controller. After 6 min, the absorbance reading was taken. The absorbance of ABTS<sup>•+</sup> with 10- $\mu$ L ethanol added was the control sample and appropriate solvent blanks were run for each assay. Blackberry fractions were diluted in ethanol such that they produced between a 20 to 80% inhibition of the

ABTS<sup>•+</sup> working solution. TEAC values were expressed as  $\mu\text{mol Trolox equivalents}/100\text{-mg}$  fraction from triplicate samples as per the following formula:

$$\% \text{ Inhibition} = [(AC - AS)/AC] \times 100$$

$$\text{TEAC value} = \% \text{ Inhibition}/m$$

where AC is the  $\text{Abs}_{734\text{nm}}$  of the control at  $t = 6$  min; AS is the  $\text{Abs}_{734\text{nm}}$  of the test sample or Trolox standard at  $t = 6$  min; and  $m$  is the slope of the generated standard curve.

**Statistical Analysis.** System software (SAS, Version 9.1) was used for statistical analysis of data. All observations were expressed as the mean  $\pm$  standard deviation ( $n = 3$ ). Statistical evaluations used  $t$ -tests and one-way analysis of variance (ANOVA) with cultivar as the variable factor and post-hoc tests for significance of differences by Duncan's multiple comparison test. Statistical significance was considered at  $p < 0.05$ . The SAS software was also used to determine correlation coefficients between different antioxidant capacity assays [significance level of 5% ( $p < 0.05$ )].

## RESULTS

**Fractionation.** A flow diagram summarizing the extraction and chromatography steps is depicted in **Figure 4.3**. A comprehensive discussion of this process and the partial characterization of the bioactives by HPLC and mass spectrometries are given in Chapter 3. Based on absorption readings at 255, 280, 320, 360, and 520 nm, eluent from the Sephadex LH-20 column was pooled into a total of seven fractions for each of the three blackberry PPEs being examined. Fractions I to VI were eluted using 95% (v/v) ethanol as the mobile phase, whereas

FXN-VII was eluted with 50% (v/v) acetone. The relative proportions of FXN-III, -IV, -V, and -VII were higher than those of FXN-I, -II, and -VI. The TPCs for all fractions from the Navaho, Kiowa, and Ouachita cultivars are given in **Table 4.1**. TPC values ranged from  $6.9 \pm 0.07$  to  $77.8 \pm 0.49$  mg of GAE/100-mg respective fraction with the highest TPC determined in FXN-III followed by the acetonic FXN-VII (*i.e.*,  $75.4 \pm 0.34$  mg GAE/100-mg respective fraction) isolated from the Navaho variety. Fraction I showed the lowest TPC values in all three cultivars; that is,  $11.3 \pm 0.96$ ,  $28.3 \pm 0.71$ , and  $6.9 \pm 0.07$  mg GAE/100-mg fraction for Navaho, Kiowa, and Ouachita, respectively. The ethanolic fractions chiefly consisted of low-molecular-weight phenolics including phenolic acids and their ester as well as glycoside derivatives, flavonols, and anthocyanins, whereas the aqueous acetonic fraction comprised more high-molecular-weight phenolic compounds including hydrolyzable tannins, measured as free or esters of gallic acid and ellagic acid, as well as some condensed tannins. For the purposes of this work, the TPC was expressed as GAEs.

It is difficult to compare TPC values from our studies with those presented in the literature because most researchers have utilized a variety of crude extracts in TPC investigations (53-54). Many of the preparations contained non-phenolic constituents like mono- and disaccharides as well as Vitamin C; these interfere with the colorimetric reaction involving Folin-Ciocalteu's phenol reagent. TPC values may be overestimated (55). To our knowledge no report exists concerning the composition of phenolic fractions isolated from blackberries. Hence, no direct comparisons can be made with literature values.

A partial characterization of the phenolics found in the seven fractions is presented in **Table 4.2**. Even though every possible species in each fraction is not listed, identification of selected compounds, or classes of compounds, was achieved through comparison of absorption spectra and HPLC retention times (RTs) with those of commercial standards. Qualitatively, the predominant phenolic compounds found were ellagic and gallic acid as well as their derivatives (*i.e.*, esters, glycosides, and hydrolyzable tannins of both ellagi- and gallo-type) in FXN-I, -II, and -VII, while in FXN-III thru -V, the predominant phenolics were anthocyanins like C3G. Trace flavonols such as quercetin, and most likely its glycosides, were also present in many of the fractions. Similar findings have been reported in Marion and Evergreen blackberries (53), *Rubus glaucus* and *Rubus adenotrichus* (55), as well as Oklahoma blackberry cultivars including the Navaho and Kiowa varieties (45).

**Antiglycation Activity.** Maximum glycation of BSA by D-(-)-fructose was exhibited in the control sample with no additives after three days of incubation in the 5% (v/v) CO<sub>2</sub> incubator: it appeared brown and gave the highest fluorescent intensity of all samples tested. The observed fluorescence is an index of the extent to which protein glycation has occurred. When incubated under similar conditions in the absence of fructose, the BSA solution exhibited, as expected, low fluorescence intensity. Both intensities represent the upper and lower limits of albumin glycation in the experiment, and provide the range within which the antiglycation efficacy of the phenolic treatments is estimated.

**Figure 4.4** shows the % inhibition of fructose-mediated BSA glycation by all fractions (*i.e.*, FXN-I thru -VII) isolated from the Navaho, Kiowa, and Ouachita cultivars at 50- $\mu$ g fraction/mL

incubation solution. The inhibition ranged from  $20.7 \pm 1.9\%$  (TPC =  $5.64 \mu\text{g GAE/mL}$  incubation solution) to  $79.3 \pm 2.0\%$  (TPC =  $37.7 \mu\text{g GAE/mL}$  incubation solution). The strongest inhibitory effect was observed by Navaho FXN-VII with the lowest exhibited by Navaho FXN-I. When the concentration of each blackberry fraction was diluted by half (*i.e.*,  $25\text{-}\mu\text{g}$  fraction/mL incubation solution), significant ( $p < 0.05$ ) increases in fluorescence intensity were observed for all test systems, thereby denoting a concentration-dependent reduction in the inhibition of BSA glycation. At the half concentration, Navaho FXN-VII still imparted the greatest inhibition ( $44.6 \pm 1.1\%$ ) while FXN-I for both Kiowa and Ouachita demonstrated the weakest; that is,  $13.4 \pm 1.6$  and  $10.9 \pm 0.78\%$ , respectively.

**Antioxidant Assays.** The total antioxidant capacities of all fractions were determined according to the FRAP and TEAC assays. FRAP and TEAC values are presented as **Figure 4.5** and **Figure 4.6**, respectively. For the FRAP assay, the antioxidant capacities ranged from  $0.88 \pm 0.05$  to  $6.0 \pm 0.05$  mmol  $\text{Fe}^{2+}$  equivalents/100-mg respective fraction; the overall difference in antioxidant capacity was nearly 7 fold. Based on fractionation, the FRAP values in decreasing order were as follows: FXN-VII > III >> V > IV >> VI >> II >> I for Navaho, FXN-III >> VII  $\approx$  V >> II  $\approx$  IV  $\approx$  VI >> I for Kiowa, and FXN-III > IV >> VII >> V > II > VI >> I for Ouachita. The TEAC values ranged from  $7.7 \pm 1.1$  to  $79.4 \pm 0.9$   $\mu\text{mol Trolox}$  equivalents/g fraction; the overall difference in antioxidant capacity was 10 fold. Based on fractionation, the TEAC values in decreasing order were as follows: FXN-III  $\approx$  VII >>> V >> IV >> II  $\approx$  VI >> I for Navaho, FXN-VI >> II  $\approx$  V > III > IV >> VII >>> I for Kiowa, and FXN-III >> VII >> IV >> V >> VI > II >>> I for Ouachita.

## DISCUSSION

**Fructose-mediated Albumin Glycation Assay.** Albumin, albeit from bovine serum, was chosen as the model protein because in man, albumin is a key protein found abundantly in human plasma, lymph, and humors throughout the body's essential organs. Although glucose is sometimes referred to as "blood sugar," fructose was selected as the reducing sugar because in tissues it is generally present at a concentration comparable to that of glucose; yet, it is ~10 times more reactive with protein (7). The nature of the reducing sugar will influence both the rate and extent of glycation. The formation of AGEs through the *in vitro* glycation reaction with the fructose-BSA system (57) is a well-known process of non-enzymatic protein glycation and the findings may offer theories and help design mechanistic studies to elucidate the effects and impact of protein glycation *in vivo*. To our knowledge, this is the first time the assay has been employed using blackberry preparations. The present study revealed that both low- and high-molecular-weight phenolic compounds isolated from Navaho, Kiowa, and Ouachita blackberry cultivars can inhibit fructose-mediated BSA glycation *in vitro*. All fractions demonstrated some degree of inhibition of AGE formation as depicted in **Figure 4.4**. Preliminary experiments also revealed that the effect from each fraction at curbing glycation was concentration dependent (results not shown); however, 100% inhibition was not achieved. The fact that blackberry phenolics inhibited the glycation of BSA in this model system study is an interesting finding due to importance of protein glycation in the pathogenesis of diabetic complications (3). It raises the question, what role(s) do dietary antioxidants play in preventing or managing chronic disease states such as diabetes, atherosclerosis, vascular narrowing, and cataract formation?

A number of studies have confirmed the close relationship between AGE formation with diabetes and aging (7, 19, 58). Three mechanisms have been proposed to explain how the production of intracellular AGE precursors can damage target cells. They include the following: (i) intracellular proteins modified by AGEs possess altered function; (ii) extracellular matrix components modified by AGE precursors interact abnormally with other matrix components and the receptors for matrix proteins (integrins) on cells; and (iii) plasma proteins modified by AGE precursors bind to RAGE on endothelial cells, mesangial cells and macrophages, inducing receptor-mediated production of ROS (16). Consequently, AGE formation on the extracellular matrix results in decreased elasticity, increased thickness and rigidity of tissue protein that affects the process of tissue repair and protein turnover (1). On vascular matrix proteins AGE formation can result in the quenching of NO, an endothelium-derived relaxing factor, which induces smooth muscle relaxation and is an important mediator in the regulation of vascular tone (59). It also induces the expression and release of the potent vasoconstrictor, endothelin-1 (7).

Binding of AGEs to their cellular binding sites (60) results in reduction of cellular antioxidant defense mechanism (*e.g.*, glutathione, Vitamin C) (15) and the generation of ROS. Increased cellular oxidative stress leads to the activation of the free-radical sensitive transcription factor NF- $\kappa$ B *in vitro* and *in vivo* (7). Thus, the modified protein – both glycated and oxidized – can promote enhancement of oxidative and proinflammatory cascades. Primary antioxidants such as  $\alpha$ -tocopherol (vitamin E) (61) and flavonoids (62-63) have the capacity to curb oxidation steps in the glycation of proteins by reducing sugars. Aminoguanidine, the classic inhibitor of protein glycation, is both an antioxidant (64) and capable of trapping reactive carbonyl compounds (65). Secondary antioxidants can inhibit metal ion-mediated autoxidation of glucose leading to the

formation of AGEs (63). Several mechanisms have been proposed that explain this activity. Flavonoids and other phenolics such as those found in blackberries, can participate as antioxidants via hydrogen atom transfer (HAT) or single electron transfer (SET) reactions, while select flavonoids, like quercetin, are capable of directly complexing pro-oxidative metal ions (66).

Using the Navaho cultivar as an illustration, the considerable % inhibition of AGE formation afforded by fraction III ( $74.2 \pm 1.5\%$ , based on the assay conditions described above) is believed to be due to the anthocyanins present in the fraction. Fraction III from all cultivars possessed the greatest concentration of C3G as confirmed by HPLC analyses. Cyanidin-3-*O*-rutinoside, -3-*O*-xyloside, and -3-*O*-glucoside acylated with malonic acid are examples of other anthocyanins determined in blackberry cultivars (53). Our results are in agreement with previous findings demonstrating that plant extracts possessing high anthocyanin concentrations can significantly inhibit protein glycation (67).

Matsuda et al. (68) attempted to clarify the structure–activity relation of flavonoids for AGEs formation inhibitory activity. In their study, four anthocyanins were tested (*i.e.*, cyanidins 3-*O*- $\beta$ -D-glucopyranoside and 3-*O*-rutinoside and delphinidins 3-*O*- $\beta$ -D-glucopyranoside and 3-*O*-rutinoside). The results indicated that the anthocyanins possess substantial inhibitory activities. Based on the experimental conditions employed, their activities were stronger than that of the reference compound, aminoguanidine. Some flavonoids showed marked inhibitory activity, but others did not. The authors proposed that the following structural characteristics of flavonoids were requirements in order to see marked inhibitory effects against AGE formation: (i) the



inhibitory activities of flavones become stronger as the number of hydroxy groups at the 3'-, 4'-, 5-, and 7-positions increase; (ii) the activities of flavones are stronger than those of corresponding flavonols, flavanones, and isoflavones; (iii) methylation or glycosylation of the 4'-hydroxy group of flavones, flavonols, and flavanones reduces their respective activities; (iv) methylation of the 3-hydroxy group of flavonols tends to increase the activity; (v) glycosylation of the 7-hydroxy group of flavones and isoflavones reduces the activity; and (vi) the activities of anthocyanins are stronger than those of corresponding flavonol glycosides. **Figure 4.7** depicts a summary of the structural requirements of flavonoids for protein glycation inhibition.

FXN-VII, which was eluted from the Sephadex LH-20 column with 50% (v/v) acetone, possessed the majority of the oligomeric proanthocyanidins present in the blackberry seeds (results based on the qualitative BSA precipitation assay not shown). This fraction, like that of FXN-III, demonstrated marked antiglycation activities. Strong inhibitory activities against BSA glycation have been reported for the tannin constituents of ethanolic sorghum extracts (68), as well as ellagic acid, its derivatives, and other phenolics from muscadine seeds and skins (33). Likewise, similar findings have been shown for extracts prepared from various herbs and spices (35, 69-70). Proanthocyanidin oligomers from pine bark and grapeseed have been reported to inhibit the formation of pentosidine in collagen (31). Urios et al. (31) suggested that galloylation can increase the inhibition efficacy of the catechin/epicatechin monomer; however, the exact mechanism(s) by which phenolics inhibit glycation has yet to be elucidated.

**Antioxidant Capacities.** The antioxidant capacities of the blackberries are influenced by many factors such as the types and levels of phenolics endogenous to each fraction, extraction and

chromatography methods employed, and conditions of the test used to evaluate antioxidant activity. It is generally accepted that a single method should not be used because not all assays and antioxidant sources are compatible; that is, the same antioxidant species can yield varying results in different assays (71). In this work, three assays measuring phenolic activities were employed. Determination of the TPC with Folin-Ciocalteu's phenol reagent is a SET-based assay that is routinely employed because of its simplicity and sensitivity to phenolic residues (51, 72). Numerous publications have shown excellent linear correlations between TPCs and measures of antioxidant activity (73), but the TPC assay suffers interference from non-phenolic reducing compounds like ascorbic acid. The FRAP assay is also a SET method that is simple and provides fast, reproducible results. It is versatile and can be readily applied to both aqueous and alkanolic extracts of plants. In this assay, the antioxidant activity is measured on the basis of electron-donating substances (*i.e.*, phenolics) being able to reduce a  $\text{Fe}^{3+}$ -adduct to the ferrous(II) state under acidic conditions (74). This reaction generates an intense blue color with an absorption maximum at  $\lambda = 550 \text{ nm}$  which is quantified spectrophotometrically; the results are generally expressed as  $\text{mmol Fe}^{2+}$  equivalents per gram sample. The third method utilized was the TEAC assay: it is a mixed-mode assay that encompasses both SET and HAT chemical processes. The TEAC assay is a spectrophotometric method based on the capability of antioxidants to scavenge the generated free-radical cation,  $\text{ABTS}^{\bullet+}$ , with results being expressed as  $\mu\text{mol Trolox equivalents/g fraction}$ . The TEAC assay has been recommended for use with plants extracts because the long wavelength absorption maximum at  $734 \text{ nm}$  eliminates color interference issues from pigments (75).  $\text{ABTS}^{\bullet+}$  is a nitro-radical cation; therefore, results at times may not correlate well with other antioxidant capacity assays that measure oxyl-radical scavenging.

The marked antioxidant capacity of the blackberry fractions is attributable to their high contents of phenolics including flavonoids, free phenolic acids and derivatives, as well as tannin constituents (44, 76). For all of the cultivars investigated, FXN-III thru -V exhibited strong antioxidant activity, whereas FXN-I and II were much weaker. The majority of sugars and organic acids were removed from the CBE by the Amberlite column, but traces may have remained. These non-phenolics could be concentrated in FXN-I and therefore be partially accountable for the poor antioxidant activity. C3G and other anthocyanins dominant in FXN-III are most likely responsible for the marked antioxidant activity observed. Even though pomegranate is rich in punicalagin (an ellagitannin), antioxidant and free-radical scavenging activity data revealed that the C3G isolated from pomegranate contributes significantly to its observed activity (77).

**Correlations between TPC and Antioxidant Capacity Assays.** The antioxidant capacities of the blackberry fractions, as determined by the FRAP and TEAC assays, both correlated well with the TPCs of the respective fractions. Fractions possessing a greater phenolics content were better able to reduce the ferric ion of the  $\text{Fe}^{3+}$ -adduct in the FRAP assay and to scavenge the ABTS radical cation in the TEAC assay. **Figures 4.8(A) and (B)** depict the linear correlations. Correlation coefficients ( $r^2$ ) of 0.858 and 0.815 were determined for the FRAP and TEAC correlations, respectively, with the TPCs determined for the 21 fractions collected from the Navaho, Kiowa, and Ouachita blackberry cultivars. Incidentally, when the PPE for each cultivar eluted from the Sephadex LH-20 column was collected as only two fractions (*i.e.*, an ethanolic one representing low-molecular-weight polyphenolic compounds [LMPF] and an acetonic one

representing high-molecular-weight polyphenolic compounds [HMPF]), an  $r^2$  of 0.990 was found when comparing the TPCs and FRAP values determined for the blackberry LMPFs/HMPFs. Our results are in agreement with a previous study of other blackberry cultivars which indicates that phenolic compounds do contribute significantly to the antioxidant capacities of blackberries (74).

**Correlations between TPC, Antioxidant Capacity Assays, and Antiglycation Activity.** A significant linear correlation ( $p < 0.05$ ) was determined for the TPC data of the blackberry fractions with the % inhibition of BSA glycation. An  $r^2$  of 0.815 was determined based on the mean values from the 21 fractions collected [**Figure 4.9(A)**]. These results are in agreement with previous investigations that reported phenolic compounds do contribute significantly to antiglycation activity (33-35). In addition to the TPCs, the % inhibition of BSA glycation correlated well with both antioxidant assays employed in this study: **Figure 4.9(B)** depicts the correlation for the FRAP assay. This correlation was a bit stronger than that for the TEAC assay, as correlation coefficients of 0.856 and 0.791 were determined for the FRAP and TEAC assays, respectively. Kim and Kim (78) demonstrated a significant correlation between the % inhibition of fructose-mediated BSA glycation and antioxidant capacity determined in aqueous ethanolic extracts from 25 plant tissues. Similar findings for 24 herbs and spices procured from local supermarkets were reported by Dearlove et al. (35). Hence, the determined TPC, FRAP, and TEAC values of blackberry fractions can be predictive of the samples' ability to inhibit protein glycation.

In summary, our findings suggest that the phenolic constituents of Georgia-grown blackberry cultivars possess strong antioxidant and antiglycation capacities. All three cultivars released from the University of Arkansas are valuable sources of natural antioxidants. The berries may be consumed fresh or extracts may be used in formulated dietary supplements and nutraceutical beverages. To our knowledge, there has been no prior report concerning the inhibition of protein glycation by phenolics isolated from blackberries.

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## LITERATURE CITED

- (1) Singh, R.; Barden, A.; Mori, T.; Beilin, L. Advance glycation end-products: a review. *Diabetologia* **2001**, *44*, 129–146.
- (2) Rabhar, S.; Figarola, J. L. Novel inhibitors of advanced glycation endproducts. *Arch. Biochem. Biophys.* **2003**, *419*, 63–79.
- (3) Ahmed, N. Advanced glycation endproducts-role in pathology of diabetic complications. *Diab. Res. Clin. Pract.* **2005**, *67*, 3–21.
- (4) Price, C. L.; Knight, S. C. Advanced glycation: a novel outlook on atherosclerosis. *Curr. Pharm. Des.* **2007**, *13*, 3681–3687.
- (5) Matsumoto, T.; Tsurumoto, T.; Baba, H.; Osaki, M.; Enomoto, H.; Yonekura, A.; Shindo, H.; Miyata, T. Measurement of advanced glycation endproducts in skin of patients with rheumatoid arthritis, osteoarthritis, and dialysis-related spondyloarthropathy using non-invasive methods. *Rheumatol. Int.* **2007**, *28*, 157–160.
- (6) Srikanth, V.; Maczurek, A.; Phan, T.; Steele, M.; Westcott, B.; Juskiw, D.; Münch, G. Advanced glycation endproducts and their receptor RAGE in Alzheimer's disease. *Neurobiol. Aging* **2009**, Article in press.
- (7) Bierhaus, A.; Hofmann, M. A.; Ziegler, R.; Nawroth, P. P. AGEs and their interaction with AGE-receptors in vascular disease and diabetes mellitus. I. The AGE concept. *Cardiovasc. Res.* **1998**, *37*, 586–600.
- (8) Wells-Knecht, K. J.; Brinkmann, E.; Wells-Knecht, M. C.; Litchfield J. E.; Ahmed, M. U.; Reddy, S.; Zyzak, D. V.; Thorpe, S. R.; Baynes, J. W. New biomarkers of Maillard reaction damage to proteins. *Nephrol. Dial. Transplant.* **1996**, *11*, 41–47.

