CRANBERRY FLAVONOIDS: CHARACTERIZATION, OCCURRENCE, ANTI-CANCER PROPERTY AND BIOAVAILABILITY

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ABSTRACT OF THE DISSERTATION

Cranberry Flavonoids: Characterization, Occurrence, Anti-Cancer Property and Bioavailability

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American cranberry (*Vaccinium macrocarpon*), a North American native species, possesses various secondary metabolites with important implications for human health. Flavonoid compounds, as major cranberry secondary metabolites, also occur widely across other different plant species, and have important functions for plant growth, reproduction and survival. Major cranberry flavonoids consist of anthocyanins, flavonols and flavan-3-ols (proanthocyanidins, PACs). Varied in their structures, the three flavonoid subgroups have been associated with various human health benefits both *in vitro* and *in vivo*. The aim of the dissertation is to characterize and illustrate the natural occurrence of these flavonoid compounds in cranberry, and to discuss their bioactivity against cancer disease as well as bioavailability in human body. The objectives of the dissertation were to: (1) quantify and characterize flavonoids across fruit development in cranberry, (2) better define quantification of PACs using the 4-

dimethylaminocinamaldehyde (DMAC) spectrophotometric assay, (3) evaluate cytotoxicity and molecular basis of activity of specific flavonoid compounds toward ovarian cancer cell lines, and (4) evaluate the bioavailability of cranberry flavonoids.

The dissertation will first focus on the natural occurrence and analysis of different cranberry flavonoids in cranberry. Both variety and harvest date significantly affect the levels of PACs and anthocyanins in cranberry cultivars. PACs occur as the most abundant flavonoids, with levels of decreasing during fruit development and early ripening, and slightly increasing at late fruit maturation. Anthocyanins increase sharply during fruit maturation, while flavonol concentrations remain consistent over entire season. Quantification of PACs in plant and food materials often utilizes DMAC spectrophotometric assay which has been considered to offer ease, high sensitivity and selectivity. However, in the course of research for this dissertation it was found that individual PAC monomers and oligomers with various structural variations exhibited differential molar absorption coefficients (MACs); the value of MAC is affected by both degree-of-polymerization (DP) and inter-flavan linkage type and position.

Individual cranberry PACs and flavonols showed differential cytotoxicity against two ovarian cancer cell lines. The two most active cranberry flavonoids, quercetin aglycone and PAC DP-9 (nonamer), exhibited promising *in vitro* cytotoxic and anti-proliferative properties. They induced cell apoptosis, cell cycle arrest, cellular caspase-3 activation and PARP deactivation; in addition, they increased cancer cells' sensitivity to cisplatin. Urine clearance of cranberry flavonol glycosides and PACs were determined in female subjects following cranberry juice consumption. While PACs were not detected, five flavonol glycosides common in cranberry were identified. Quercetin-3-galactoside, the most

abundant cranberry flavonol, exhibited highest peak urine concentration, followed by quercetin-3-rhamnoside, quercetin-3-arabinoside, myricetin-3-arabinoside and myricetin-3-galactoside. Quercetin-3-arabinoside showed delayed clearance compared to other flavonols. These observations suggest that both aglycone and conjugated sugar moiety structures mediate the flavonol's bioavailability.

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Chapter 1

Literature Review

1.1 American Cranberry (*Vaccinium macrocarpon* Ait.)

American cranberry (*Vaccinium macrocarpon* Ait.) is a species native to eastern North America [1]. It belongs to the Ericaceae Family, which contains other species with berry fruits, such as blueberry, lingonberry and bilberry. First cultivated in the Massachusetts around 1820 [2], farm grown cranberry cultivars were selected from the wild until the 20th century; cranberry vines with desirable traits such as high yield and large fruit size were selected and transplanted to newly established bogs [3]. In 1929, due to the problem of false blossom disease in eastern cranberry production area, the United States Department of Agriculture (USDA) together with the Massachusetts Cranberry Experiment Station and Rutgers University of New Jersey established the first cranberry breeding program in seeking of new cranberry varieties which carried resistance to the disease [3]. Crosses of different cranberry plants were made, and in 1950 from a selection of 40 hybrids three were chosen and named Stevens, Beckwith and Wilcox; these hybrids were released as first generation hybrid cranberry varieties [3, 4]. The breeding focus was then switched to improving cranberry yield and fruit qualities, and in 1962, three more selections were released and named as Pilgrim, Franklin and Bergman [2]. Since then, breeders have been focusing on bringing out new cranberry varieties from the first-generation hybrid cultivars for preferable traits; The New Jersey Agricultural Experiment Station at Rutgers University has released several new cranberry varieties including Crimson Queen®, Mullica Queen®, Demoranville®, Scarlet Knight®, Welker™

and Haines™. Each variety carries improved traits, such as early fruit development, high fruit anthocyanins, large fruit size and vigorous growth.