- (9) Baynes, J. W.; Thorpe, S. R. Role of oxidative stress in diabetic complications. A new perspective on an old paradigm. *Diabetes* **1999**, *48*, 1–9.
- (10) Thornalley, P. J. Pharmacology of methylglyoxal: Formation, modification of proteins and nucleic acids, and enzymatic detoxification - A role in pathogenesis and antiproliferative chemotherapy. *Gen. Pharmac.* **1996**, *27*, 565–573.
- (11) Thornalley, P. J.; Westwood, M.; Lo, T. W.; McLellan, A. C. Formation of methylglyoxal-modified proteins in vitro and in vivo and their involvement in AGE-related processes. *Contrib. Nephrol.* **1995**, *112*, 24–31.
- (12) Miyata, T.; van Ypersele de Strihou, C.; Kurokawa, K.; Baynes, J. W. Alterations in nonenzymatic biochemistry in uremia: Origin and significance of “carbonyl stress” in long-term uremic complications. *Kidney Int.* **1999**, *55*, 389–399.
- (13) Requena, J. R.; Fu, M.-X.; Ahmed, M. U.; Jenkins, A. J.; Lyons, T. J.; Thorpe, S. R. Lipoxidation products as biomarkers of oxidation damage to proteins during lipid peroxidation reactions. *Nephrol. Dial. Transplant.* **1996**, *11*, 48–53.
- (14) Riel, A.; Németh, J.; Angel, P.; Hess, J. The receptor RAGE: Bridging inflammation and cancer. *J. Cell Commun. Signal.* **2009**, *7*, 1–7.
- (15) Bierhaus, A.; Chevion, S.; Chevion, M.; Hofmann, M.; Quehenberger, P.; Illmer, T.; Luther, T.; Berentshtein, E.; Tritschler H.; Müller, M.; Wahl, P.; Ziegler, R.; Nawroth, P. Advanced glycation end product-induced activation of NF- $\kappa$ B is suppressed by  $\alpha$ -lipoic acid in cultured endothelial cells. *Diabetes* **1997**, *46*, 1481–1490.
- (16) Brownlee, M. Biochemistry and molecular cell biology of diabetic complications. *Nature* **2001**, *414*, 813–820.

- (17) Vlassara, H.; Cai, W.; Crandall, J.; Goldberg, T.; Oberstein, R.; Dardaine, V.; Peppas, M.; Rayfield, E. J. Inflammatory mediators are induced by dietary glycotoxins, a major risk factor for diabetic angiopathy. *Proc. Nat. Acad. Sci. USA* **2002**, *99*, 15596–15601.
- (18) Esposito, C.; Gerlach, H.; Brett, J.; Stern, D.; Vlassara H. Endothelial receptor-mediated binding of glucose-modified albumin is associated with increased monolayer permeability and modulation of cell surface coagulant properties. *J. Exp. Med.* **1989**, *170*, 1387–1407.
- (19) Schmidt, A. M.; Yan, S. D.; Stern, D. M. The dark side of glucose. *Nature Med.* **1995**, *1*, 1002–1004.
- (20) Vlassara, H.; Fuh, H.; Donnelly, T.; Cybulsky, M. Advanced glycation endproducts promote adhesion molecule (VCAM-1, ICAM-1) expression and atheroma formation in normal rabbits. *Mol. Med.* **1995**, *1*, 447–456.
- (21) Fu, M.-X.; Wells-Knecht, K. J.; Blackledge, J. A.; Lyons, T. J.; Thorpe, S. R.; Baynes, J. W. Glycation, glycooxidation, and cross-linking of collagen by glucose. Kinetics, mechanisms, and inhibition of late stages of the Maillard reaction. *Diabetes* **1994**, *43*, 676–683.
- (22) Rouanet, J.-M.; Décorde, K.; del Rio, D.; Auger, C.; Borges, G.; Cristol, J.-P.; Lean, M. E. J.; Crozier, A. Berry juices, teas, antioxidants and the prevention of atherosclerosis in hamsters. *Food Chem.* **2010**, *118*, 266–271.
- (23) Visioli, F.; Hagen, T. M. Nutritional strategies for healthy cardiovascular aging: Focus on micronutrients. *Pharmacol. Res.* **2007**, *55*, 199–206.
- (24) Dashwood, R. H. Frontiers in polyphenols and cancer prevention. *J. Nutr.* **2007**, *137*, 267S–269S.



- (25) Banini, A. E.; Boyd, L. C.; Allen, J. C.; Allen, H. G.; Sauls, D. L. Muscadine grape products intake, diet and blood constituents of non-diabetic and type 2 diabetic subjects. *Nutrition* **2006**, *22*, 1137–1145.
- (26) Sun, A. Y.; Wang, Q.; Simonyi, A.; Sun, G. Y. Botanical phenolics and brain health. *Neuromolecular Med.* **2008**, *10*, 259–274.
- (27) Rice-Evans, C. A.; Miller, N. J.; Bolwell, P. G.; Bramley, P. M.; Pridham, J. B. The relative antioxidant activities of plant-derived polyphenolic flavonoids. *Free Radic. Res.* **1995**, *22*, 375–383.
- (28) Ding, M.; Feng, R.; Wang, S. Y.; Bowman, L.; Lu, Y.; Qian, Y.; Castranova, V.; Jiang, B. H.; Shi, X. Cyanidin-3-glucoside, a natural product derived from blackberry, exhibits chemopreventive and chemotherapeutic activity. *J. Biol. Chem.* **2006**, *281*, 17359–17368.
- (29) Shen, S. Q.; Zhang, Y.; Xiang, J.-J.; Xiong, C.-L. Protective effect of curcumin against liver warm ischemia/reperfusion injury in rat model is associated with regulation of heat shock protein and antioxidant enzymes. *World J. Gastroenterol.* **2007**, *13*, 1953–1961.
- (30) Chen, C.; Yu, R.; Owuor, E. D.; Kong, A. N. Activation of antioxidant response element (ARE), mitogen-activated protein kinases (MAPKs) and caspases by major green tea polyphenol components during cell survival and death. *Arch. Pharm. Res.* **2000**, *23*, 605–612.
- (31) Urios, P.; Grigorova-Borsos, A. M.; Sternberg, M. Flavonoids inhibit the formation of the cross-linking AGE pentosidine in collagen incubated with glucose, according to their structure. *Eur. J. Nutr.* **2007**, *46*, 139–146.

- (32) Tsuji-Naito, K.; Saeki, H.; Hamano, M. Inhibitory effects of *Chrysanthemum* species extracts on formation of advanced glycation end products. *Food Chem.* **2009**, *116*, 854–859.
- (33) Farrar, J. L.; Hartle, D. K.; Hargrove, J. L.; Greenspan, P. Inhibition of protein glycation by skins and seeds of the muscadine grape. *BioFactors* **2007**, *30*, 193–200.
- (34) Wu, J.-W.; Hsieh, C.-L.; Wang, H.-Y.; Chen, H.-Y. Inhibitory effects of guava (*Psidium guajava* L.) leaf extracts and its active compounds on the glycation process of protein. *Food Chem.* **2009**, *113*, 78–84.
- (35) Dearlove, R. P.; Greenspan, P.; Hartle, D. K.; Swanson, R. B.; Hargrove, J. L. Inhibition of protein glycation by extracts of culinary herbs and spices. *J. Med. Food* **2008**, *11*, 275–281. Saraswat, M.; Reddy, P. Y.; Muthenna, P.; Reddy, G. B. Prevention of non-enzymic glycation of protein by dietary agents: prospects for alleviating diabetic complications. *Br. J. Nutr.* **2009**, *101*, 1714–1721.
- (37) Sajithlal, G. B.; Chithra, P.; Chandrakasan, G. Effect of curcumin on the advanced glycation and cross-linking of collagen in diabetic rats. *Biochem. Pharmacol.* **1998**, *56*, 1607–1614.
- (38) Babu, P. V. A.; Sabitha, K. E.; Shyamaladevi, C. S. Therapeutic effect of green tea extract on advanced glycation and cross-linking of collagen in the aorta of streptozotocin diabetic rats. *Clin. Exp. Pharmacol. Physiol.* **2006**, *33*, 351–357.
- (39) Hanumura, T.; Aoki, H. Toxicological evaluation of polyphenol extract from acerola (*Malpighia emarginata* DC.) fruit. *J. Food Sci.* **2008**, *73*, T55–T61.

- (40) Halvorsen, B. L.; Carlsen, M. H.; Phillips, K. M.; Bøhn, S. K.; Holte, K.; Jacobs, D. R.; Blomhoff, R. Content of redox-active compounds (ie, antioxidants) in foods consumed in the United States. *Am. J. Clin. Nutr.* **2006**, *84*, 95–135.
- (41) Bagchi, D.; Sen, C. K.; Bagchi, M.; Atalay, M. Anti-angiogenic, antioxidant, and anti-carcinogenic properties of a novel anthocyanin-rich berry extract formula. *Biochem. (Moscow)* **2004**, *69*, 75–80.
- (42) Seeram, N. P.; Adams, L. S.; Zhang, Y.; Lee, R.; Sand, D.; Scheuller, H. S.; Heber, D. Blackberry, black raspberry, blueberry, cranberry, red raspberry, and strawberry extracts inhibit growth and stimulate apoptosis of human cancer cells in vitro. *J. Agric. Food Chem.* **2006**, *54*, 9329–9339.
- (43) Nijveldt, R. J.; van Nood, E.; van Hoorn, D. E. C.; Boelens, P. G.; van Norren, K.; van Leeuwen, P. A. M. Flavonoids: a review of probable mechanisms of action and potential applications. *Am. J. Clin. Nutr.* **2001**, *74*, 418–425.
- (44) Pellegrini, N.; Serafini, M.; Colombi, B.; del Rio, D.; Salvatore, S.; Bianchi, M.; Brighenti, F. Total antioxidant capacity of plant foods, beverages and oils consumed in Italy assessed by three different in vitro assays. *J. Nutr.* **2003**, *133*, 2812–2819.
- (45) Fan-Chiang, H.-J.; Wrolstad, R. E. Anthocyanin pigment composition of blackberries. *J. Food Sci.* **2005**, *70*, C198–C202.
- (46) Hager, T. J.; Howard, L. R.; Liyanage, R.; Lay, J. O.; Prior, R. L. Ellagitannin composition of blackberry as determined by HPLC-ESI-MS and MALDI-TOF-MS. *J. Agric. Food Chem.* **2008**, *56*, 661–669.

- (47) Hassimotto, N. M. A.; da Silva Pinto, M.; Lajolo, F. M. Antioxidant status in humans after consumption of blackberry (*Rubus fruticosus* L.) juices with and without defatted milk. *J. Agric. Food Chem.* **2008**, *56*, 11727–11733.
- (48) Singleton, V. L.; Rossi, J. A., Jr. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* **1965**, *16*, 144–158.
- (49) McPherson, J. D.; Shilton, B. H.; Walton, D. J. Role of fructose in glycation and cross-linking of proteins. *Biochem.* **1988**, *27*, 1901–1907.
- (50) Pulido, R.; Bravo, L.; Saura-Calixto, F. Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. *J. Agric. Food Chem.* **2000**, *48*, 3396–3402.
- (51) Liyana-Pathirana, C. M.; Shahidi, F. Antioxidant properties of commercial soft and hard winter wheats (*Triticum aestivum* L.) and their milling fractions. *J. Sci. Food Agric.* **2006**, *86*, 477–485.
- (52) Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic. Biol. Med.* **1999**, *26*, 1231–1237.
- (53) Siriwoharn, T.; Wrolstad, R. E. Polyphenolic composition of Marion and Evergreen blackberries. *J. Food Sci.* **2004**, *69*, FCT233–FCT240.
- (54) George, S.; Brat, P.; Alter, P.; Amiot, M. J. Rapid determination of polyphenols and vitamin C in plant-derived products. *J. Agric. Food Chem.* **2005**, *53*, 1370–1373.

- (55) Mertz, C.; Cheynier, V.; Günata, Z.; Brat, P. Analysis of phenolic compounds in two blackberry species (*Rubus glaucus* and *Rubus adenotrichus*) by high-performance liquid chromatography with diode array detection and electrospray ion trap mass spectrometry. *J. Agric. Food Chem.* **2007**, *55*, 8616–8624.
- (56) Rahimi, R.; Nikfar, S.; Larijani, B.; Abdollahi, M. A review on the role of antioxidants in the management of diabetes and its complications. *Biomed. Pharmacother.* **2005**, *59*, 365–373.
- (57) Wang, J.; Sun, B.; Cao, Y.; Tian, Y. Protein glycation inhibitory activity of wheat bran feruloyl oligosaccharides. *Food Chem.* **2009**, *112*, 350–353.
- (58) Vlassara, H.; Bucala, R.; Striker, L. Pathogenic effects of advanced glycosylation: biochemical, biologic and clinical implications for diabetes and aging. *Lab Invest.* **1994**, *70*, 138–151.
- (59) Uhlmann, S.; Rezzoug, K.; Friedrichs, U.; Hoffmann, S.; Wiedemann, P. Advanced Glycation end products quench nitric oxide in vitro. *Graefe's Arch. Clin. Exp. Ophthalmol.* **2002**, *240*, 860–866.
- (60) Schmidt, A. M.; Hori, O.; Brett, J.; Yan, S. D.; Wautier, J. L.; Stern, D. Cellular receptors for advanced glycation end products. Implications for induction of oxidant stress and cellular dysfunction in the pathogenesis of vascular lesions. *Arterioscler. Thromb. Vasc. Biol.* **1994**, *14*, 1521–1528.
- (61) Ceriello, A.; Giugliano, D.; Quatraro, A.; Dello Russo, P.; Torella, R. A preliminary note on inhibiting effect of alpha-tocopherol (vit. E) on protein glycation. *Diabete Metab.* **1988**, *14*, 40–42.

- (62) Cervantes-Laurean, D.; Schramm, D. D.; Jacobson, E. L.; Halaweish, I.; Bruckner, G. G.; Boissonneault, G. A. Inhibition of advanced glycation end product formation on collagen by rutin and its metabolites. *J. Nutr. Biochem.* **2006**, *17*, 531–540.
- (63) Wu, C.-H; Yen, G.-C. Inhibitory effect of naturally occurring flavonoids on the formation of advanced glycation endproducts. *J. Agric. Food Chem.* **2005**, *53*, 3167–3173.
- (64) Giardino, I.; Fard, A. K.; Hatchell, D. L.; Brownlee, M. Aminoguanidine inhibits reactive oxygen species formation, lipid peroxidation, and oxidant-induced apoptosis. *Diabetes* **1998**, *47*, 1114–1120.
- (65) Liggins, J.; Furth, A. J. Role of protein-bound carbonyl groups in the formation of advanced glycation endproducts. *Biochim. Biophys. Acta* **1997**, *1361*, 123–130.
- (66) Leopoldini, M.; Russo, N.; Chiodo, S.; Toscano, M. Iron chelation by the powerful antioxidant flavonoid quercetin. *J. Agric. Food Chem.* **2006**, *54*, 6343–6351.
- (67) Matsuda, H.; Wang, T.; Managi, H.; Yoshikawa, M. Structural requirements of flavonoids for inhibition of protein glycation and radical scavenging activities. *Bioorg. Med. Chem.* **2003**, *11*, 5317–5323.
- (68) Farrar, J. L; Hartle, D. K.; Hargrove, J. L.; Greenspan, P. A novel nutraceutical property of select Sorghum (*Sorghum bicolor*) brans: Inhibition of protein glycation. *Phytother. Res.* **2008**, *22*, 1052–1056.
- (69) Ahmad, M. S.; Ahmed, N. Antiglycation properties of aged garlic extract: Possible role in prevention of diabetic complications. *J. Nutr.* **2006**, *136*, 796S–799S.
- (70) Bae, J. W.; Lee, M. H. Effect and putative mechanism of action of ginseng on formation of glycated hemoglobin in vitro. *J. Ethnopharmacol.* **2004**, *91*, 137–140.

- (71) Wong, S. P.; Leong, L. P.; Koh, J. H. W. Antioxidant activities of aqueous extracts of selected plants. *Food Chem.* **2006**, *99*, 775–783.
- (72) Prior, R. L.; Wu, X.; Schaich, K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J. Agric. Food Chem.* **2005**, *53*, 4290–4302.
- (73) Huang, D.; Ou, B.; Prior, R. L. The chemistry behind antioxidant capacity assays. *J. Agric. Food Chem.* **2005**, *53*, 1841–1856.
- (74) Nilsson, J.; Pillai, D.; Önning, G.; Persson, C.; Nilsson, Å.; Åkesson, B. Comparison of the 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and ferric reducing antioxidant power (FRAP) methods to assess the total antioxidant capacity in extracts of fruit and vegetables. *Mol. Nutr. Food Res.* **2005**, *49*, 239–246.
- (75) Awika, J. M.; Rooney, L. W.; Wu, X.; Prior, R. L.; Cisneros-Zevallos, L. Screening methods to measure antioxidant activity of sorghum (*Sorghum bicolor*) and sorghum products. *J. Agric. Food Chem.* **2003**, *51*, 6657–6662.
- (76) Häkkinen, S.; Heinonen, M.; Kärenlampi, S.; Mykkänen, H.; Ruuskanen, J.; Törrönen, R. Screening of selected flavonoids and phenolic acids in 19 berries. *Food Res. Int.* **1999**, *32*, 345–353.
- (77) Noda, Y.; Kaneyuki, T.; Mori, A.; Packer, L. Antioxidant activities of pomegranate fruit extract and its anthocyanidins: Delphinidin, cyanidin, and pelargonidin. *J. Agric. Food Chem.* **2002**, *50*, 166–171.
- (78) Kim, H. Y.; Kim, K. Protein glycation inhibitory and antioxidative activities of some plant extracts in vitro. *J. Agric. Food Chem.* **2003**, *51*, 1586–1591.

## FIGURE CAPTIONS

**Figure 4.1** A classical pathway of advanced glycation endproducts (AGEs) formation in the Maillard reaction: carbonyl groups of reducing sugars condense with amino groups of macromolecules to form reversible Schiff base adducts. Intramolecular rearrangements lead to chemically-stabilized Amadori products. These products give rise to a number of complex reactions (*e.g.*, dehydration, oxidation, cyclization, scission) leading to the generation of AGEs (Structures have been adapted from [13]).

**Figure 4.2** Chemical structures of selected AGEs: (a) N<sup>ε</sup>-carboxymethyllysine; (b) pentosidine; (c) crossline; and (d) glyoxal-lysine dimer (Structures have been adapted from [15]).

**Figure 4.3** Schematic process flow diagram detailing the isolation of ethanolic and acetonetic fractions from lyophilized blackberries using extraction and column chromatography strategies. Abbreviations: CBE, crude blackberry extract; PPE, polyphenolic extract; FXN I – VII, fractions I thru VII.



**Figure 4.4** Percent inhibition of fructose-mediated bovine serum albumin (BSA) glycation by blackberry fractions. The *X*-axis represents fractions from the Navaho, Kiowa, and Ouachita cultivars. The *Y*-axis gives % inhibition of BSA glycation. Values are means of triplicate determinations  $\pm$  standard deviation. When one-way ANOVA was significant, differences in % inhibition of fractions amongst cultivars were determined using Duncan's multiple comparison test ( $p < 0.05$ ). Means with the same letters are not significantly ( $p > 0.05$ ) different.

**Figure 4.5** Ferric reducing antioxidant power (FRAP) values of blackberry fractions. The *X*-axis represents fractions from Navaho, Kiowa, and Ouachita cultivars. The *Y*-axis gives FRAP values as mmol Fe<sup>2+</sup> equivalents/100-mg fraction (d.w., dry weight). Values are means of triplicate determinations  $\pm$  standard deviation. When one-way ANOVA was significant, differences in FRAP values of fractions amongst cultivars were determined using Duncan's multiple comparison test ( $p < 0.05$ ). Means with the same letter are not significantly ( $p > 0.05$ ) different.

**Figure 4.6** Trolox equivalent antioxidant capacity (TEAC) values of blackberry fractions. The *X*-axis represents fractions from Navaho, Kiowa, and Ouachita cultivars. The *Y*-axis gives TEAC values as  $\mu$ mol Trolox equivalents/g of fraction (d.w., dry weight). Values are means of triplicate determinations  $\pm$  standard deviation. When one-way ANOVA was significant, differences in TEAC values of fractions amongst fractions were determined using Duncan's multiple comparison test ( $p < 0.05$ ). Means with the same letter are not significantly ( $p > 0.05$ ) different.

**Figure 4.7** Structural requirements of flavonoids for inhibition of protein glycation and radical-scavenging capacity (Adapted from [67]).

**Figure 4.8** Correlations established between the total phenolics contents (TPCs) and **(A)** ferric reducing antioxidant power (FRAP) values and **(B)** Trolox equivalent antioxidant capacity (TEAC) values of blackberry fractions from the Navaho, Kiowa, and Ouachita cultivars. All points represent mean values based on three determinations.

**Figure 4.9** Correlations established between the percent inhibition of fructose-mediated bovine serum albumin (BSA) glycation and **(A)** the total phenolics contents (TPCs) and **(B)** ferric reducing antioxidant power (FRAP) values of blackberry fractions from the Navaho, Kiowa, and Ouachita cultivars. All points represent mean values based on three determinations.

**Table 1.** Total phenolics content of ethanolic and acetonetic fractions isolated from Navaho, Kiowa, and Ouachita cultivars of Georgia-grown blackberries using a Sephadex LH-20 column<sup>1</sup>

Total Phenolics Content (TPC) (mg GAE/100-mg respective fraction, d.w.) <sup>2</sup>			
Fractions	Navaho	Kiowa	Ouachita
I	11.3 ± 0.96 b	28.2 ± 0.71 a	6.9 ± 0.07 c
II	16.9 ± 0.12 b	65.7 ± 0.99 a	17.4 ± 0.29 b
III	77.8 ± 0.49 a	62.1 ± 0.74 c	75.0 ± 0.33 b
IV	51.3 ± 0.44 c	61.1 ± 0.77 b	69.2 ± 0.13 a
V	62.7 ± 0.49 b	66.0 ± 0.35 a	46.3 ± 0.57 c
VI	35.6 ± 0.23 b	68.9 ± 0.64 a	22.7 ± 0.98 c
VII	75.4 ± 0.34 a	48.6 ± 0.85 c	57.6 ± 0.95 b

<sup>1</sup>Fractions I thru VI were eluted from the Sephadex LH-20 column using 95% (v/v) ethanol as the mobile phase, whereas fraction VII was eluted with 50% (v/v) acetone:water.