As one of the major commercial crops in North America, total cranberry production in United States at 2014 reached 8.72 million barrels, with a total value of 266 million dollars [5]. Wisconsin (50% in 2014), Massachusetts (31%), New Jersey (8%), Oregon (7%) and Washington (4%) are the top five States of cranberry production [5].

Cranberry fruit contains various phytochemicals that contribute to the fruits' appearance (color), nutrients, and flavor as well as potential health beneficial values. Mature fresh fruit consists of 88% water, 4.2% sugars, 2.4% acids and other components with less than 1% proportions such as protein, fiber, fat and pectin [6]. Anthocyanins accumulate during cranberry fruit development and ripening and account for the red fruit color [7]. The sour taste of cranberry fruits results from high content (~1%) of three major organic acids - quinic, malic and citric acids. Cranberry fruits also contain various vitamins such as vitamin A (40 I.U./100 g), vitamin C (7.5-10.5 mg/100 g), Thiamine (vitamin B1), riboflavin (vitamin B2) and pyridoxine (vitamin B6) [6]. Besides anthocyanins, cranberry also possesses a rich profile of diverse flavonoids, most notably flavan-3-ols (proanthocyanidins) and flavonols; there has been a considerable research interest of cranberry flavonoids due to their potential health beneficial bioactivities [8, 9].

The medicinal benefits of cranberry were first explored by Native Americans [10]. Particularly, cranberry's health benefit against urinary tract infection (UTI) has long been recorded as a folklore remedy. Since 1966, many clinical studies have been conducted to evaluate the efficacy of cranberry product consumption on the prevention or reduction

against UTI and its symptoms [11]. Several studies have reported that ingestion of cranberry products is statistically effective for prevention reoccurrence of UIT in female patients [12-14]. Others showed that cranberry product consumption reduce instances of urine bacteriuria with pyuria in older females [15, 16]. Previously it was believed that cranberry caused an increase of urinary hippuric acid and reduction of urine pH thus resulting in bacteriostatic effects [17-19]. However, more recent research indicates that certain cranberry phenolics, specifically flavan-3-ols, due to their anti- adherent properties against uropathogens, contribute to cranberry's protection against UTI [20, 21].

Beyond protection against UTI, cranberries also exhibit various beneficial properties for human health. Consumption of cranberry juice significantly increased plasma's antioxidant capacity of health female subjects, presumably through accumulation of vitamin C [22]. A similar result was also observed in male subjects where short-term consumption of cranberry juice significantly increased antioxidant capacity and reduced oxidized low density lipoprotein concentrations of plasma [23]. Long-term cranberry juice consumption was found to reduce aortic stiffness on patients with coronary artery disease [24]. In addition, consumption of low-calorie cranberry juice was associated with a favorable postprandial glycemic response in healthy human subjects, which is potentially beneficial for patients with glucose tolerance problems [25].

While cranberry's various specific health benefits have been investigated in many studies, there is also a growing interest on its comprehensive composition of diverse phytochemicals and their contribution to the overall human health. Efforts on the qualitative and quantitative analysis of cranberry phytochemicals, particularly flavonoid

compounds, and those compounds' bioactivities and bioavailability, will add great value to the overall understanding of cranberry's nutritional and health benefit values.

1.2 Occurrence and Biological Functions of Plant Flavonoids

Flavonoids are a group of phenolic compounds that naturally occurr across different plants species and organs such as fruits and vegetables. The name "flavonoid" originated from Latin word "*flavus*" which means yellow, as many of the compounds within the group have yellow colors. As major plant secondary metabolites flavonoids share a 2-phenylchromen-4-one flavone backbone and can be divided into several sub-groups including flavonols, flavanones, flavanonols, flavones, flavan-3ols (flavanols) and anthocyanins [26]. Over 6500 flavonoid compounds have been identified in various plants [27], and the estimated number of flavonoid structures could be over 8000 [28, 29]. Numerous flavonoid compounds carry important biological functions for plant survival and reproduction by various mechanisms.