<sup>2</sup>Values are means of triplicate analyses ± standard deviation. When one-way ANOVA was significant, differences in TPCs of fractions amongst cultivars were determined using Duncan's multiple comparison test ( $p < 0.05$ ). Means with the same letter in a row are not significantly different ( $p > 0.05$ ). Abbreviations: GAE, gallic acid equivalents and d.w., dry weight.

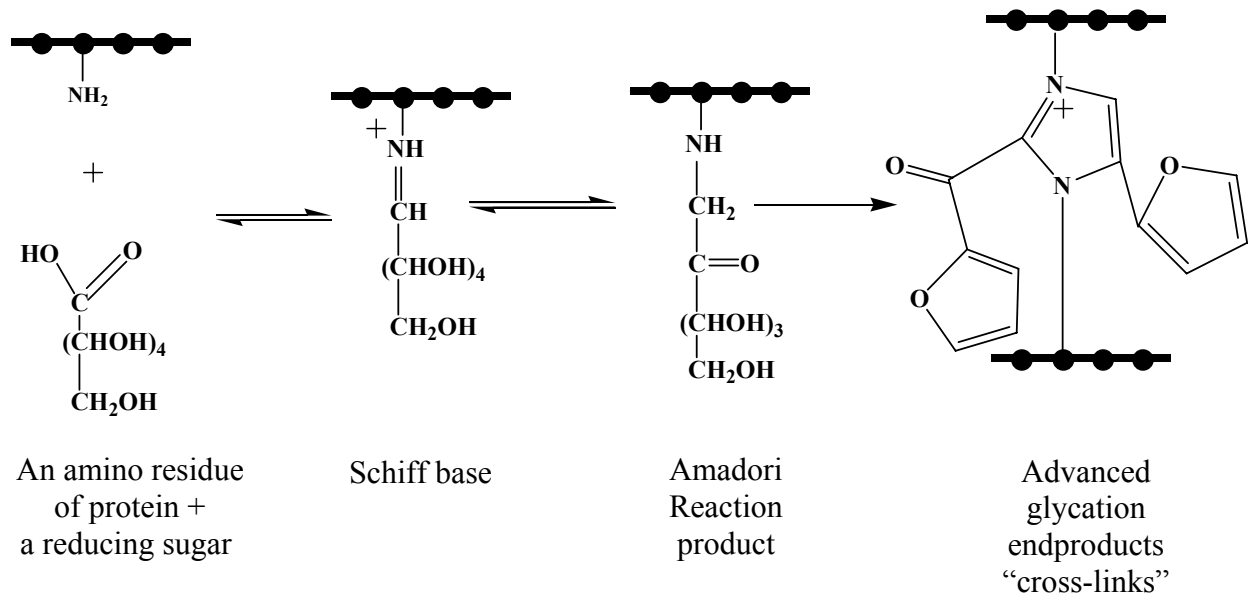
**Table 4.2.** Phenolic compounds identified in ethanolic and acetonic fractions isolated from Navaho, Kiowa, and Ouachita cultivars of Georgia-grown blackberries using a Sephadex LH-20 column<sup>1</sup>

Fractions	Navaho	Kiowa	Ouachita
I	gallic acid; ellagic acid; <i>p</i> -coumaric acid	gallic acid; ellagic acid; <i>trans</i> -cinnamic acid	gallic acid; ellagic acid; catechin
II	gallic acid; ellagic acid; <i>trans</i> -cinnamic acid; <i>p</i> - coumaric acid; catechin; epicatechin	gallic acid; ellagic acid; <i>trans</i> -cinnamic acid; cyanidin-3- <i>O</i> -glucoside	gallic acid; ellagic acid; <i>trans</i> -cinnamic acid; catechin; cyanidin-3- <i>O</i> - glucose
III	ellagic acid; quercetin; cyanidin-3- <i>O</i> -glucoside	ellagic acid; <i>trans</i> - cinnamic acid; cyanidin- 3- <i>O</i> -glucoside	gallic acid; ellagic acid; caffeic acid; <i>p</i> -coumaric acid; quercetin; cyanidin- 3- <i>O</i> -glucoside
IV	ellagic acid; catechin; quercetin; cyanidin-3- <i>O</i> - glucoside + additional anthocyanins	ellagic acid; cyanidin-3- <i>O</i> -glucoside + additional anthocyanins	gallic acid; ellagic acid; quercetin; cyanidin-3- <i>O</i> - glucoside + additional anthocyanins
V	ellagic acid; quercetin; cyanidin-3- <i>O</i> -glucoside + additional anthocyanins	ellagic acid; cyanidin-3- <i>O</i> -glucoside + additional anthocyanins	ellagic acid; cyanidin-3- <i>O</i> -glucoside + additional anthocyanins
VI	ellagic acid; quercetin; cyanidin-3- <i>O</i> -glucoside + additional anthocyanins	ellagic acid; cyanidin-3- <i>O</i> -glucoside + additional anthocyanins	ellagic acid; cyanidin-3- <i>O</i> -glucoside + additional anthocyanins
VII <sup>2</sup>	ellagic acid & derivatives ( <i>i.e.</i> , ellagitannins); gallic acid & derivatives ( <i>i.e.</i> , gallotannins)	ellagic acid & derivatives ( <i>i.e.</i> , ellagitannins); gallic acid & derivatives ( <i>i.e.</i> , gallotannins)	ellagic acid & derivatives ( <i>i.e.</i> , ellagitannins); gallic acid & derivatives ( <i>i.e.</i> , gallotannins)

<sup>1</sup>Fractions I thru VI were eluted from the Sephadex LH-20 column using 95% (v/v) ethanol as the mobile phase, whereas fraction VII was eluted with 50% (v/v) acetone:water.

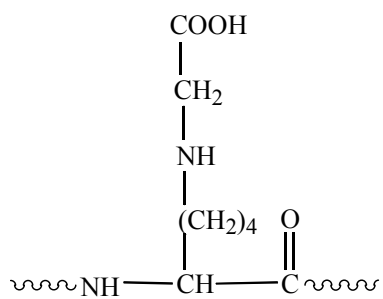
<sup>2</sup>The presence of tannins was confirmed by the qualitative BSA precipitation assay.

Figure 4.1

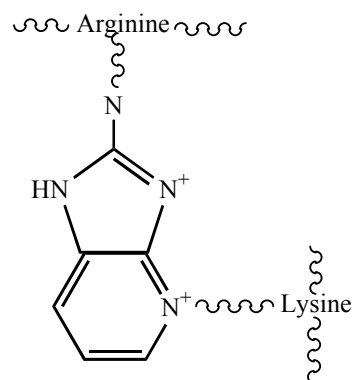


**Figure 4.2**

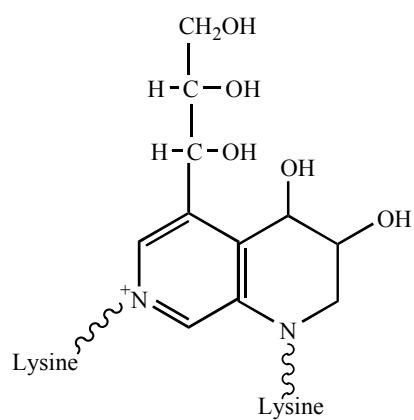
**(A)**



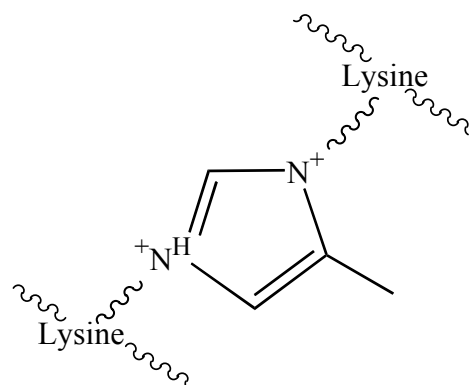
**(B)**



**(C)**



**(D)**



**Figure 4.3**  
**Extraction**

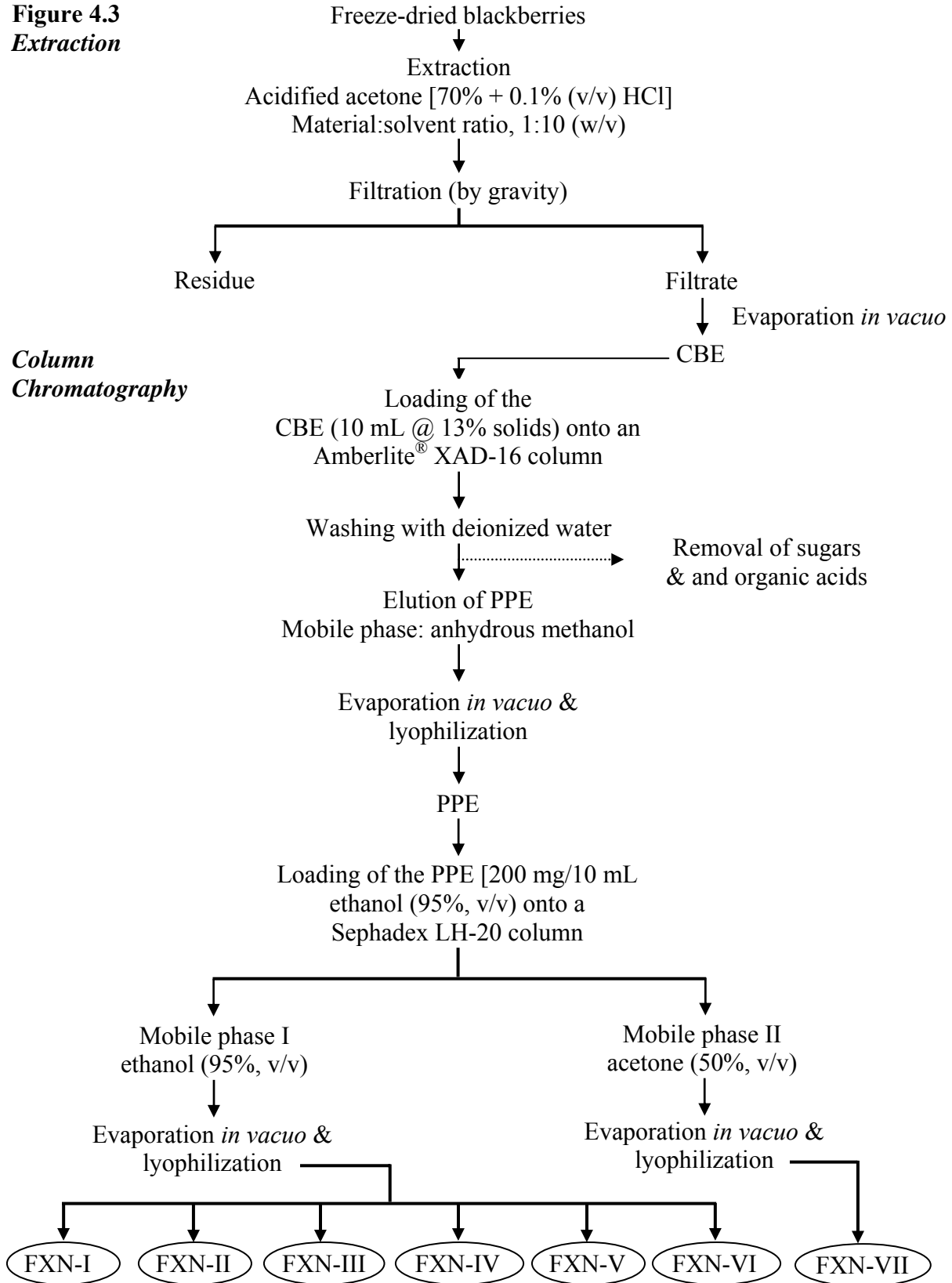


Figure 4.4

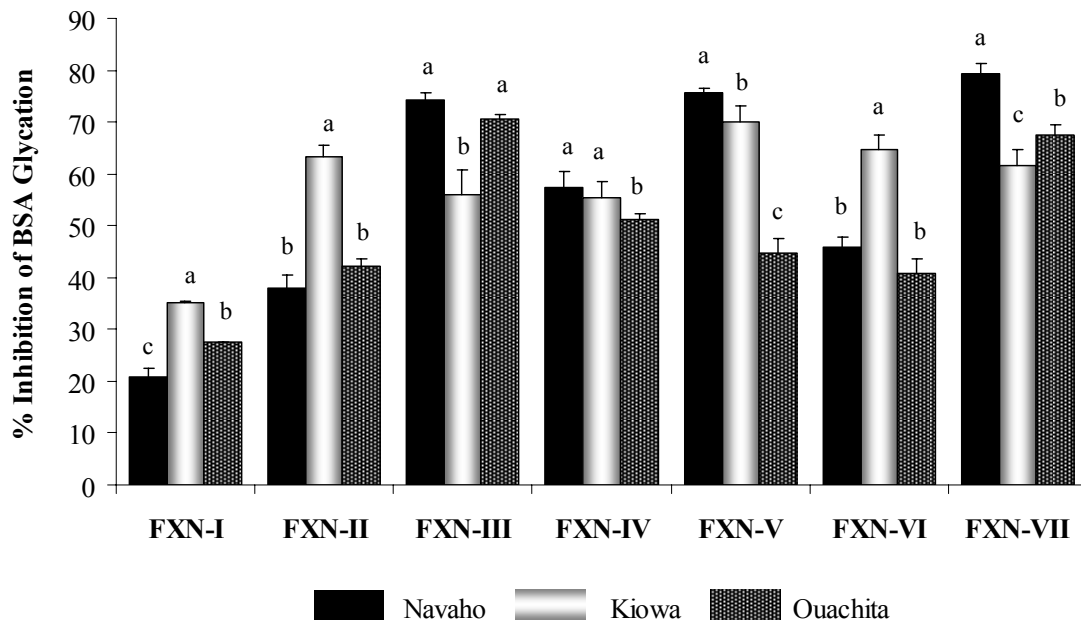




Figure 4.5

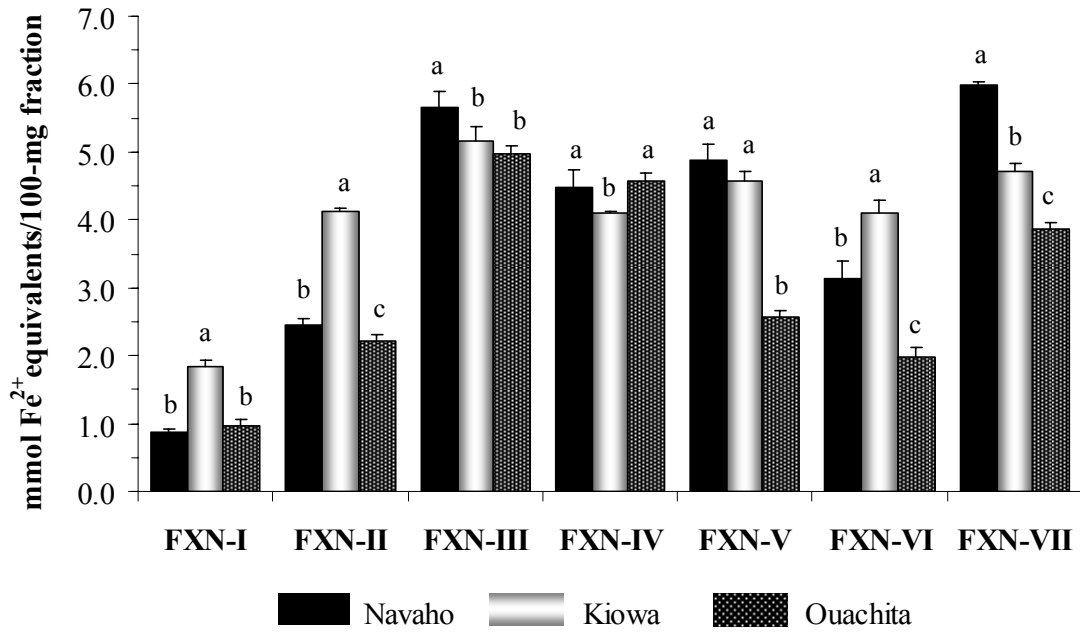


Figure 4.6

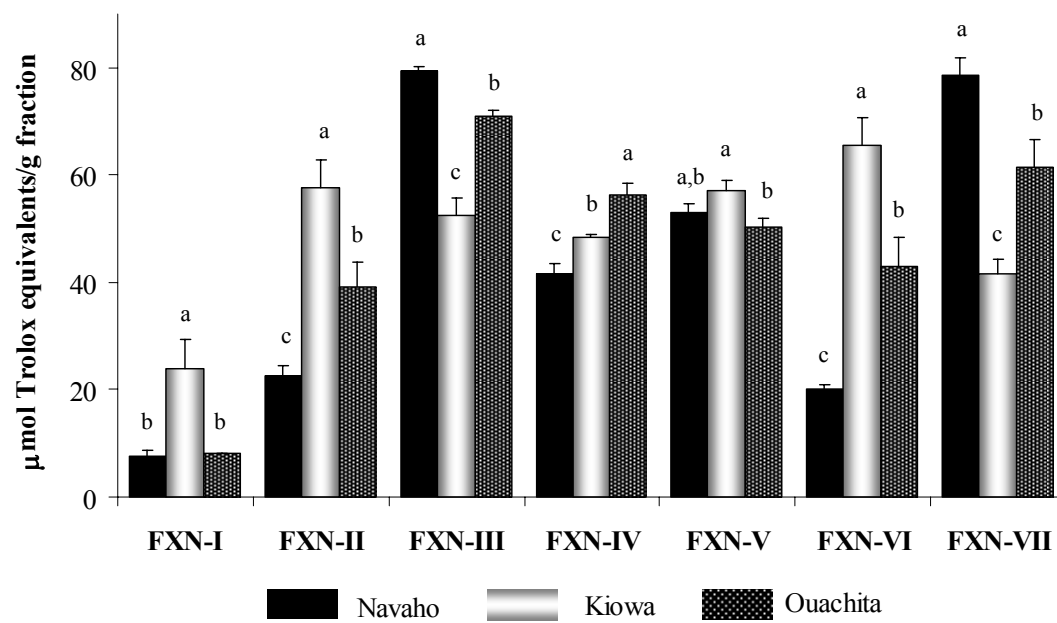


Figure 4.7

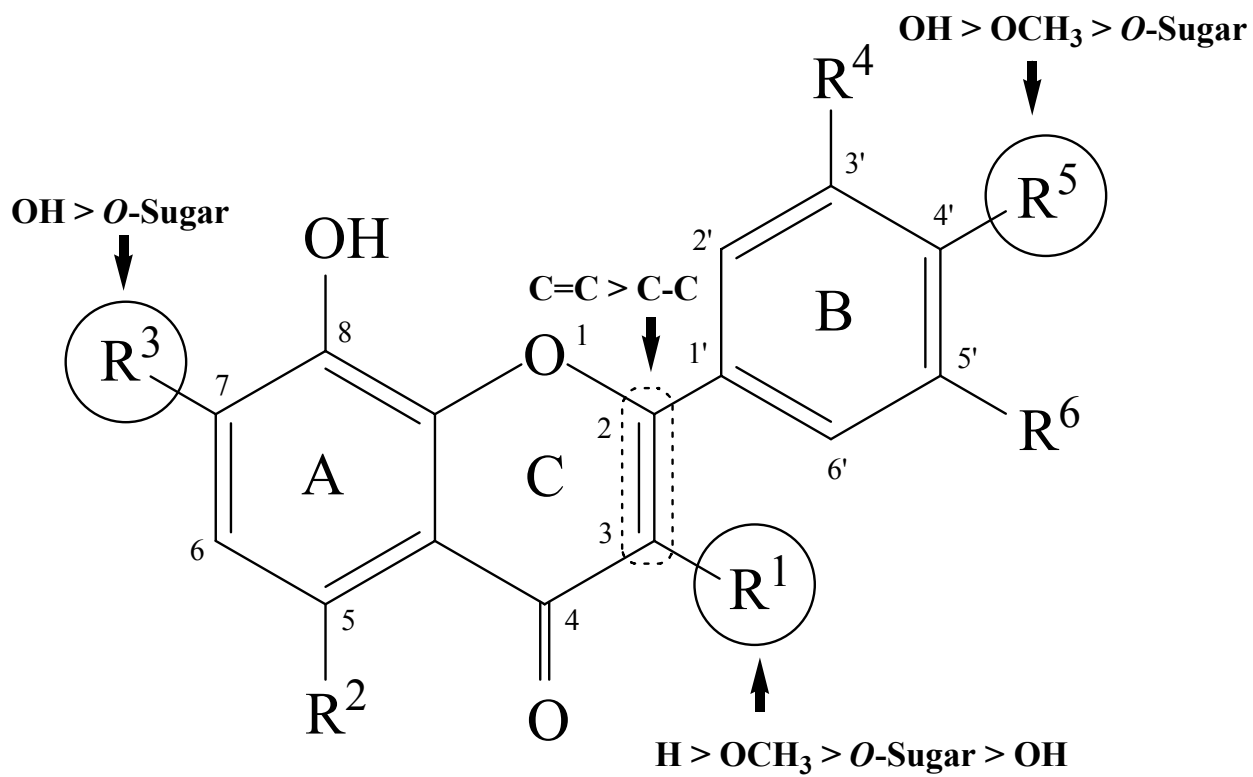


Figure 4.8(A) and (B)

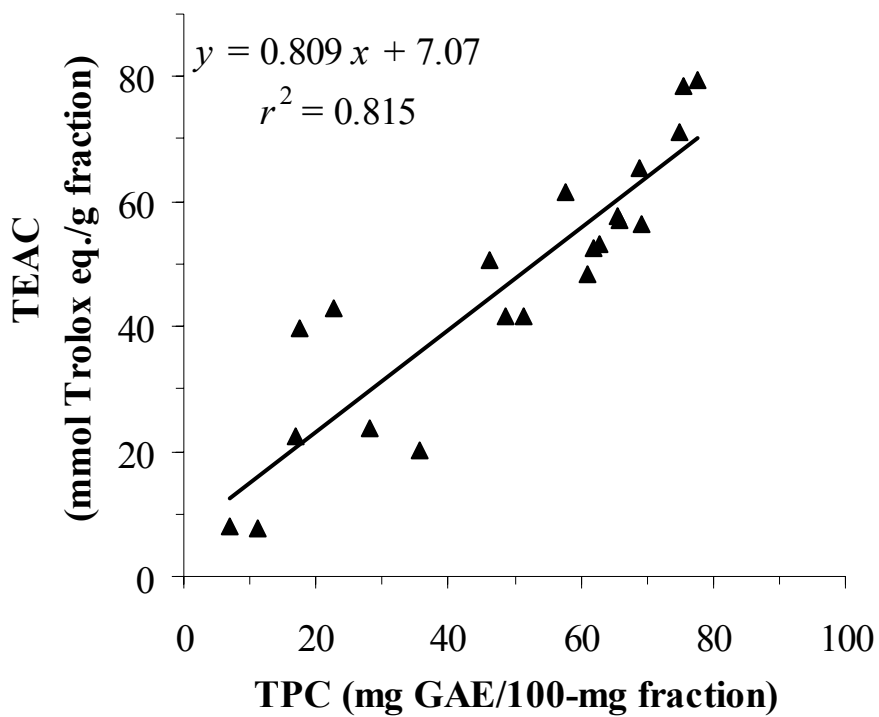
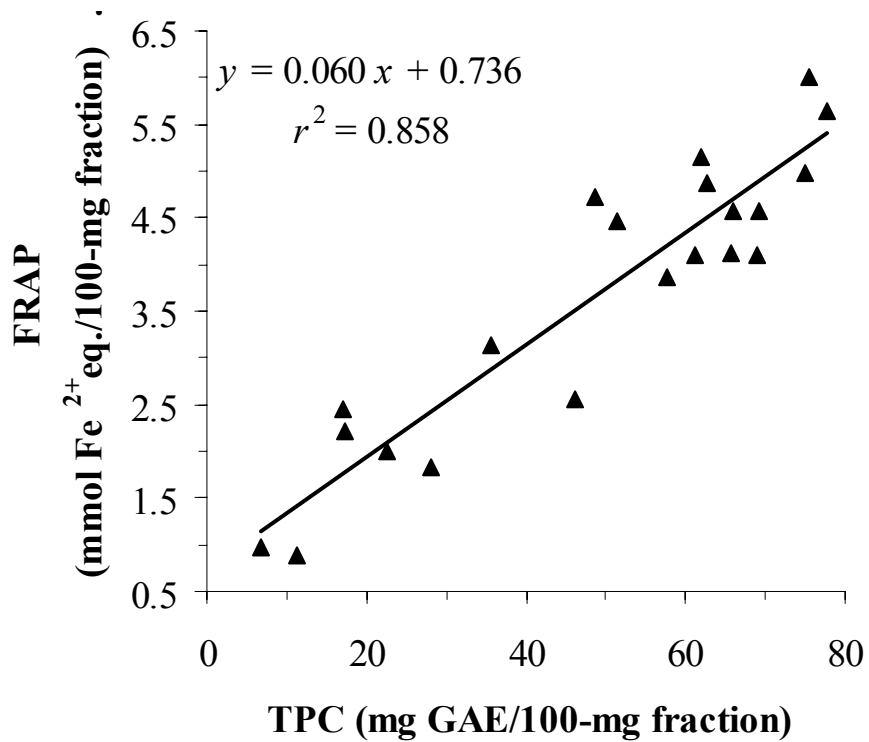
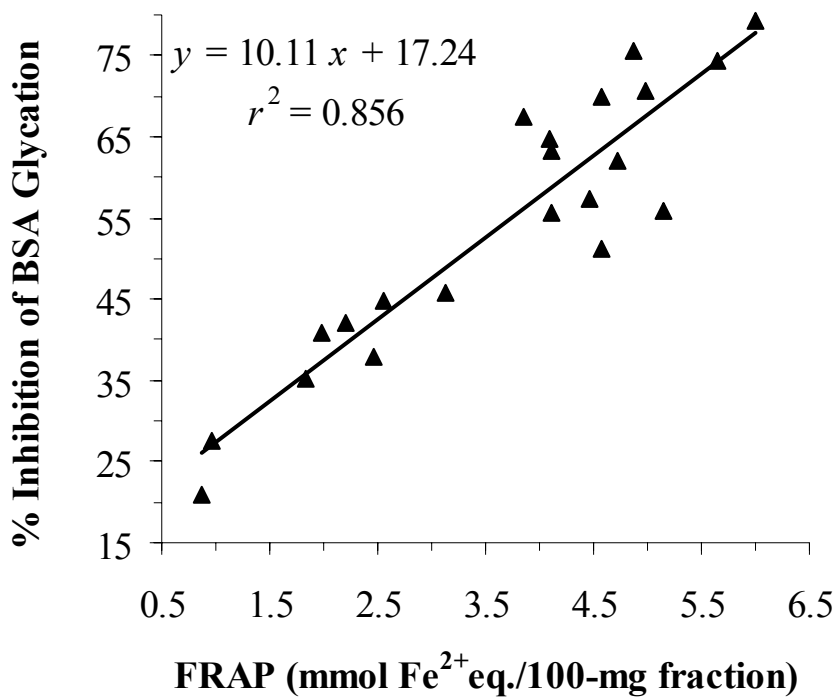
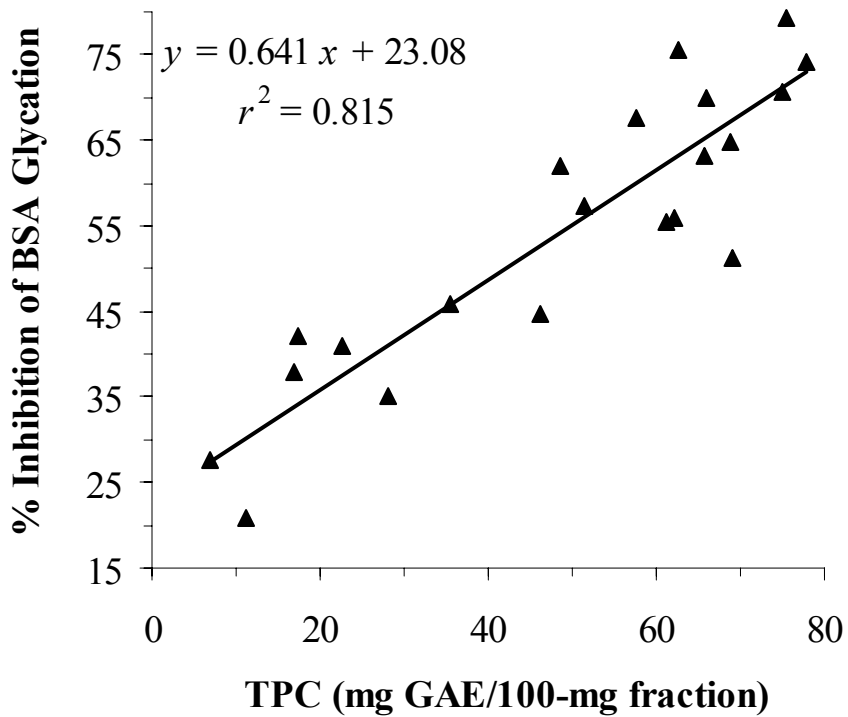


Figure 4.9(A) and (B)



## CHAPTER 5

# TOPICAL ANTI-INFLAMMATORY ACTIVITIES OF POLYPHENOLICS EXTRACTED FROM SOUTHEASTERN U.S. RANGE BLACKBERRY CULTIVARS USING THE 12-*O*- TETRADECANOYLPHORBOL-13-ACETATE (TPA) MODEL OF MOUSE EAR INFLAMMATION

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## ABSTRACT

The antioxidant and anti-inflammatory activities of low- and high-molecular-weight phenolic fractions (LMPF and HMPF, respectively) isolated from three newer blackberry cultivars (*i.e.*, Navaho, Kiowa, and Ouachita), bred to tolerate the warm and humid climatic conditions of the southeastern U.S., were investigated by the *in vitro* ferric reducing antioxidant power (FRAP) assay and an *in vivo* mouse ear edema model. Seventy percent (v/v) acidified acetone was employed to extract phenolics from the Georgia-grown blackberry cultivars, which were subsequently cleaned up on an Amberlite XAD-16 column and then further fractionated with Sephadex LH-20 to LMPF and HMPF. The anti-inflammatory response from topical application of solutions of the LMPF and HMPF as well as indomethacin, a non-steroidal anti-inflammatory drug (NSAID), was assessed in the TPA mouse ear model. All treatments significantly ( $p < 0.05$ ) reduced the TPA-induced injury. Correlation coefficients of 0.856 and 0.852 were found when the anti-inflammatory activities of the blackberry fractions were compared to their total phenolics contents and antioxidant activities (*i.e.*, FRAP values), respectively. Mouse ear myeloperoxidase (MPO) activity, an indicator of polymorphonuclear leukocyte infiltration, was significantly ( $p < 0.05$ ) reduced by indomethacin and all blackberry preparations. Inhibition of both edema and MPO activity indicated marked anti-inflammatory activities by the polyphenolics in blackberries.

**Key words:** Blackberries, Phenolics, Anti-inflammatory activity, Antioxidant activity, Edema, Myeloperoxidase activity, Polymorphonuclear leukocyte infiltration

## INTRODUCTION

Epidemiological studies agree that the consumption of fruits and vegetables confer health benefits to man, and reduce the risk of developing chronic and neurodegenerative diseases such as cardiovascular diseases (Esposito and Gagliano, 2006; Takachi et al. 2008), cancers (Liu, 2004; Thomasset et al. 2007), and Alzheimer's disease (Dai et al. 2006). Fruits and vegetables contain numerous phytochemicals; these bioactive compounds possess antioxidant, radical-scavenging, anti-inflammatory, and anticarcinogenic properties, which contribute to their chemopreventative potential (Nijveldt et al. 2001; Liu, 2004; Surh et al. 2005). *In vitro* and *in vivo* studies suggest that the antioxidant and anti-inflammatory capacities of fruits and vegetables are attributed, at least partly, to their polyphenolic constituents including flavonoids like anthocyanins (Nijveldt et al. 2001; Bagchi et al. 2004). Although the exact mechanisms for the observed beneficial effects of polyphenolics are largely undetermined, sufficient evidence supports the fact that daily consumption of fruits and vegetables is highly protective of health. Berries are among fifty products that are ranked highest in antioxidant levels (Halvorsen et al. 2006), and therefore are worthy of further study.

Our understanding of the biochemical role of antioxidants, both from endogenous and exogenous sources, in the body at retarding inflammation is still in its infancy. A number of *in vitro* studies have shown anti-inflammatory activities of polyphenolics. For example, a muscadine pomace extract inhibited the release of the superoxide anion radical from phorbol myristate acetate-activated neutrophils and the release of the cytokines; *i.e.*, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-6 (IL-6), and interleukin-1 $\beta$  (IL-1 $\beta$ ) in lipopolysaccharides (LPS)-activated peripheral blood mononuclear cells (Greenspan et al. 2005). Anthocyanidins, the aglycones of anthocyanins, contributed to the inhibition of tumorigenesis by blocking the



activation of the mitogen-activated protein kinase (MAPK) pathway in 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced tumor mouse JB6 cells (Hou et al. 2004). Additionally, anthocyanin-rich berry extracts showed considerable inhibitory effects on nitric oxide (NO) production in LPS/interferon- $\gamma$  (IFN- $\gamma$ ) activated RAW 264.7 macrophages (Wang and Mazza, 2002): NO is known to be an important mediator of acute and chronic inflammation. Quercetin, a flavonol found in berries, suppresses TNF- $\alpha$  induced expression of interleukin-8 (IL-8) and the monocyte chemoattractant protein (MCP-1) due to its ability to inhibit the activation of nuclear factor-kappa B (NF- $\kappa$ B). NF- $\kappa$ B is a protein complex that controls the transcription of DNA and is a cell-signaling molecule for inflammation. It can act on/regulate genes (*e.g.*, the cyclooxygenase-2 [COX-2]-encoding gene) inducing the expression of proinflammatory prostaglandins, chemokines (*i.e.*, chemotactic cytokines that attract inflammatory cells to the sites of inflammation), enzymes that generate mediators of inflammation, immune receptors, and adhesion molecules which play a key role in the initial recruitment of neutrophils to the sites of inflammation. The flavonoids found in berries are capable of reducing redox activation of NF- $\kappa$ B. Therefore, diets rich in phenolic antioxidants from fruits (*e.g.*, blackberries) and vegetables may assist in keeping NF- $\kappa$ B at bay.

Animal studies have demonstrated that the consumption of polyphenols can alter the progression of experimental disease states. For example, anti-tumor activities have been reported for resveratrol, a stilbene found in grape skins (Mittal et al. 2003; Kundu et al. 2004), and (-)-epigallocatechin (EGC) from green tea (Meeran et al. 2006). Studies have shown that the anthocyanins from tart cherries have the ability to suppress inflammation-induced pain indices in rats (Tall et al. 2004). Rats fed a diet of 5% (w/w) muscadine grape skin exhibited anti-inflammatory activity with a 50% reduction in hind paw edema in carrageenan-treated animals

(Greenspan et al. 2005). Topical application of anthocyanins and a hydrolyzable tannin-rich pomegranate fruit extract on mouse skin significantly inhibited phosphorylation by mitogen-activated protein kinases (MAPKs), activation of NF- $\kappa$ B, and activity of COXs; the later being an important enzyme involved in mediating the inflammatory process (Afaq et al. 2005).

Neutrophil granulocytes or polymorphonuclear (PMN) leukocytes are the first inflammatory cell type to migrate to the site of inflammation due to chemical attractant signals such as IL-8 and INF- $\gamma$  (a process known as chemotaxis). Myeloperoxidase is a heme-containing peroxidase of PMN leukocytes that functions in body-defense mechanisms against a broad range of organisms (*e.g.*, a bacterial infection). Activation of PMN leukocytes by inflammatory stimuli results in the release of lysosomal enzymes and MPO. MPO catalyzes H<sub>2</sub>O<sub>2</sub> to produce hypochlorous acid (HOCl), a strong oxidant generated in the presence of chloride ions (Weiss, 1989). Hypochlorous acid is an antimicrobial oxidant is a harmful agent that participates in an increasing number of inflammatory-mediated disorders (Nauseef, 2001). For example, Baldus et al. (2003) demonstrated that there is a significant correlation between serum MPO levels and cardiovascular diseases.

Activation of PMN leukocytes and the generation of oxidant species such as HOCl play a significant role in the pathophysiology of vascular and other inflammatory diseases (Ximenes et al. 2005). Kato et al. (2003) reported that commercially-available phenolic antioxidants such as epigallocatechin gallate (EGCG), epicatechin gallate (ECG), and curcumin functioned as strong inhibitors of MPO activity. A significant reduction in MPO activity by a muscadine extract of skins and seeds was reported in the TPA-induced inflammation mouse ear model (Bralley et al. 2007). Limited knowledge is available on the effectiveness of blackberries at curbing inflammation. Duthie (2007) reported that black raspberry extracts altered the expression of

genes associated with inflammation and carcinogenesis. He reported that COX-2 gene expression and subsequent prostaglandin production as well as NO synthase activity were inhibited in premalignant rat esophageal cells following feeding with the berry extract.

Blackberries (*Rubus* spp.) are an important small fruit crop in the United States, and are commercially produced over a wide geographic range. The blackberry is a rich source of anthocyanins and other polyphenolic antioxidants including ellagic acid and its derivatives. In particular, cyanidin-based compounds have been found to be the major anthocyanins in blackberries (Siriwoharn et al. 2004). With regard to the tannin composition, blackberries predominantly contain hydrolyzable tannins; *i.e.*, ellagitannins and gallotannins (Seeram et al. 2006). The fruit, therefore, possesses a considerable potential for extraction of biologically-active compounds that could, when consumed at appropriate levels, potentially alleviate or attenuated symptoms of inflammatory diseases.

The goal of this study was to investigate the antioxidant and anti-inflammatory capacities of blackberry phenolics from three cultivars (*i.e.*, Navaho, Kiowa, and Ouachita) bred for the hot, humid climatic conditions of the southeastern U.S. using the *in vitro* ferric reducing antioxidant power (FRAP) assay and an *in vivo* mouse ear edema model, respectively.

## **MATERIALS AND METHODS**

Phenolic compounds including (+)-catechin, (-)-epicatechin, myricetin, quercetin, and a series of phenolic acids comprising gallic, ellagic, vanillic, caffeic, *p*-coumaric, *trans*-cinnamic, protocatechuic, syringic, chlorogenic, and *p*-hydroxybenzoic acids were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). A pure standard of kuromanin chloride (*i.e.*, cyanidin-3-*O*-glucoside chloride) was acquired from Indofine Chemical Co. (Hillsborough, NJ). Chemicals

for analytical and anti-inflammatory assays included 12-*O*-tetradecanoylphorbol-13-acetate (TPA), hexadecyltrimethylammonium bromide, indomethacin, 3,3',5,5'-tetramethylbenzidine dihydrochloride, *N,N*-dimethylformamide, hydrogen peroxide solution (30% w/v), Folin-Ciocalteu's phenol reagent, sodium carbonate, 2,4,6-tri(2-pyridyl)-*s*-triazine (TPTZ), ferric chloride hexahydrate, ferrous sulfate heptahydrate, sodium acetate, acetic acid, triethanolamine, sodium dodecyl sulfate (SDS), monobasic potassium phosphate and dibasic potassium phosphate; all were obtained from Sigma-Aldrich. The HPLC-grade solvents acetonitrile, water, and methanol were purchased from Fisher Scientific Co. (Suwanee,, GA) as were ACS-grade acetone, 95% ethanol (v/v), glacial acetic acid, and hydrochloric acid.

### **Sample collection**

Mature blackberries (*Rubus* spp.) were harvested from Jacob W. Paulk Farms, Inc. (Wray, GA) industrial operation in May 2006 and 2007. The three blackberry cultivars collected over two crops years were as follows: Navaho, an erect thornless variety; Kiowa, an erect thorny variety; and Ouachita, a very erect cane and thornless variety. All three cultivars, which grow particularly well in the hot, humid conditions of the southeastern U.S., were patented by and released from the University of Arkansas. These cultivars are different from those in the dominant blackberry production areas of the northwest U.S. Hand-picked blackberries were transported to the Department of Food Science & Technology, UGA, in Athens, GA. The berries were sorted, cleaned, and frozen in polyethylene pouches at  $-40\text{ }^{\circ}\text{C}$ . Representative samples from each cultivar were lyophilized using a UNITOP 600L VirTis™ freeze dryer (The VirTis Company, Inc., Gardiner, NY), transferred to polyethylene pouches, and then stored at  $-40\text{ }^{\circ}\text{C}$  until analyzed.

### **Preparation of crude blackberry extracts (CBEs)**

Freeze-dried blackberry samples (*i.e.*, containing fruit receptacles, skins and seeds) from each cultivar were ground in a commercial coffee mill (KitchenAid, St Joseph, MI). Fifteen grams of blackberry powder were mixed with 150 mL of 70% (v/v) acidified acetone (containing 0.1% [v/v] HCl) and blended using a PT-3100 Polytron™ homogenizer (Brinkmann Instruments, Westbury, NY) at 15,000 rpm for 10 min. The slurry was then filtered by gravity through fluted P8 filter paper (Fisher Scientific). This extraction process was repeated 2× as described above. All filtrates were pooled and acetone was evaporated with a Büchi Rotavapor R-210 using a V-700 vacuum pump connected to a V-850 vacuum controller (Büchi Corporation, New Castle, DE) at 40 °C. Sample extractions of each cultivar were performed in triplicate.

### **Column Chromatography**

#### **Preparation of polyphenolic extracts (PPEs)**

Ten milliliters of each CBE (containing 13% solids) were applied to the top of a chromatographic column (30 mm *i.d.* × 340 mm *e.l.*, Kontes, Vineland, NJ) packed with Amberlite XAD-16 [(bead size: 20-60 mesh), Sigma-Aldrich] and washed with ~300 mL of deionized water to remove sugars and organic acids (Mazza et al. 2004). After the first 100 mL, the pH of the eluent was checked with pH paper test strips every 20 mL until a neutral pH was reached. The polyphenolic extract (PPE) was then eluted from the column with anhydrous methanol (~300 mL) as the mobile phase. Methanol was evaporated using the Büchi Rotavapor at 40 °C. The PPE was lyophilized using a FreeZone® 2.5-L bench-top freeze dryer (Labconco

Corporation, Kansas City, MO) to ensure all traces of moisture were removed and then stored in amber-glass bottles in a 4 °C refrigerator.

### **Preparation of a low-molecular-weight phenolic fraction (LMPF) and a high-molecular-weight phenolic fraction (HMPF)**

For each cultivar, 200 mg of lyophilized PPE were dissolved in 10 mL of 95% (v/v) ethanol, sonicated to facilitate dissolution, and then applied to the top of a chromatographic column (30 mm *i.d.* × 360 mm *e.l.*, Kontes, Vineland, NJ) packed with Sephadex LH-20 [(bead size: 25-100 µm), Sigma-Aldrich]. Five hundred milliliters of ethanol (95% [v/v]) were used as the mobile phase to elute the LMPF. The system was changed over to 50% (v/v) acetone, and ~300 mL were required to elute the HMPF from the Sephadex LH-20 column. The bovine serum albumin (BSA) assay (a detailed procedure for this assay is described below) was performed to check qualitatively for the presence of high-molecular-weight phenolics (*i.e.*, hydrolyzable and condensed tannins). For the LMPF and HMPF, ethanol and acetone were evaporated with the Büchi Rotavapor at 40 °C, respectively, and each residue was lyophilized. Freeze-dried fractions were stored in amber-glass bottles at 4 °C until analyzed.