Flavonoids have been connected to adaptation of plants to severe environmental conditions. The immobility of plants requires them to develop specific survival mechanisms under environmental stresses such as heat, drought, frost and light radiation. Anti-oxidative flavonoids have been proposed to provide protective functions to plants under drought condition [30]. In addition, distribution of flavonoids in cell membranes and cell walls could contribute to plants' resistance to frost stress [31]. Most importantly, many flavonoid compounds possess UV absorption activity and are believed to provide photoprotection on plants [32, 33].

Other studies also indicate flavonoids act as defensive agents that contribute to various survival mechanisms. It has been suggested that flavonoids play important roles in plant-insect interaction, as different insects have been found to be sensitive to flavonoids, which potentially function as digestion reducers, toxins or antifeedants [34-36]. Due to their protein-binding activity, the flavan-3-ols' protection against large herbivores also has been well documented [37]. Certain flavonoids also possess antimicrobial activities through different mechanisms and have been proposed to contribute to plants' disease resistance [38, 39]. Enhanced flavonoid biosynthesis has been observed after pest/pathogen injury of plants [40, 41] and indicates their roles in plant defense mechanisms.

Flavonoids were also found to function as signaling molecules in plant-microbe interaction to promote symbiosis and plant growth. Flavonoids secreted from roots of legumes induced gene transcription of N_2-fixing bacteria, which protein products are required for infection [42, 43]. The flavonoid naringin was found to induce gene expression in cyanobacterium to benefit symbiosis [44], as well as promote diazotrophic bacteria colonization in wheat roots [45]. Besides the aforementioned functions, flavonoids also play important role in plant reproduction, e.g. accumulation of colored flavonoid compounds such as anthocyanins in insect-pollinated plant flowers to recruit pollinators and seed dispersers [46]. Other studies have suggested that flavonoids can act as stimulators for pollen germination [47] or regulate auxin transport [27]. Evidences also suggested that anti-microbial flavonoids can be utilized as allelochemicals by plants to inhibit growth of other competitors through plant-plant interaction [48].

Because of their wide distribution across different plants and important biological functions on both plants and human being, efforts have been made to elucidate the biosynthetic pathways of flavonoids in plants. Phenylpropanoid metabolism is proposed to convert phenylalanine into various plant secondary polyphenol metabolites. The metabolic pathway is believed to be involved by around 20% of the carbon fixed by photosynthesis and produce most of the naturally occurred phenolic compounds such as lignins and flavonoids [49]. In this metabolism pathway, phenylalanine, as one of the end aromatic amino acid products of shikimate pathway [50], is first transformed into *trans*-cinnamate, and then *p*-coumarate; the latter is further converted into flavan backbone [26], which forms the basic structure of flavonoids. Chemical modifications such as glycosylation and polymerization are then introduced to generate different groups of flavonoid with structural variations.

Biosynthesis of phenylpropanoid products has been found to be activated in both tissue/cell specific and development specific manners [51], and can be also induced by environmental stresses, such as pathogen attack, UV irradiation or wounding [52]. Although progress has been continuously made in identifying enzymes and genes involved in flavonoids biosynthesis [53], many important questions still remain unanswered regard to some of the key synthetic pathways, such as the polymerization of flavan-3-ol monomers into oligomers and polymers and their transportation [54].

With regards to dietary flavonoids, flavan-3-ols, flavonols, flavanones, and anthocyanins are the most consumed flavonoid groups in American diet [55].