### **Bovine serum albumin (BSA) precipitation assay**

The effect of blackberry tannins resulting in the formation of an insoluble tannin-protein complex was measured qualitatively by the BSA precipitation assay of Hagerman and Butler (1978). Briefly, one milliliter of a methanolic solution (1.0 mg/mL) of each fraction (*i.e.*, LMPF and HMPF) was pipetted into a 15-mL Falcon<sup>®</sup> centrifuge tube. To each, 2.0 mL of a standard protein solution [*i.e.*, 1.0 mg of BSA/mL of 0.2 M acetate buffer (pH 5.0, containing 0.17 M

NaCl)] were added. The solutions were vortexed, allowed to stand at room temperature for ~15 min, and then centrifuged using a Centrifuge (Model 1228, Fisher Scientific, Pittsburgh, PA) for ~15 min at  $5000 \times g$ . The supernate was discarded. The surface of the residual pellet and walls of the tube were carefully rinsed with 1 mL of 0.2 M acetate buffer (pH 4.0) without disturbing the pellet. The pellet was then dissolved in 4 mL of SDS-triethanolamine solution [1% (w/v) sodium dodecyl sulfate (SDS) and 5% (v/v) triethanolamine in deionized water]. One milliliter of  $\text{FeCl}_3$  reagent (0.01 M  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  in 0.01 N HCl) was added, and the solution was vortexed. Approximately 20 min after addition of the iron(III) reagent, sample absorbance readings were measured at  $\lambda = 510$  nm with an Agilent 8453 photodiode array spectrophotometer (Agilent Technologies, Wilmington, DE). The average  $A_{510}$  of triplicate samples of the SDS-triethanolamine solution plus  $\text{FeCl}_3$  reagent was subtracted from the  $A_{510}$  of each sample to correct for background absorbance.

### **Total phenolics content (TPC) assay**

The TPC was determined for the LMPF and HMPF colorimetrically using the classical Folin-Ciocalteu assay (Singleton and Rossi, 1965). This assay is based on the reduction of a heteropolyphosphotungstate-molybdate complex by phenolic compounds under alkaline conditions yielding a blue color. Briefly, 0.5 mL of a methanolic solution (5  $\mu\text{g}/\text{mL}$ ) of each fraction was pipetted into a test tube followed by the addition of 8.0 mL of deionized water, 0.5 mL of 2 N Folin-Ciocalteu's phenol reagent, and 1.0 mL of a saturated  $\text{Na}_2\text{CO}_3$  solution. The contents were vortexed for 15 s. After an incubation period of 60 min at room temperature to allow for optimal color development of the samples, absorbance readings were taken at  $\lambda = 750$  nm with the Agilent spectrophotometer. Quantification was based on a standard curve generated

with gallic acid as described by Singleton and Rossi (1965). The TPCs were determined from the standard curve, and results were expressed as mg gallic acid equivalents/100-mg respective fraction.

### **FRAP (Ferric Reducing Antioxidant Power) assay**

The antioxidant capacity of each fraction was estimated according to the procedure described by Benzie and Strain (1996). Briefly, the FRAP reagent was prepared fresh each day by adding 2.5 mL of a 10 mM TPTZ [2,4,6-tri(2-pyridyl)-s-triazine] solution in 40 mM HCl, 2.5 mL of 20 mM  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , and 25 mL of acetate buffer (300 mM, pH 3.6). The FRAP reagent was kept warm at 37 °C in a Precision Model-182 water bath (Precision Scientific Inc., Chicago, IL) until further use. Three hundred microliters of the FRAP reagent and 10  $\mu\text{L}$  of each sample solution [*i.e.*, 50  $\mu\text{g}/\text{mL}$  of 50% (v/v) ethanol] were pipetted into a borosilicate glass cuvette and mixed well by pumping the mixture through the pipette tip. After a 6 min quiescent period, 340  $\mu\text{L}$  of deionized water were added to the mixture. Absorbance readings were measured at  $\lambda = 593$  nm using a UV/VIS spectrophotometer (Beckman DU-650, Beckman Instruments, Inc., Fullerton, CA). Aqueous solutions of known Fe(II) concentrations in the range of 0.15 to 1.5 mM were employed to construct a calibration curve. The FRAP values were calculated from a standard curve of  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  and expressed as mmol Fe(II) equivalents/100-mg respective fraction.

### **HPLC analysis**

An Agilent 1200 Series HPLC system consisting of a quaternary pump with degasser, autosampler, thermostatted column compartment, UV/VIS diode array detection (DAD) with standard flow cell, and 3D ChemStation software (Agilent Technologies) was used for the



chromatography. A reversed-phase Luna C<sub>18</sub>(2) column (4.6 × 250 mm, 5 μm; Phenomenex, Torrance, CA) was used. A gradient elution consisting of mobile phase A (H<sub>2</sub>O:CH<sub>3</sub>CN:CH<sub>3</sub>COOH; 93:5:2, v/v/v) and mobile phase B (H<sub>2</sub>O:CH<sub>3</sub>CN:CH<sub>3</sub>COOH; 58:40:2, v/v/v) from 0% to 100% B over a 50-min period at a flowrate of 1 mL/min was employed. Before subsequent injections, the system was re-equilibrated for 10 min using 100% A giving a total run time of 60 min. The injection volume of each fraction (0.5 mg/mL methanol) was 20 μL. Detection wavelengths employed were 255 nm (ellagic acid and ellagic acid derivatives), 280 nm (phenolic acids, catechin, epicatechin), 320 nm (phenolic acids), 360 nm (flavonols), and 520 nm (anthocyanins). Tentative identification of separated components was made by matching UV/VIS spectra and retention time mapping with authentic standards.

### **Animal model studies**

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at UGA and conducted according to IACUC guidelines. The sample size ( $n$ ) of eight animals for each test group was justified on the basis of preliminary experiments showing that sample standard deviation ( $s$ ) for determinations of ear edema was ~5% of the measured value and the average expected difference ( $d$ ) between TPA-treated ears and PPE-treated ears was about 0.2 mm. Assuming that  $\alpha = 0.05$  and  $1 - \beta = 0.9$ , the formula employed was  $n = 1 + 21 \times (s/d)^2$  (Dell et al. 2002). The formula gave 6.25, which was increased to eight in case of unexpected experimental problems.

Male Swiss Webster mice (Harlan Laboratories, Indianapolis, IN), weighing between 22 and 25 g, were housed in groups of four in large shoebox cages. All groups were fed a standard rodent diet (TestDiet<sup>®</sup> 570B, Purina Mills, St. Louis, MO) *ad libitum* with free access to water.

Animals were in the fed-condition throughout the experiment. Photoperiods equaled 12 h of daylight and 12 h of darkness daily, with the environmental temperature maintained at 21 °C.

### **12-*O*-Tetradecanoylphorbol-13-acetate (TPA)-induced mouse ear edema**

All of the animals were divided into nine groups, and each group contained eight animals. Group-1 was designated as the control group in which 10 µL of acetone was applied to both the inner and outer ear surfaces. Thirty minutes after applying the acetone, 10 µL of ethanol (50%, v/v) was pipetted on each ear without any anti-inflammatory intervention. For groups 2 thru 9, edema was induced by topical application of 10 µL of an acetic TPA solution (0.1 mg/mL) to each side of the ear. Group-2 was the vehicle control group, also called the TPA-positive control. Thirty minutes after the TPA application, 10 µL of 50% (v/v) ethanol was applied to each ear without any anti-inflammatory treatment. Group-3 was the treatment-positive control group; *i.e.*, 30 min after the TPA application, 10 µL of a non-steroidal anti-inflammatory drug (NSAID; indomethacin, 25 mg/mL acetone) was applied to each ear. For treatment groups 4 thru 9, 10 µL of LMPF and HMPF solutions [*i.e.*, 50 mg/mL of ethanol (50%, v/v) from Kiowa, Navaho, and Ouachita cultivars] were pipetted on each ear, respectively, 30 min after the TPA application.

Edema was expressed as the increase in ear thickness due to inflammation. The thickness of each ear was measured using a micrometer (Mitutoyo Series IP65, Mitutoyo America, Aurora, IL) at 0, 4, and 24 h after the TPA application. The micrometer was applied near the tip of the ear just distal to the cartilaginous ridges, and the thickness was recorded in mm.

At 24 h, each animal was sacrificed with CO<sub>2</sub> inhalation by the IACUC-approved protocol. Ear punch biopsies (*i.e.*, 6-mm diameter hole punch) were taken immediately,

weighed, and stored in an ultra-low temperature freezer at  $-80\text{ }^{\circ}\text{C}$ . A single investigator performed all ear measurements and biopsies in order to standardize the procedure and to reduce experimental error. The percent inhibition was calculated as per the formula given below:

$$\% \text{ Inhibition} = \left( 1 - \frac{\text{Difference in thickness (mm) of inflamed treated ear} - \text{Difference in thickness (mm) of control ear}}{\text{Difference in thickness (mm) of inflamed ear} - \text{Difference in thickness (mm) of control ear}} \right) \times 100$$

### **Myeloperoxidase (MPO) assay**

Tissue MPO (MPO, E.C. 1.11.1.7) activity was measured in biopsies taken from both ears 24 h after TPA administration using the method of Suzuki et al. (1983) as modified by De Young et al. (1989). This assay is based on the oxidation of 3,3',5,5'-tetramethylbenzidine•dihydrochloride (TMB) in the presence of  $\text{H}_2\text{O}_2$  catalyzed by the MPO enzyme. When oxidized, TMB produces a chromophore giving a blue color. Because the extent of TMB oxidation is dependent on the MPO concentration (Andrews and Krinsky, 1986), the formation of oxidized product was related to the MPO activity by an increase in absorbance. Each mouse ear biopsy (~8 to 18 mg) was placed in a 5-mL test tube to which 0.75 mL of a HTAB solution (80 mM phosphate-buffered saline (PBS) [pH 5.4, containing 0.5% (v/v) hexadecyltrimethylammonium bromide (HTAB)]) was added. Each sample was homogenized for 45 s at  $4\text{ }^{\circ}\text{C}$  with a tissue homogenizer (Tekmar Tissumizer, Model SDT-1810, Tekmar Co, Cincinnati, OH). The homogenate was transferred quantitatively to a microcentrifuge tube with rinsing using an additional 0.75 mL of the HTAB solution. The 1.5-mL sample was centrifuged in an Eppendorf<sup>®</sup> Model 5417R refrigerated microcentrifuge (Eppendorf AG, Hamburg, Germany) at

12,000 × g for 15 min, maintained at 4 °C. Triplicate 30-μL aliquots of the resulting supernate were added to a COSTAR® 96-well microtiter plate (Corning Incorporated, Corning, NY). For the MPO assay, 200 μL of a mixture containing 100 μL of 80 mM PBS (pH 5.4), 85 μL of 0.22 M PBS (pH 5.4), and 15 μL of 0.017% (v/v) H<sub>2</sub>O<sub>2</sub> were added to each well. Twenty microliters of 18.4 mM TMB in 8% (v/v) aqueous *N,N*-dimethylformamide were added to initiate the reaction. Microtiter plates were incubated in an Isotemp Plus incubator (Fisher Scientific) at 37 °C for 3 min, after which they were immediately placed on ice. The reaction was then stopped by the addition of 30 μL of 1.46 M sodium acetate, pH 3.0. Myeloperoxidase enzyme activity was measured colorimetrically using an ELx800 Universal Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT) at λ = 630 nm by top scanning. MPO activity was expressed as optical density (OD) at 630 nm/biopsy.

$$\% \text{ Inhibition} = \left( 1 - \frac{\text{Abs @ 630 nm/biopsy inflamed treated ear} - \text{Abs @ 630 nm/biopsy control ear}}{\text{Abs @ 630 nm/biopsy inflamed ear} - \text{Abs @ 630 nm/biopsy control ear}} \right) \times 100$$

### Statistical analysis

System software (SAS, Version 9.1) was used for statistical analysis of data. All observations were expressed as the mean ± standard deviation (*n* = 8). Statistical evaluations used *t*-tests and one-way analysis of variance (ANOVA) with post-hoc tests for significance of differences by Duncan's multiple comparison test. Statistical significance was considered at *p* < 0.05. The SAS software was also used to determine correlation coefficients between different TPC and % inhibition of edema or MPO activity [significance level of 5% (*p* < 0.05)].

## RESULTS AND DISCUSSION

### Fractionation of Blackberry Phenolics

A flow diagram summarizing the extraction and chromatography steps is depicted in Figure 5.1. A comprehensive discussion of this process and the partial characterization of the bioactives by HPLC and mass spectrometries are given in Chapter 3.

The CBE was obtained using a two-stage extraction with 70% (v/v) acidified acetone (containing 0.1% [v/v] HCl) based on a method described by Naczki and Shahidi (2004). Seventy percent acetone is known to assist with the extraction of tannin constituents, important antioxidant compounds. The CBE was partially purified using Amberlite XAD-16 particles to remove sugars and organic acids. Though some low-molecular-weight polar phenolics might have been removed in the aqueous washing step, the bulk of the polyphenolic constituents were retained on the column until eluted with anhydrous methanol; the resultant product was a crude PPE. The PPE for each cultivar was further fractionated into a LMPF and a HMPF via Sephadex LH-20 column chromatography, as has been described in the Methods section. The extraction and chromatography relative yields for CBE, PPE, LMPF, and HMPF are listed in Table 5.1.

### Partial Characterization of Blackberry Phenolics and Their Antioxidant Activities

The TPCs of each fraction (*i.e.*, LMPF and HMPF) from the Navaho, Kiowa, and Ouachita cultivars are provided in Table 5.2. The results are reported as mg gallic acid equivalents (GAE)/100-mg respective fraction as opposed to (+)-catechin or (-)-epicatechin equivalents, because blackberries contain more hydrolyzable than condensed tannins (Hager et al. 2008). As evident from Table 5.2, the TPCs ranged from  $68.0 \pm 1.9$  to  $89.9 \pm 1.9$  mg GAE/100-mg

respective fraction. Of the three cultivars investigated, the Navaho HMPF possessed the highest TPC, but it was not significantly ( $p > 0.05$ ) different from that of its Kiowa counterpart. It is somewhat difficult to compare our results with those reported in the literature, because most researchers have not removed sugars, organic acids, and water-soluble compounds (like vitamin C) which can interfere with the reaction involving Folin-Ciocalteu's phenol reagent (Georgé et al. 2005). When factoring in extraction and chromatography yields from the mass balances, the blackberries for the three cultivars investigated in this study contain ~545 mg GAE/100-g fresh berries: our results are ~2× those reported by Kao et al. (2007) for the Navaho variety grown in Alabama (232.6 mg GAE/100-g fresh berries), and are comparable to the TPC reported by Sellappan et al. (2002) (417.8 mg/100-g fresh berries) in Kiowa. Variations may be due to differences in extraction methodologies employed for the phenolics; the presence of non-phenolic compounds such as ascorbic acid, monosaccharides/disaccharides, and organic acids; as well as differences in blackberry cultivars, stage of maturity, and environmental factors such as light, temperature, agronomic practices, and geographical area.

The BSA precipitation assay was used as a qualitative tool to detect the presence of tannins, as this assay is applicable for both hydrolyzable and condensed tannins (Hagerman and Butler, 1978; Kawamoto et al. 1996). The precipitation assay is based on the capacity of tannins to interact with protein in a manner that results in precipitation. It is important to note, however, that not all tannin-protein complexes will precipitate from solution. Absorbance readings at  $\lambda = 510$  nm of the tannin-protein precipitate, dissolved in SDS-ethanolamine to which the ferric chloride reagent was added, for the LMPF and HMPF of all three cultivars are given in Figure 5.2. As expected, the high optical densities for the HMPFs confirmed the presence of tannins, while the LMPFs were low in hydrolyzable and condensed tannins.

Antioxidant activity was measured for the LMPF and HMPF from the blackberry cultivars by the classical FRAP assay. FRAP values ranged from  $2.03 \pm 0.02$  to  $3.92 \pm 0.24$  mmol Fe<sup>2+</sup> equivalents/100-mg respective fraction. The Navaho HMPF showed the highest FRAP value as compared to HMPFs from Ouachita and Kiowa. Pellegrini et al. (2003) reported that berries, as a general category (*i.e.*, blackcurrant, strawberry, raspberry, and blueberry), possessed marked antioxidant capacity and that the blackberry was most effective at 5.15 mmol Fe<sup>2+</sup> equivalents/100-g fresh weight).

An HPLC profile/fingerprint of a LMPF from one of the blackberry cultivars with DAD at  $\lambda = 520$  nm is depicted in Figure 5.3. Phenolic compounds were quantified using response factors of the representative standard near their characteristic wavelength of maximum absorption (*i.e.*, hydroxybenzoic acids and flavan-3-ol at 280 nm; hydroxycinnamic acids at 320 nm; flavonol & flavonol glycosides at 360 nm; and anthocyanins at 520 nm). The LMPF contained a dominant single peak that eluted at a retention time (RT) of 11.4 min. This peak corresponded to the cyanidin-3-*O*-glucoside standard and was confirmed by ESI-TOF-MS. Cyanidin-3-*O*-glucoside was the primary anthocyanin found in the LMPFs of each blackberry cultivar examined; it ranged from  $229 \pm 0.1$  to  $309 \pm 0.2$   $\mu\text{g}/\text{mg}$  LMPF. This is significant, as cyanidin-3-*O*-glucoside has been reported to have the highest antioxidant capacity of 14 different anthocyanins tested (Mazza and Miniati 1993; Wang et al. 1996). The content of cyanidin-3-*O*-glucoside in the LMPF was present in the following order: Kiowa > Navaho > Ouachita. Gallic acid and *p*-coumaric acid were also detected in the LMPF of the Navaho cultivar and tentatively identified by comparison of their UV-spectra and retention times with those of commercially-available standards. Traces of flavan-3-ols [*i.e.*, (+)-catechin and (-)-epicatechin, the monomers of proanthocyanidins (PACs)], and flavonols (*i.e.*, quercetin) were also found in the LMPF. The

HMPF for each cultivar was characterized by the presence of ellagic acid, its derivatives, and ellagitannins. The total amount of ellagic acid and derivatives ranged from  $164 \pm 1.5$  to  $552 \pm 0.4$   $\mu\text{g}/\text{mg}$  HMPF. The greatest content of these was found in the Navaho cultivar followed by Kiowa and then Ouachita. A minor peak was also present in the chromatograms that could not be identified based on spectral characteristics of the available standards.

Our results are in agreement with other studies indicating that cyanidin-3-*O*-glucoside is the primary anthocyanin in blackberry (Mazza and Miniati 1993; Siriwoharn et al. 2004; Fan-Chiang and Wrolstad 2005). In addition to this particular anthocyanin, Wu and Prior (2005) identified cyanidin-3-*O*-xyloside, cyanidin-3-(6''-malonyl)-glucoside, and cyanidin-3-*O*-dioxaloylglucoside in blackberry using HPLC-ESI-MS/MS. Dugo et al. (2001) also found the presence of additional anthocyanins in trace amounts; that is, cyanidin-3-*O*-rutinoside and malvidin-3-*O*-glucoside. Siriwoharn and Wrolstad (2004) reported the presence of flavonols (primarily quercetin glycosides) and procyanidins (catechin- and epicatechin-based) in the seeds of blackberries, whereas Vrhovsek et al. (2006) reported on the presence of ellagic acid and its derivatives.

### **Anti-inflammatory Activity of Blackberry Phenolics**

12-*O*-Tetradecanoylphorbol-13-acetate (TPA) can act as an inducer of epidermal hyperplasia, a tumor promoter, and an activator of various biological systems. *In vivo* TPA-induced ear edema is an acute inflammatory model; it offers a simple and useful assay for screening the efficacy of topical anti-inflammatory capacities of plant extracts, in this case blackberry preparations (see Table 5.3 for experimental design used). The exact mechanism by which topical application of the phorbol ester induces an inflammatory response is not completely understood; however, the



activation of protein kinase C with subsequent cytosolic phospholipase A<sub>2</sub> stimulation, arachidonic acid (AA) mobilization, and biosynthesis of prostaglandins and leukotrienes is involved (Nishizuka 1984). TPA applied to mouse ears initiates a cascade of events leading to inflammatory processes such as increased vascular permeability, edema, and mast cell infiltration. Moreover, one of the early hallmarks of skin irritation and local inflammation is the thickening/swelling of the skin within the dermis (De Vry et al. 2005). The TPA-induced injury was very obvious when examining the mouse ears during the course of this study. According to Carlson et al. (1985), edema formation will begin less than 1 h post-TPA administration and reach its peak within 4 to 6 h. In our study, topical application of LMPF and HMPF from all three blackberry cultivars significantly attenuated the TPA-induced inflammation in the ears of treated mice; *i.e.*, both in terms of edema and the migration of PMN leukocytes to the site of inflammation, which was assessed by measuring MPO activity.

The experiment showed that the NSAID (*i.e.*, indomethacin) and treatments of the LMPF and HMPF from all blackberry cultivars significantly ( $p < 0.05$ ) reduced the TPA-induced skin inflammatory response; Figure 5.4 depicts the changes in ear thickness at both the 4-h and 24-h test periods. At 4 h, the LMPF from Navaho, Kiowa, and Ouachita blackberries had reduced acute edema in the mouse ears by 45.7, 48.0 and 32.0%, respectively, as compared to the TPA-positive control group (See Figure 5.4: a greater change in ear thickness denotes more acute inflammation). A similar finding was noted for treatment of the HMPF from Navaho, Kiowa, and Ouachita blackberries; that is, the TPA-induced skin inflammatory response was reduced in the mouse ears by 49.0, 43.9, and 36.1%, respectively. Even though the HMPF possesses a greater content of tannins (particularly hydrolyzable tannins with numerous gallic acid residues) relative to the LMPF (see Figure 5.2), no marked differences at inhibiting the acute edema were

evident between these two fractions. Based on the concentrations employed for all treatments, indomethacin, was the most effective at reducing edema in the mouse ears by 57.0 and 59.9 % after 4 and 24 h, respectively. At the 24-h point, edema was significantly ( $p < 0.05$ ) reduced by 49.1, 49.4, and 39.4% in the groups treated with HMPF from Navaho, Kiowa, and Ouachita blackberries, respectively, and not very different from that observed at 4-h. Mice treated with the LMPF from Navaho, Kiowa, and Ouachita blackberries reduced the edema at 24 h by 42.6, 48.3, and 42.5%, respectively. When one examines the mass of the collected ear punches at 24 h, reductions in the TPA-induced injury by 52.5% from indomethacin, 36.6, 42.6, and 35.2% from the LMPF of Navaho, Kiowa, and Ouachita, respectively, as well as 47.7, 35.3, and 40.9% from the HMPF of Navaho, Kiowa, and Ouachita, respectively, were observed (See Figure 5.5; a lesser mass denotes a greater lessening of the inflammation). In all groups, the inhibition percentage was compared against the edema evident at the same time period in the TPA-positive control group.

TPA releases AA from phospholipid pools in the mice resulting in its subsequent metabolism by cytosolic phospholipase A<sub>2</sub> and generation of prostaglandin and leukotriene mediators by the action of COX and lipoxygenase (LOX) enzymes. In particular, proinflammatory prostaglandins of the 2-series and leukotrienes of the 4-series are synthesized from AA metabolism and elicit vascular permeability leading to edema during inflammatory responses. These mediators are also responsible for the recruitment of macrophages, neutrophils, and other leukocytes that release histamine and bradykinin, thereby promoting inflammation. Phytochemical-based phospholipase A<sub>2</sub> inhibitors have been shown effective at reducing leukocyte infiltration and edema in the TPA model of ear inflammation by preventing the release of AA from membrane bilayer (Tramposch et al. 1992; Lim et al. 2006). Phenolic acids have

been reported to inhibit COX and LOX enzyme activities resulting in a reduction in TPA-induced injury (Park et al. 2006; Silva et al. 2007); however, the efficacy of the phenolics depends directly upon their chemical structures.

Mast cells can react quickly in acute inflammatory responses and release mediators such as histamine and serotonin as well as cytokines (*i.e.*, TNF- $\alpha$ , IL-6, IL-1 $\beta$ ), that increase vascular permeability and promote neutrophils influx (Rao et al. 1993). This process takes anywhere from 4 to 6 h to develop and can persist up to 24 h after administration of an inflammatory stimuli such as TPA (Carlson et al. 1985; Kumar et al., 2007). Neutrophil infiltration to the dermis is generally at its maximum within 24 h following TPA-induced injury. PMN leukocyte recruitment is a multi-step cellular event of inflammation involving rolling, adhesion, and transmigration of neutrophils to the inflammatory site (Kumar et al. 2007). The MPO assay is an index of granulocyte infiltration and the % inhibition of MPO activity relative to a TPA-positive control sample is indicative of anti-inflammatory action (Bradley et al. 1982; Ajuebor et al. 2000). This neutrophil enzyme plays an essential part in the innate immune system by catalyzing the production of HOCl from H<sub>2</sub>O<sub>2</sub>. Flavonoids with high antioxidant activity have been reported to potently reduce inflammation and the production of H<sub>2</sub>O<sub>2</sub> in this animal model (Chung et al. 2001).

In this study, MPO activity was measured in the mouse ear biopsies taken 24 h after TPA administration (*i.e.*, treatments 2 thru 9, see Figure 5.6), as an index of neutrophil infiltration. MPO activity was significantly ( $p < 0.05$ ) reduced by 57.1% from indomethacin, 42.9, 44.8, and 39.4% from the LMPF of Navaho, Kiowa, and Ouachita, respectively, as well as 47.5, 42.0, and 41.9% from the HMPF of Navaho, Kiowa, and Ouachita, respectively, compared with the TPA-positive control group. Huang et al. (2006) reported that the potential degree of anti-

inflammatory activity attributed to phenolics is proportional to the number of hydroxy groups residing in the bioactives (Huang et al. 2006). In our study, however, no statistically significant ( $p > 0.05$ ) differences were observed in ear edema or MPO activity amongst the groups treated with the LMPF and HMPF from the three cultivars. Less effective transdermal penetration of tannin constituents in the HMPF due to their larger molecular weights relative to phenolic compounds in the LMPF might decrease the level of the phenolics within the ear tissue for HMPF treatments. Yet, Rocha et al. (2007) did confirm the penetration of proanthocyanidin B<sub>2</sub> (C<sub>30</sub>H<sub>26</sub>O<sub>12</sub>, FW = 578.5 g/mol) through the skin up to the dermis layer.

Correlations between the anti-inflammatory response from the blackberry LMPFs/HMPFs to the TPA-induced injury (as measured by the percent inhibition in edema) with the colorimetrically-determined TPCs and antioxidant activities (as assessed by the FRAP assay) were established. Figure 5.7(A) and (B) depicts the positive linear correlations found. Correlation coefficients,  $r^2$ , of 0.856 and 0.852 were determined for the anti-inflammatory activity of the blackberry samples with both the TPCs and FRAP values. These results suggest that the anti-inflammatory effects observed from blackberries directly related to the level of total phenolics present. An  $r^2$  of 0.990 was found when comparing the TPCs and FRAP values determined for the blackberry LMPFs/HMPFs.

Why investigate the anti-inflammatory activity of blackberry phenolics? Due to undesirable side effects from many common inflammatory drugs, dietary intervention strategies for the treatment of chronic inflammatory states are gaining interest. Polyphenols found in fruits, vegetables, herbs, and spices have been shown to impart an inhibitory effect on inflammatory responses (Yoon and Baek, 2005), and flavonoids specifically have been found to inhibit histamine release from human mast cells (Middleton et al. 2000). Chung et al. (2001) reported

that edema formation may also be regulated by H<sub>2</sub>O<sub>2</sub> generation, as evident from the anti-inflammatory activity of several phenolic antioxidant compounds against TPA-induced inflammation (Hara et al. 1992; Cui et al. 2006). For example, peonidin was shown to inhibit TPA-induced COX-2 expression (Kwon et al. 2007). Theaflavins and their derivatives exhibited a strong anti-inflammatory activity due to their ability to retard AA metabolism and inhibit overexpression of proinflammatory cytokines like IL-1 $\beta$  and IL-6. Serraino et al. 2003 demonstrated that cyanidin-3-*O*-glucoside, which represents > 85% of the total anthocyanins in blackberry juice, prevents vascular hyporeactivity and endothelial dysfunction by scavenging peroxynitrite (ONOO<sup>-</sup>); the reaction product between NO and the superoxide anion radical, is known to cause cellular and tissue injury in a variety of inflammatory states.

It is difficult to speculate what the precise mechanism(s) is by which the LMPF and HMPF from blackberries exert their anti-inflammation. Possibly the phenolics within these fractions can interact with protein-based compounds like COX-2 and NF- $\kappa$ B, or inhibit the release of proinflammatory cytokine interleukins. The proinflammatory cytokines can trigger the up-regulation of other proinflammatory cytokines and chemokines, as well as increase the expression of many cellular adhesion molecules, selectins, integrins, and immunoglobulins (Saklatvala et al. 2003). Cells in injured skin, such as dermal dendritic cells, epidermal Langerhans cells, melanocytes, fibroblasts, and migrating leukocytes, are known as the source and target of interleukins (Gröne, 2002). Moreover, the IL-1 pathway is the induced transcription of a series of genes, including adhesion molecules, chemokines, secondary cytokines, NO synthase, and COX, all relevant to skin inflammation and play an important role in the modulation of inflammation (Murphy et al. 2000).

The present study demonstrated that topical application of the LMPF (cyanidin-3-*O*-glucoside was the predominant anthocyanin) and the HMPF (ellagic acid and its derivatives were the main components) from Georgia-grown blackberry cultivars can impart a significant inhibition of *in vivo* TPA-induced ear edema in mice. Even though there were different classes of phenolic constituents present with varying molecular weights, transdermal penetration of these compounds into the ear tissue was found effective; that is, inflammatory markers were reduced in all treatment groups. We believe that this demonstration of potent anti-inflammatory properties adds to the other known benefits of blackberries and can significantly enhance this fruit's market potential as a constituent in functional food/nutraceutical beverage formulations.

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## REFERENCES

- Afaq, F., Saleem, M., Krueger, C. G., Reed, J. D., and Mukhtar, H. Anthocyanin- and hydrolyzable tannin-rich pomegranate fruit extract modulates MAPK and NF- $\kappa$ B pathways and inhibits skin tumorigenesis in CD-1 mice, *International Journal of Cancer* 113 (2005), pp. 423–433.
- Ajuebor, M. N., Singh, A., and Wallace, J. L. Cyclooxygenase-2-derived prostaglandin D<sub>2</sub> is an early anti-inflammatory signal in experimental colitis, *American Journal of Physiology-Gastrointestinal and Liver Physiology* 279 (2000), pp. G238–G244.
- Andrews, P. C. and Krinsky, N. I. Human myeloperoxidase and hemi-myeloperoxidase, *Methods in Enzymology* 132 (1986), pp. 369–378.
- Bagchi, D., Sen, C. K., Bagchi, M., and Atalay, M. Anti-angiogenic, antioxidant, and anti-carcinogenic properties of a novel anthocyanin-rich berry extract formula, *Biochemistry (Moscow)* 69 (2004), pp. 75–80.
- Baldus, S., Heeschen, C., Meinertz, T., Zeiher, A. M., Eiserich, J. P., Münzel, T., Simoons, M. L., and Hamm, C. W. Myeloperoxidase serum levels predict risk in patients with acute coronary syndromes, *Circulation* 108 (2003), pp. 1440–1445.
- Benzie, I. F. F. and Strain, J. J. The ferric reducing ability of plasma (FRAP) as a measure of “antioxidant power”: The FRAP assay, *Analytical Biochemistry* 239 (1996), pp. 70–76.
- Bradley, P. P., Priebat, D. A., Christensen, R. D., and Rothstein, G. Measurement of cutaneous inflammation: Estimation of neutrophil content with an enzyme marker, *Journal of Investigative Dermatology* 78 (1982), pp. 206–209.

- Bralley, E. E., Hargrove, J. L., Greenspan, P., and Hartle, D. K. Topical anti-inflammatory activities of *Vitis rotundifolia* (Muscadine Grape) extracts in the tetradecanoylphorbol acetate model of ear inflammation, *Journal of Medicinal Food* 10 (2007), pp. 636–642.
- Carlson, R. P., O'Neill-Davis, L., Chang, J., and Lewis, A. J. Modulation of mouse ear edema by cyclooxygenase and lipoxygenase inhibitors and other pharmacologic agents, *Agents Actions* 17 (1985), pp. 197–204.
- Chung, W.-Y., Jung, Y.-J., Surh, Y.-J., Lee, S.-S., and Park, K.-K. Antioxidative and antitumor promoting effects of [6]-paradol and its homologs, *Mutation Research* 496 (2001), pp. 199–206.
- Cui, X.-Y., Kim, J.-H., Zhao, X., Chen, B.-Q., Lee, B.-C., Pyo, H.-B., Yun, Y.-P., and Zhang, Y.-H. Antioxidative and acute anti-inflammatory effects of *Campsis grandiflora* flower, *Journal of Ethnopharmacology* 103 (2006), pp. 223–228.
- Dai, Q., Borenstein, A. R., Wu, Y., Jackson, J. C., and Larson, E. B. Fruit and vegetable juices and Alzheimer's disease: The *Kame* project, *The American Journal of Medicine* 119 (2006), pp. 751–759.
- De Vry, C. G., Valdez, M., Lazarov, M., Muhr, E., Buelow, R., Fong, T., and Iyer, S. Topical application of a novel immunomodulatory peptide, RDP58, reduces skin inflammation in the phorbol ester-induced dermatitis model, *Journal of Investigative Dermatology* 125 (2005), pp. 473–481.
- De Young, L. M., Kheifets, J. B., Ballaron, S. J., and Young, J. M. Edema and cell infiltration in the phorbol ester-treated mouse ear are temporally separate and can be differentially modulated by pharmacologic agents, *Agents Actions* 26 (1989), pp. 335–341.



- Dell, R. B., Holleran, S., and Ramakrishnan, R. (2002). Sample size determination, *ILAR Journal* 43 (2002), pp. 207–213.
- Dugo, P., Mondello, L., Errante, G., Zappia, G., and Dugo, G. Identification of anthocyanins in berries by narrow-bore high-performance liquid chromatography with electrospray ionization detection, *Journal of Agricultural and Food Chemistry* 49 (2001), pp. 3987–3992.
- Duthie, S. J. Berry phytochemicals, genomic stability and cancer: Evidence for chemoprotection at several stages in the carcinogenic process, *Molecular Nutrition and Food Research* 51 (2007), pp. 665–674.
- Esposito, K. and Giugliano, D. Diet and inflammation: a link to metabolic and cardiovascular diseases, *European Heart Journal* 27 (2006), pp. 15–20.
- Fan-Chiang, H. and Wrolstad, R. E. Anthocyanin pigment composition of blackberries, *Journal of Food Science*, 70 (2005), pp. C198–C202.
- Georgé, S., Brat, P., Alter, P., and Amiot, M. J. Rapid determination of polyphenols and vitamin C in plant-derived products, *Journal of Agricultural and Food Chemistry* 53 (2005), pp. 1370–1373.
- Greenspan, P., Bauer, J. D., Pollock, S. H., Gangemi, J. D., Mayer, E. P., Ghaffar, A., Hargrove, J. L., and Hartle, D. K. Antiinflammatory properties of the muscadine grape (*Vitis rotundifolia*), *Journal of Agricultural and Food Chemistry* 53 (2005), pp. 8481–8484.
- Gröne, A. Keratinocytes and cytokines, *Veterinary Immunology and Immunopathology* 88 (2002), pp. 1–12.
- Hager, T. J., Howard, L. R., Liyanage, R., Lay, J. O., and Prior, R. L. Ellagitannin composition of blackberry as determined by HPLC-ESI-MS and MALDI-TOF-MS, *Journal of Agricultural and Food Chemistry* 56 (2008), pp. 661–669.

- Hagerman, A. E. and Butler, L. G. Protein precipitation method for the quantitative determination of tannins, *Journal of Agricultural and Food Chemistry* 26 (1978), pp. 809–812.
- Halvorsen, B. L., Carlsen, M. H., Phillips, K. M., Bøhn, S. K., Holte, K., Jacobs, D. R., Jr., and Blomhoff, R. Content of redox-active compounds (ie, antioxidants) in foods consumed in the United States, *American Journal of Clinical Nutrition* 84 (2006), pp. 95–135.
- Hara, H., Sukamoto, T., Ohtaka, H., Abe, K., Tatumi, Y., Saito, Y., Suzuki, A., and Tsukamoto, G. Effects of baicalein and  $\alpha$ -tocopherol on lipid peroxidation, free radical scavenging activity and 12-O-tetradecanoylphorbol acetate-induced ear edema, *European Journal of Pharmacology* 221 (1992), pp. 193–198.
- Hou, D-X., Kai, K., Li, J-J., Lin, S., Terahara, N., Wakamatsu, M., Fujii, M., Young, M. R., and Colburn, N. Anthocyanidins inhibit activator protein 1 activity and cell transformation: structure–activity relationship and molecular mechanisms, *Carcinogenesis* 25 (2004), pp. 29–36.
- Huang, M.-T., Liu, Y., Ramji, D., Lo, C.-Y., Ghai, G., Dushenkov, S., and Ho, C.-T. Inhibitory effects of black tea theaflavin derivatives on 12-O-tetradecanoylphorbol-13-acetate-induced inflammation and arachidonic acid metabolism in mouse ears, *Molecular Nutrition Food Research* 50 (2006), pp. 115–122.
- Kao, M.-W. S., Woods, F. M., Dozier, W. A., Jr., Ebel, R. C., Nesbitt, M., Jee, J., and Fields, D. Phenolic content and antioxidant capacities of Alabama - grown thornless blackberries, *International Journal of Fruit Science* 7 (2007), pp. 33–46.

- Kato, Y., Nagao, A., Terao, J., and Osawa, T. Inhibition of myeloperoxidase-catalyzed tyrosylation by phenolic antioxidants *in vitro*, *Bioscience Biotechnology Biochemistry* 67 (2003), pp. 1136–1139.
- Kawamoto, H., Nakatsubo, F., and Murakami, K. Stoichiometric studies of tannin-protein co-precipitation, *Phytochemistry* 41 (1996), pp. 1427–1431.
- Kumar V., Abbas A. K., Fausto N., and Mitchell R. N. Acute and chronic inflammation. In *Robbins' Basic Pathology, 8<sup>th</sup> Edition*, Saunders/Elsevier: Philadelphia, PA, 2007, pp. 31–58.
- Kundu, J. K., Chun, K.-S., Kim, S. O., and Surh, Y.-J. Resveratrol inhibits phorbol ester-induced cyclooxygenase-2 expression in mouse skin: MAPKs and AP-1 as potential molecular targets, *BioFactors* 21 (2004), pp. 33–39.
- Kwon, J. Y., Lee, K. W., Hur, H. J., and Lee, H. J. Peonidin inhibits phorbol-ester-induced COX-2 expression and transformation in JB6 P<sup>+</sup> cells by blocking phosphorylation of ERK-1 and -2, *Annals of the New York Academy of Sciences* 1095 (2007), pp. 513–520.
- Lim, H., Son, K. H., Chang, H. W., Kang, S. S., and Kim, H. P. Effects of anti-inflammatory biflavonoid, ginkgetin, on chronic skin inflammation, *Biological & Pharmaceutical Bulletin* 29 (2006), pp. 1046–1049.
- Liu, R. H. Potential synergy of phytochemicals in cancer prevention: Mechanism of action, *The Journal of Nutrition* 134 (2004), pp. 3479S–3485S.
- Mazza, G. and Miniati, E. Anthocyanins in Fruits, Vegetables, and Grains, CRC Press: Boca Raton, FL, 1993.
- Mazza, G., Cacace, J. E., and Kay, C. D. Methods of analysis for anthocyanins in plants and biological fluids, *Journal of AOAC International* 87 (2004), pp. 129–145.

- Meeran, S. M., Mantena, S. K., and Katiyar, S. K. Prevention of ultraviolet radiation-induced immunosuppression by (-)-epigallocatechin-3-gallate in mice is mediated through interleukin 12-dependent DNA repair, *Clinical Cancer Research* 12 (2006), pp. 2272–2280.
- Middleton, E., Jr., Kandaswami, C., and Theoharides, T. C. The effects of plant flavonoids on mammalian cells: Implications for inflammation, heart disease, and cancer, *Pharmacological Reviews* 52 (2000), 673–751.
- Mittal, A., Elmets, C. A., and Katiyar, S. K. Dietary feeding of proanthocyanidins from grape seeds prevents photocarcinogenesis in SKH-1 hairless mice: relationship to decreased fat and lipid peroxidation, *Carcinogenesis* 24 (2003), pp. 1379–1388.
- Murphy, J.-E., Robert, C., and Kupper, T. S. Interleukin-1 and cutaneous inflammation: A crucial link between innate and acquired immunity, *Journal of Investigative Dermatology* 114 (2000), pp. 602–608.
- Naczki, M. and Shahidi, F. Extraction and analysis of phenolics in food, *Journal of Chromatography A* 1054 (2004), pp. 95–111.
- Nauseef, W. M. Contributions of myeloperoxidase to proinflammatory events: More than an antimicrobial system, *International Journal of Hematology* 74 (2001), pp. 125–133.
- Nijveldt, R. J., van Nood, E., van Hoorn, D. E. C., Boelens, P. G., van Norren, K., and van Leeuwen, P. A. M. Flavonoids: a review of probable mechanisms of action and potential applications, *American Journal of Clinical Nutrition* 74 (2001), pp. 418–425.
- Nishizuka, Y. The role of protein kinase C in cell surface signal transduction and tumour promotion, *Nature* 308 (1984), pp. 693–698.
- Park, H., Kim, Y. H., Chang, H. W., and Kim, H. P. Anti-inflammatory activity of the synthetic C–C biflavonoids, *Journal of Pharmacy and Pharmacology* 58 (2006), pp. 1661–1667.

- Pellegrini, N., Serafini, M., Colombi, B., del Rio, D., Salvatore, S., Bianchi, M., and Brighenti, F. Total antioxidant capacity of plant foods, beverages and oils consumed in Italy assessed by three different in vitro assays, *Journal of Nutrition* 133 (2003), pp. 2812–2819.
- Rao, T. S., Currie, J. L., Shaffer, A. F., and Isakson, P. C. Comparative evaluation of arachidonic acid (AA)- and tetradecanoylphorbol acetate (TPA)-induced dermal inflammation, *Inflammation* 17 (1993), pp. 723–741.
- Rocha, J. C. B., Pedrochi, F., Hernandez, L., DeMello, J. C. P., and Baesso, M. L. *Ex vivo* evaluation of the percutaneous penetration of proanthocyanidin extracts from *Guazuma ulmifolia* using photoacoustic spectroscopy, *Analytica Chimica Acta* 587 (2007), pp. 132–136.
- Saklatvala, J., Dean, J., and Clark, A. Control of the expression of inflammatory response genes, *Biochemical Society Symposia* 70 (2003), pp. 95–106.
- Seeram, N. P., Adams, L. S., Zhang, Y., Lee, R., Sand, D., Scheuller, H. S., and Heber, D. Blackberry, black raspberry, blueberry, cranberry, red raspberry, and strawberry extracts inhibit growth and stimulate apoptosis of human cancer cells in vitro, *Journal of Agricultural and Food Chemistry* 54 (2006), pp. 9329–9339.
- Sellappan, S., Akoh, C. C., and Krewer, G. Phenolic compounds and antioxidant capacity of Georgia-grown blueberries and blackberries, *Journal of Agricultural and Food Chemistry* 50 (2002), pp. 2432–2438.
- Serraino, I., Dugo, L., Dugo, P., Mondello, L., Mazzon, E., Dugo, G., Caputi, A. P., and Cuzzocrea, S. Protective effects of cyanidin-3-*O*-glucoside from blackberry extract against peroxynitrite-induced endothelial dysfunction and vascular failure, *Life Sciences* 73 (2003), pp. 1097–1114.