Flavan-3-ols

Flavan-3-ols, also referred as flavanols, are characterized by a 2-phenyl-3,4-dihydro-2H-chromen-3-ol backbone. Naturally occurred monomeric flavan-3-ols include epicatechin, catechin, epigallocatechin, gallocatechin and their esters formed with gallic acid, such as epigallocatechin gallate [56]. Flavan-3-ols in plants are also characterized by their polymeric structures; monomer flavan-3-ols tend to from oligomers and polymers [57]. Polymerized flavan-3-ols, referred to proanthocyanidins (PACs), are widely distributed in plants and food such as fruits, vegetables, tea and wine [57]. While the most common linkage between monomer flavan-3-ols is C-C bond (B-type) [57], in cranberry and few other plant species, a C-O-C bond is also presented occasionally to form a double linkage (A-type) with the C-C bond [58], making them structurally unique compared with B-type PACs. Estimated PAC concentration in cranberry can be as high as > 400 mg/100 g fresh fruit, higher than other commonly consumed fruits such as blueberry (~250 mg/100 g), strawberry (~150 mg/100 g), apple (~100 mg/100 g) and peach (~ 70 mg/ 100 g) [59], making it an ideal source of dietary PACs.

Flavonols

Flavonols are another important class of flavonoids. Different flavonol compound share a 3-hydroxy-2-phenylchromen-4-one backbone, which contains an extra ketone group compared to the flavan-3-ols' 2-phenyl-3,4-dihydro-2H-chromen-3-ol backbone. Unlike polymeric flavan-3-ols, most flavonols occurred as monomer glycosides with conjugated sugar moieties (primarily in the 3 position) in plants or diets [26, 60]. It is believed that glycosylated flavonols have higher water solubility and mobility than their aglycone forms, and also less likely to interfere with other critical cellular metabolisms when stored in plant cells [26]. Different fruits or vegetables have differential profiles of

dietary flavonol glycosides with regard to their number, position and type of conjugated sugars. As a rich source of flavonols, onion contains primarily quercein-3,4-diglucoside and quercetin-4-glucoside which account for over 85% of total onion flavonols [61]. Cranberry, on the other hand, contains very limited quercetin-3-glucoside, compared with major glycosides such as galactoside, arabinoside, rhamnoside and xyloside conjugated with quercetin or myricetin [62]. Varied across different studies, estimated cranberry flavonol concentrations range from 40-50 to ~150 mg/100 g fresh fruit [62, 63], more than other common fruits such as blueberry, grape and apple [64, 65].

Anthocyanins

Anthocyanins are glycosides of anthocyanidins, which are oxygenated derivatives of flavylium (2-phenylchromenylium) salts. They account for the cyanic colors ranging from pink and red to violet and blue of most of the fruits, flowers and leaves of vascular plants and can be also accumulated in other plant tissues such as roots and stems [66]. Anthocyanins, or glycosylated anthocyanidins, account for the majority (over 97%) of naturally occurred anthocyanidins [67], due to increased compound stability after glycosylation. Although over 500 anthocyanins have been reported in plants, most of them are derived from the 6 most common anthocyanidin aglycones including pelargonidin, cyaniding, peonidin, delphinidin, petunidin and malvidin [66]. Cranberry contains 6 major anthocyanins as the galactoside, glucoside and arabinoside of cyanidin or peonidin, and concentration of total anthocyanins has been estimated at 70-100 mg/100 g fresh ripe fruit [62].

1.3 Quantitative and Qualitative Analysis of Plant Flavonoids

Qualitative and quantitative analyses of flavonoids in plants and food with plant origin have been the foci in agricultural and food chemistry. Among the different flavonoid groups, flavan-3-ols, flavonols and anthocyanins have received most of the research interest for their high dietary consumption levels and perhaps more importantly their potential human health benefits.

Anthocyanin levels have been quantified in fruits and beverage products like cranberry, blueberry, apple, grape, juice and wine [68-71] and are regarded as important measures of food quality. Structural characterization of anthocyanins can be achieved by different primary or advanced analytical techniques, most commonly *via* high performance liquid chromatography (HPLC) coupled with a photodiode array (PDA) detector to monitor the UV-Visible absorbance of anthocyanins [72]. With the addition of more advanced detection techniques, such as mass spectrometry (MS) or nuclear magnetic resonance (NMR) spectrometry, more structure details such as glycosylation position can be revealed, with less demand for authentic standards [72].

Accordingly, chromatographic techniques such as HPLC and MS can be applied in anthocyanin quantification, with a great advantage in providing detail information of the composition of different anthocyanins within material. In the food industry and in laboratories, however, due to their low cost and simplicity, spectrophotometric methods are often used in measuring anthocyanin levels based on the spectral characteristics [73]. Many different yet similar spectrophotometric anthocyanin quantification methods have been developed, which all utilize the 520-535 nm visible light absorbance of these molecules [73-75]. Among them a pH differential spectrophotometric method has been validated and approved by Association of Analytical Communities (AOAC) as an official

quantification method for total monomeric anthocyanins in fruit juices, wines and other beverages [76]. The method, utilizing the color difference of monomeric anthocyanins in different pH conditions (pH 1.0 and pH 4.5), exhibited high correlation ($R \geq 0.925$, $p \leq 0.05$) with an HPLC method [77].

Similar chromatographic instrumentations have been also widely used in structural characterization and quantification of flavonols. Different HPLC methods have been developed for flavonol identification and quantification in different plant and food materials such as apple [78], grape [79], onion, tomato and lettuce [80]. With the incorporation of MS and/or NMR spectrometry and non-hydrolysis sample extraction methods, detail structures of flavonol glycosides, especially the structure and position of sugar moieties, have been revealed in various studies with focuses on tea, strawberry, onion, citrus, raspberry as well as on other crops [81-84]. In 2004 Vvedenskaya et al. reported the identification of 19 different flavonol glycosides in cranberry powder, including six newly discovered flavonols [85]. With the same HPLC method, Vvedenskaya and Vorsa [62] quantified the composition of cranberry flavonol glycosides during fruit development and ripening in two cranberry cultivars.

Characterization and quantification of flavan-3-ols have been a major challenge in flavonoid analysis primarily because of the array of polymeric structures. The wide range of molecular sizes and weights across different flavan-3-ol oligomers and polymers has been a major hindrance for proper peak separation in HPLC as well as in compound identification using either UV absorbance detection (PDA) or MS/NMR. Flavan-3-ol polymers with increased degree-of-polymerization (DP) exhibit wider HPLC peaks with reduced UV absorbance intensity, which often leads to poorly separated, overlapped

peaks. Large compounds with DP > 10 (molecular weight > 2800) cannot be separated from each other and exhibit only one peak in HPLC-MS analysis, making it impossible to determine its composition of individual flavan-3-ols [86]. Moreover, flavan-3-ol molecules with molecular weight over 2000 (DP > 7) are poorly ionized with common MS ionization methods, such as electrospray ionization (ESI) and atmospheric-pressure chemical ionization (APCI); thus to often caused insufficient fragmentation and detection of such molecules in regular MS system. Several studies have utilized matrix-assisted laser desorption/ionization coupled with time-of-flight (MALDI-TOF) mass spectrometry for the characterization of flavan-3-ols from materials such including cranberry, apple, grape and sorghum [87-89]. With proper ionization of large-molecular weight flavan-3-ols, their DP, the structure of monomer building unit and type of inter-flavan linkage (A/B-type) can be determined.

HPLC methods with either PDA (UV) or fluorescence detection are the most commonly used chromatographic methods for flavan-3-ol quantification [90-92]. They can provide quantitative information for individual flavan-3-ol compounds as well as the composition of different flavan-3-ols in the analyzed material. However, certain sample extraction and purification procedures are needed in such methods, which can be both time-consuming and costly. Before the advancement of chromatographic techniques, several flavan-3-ol quantification methods were developed historically based on the biological or chemical activity of flavan-3-ol molecules. Some of those are still utilized in flavan-3-ol quantification as alternative or complementary methods for HPLC analysis.

The ability of flavan-3-ols to bind and precipitate protein has been utilized in their quantification [93]. Several protein precipitation methods were early developed and

widely used, such as the “Bate-Smith” method, in which flavan-3-ols react with proteins of hemolyed blood and the remaining hemoglobin is measured [94] as well as the “Hagerman and Butler” method, which involves the precipitation of bovine serum albumin (BSA) by flavan-3-ols [95]. Newly developed methods include using methylcellulose rather than proteins to react with flavan-3-ols, as the former offers higher selectivity of precipitation with flavan-3-ols [96].