- Silva, D. H. S., Zhang, Y., Santos, L. A., Bolzani, V. S., and Nair, M. G. Lipoperoxidation and cyclooxygenases 1 and 2 inhibitory compounds from *Iryanthera juruensis*, *Journal of Agricultural and Food Chemistry* 55 (2007), 2569–2574.
- Singleton, V. L. and Rossi, J. A., Jr. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents, *American Journal of Enology and Viticulture* 16 (1965), pp. 144–158.
- Siriwoharn, T. and Wrolstad, R. E. Phenolic composition of Marion and evergreen blackberries, *Journal of Food Science* 69 (2004), pp. FCT233–FCT240.
- Siriwoharn, T., Wrolstad, R. E., Finn, C. E., and Pereira, C. B. Influence of cultivar, maturity, and sampling on blackberry (*Rubus* L. hybrids) anthocyanins, polyphenolics, and antioxidant properties, *Journal of Agricultural and Food Chemistry* 52 (2004), pp. 8021–8030.
- Surh, Y.-J., Kundu, J. K., Na, H.-K., and Lee, J.-S. Redox-sensitive transcription factors as prime targets for chemoprevention with anti-inflammatory and antioxidative phytochemicals, *The Journal of Nutrition* 135 (2005), pp. 2993S–3001S.
- Suzuki, K., Ota, H., Sasagawa, S., Sakatani, T., and Fujikura, T. Assay method for myeloperoxidase in human polymorphonuclear leukocytes, *Analytical Biochemistry* 132 (1983), pp. 345–352.
- Takachi, R., Inoue, M., Ishihara, J., Kurahashi, N., Iwasaki, M., Sasazuki, S., Iso, H., Tsubono, Y., and Tsugane, S. Fruit and vegetable intake and risk of total cancer and cardiovascular disease, *American Journal of Epidemiology* 167 (2008), pp. 59–70.
- Tall, J. M., Seeram, N. P., Zhao, C., Nair, M. G., Meyer, R. A., and Raja, S. N. Tart cherry anthocyanins suppress inflammation-induced pain behavior in rat, *Behavioural Brain Research* 153 (2004), pp. 181–188.

- Thomasset, S. C., Berry, D. P., Garcea, G., Marczylo, T., Steward, W. P., and Gescher, A. J. Dietary polyphenolic phytochemicals--promising cancer chemopreventive, agents in humans? A review of their clinical properties, *International Journal of Cancer* 120 (2007), pp. 451–458.
- Tramposch, K. M., Steiner, S. A., Stanley, P. L., Nettleton, D. O., Franson, R. C., Lewin, A. H., and Carroll, F. I. Novel inhibitor of phospholipase A<sub>2</sub> with topical anti-inflammatory activity, *Biochemical and Biophysical Research Communications* 189 (1992), pp. 272–279.
- Vrhovsek, U., Palchetti, A., Reniero, F., Guillou, C., Masuero, D., and Mattivi, F. Concentration and mean degree of polymerization of *Rubus* ellagitannins evaluated by optimized acid methanolysis, *Journal of Agricultural and Food Chemistry* 54 (2006), pp. 4469–4475.
- Wang, H., Cao, G., and Prior, R. L. Total antioxidant capacity of fruits, *Journal of Agricultural and Food Chemistry* 44 (1996), pp. 701–705.
- Wang, J. and Mazza, G. Inhibitory effects of anthocyanins and other phenolic compounds on nitric oxide production in LPS/IFN- $\gamma$ -activated RAW 264.7 macrophages, *Journal of Agricultural and Food Chemistry* 50 (2002), pp. 850–857.
- Weiss, S. J. Tissue destruction by neutrophils, *New England Journal of Medicine* 320 (1989), pp. 365–376.
- Wu, X. and Prior, R. L. Systematic identification and characterization of anthocyanins by HPLC-ESI-MS/MS in common foods in the United States: Fruits and berries, *Journal of Agricultural and Food Chemistry* 53 (2005), pp. 2589–2599.
- Ximenes, V. F., Paino, I. M. M., de Faria-Oliveira, O. M. M., da Fonseca, L. M., and Brunetti, I. L. Indole ring oxidation by activated leukocytes prevents the production of hypochlorous acid, *Brazilian Journal of Medical and Biological Research* 38 (2005), pp. 1575–1583.

Yoon, J.-H. and Baek, S. J. Molecular targets of dietary polyphenols with anti-inflammatory properties, *Yonsei Medical Journal* 46 (2005), pp. 585–596.



## Figure Captions

**Figure 5.1** Process flow diagram detailing the isolation and separation of the LMPF and HMPF from blackberry cultivars. Abbreviations: CBE, crude blackberry extract; PPE, polyphenolic extract; LMPF, low-molecular-weight phenolic fraction; and HMPF, high-molecular-weight phenolic fraction.

**Figure 5.2** Relative tannin contents in the LMPF and HMPF of three blackberry cultivars. Abbreviations: N/LMPF & N/HMPF, Navaho low- and high-molecular-weight phenolic fractions, respectively; K/LMPF & K/HMPF, Kiowa low- and high-molecular-weight phenolic fractions, respectively; and O/LMPF & O/HMPF, Ouachita low- and high-molecular-weight phenolic fractions, respectively.

**Figure 5.3** HPLC chromatogram with diode array detection at  $\lambda = 520$  nm of a LMPF from one of the blackberry cultivars examined.

**Figure 5.4** Change in ear thickness (mm) of mice after 4 and 24 h of TPA-induced injury. Ear thickness was measured with a digital micrometer. Values are means of triplicate determinations  $\pm$  standard deviation. When one-way ANOVA was significant, differences in ear thickness for either the 4-h or 24-h group were determined using Duncan's multiple comparison test ( $p < 0.05$ ). Means with the same letter at 4 h (a,b,c,d,e) and 24 h (w,x,y,z) are not significantly ( $p > 0.05$ ) different.

**Figure 5.5** Change in mass (mg) of 6-mm ear punches after 24 h of TPA-induced injury. Values are means of triplicate determinations  $\pm$  standard deviation. When one-way ANOVA was significant, differences in the masses of ear punches for different groups were determined using Duncan's multiple comparison test ( $p < 0.05$ ). Means with the same letter are not significantly ( $p > 0.05$ ) different.

**Figure 5.6** Colorimetric assay determination of myeloperoxidase (MPO) activity for biopsies of mouse ears after 24 h of TPA-induced injury. Values are means of triplicate determinations  $\pm$  standard deviation. When one-way ANOVA was significant, differences in MPO activity for different groups were determined using Duncan's multiple comparison test ( $p < 0.05$ ). Means with the same letter are not significantly ( $p > 0.05$ ) different.

**Figure 5.7** Correlations established between the percent inhibition of edema and (A) the total phenolics contents (TPCs) and (B) ferric reducing antioxidant power (FRAP) values of the LMPFs and HMPFs from blackberries.

**Table 5.1** – Relative yield (%) of CBE, PPE, LMPF, and HMPF from lyophilized Georgia-grown blackberry cultivars (Navaho, Kiowa, and Ouachita).

Blackberry Cultivars	CBE	PPE	LMPF	HMPF
Navaho	1.85 ± 0.18	1.27 ± 0.03	0.75 ± 0.06	0.38 ± 0.01
Kiowa	1.25 ± 0.09	0.80 ± 0.11	0.44 ± 0.07	0.24 ± 0.04
Ouachita	1.36 ± 0.26	0.90 ± 0.30	0.52 ± 0.14	0.25 ± 0.10

Values are means of triplicate analyses ± standard deviation.

Abbreviations: CBE: crude blackberry extract; PPE: polyphenolic extract; LMPF: low-molecular-weight phenolic fraction; and HMPF: high-molecular-weight phenolic fraction.

**Table 5.2** – Total phenolics content and antioxidant activity of the LMPF and HMPF from Georgia-grown blackberry cultivars (Navaho, Kiowa, and Ouachita).<sup>a</sup>

Blackberry Cultivars	TPC (mg GAE/100-mg respective fraction)	FRAP (mmol Fe <sup>2+</sup> eq./100-mg respective fraction)
Navaho (LMPF)	72.6 ± 2.1	2.34 ± 0.15
Navaho (HMPF)	89.9 ± 1.9	3.92 ± 0.24
Kiowa (LMPF)	77.5 ± 2.1	2.70 ± 0.03
Kiowa (HMPF)	83.8 ± 1.0	3.45 ± 0.42
Ouachita (LMPF)	68.0 ± 1.9	2.03 ± 0.02
Ouachita (HMPF)	76.9 ± 1.2	2.21 ± 0.03

Values are means of triplicate analyses ± standard deviation.

Abbreviations: TPC: total phenolics content; GAE: gallic acid equivalents; FRAP: ferric reducing antioxidant power; LMPF: low-molecular-weight phenolic fraction; and HMPF: high-molecular-weight phenolic fraction.

**Table 5.3** – Experimental design for the TPA-induced mouse ear edema study.<sup>a</sup>

Animal group	Treatments	Topical application of solution on both the inner & outer mouse ear surfaces at $t = 0$ min	Topical application of solution on both the inner & outer mouse ear surfaces at $t = 30$ min
1	Control group	Acetone	ethanol (50% , v/v)
2	TPA-positive control group	TPA solution	ethanol (50% , v/v)
3	Treatment-control group	TPA solution	indomethacin solution <sup>b</sup>
4	Treatment group	TPA solution	N/LMPF solution <sup>c</sup>
5	Treatment group	TPA solution	N/HMPF solution <sup>d</sup>
6	Treatment group	TPA solution	K/LMPF solution
7	Treatment group	TPA solution	K/HMPF solution
8	Treatment group	TPA solution	O/LMPF solution
9	Treatment group	TPA solution	O/HMPF solution

<sup>a</sup>Each group comprised 8 mice. Ten microliters of a 0.1 mg/mL TPA solution were applied to both the inner and outer ears of mice in groups 2 thru 9.

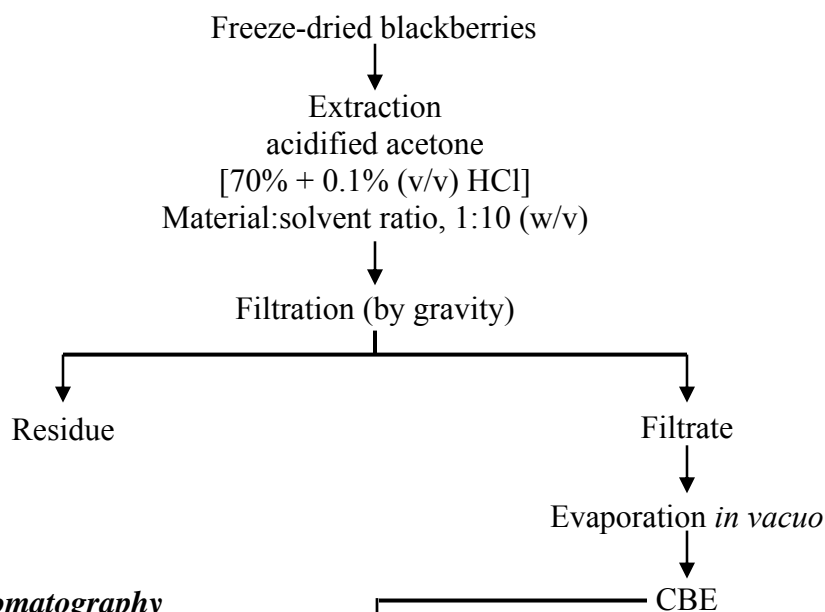
<sup>a</sup>10  $\mu$ L of a 25 mg/mL indomethacin solution in acetone were applied to both the inner and outer ears of mice.

<sup>a</sup>10  $\mu$ L of 50 mg/mL LMPF in ethanol (50%, v/v) from each cultivar were applied to both the inner and outer ears of mice.

<sup>a</sup>10  $\mu$ L of 50 mg/mL HMPF in ethanol (50%, v/v) from each cultivar were applied to both the inner and outer ears of mice.

Abbreviations: TPA: 12-*O*-tetradecanoylphorbol-13-acetate;  $t$ : time; N/LMPF and N/HMPF: Navaho low-molecular-weight phenolic fraction and high-molecular-weight phenolic fraction, respectively; K/LMPF and K/HMPF: Kiowa low-molecular-weight phenolic fraction and high-molecular-weight phenolic fraction, respectively; and O/LMPF and O/HMPF: Ouachita low-molecular-weight phenolic fraction and high molecular-weight phenolic fraction.