Several spectrophotometric methods offer flavan-3-ol quantification by measuring colored reactants resulted from different chemical reaction between flavan-3-ols and certain reagents. The acid-butanol assay, as indicated by its name, uses HCl acidified butanol to hydrolyze flavan-3-ols into monomer anthocyanins and then quantify flavan-3-ols by measuring the anthocyanin product (~550nm) [97]. However, flavan-3-ol molecules with different inter-flavan linkage, e.g. 4β-8 versus 4β-6 C-C linkage, were found to have different reaction efficacy and kinetics in the assay [98]. Moreover, as flavan-3-ol polymers with higher DPs yield more anthocyanidins than smaller molecules such as dimers and trimers, the quantification result strongly depends on the selected reference standard.

The vanillin assay as another popular spectrophotometric method uses vanillin to react with flavan-3-ols in strong acidic conditions, which yields a colored compound with absorbance at 500 nm [99]. Anthocyanins with similar absorbance profiles can potentially interfere with the measurement, as does chlorophyll which was found to affect the color development [100]. Flavan-3-ols with different DPs have different molar absorption coefficient (MAC) as additional or side reactions appear to take place at sites other than the terminal units [101].

The 4-dimethylaminocinnamaldehyde (DMAC) assay, first reported by McMurrough and McDowell in 1978 [102], has been popular in flavan-3-ol quantification of plant and food materials. The DMAC reagent is proposed to only react with the A-ring of the terminal unit of flavan-3-ol molecule, forming a blue-colored product with the absorbance at 640 nm [103]. As a result, consistent MACs across different flavan-3-ol oligomers and polymers have been suggested [59]. This reaction mechanism indicates a high accuracy of DMAC assay in flavan-3-ol quantification compared with other spectrophotometric methods. A multi-laboratory validation study reported consistent quantification results of cranberry total flavan-3-ols using a standardized DMAC assay protocol [104]. DMAC reagent was also utilized in post-column derivatization of HPLC analysis for detection of flavan-3-ols [105] or in microscopy studies for flavan-3-ol localization [106]. Compared with chromatographic methods, the conventional spectrophotometric methods, particularly DMAC assay, hold several important advantages such as easy experiment setup, simple sample preparation, high efficiency and low cost. As such they are still used in agriculture and food industry for rapid determination of total flavan-3-ols.

Various chemical analysis methods for anthocyanins, flavonols or flavan-3-ls are summarized in Table 1.1.

1.4 Health Beneficial Activities of Plant Flavonoids

Flavonoids from plant or food sources have received plentiful research interest predominantly because of their various potential human health benefits. Over the past decades studies have focused on many different aspects of flavonoids' bioactivities,

including their antioxidant, anti-inflammatory, anti-bacterial and anti-cancer activities as well as cardiovascular benefits.

1. Antioxidant activity

The anti-oxidant activity of flavonoids has been considered to be directly connected with many of their other health promoting properties such as protection against cardiovascular disorders, viral and bacterial infections and cancer diseases [107]. They contribute to the antioxidant values of many fruits and vegetables, as well as other food and beverage products such as plant oils, wines, teas and chocolates [107]. The hydroxyl groups and conjugated aromatic ring structures shared by the compound group contribute greatly to their antioxidant properties [108]. Potential mechanisms include metal chelating, enzyme inhibition, free radical chain breaking and quenching of singlet oxygen [109]. However the anti-oxidant assay is an *in vitro* measure and may not reflect *in vivo* activity [110].

2. Anti-viral and bacterial activities

Cranberry's protection effect against UTI has been attributed to its high level of flavan-3-ols. Purified cranberry flavan-3-ols, also named proanthocyanidins, inhibited the adherence of uropathogenic bacteria P-fimbriated *Escherichia coli* to uroepithelial-cell surfaces *in vitro* [111]. The inhibition effect was later found structure-specific as A-type flavan-3-ol trimers exhibited stronger activity than dimer and monomers [112]. Cranberry flavonol and flavan-3-ol extracts also inhibited the biofilm development, acid production and enzyme activities of the dental cavity pathogenic bacteria *Streptococcus mutans* [113]. Plant originated flavonoids also exhibited anti-virus activities in different studies. Flavonoid ellagic acid isolated from medicinal plants exhibited anti- hepatitis B virus

effect through blocking its antigen secretion in infected hepatocytes [114]. Flavonoid baicalin was found to inhibit leukemia virus through inhibition of reverse transcriptase activity [115]. Flavonol aglycone quercetin suppressed hepatitis C virus through inhibition of nonstructural protease activity [116].

3. Anti-inflammatory activities.

Plant flavonoids also exhibited anti-inflammatory effects both *in vitro* and *in vivo*. Flavonoids rutin, quercetin and hesperidin inhibited both acute and chronic inflammation in a rat arthritis model [117]. Other flavonoids apigenin, luteolin and chrysin induced decreased pro-inflammatory cytokine production in lipopolysaccharide-stimulated human peripheral blood mononuclear cells [118]. Proposed mechanisms responsible for flavonoids' *in vivo* anti-inflammatory properties include inhibition of eicosanoid generating enzymes, regulation of proinflammatory gene expression [119].

4. Cardiovascular benefits.

The cardiovascular benefits of dietary flavonoids have been investigated by many studies on different populations and diet profiles. Negative correlation between dietary flavonoids intake and risk of cardiovascular disease was observed in several different epidemiologic studies [120-125] indicates flavonoids' potential protective activities on human cardiovascular health. A study utilizing normal and obese mice models reports improvement of insulin resistance and plasma lipid profile, as well as reduction of visceral fat mass in obese supplemented with cranberry flavonoids, and suggests the adiponectin-AMPK pathway and hepatic cholesterol synthesis pathway were being regulated [126]. While more studies are needed to reveal the action mechanisms of

flavonoids which account for their cardiovascular benefits, it is believed that their anti-inflammatory and anti-oxidant activities, as well as capability to induce apoptosis, contribute to the potential cardioprotective properties [127].

5. Anti-cancer properties.

Cancer is one group of diseases caused by uncontrolled, non-stopped division of cells and their invasion into nearby tissues or organs [128]. All cancers are genetically initiated, developing from mutations on genes responsible for normal cell function, such as cell growth regulation and cellular DNA repair [129, 130]. Most of the genetic mutations responsible for cancer development are induced by exposure to certain environmental conditions, such as chemicals, irradiations or pathogens, and risk of such mutations increases with peoples' age due to long-term environmental exposure and/or increased chance of DNA replication error [131, 132]. Some cancers are grouped as "family cancers", as members in certain families prone to develop such cancers without noticeable exposure to known carcinogens, which can be resulted from inherited genetic profiles linked with high cancer risk [132].

As one of the most epidemic diseases cancer has major social impact in the US and across the world. Over 1.6 million new cases of cancer are estimated to be diagnosed in US during 2016, accompanied with almost 0.6 million death from the disease. The most common cancer types in 2016 are predicted to be breast cancer, followed with lung and bronchus cancer, prostate cancer, colon and rectum cancer and bladder cancer [133].

Besides traditional cancer therapeutic strategies such as direct surgery, chemotherapy and radiation therapy, modern studies on cancer have focused on different aspects of its

molecular and cellular characteristics for the purpose of developing effective therapeutic strategies for cancer treatment. Important cell functions which are modified or lost in cancer cells due to genetic mutations, such as cell apoptosis, cell cycle progression and cell metabolism have been investigated by many studies which provide critical insights on potent cancer therapeutic agents [128, 134-136].

Relationship between fruit and vegetable consumption and cancer risk has been investigated by many studies focused on different cancer types. Statistical significant protective effects of fruit and vegetable consumption against cancer risk have been showed by many individual studies on cancer types such as lung cancer, pancreas and stomach cancer, colorectal cancer, bladder cancer, breast cancer and ovarian cancer [137]. As one of the major constituents in dietary fruits and vegetables, flavonoids received considerable research interests and many epidemiological studies indicate the beneficial effects of dietary flavonoids on cancer prevention [138, 139].

Apart from epidemiological studies, flavonoid compounds' anti-cancer properties have been directly revealed by many *in vitro* and *in vivo* studies. Both purified anthocyanins and anthocyanin-rich plant extracts have exhibited potent anti-proliferative property against multiple cancer cell lines [140-143]. An anthocyanin-rich extract was found to specifically inhibit the growth of colon cancer cells, but not normal colon cells, through modulation of cell cycle regulatory genes including p21, p27, cyclin A and cyclin B [144]. Other studies revealed the induction of cancer cell apoptosis by anthocyanin-rich extracts though both intrinsic and extrinsic pathways [145, 146]. Anthocyanins are also found to potentially prevent tumor growth and metastasis through their anti-angiogenesis, anti-invasiveness or cell differentiation induction activities against cancer cells [147-149]. *In*

vivo studies on anthocyanins also provide evidences of their abilities to inhibit cancer growth in model animals. Purified anthocyanins or anthocyanin-rich fractions have showed ability to inhibit development of skin cancer, colon cancer, lung cancer and esophageal cancer on animals treated by carcinogen or with tumor cell xenotransplantation [150-153]. Further studies are still needed to elucidate the mechanisms of the *in vivo* anti-cancer effects of anthocyanins.