## Extraction



## Column Chromatography

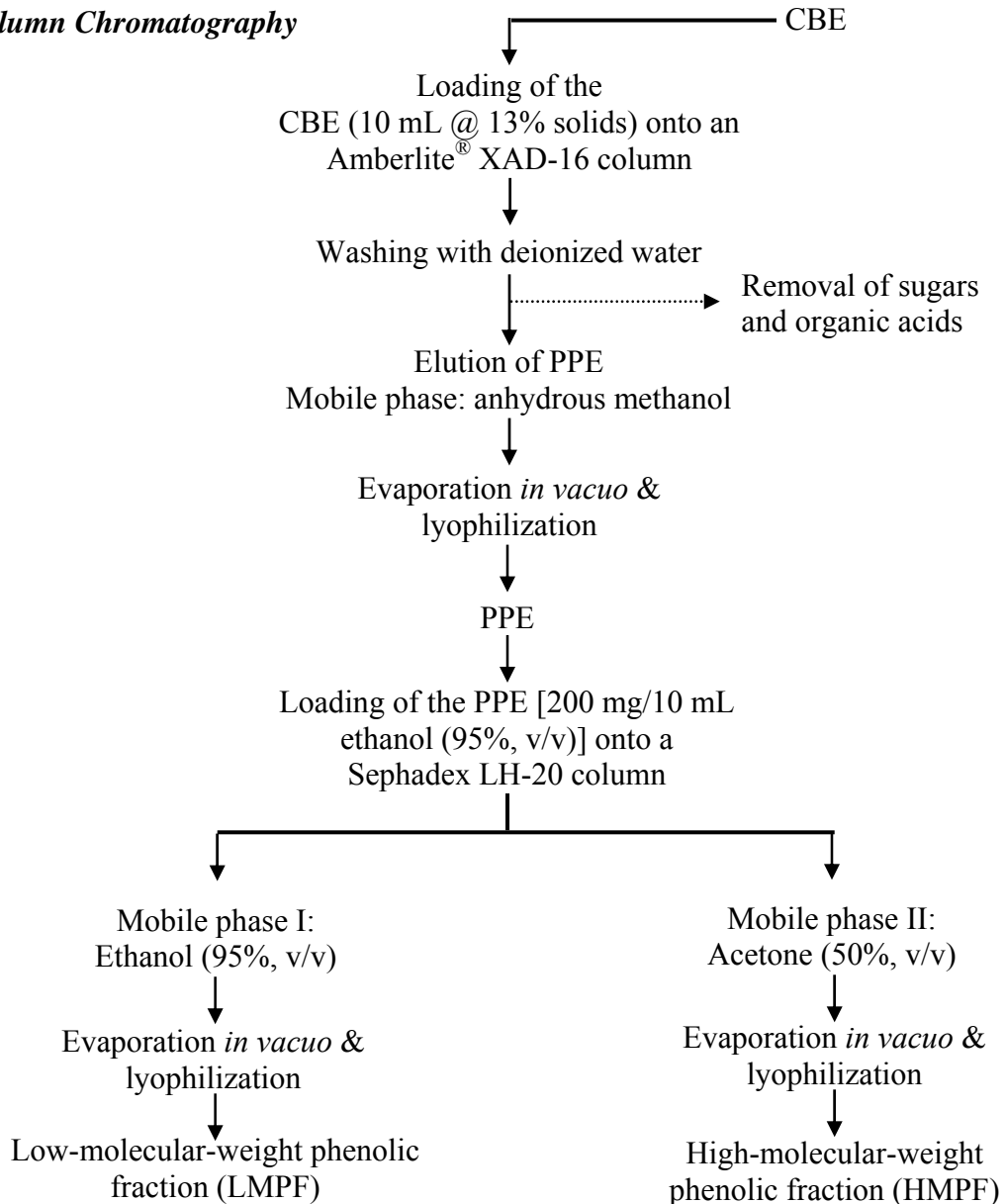


Figure 5.2

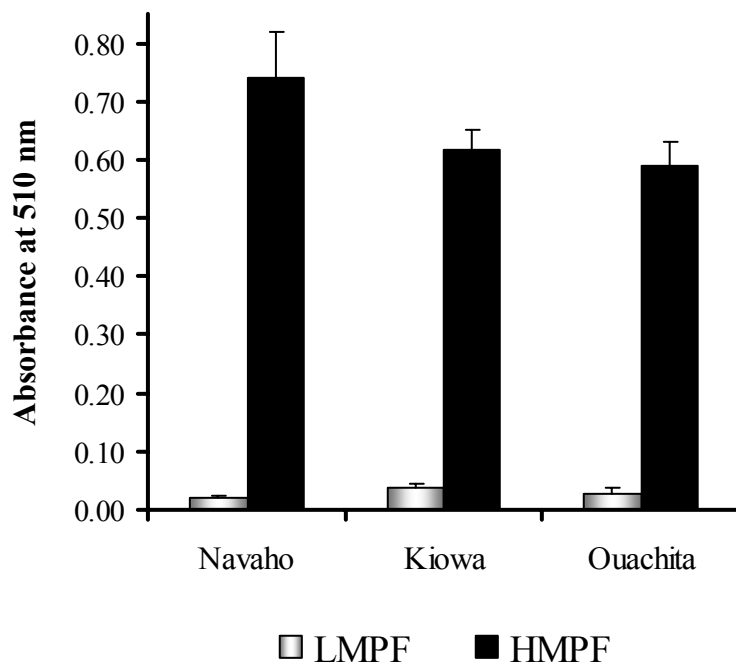


Figure 5.3

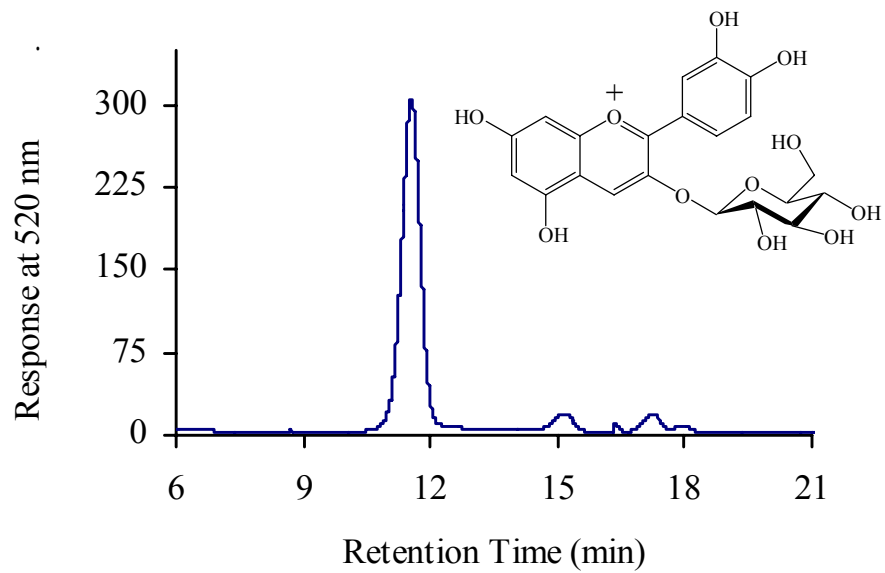




Figure 5.4

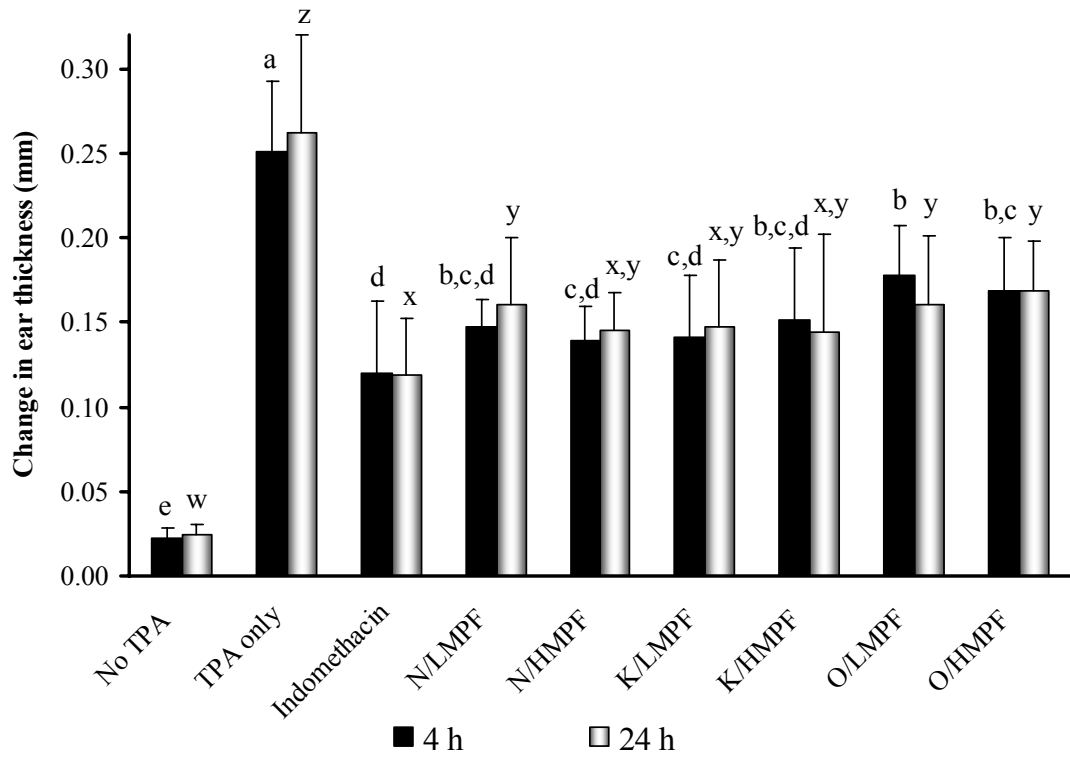


Figure 5.5

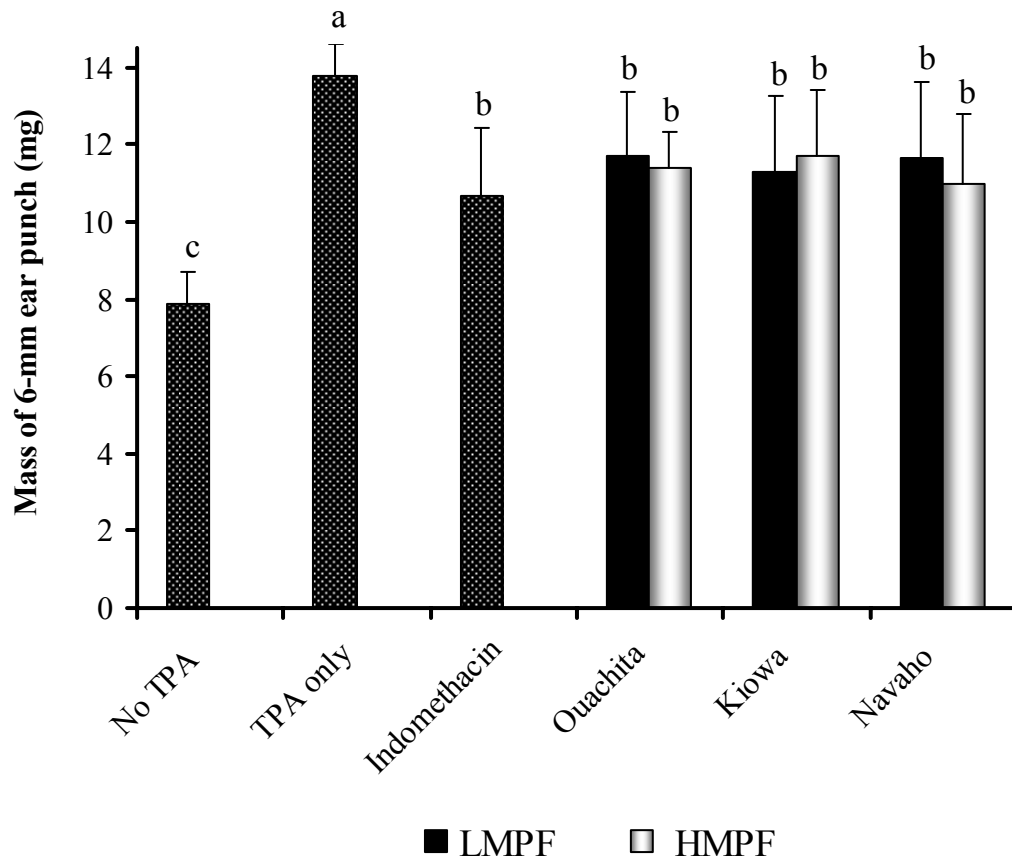


Figure 5.6

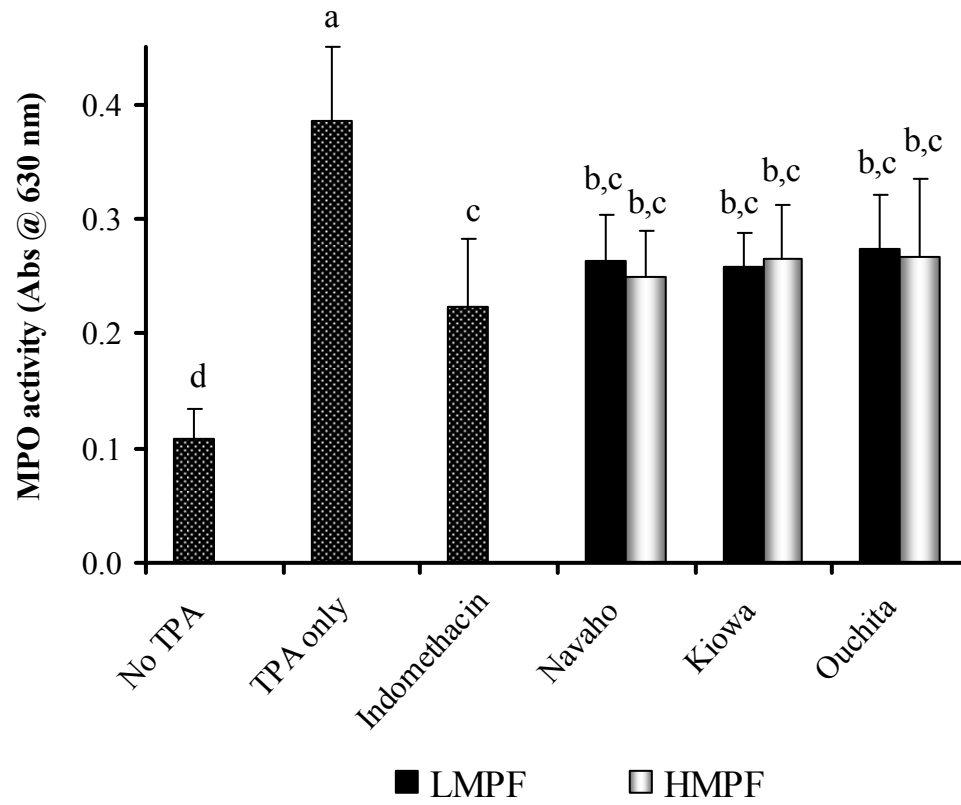


Figure 5.7(A)

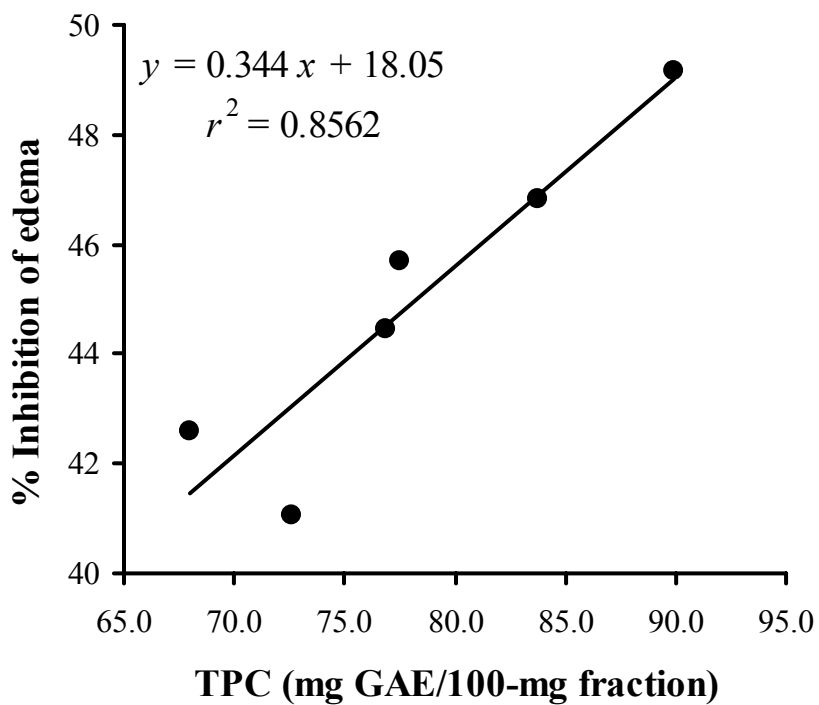
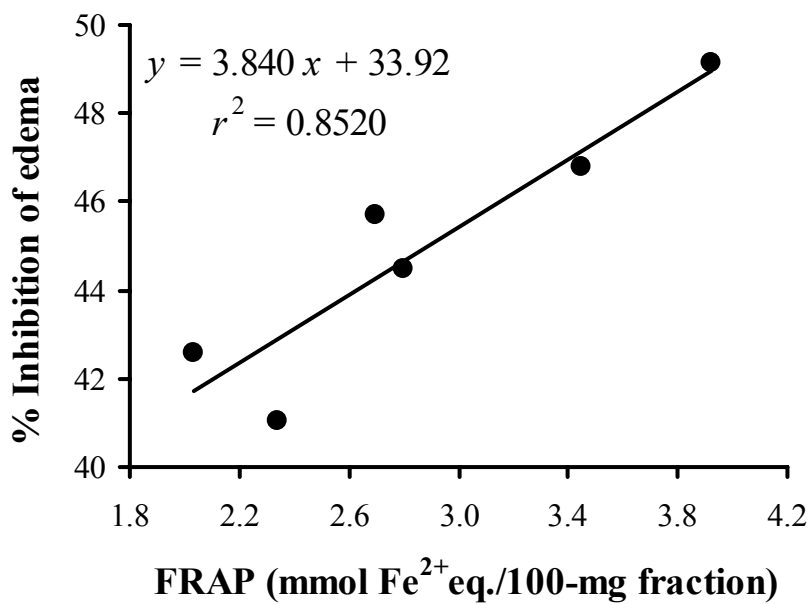


Figure 5.7(B)



## CHAPTER 6

### SUMMARY AND CONCLUSION

Blackberries have been shown to be a rich source of anthocyanins and other phenolic compounds with high antioxidant, radical-scavenging, antiglycation, and anti-inflammatory activities. In this study, anthocyanins, ellagitannins, ellagic acid and its derivatives were found to be the major phenolic classes present in Navaho, Kiowa, and Ouachita blackberry cultivars examined.

In total, VII fractions were partitioned by way of classical preparative column chromatography with Sephadex LH-20. UV absorbance of fractions I and II at 280 and 320 nm confirmed the presence of phenolic acids and flavonoids while, UV absorbance of fractions III, IV and V at 360 and 520 nm clearly indicated the presence of flavonoids. The majority of flavonoids in blackberry fractions were eluted in fractions III, IV, and V. Fraction VII was a high-molecular-weight phenolic fraction eluted with aqueous acetone (50% v/v).

Full scan ESI-MS data exhibited cyanidin-3-*O*-glucoside as the dominant component present in all three cultivars. Cyanidin-3-*O*-rutinoside and cyanidin-3-*O*-xylosylrutinoside were also found in the Kiowa cultivar. MALDI-TOF-MS confirmed the presence of several large molecular mass compounds. Pedunculagin (bis-HHDP-glucose) was detected in the Navaho cultivar, but absent in Kiowa and Ouachita. The dimer and trimer of galloyl-bis-HHDP-glucopyranose; *i.e.*, sanguine H-6/lambertianin A and lambertianin C, respectively, were found in all three Georgia-grown blackberry cultivars. The highest molecular mass compound,

lambertianin D isomer, was present only in the Kiowa variety. The ethanolic fraction primarily contained cyanidin-3-*O*-glucoside, ellagic acid, gallic acid, catechin, traces of *trans*-cinnamic acid and *p*-coumaric acid. This study establishes, with confidence, that blackberries hold significant potential as healthy fruits and suitable raw materials for bioactives in functional food categories.

The antioxidant capacities of blackberry fractions determined by FRAP and TEAC assays were well correlated, which implied that antioxidants in these fractions were capable of scavenging free radicals (ABTS<sup>•+</sup>) and reducing oxidants (Fe<sup>3+</sup>). The phenolic characterization suggested that different phenolics in the fractions like gallic acid, ellagic acid (free and ester forms), and anthocyanins (cyanidin-3-*O*-glucoside) may have attributed to the antioxidant action. Significant correlations ( $p < 0.05$ ) were detected in all fractions for FRAP, TEAC, and TPC assays. Positive, linear correlation was observed between the TPC values and inhibition percentage of protein glycation.

Phenolic constituents in Georgia-grown blackberry cultivars possess strong antioxidant and anti-inflammatory capacities, and are effective inhibitors of AGEs formation. Consequently, all three cultivars were found to be valuable sources of natural antioxidants, and may be consumed as a fruit or purified fraction with concentrated sources of anthocyanins (FXN-III thru -V) and ellagic acid (FXN-VII) with potential use in functional food preparations. To our knowledge, there is no prior reporting with regards to the inhibition of protein glycation by isolated fractions from blackberry. The present study gives valuable data on the marked antioxidant capacity of blackberries. Isolation and characterization of the cultivars individual active components and *in vivo* relevance of such activity, awaits further comprehensive studies.

The high molecular-weight phenolic fractions such as, ellagic acid/ellagic acid derivatives as primary active components and cyanidin-3-*O*-glucoside as predominant anthocyanins from the Georgia-grown blackberry cultivars, resulted in a significant inhibition of an important process related to TPA-induced skin inflammatory response, both edema and migration of polymorphonuclear leukocytes were measured as MPO activity. Inflammatory markers were reduced in all treatment groups treated with transdermal penetration of HMPF and LMPF into the ear tissue. The average values of %inhibition of edema showed a positive correlation with average values of TPCs. A linear relationship was observed between TPC, and inhibition of edema or MPO activity.

**Suggestions for continued research:**

Georgia grown blackberry cultivars contain ellagic acid derivatives and multiple isomers of which most ellagitannins can be separated by chromatography, however, each peak will require further evaluation by NMR of the analytes separated and recovered by HPLC to determine the elution order and structure of each isomer.

*In-vitro* and *in-vivo* studies have confirmed antioxidant and anti-inflammatory activities by phenolic compounds isolated from Georgia-grown blackberry cultivars. These studies were performed in a controlled environment; human studies would be required to further confirm the process within complex biological environments.

## INDEX OF ABBREVIATIONS

@	at
[M] <sup>+</sup>	molecular cation
[M+H] <sup>+</sup>	parent molecular ion with an hydrogen proton
[M+Na] <sup>+</sup>	sodium adduct of the parent molecular ion
~	approximately
®	registered trademark
•OH	hydroxyl radical
<sup>1</sup> O <sub>2</sub>	singlet state oxygen
3-DG	3-deoxyglucosone
3GT	Flavonoid 3-glucosyltransferase
5GT	Anthocyanin 5- <i>O</i> -glucosyltransferase
5-HETE	5 hydroxyeicosatetraenoic acid
5-HPETE	5-hydroperoxyeicosatetraenoic acid
A <sub>512nm</sub>	absorbance at 512 nanometers
A <sub>700nm</sub>	absorbance at 700 nanometers
A <sub>765nm</sub>	absorbance at 765 nanometers
AA	arachidonic acid
AAPH	2,2'-azobis-(2-amidinopropane) dihydrochloride
ABTS	2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)



ABTS <sup>•+</sup>	2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) radical cation
AGEs	Advanced glycation end products
AH <sup>•</sup>	antioxidant in the non-radical (reduced) state
ALA	$\alpha$ lipoic acid
AMP	adenosine monophosphate
ANOVA	analysis of variance
ANS	anthocyanidin synthase
AP-1	activator protein-1
ArO <sup>•</sup>	aromatic hydroxyl (phenoxy) radical
ArOH	aromatic hydroxyl
ArOH	antioxidant
AS	absorption of sample
Asc <sup>•</sup>	ascorbate radical
AscH <sup>-</sup>	ascorbate monoanion
ATP	adenosine triphosphate
AUC	area under the curve
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene
BSA	bovine serum albumin
C3G	cyranidin-3- <i>O</i> -glucoside
CAT	catalase
CBE	crude blackberry extract
CCC	classical column chromatography

CHS	chalcone synthase
CML	N <sup>ε</sup> -carboxymethyllysine
CoA	coenzyme A
CoAS-	Coenzyme A thiol anion
COX	cyclooxygenase
COX-2	cyclooxygenase-2
d.w.	dry weight
DAD	diode array detector
DAG	diacylglycerol
DF	dilution factor
DF	dilution factor
DFR	dihydroflavonol-4-reductase
DHK	dihydrokaempferol
DHLA	dihydrolipoic acid
DHM	dihydromyricetin
DHQ	dihydroquercetin
DNA	deoxyribonucleic acid
DP	degree of polymerization
DPPH	2,2'-diphenyl-1-picrylhydrazyl
DPPH <sup>•</sup>	2,2'-diphenyl-1-picrylhydrazyl radical
ε	molar absorptivity
ECG	epicatechin gallate
ECM	extracellular matrix

EGC	epigallocatechin
EGCG	epigallocatechin gallate
eq.	equivalents
ERK	extracellular signal-regulated protein kinase
ESI	electrospray ionization
ESI-MS	Electrospray ionization mass spectrometry
ETs	ellagitannins
f.w.	fresh weight
F3'5'H	flavonoid 3'5'-hydroxylase
F3H	flavanone 3-hydroxylase
F3'H	flavonoid 3'-hydroxylase
FAB	fast atom bombardment
FCR	Folin-Ciocalteu's phenol reagent
FLS	flavonol synthase
FRAP	ferric reducing antioxidant power
FXN-I to -VII	fractions I to VII
GAE	gallic acid equivalence
GC	gas chromatography
Glc	glucose
GLUT2	glucose transporter 2
GOLD	glyoxal-lysine dimer
G <sub>Red</sub>	glutathione reductase
GSH	glutathione

GSH	reduced glutathione
GSHPx	glutathione peroxidase
GSSG	oxidized glutathione
GST	glutathione- <i>s</i> -transferase
GTs	gallotannins
HAT	hydrogen atom transfer
HDL	high density lipoprotein
HHDP	hexahydroxydiphenic acid
HMPF	high-molecular-weight phenolic fractions
HMW	high-molecular weight
HOCl <sup>•</sup>	hypochlorous radical
HOO <sup>•</sup>	hydroperoxyl
HPA	hypothalamus-pituitary-adrenal
HPLC	high performance liquid chromatography
HTAB	hexadecyltrimethylammonium bromide
<i>i d.</i>	internal diameter
IACUC	Institutional Animal Care and Use Committee
ICAM-1	intercellular adhesion molecule 1
IFN- $\gamma$	interferon- $\gamma$
IL-1 $\beta$	interleukin-1 $\beta$
IL-6	interleukin-6
IQF	individually quick-frozen
I $\kappa$ B	inhibitors of kappa B

I $\kappa$ B $\alpha$	inhibitors of kappa B $\alpha$
I $\kappa$ B $\beta$	inhibitors of kappa B $\beta$
JNK	c-jun N-terminal kinase
K/HMPF	Kiowa high-molecular-weight phenolic fraction
K/LMPF	Kiowa low-molecular-weight phenolic fraction
L $\bullet$	lipid radical
LC	liquid chromatography
LDL	low density lipoprotein
LMPF	low-molecular-weight phenolic fractions
LO $\bullet$	lipid alkoxy radical
LOO $\bullet$	lipid peroxy radical
LOX	lipooxygenase
LPH	lactasephlorhizin-hydrolase
LPS	lipopolysaccharide
LPS	lipopolysaccharides
LTB <sub>4</sub>	leukotrienes B <sub>4</sub>
LTC <sub>4</sub> ,	leukotrienes C <sub>4</sub>
LTD <sub>4</sub> ,	leukotrienes D <sub>4</sub>
LTE <sub>4</sub> ,	leukotrienes E <sub>4</sub>
LXA <sub>4</sub> ,	lipoxin A <sub>4</sub>
LXB <sub>4</sub> ,	lipoxin B <sub>4</sub>
<i>m/z</i>	mass-to-charge ratio
MALDI-TOF	matrix-assisted laser desorption/ionization time-of-flight

MAPK	mitogen-activated protein kinase
mAU	milli-absorbance units
MCP-1	monocyte chemoattractant protein-1
MDA	malonaldehyde
MMP-9	matrix metalloproteinase-9
MOLD	methylglyoxal-lysine dimer
MPO	myeloperoxidase
mRNA	messenger ribonucleic acid
MS	mass spectrometry
MW	molecular weight
N/HMPF	Navaho high-molecular-weight phenolic fraction
N/LMPF	Navaho low-molecular-weight phenolic fraction
NAD	nicotine adenine dinucleotide
NADPH	nicotine adenine dinucleotide phosphate (reduced form)
ND	not detected
NF- $\kappa$ B	nuclear factor-kappa B
No.	number
NO $\bullet$	nitric oxide
NSAIDs	non-steroidal anti-inflammatory drugs
O/HMPF	Ouachita high-molecular-weight phenolic fraction
O/LMPF	Ouachita low-molecular-weight phenolic fraction
O $_2^{\bullet-}$	superoxide anion radical
OD	optical density

ODS	octyldecylsilyl
ONOO <sup>•</sup>	peroxynitrite
ORAC	oxygen radical antioxidant capacity
PAL	phenylalanine ammonia lyase
PACs	proanthocyanidins
PBS	phosphate-buffered saline
PC	paper chromatography
PG	propyl gallate
PGD <sub>2</sub>	prostaglandins D <sub>2</sub>
PGE <sub>2</sub>	prostaglandins E <sub>2</sub>
PGF <sub>2</sub>	prostaglandins F <sub>2</sub>
PGG <sub>2</sub>	prostaglandins G <sub>2</sub>
PGH <sub>2</sub>	prostaglandins H <sub>2</sub>
PGI <sub>2</sub>	Prostacyclin
PIP <sub>2</sub>	phosphatidylinositol bisphosphate
PKC	protein kinase C
PLA <sub>2</sub>	Phospholipase A <sub>2</sub>
PMN	polymorphonuclear
PPE	Polyphenolic extract
$r^2$	correlation coefficient
RAGE	advanced glycation endproduct receptor
RC	relative content
Rha	Rhamnose

RO•	alkoxy radical
ROO•	peroxyl radical
ROOH	alkyl peroxide
ROS	reactive oxygen species
RP	reversed-phase
SAS	System software
SD	standard deviation
SET	single-electron transfer
SGLT1	sodium-dependent glucose transporter 1
SOD	superoxide dismutase
SPE	solid-phase extraction
TA	titratable acidity
TACY	total anthocyanins content
TAL	tyrosine ammonia lyase
TBHQ	<i>tert</i> -butylhydroquinone
TEAC	Trolox equivalence antioxidant capacity
TLC	thin layer chromatography
TMB	3,3',5,5'-tetramethylbenzidine dihydrochloride
TNF- $\alpha$	Tumor necrosis factor- $\alpha$
T-O	vitamin E
T-O•	Vitamin E radical
TPA	12- <i>O</i> -tetradecanoylphorbol-13-acetate
TPC	total phenolics content



TRAP	total radical-trapping antioxidant parameter
TSS	total soluble solids
TXA <sub>2</sub>	thromboxane A <sub>2</sub>
USDA	United States Department of Agriculture
UV	ultraviolet
UV/VIS	ultraviolet/visible
v/v	volume per volume
v/v/v	volume per volume per volume
VCAM-1	vascular cell adhesion molecule 1
VIS	visible
$\lambda_{\max}$	maximum wavelength