Among different dietary flavonols, quercetin aglycone has been most extensively studied for anti-cancer property due to its wide distribution and high content across different food sources. Many studies reported the *in vitro* anti-proliferation property of quercetin aglycone on different cancer cell line models, including colon cancer, breast cancer, lung cancer, prostate cancer, ovarian cancer and leukemia [154-159]. The action mechanisms of quercetin aglycone's cytotoxicity on the aforementioned cancer cells include inhibition of cyclin D1 and survivin expression in colon cancer cells [154], induction of cell cycle arrest at G2/M transition and apoptosis in breast cancer cells[155], activation of MEK-ERK signaling pathway of lung cancer cells [156], inhibition of androgen receptor in prostate cancer cells [157], and inhibition of protein kinase C and/or tyrosine protein kinase activity on leukemia cells [158].

Other flavonol aglycones, mainly myricetin and kampferol, are also reported to have anti-cancer activities on different cancer cell models. Myricetin was found to suppress skin, bladder and pancreatic cancer cells through different mechanisms [160-162]; kaempferol was reported to have growth inhibition effect on pancreatic, lung, ovarian and colon cancer cells by multiple studies [163-166]. Flavonol glycosides, on the other hand, receive less research interest on their anti-cancer activity, possibly due to their believed

low human absorption. Quercetin galactoside was reported to induce apoptosis in pancreatic, colon and lung cancer cells [167-169]. Rutin, as quercetin rutinoside, exhibited anti-colon cancer activities *in vitro* [169].

Flavan-3-ols monomers and polymers, also referred as condensed tannins or proanthocyanidins (PACs), have been considered as important dietary flavonoids with anti-cancer potential B-type PACs isolated from grape seeds have been most extensively studied, and many report evidence of their *in vitro* and *in vivo* anti-cancer properties. These PAC isolations have been shown to induce apoptosis of skin cancer cells through a p53-dependent mechanism with activation of apoptosis regulation genes/proteins Bax/Bcl-2 and caspase-3 [170]. Their anti-proliferation effect against human prostate cancer cells through inactivation of MAPK and NF-κB signaling pathways was also reported [171]. Their anti-proliferation activities against human breast, lung and colon cancer cells have also been revealed by different studies [172-174]. In *In vivo* tests, dietary feeding of PACs isolated from grape seeds inhibited carcinogenesis of UVB-induced skin cancer on hairless mice [175], and reduced the incidence of carcinogen-induced mammary, colon or breast cancer in rats [176-178].

Cranberry flavan-3-ols which primarily consist of A-type PACs also exhibit anti-cancer activity. A cranberry PAC fraction primarily contained A-type PACs with DP ranged from 4 to 7 was shown to inhibit growth of lung cancer, colon cancer and leukemia cells [89]. In another study, A-type cranberry PACs with DP ranged from 2 to 8 selectively inhibited proliferation of human ovarian, neuroblastoma and prostate cancer cells over normal lung fibroblast cells and sensitized the platinum-resistant ovarian cancer cells to chemotherapy drug cisplatin [179]. The same PAC fraction was further reported to

induce G2/M cell cycle arrest, apoptosis and intracellular ROS production in ovarian cancer cells as well as inhibited angiogenesis in model human umbilical vein endothelial cells [180].

1.5 Bioavailability of Dietary Flavonoids

Flavonoids' high dietary intake values and diverse health benefit potentials toward human makes it important to evaluate their bioavailability in human body. Bioavailability, which can be defined in different ways, usually refers to the proportion of nutrients/compounds that is absorbed and metabolized through normal *in vivo* pathways. The absorbance efficiency, metabolic transition and excretion status of dietary flavonoids not only determine the potential nutritional and health beneficial values of flavonoids containing foods, but also provide critical information to illustrate the relationship between flavonoids' structure and their bioactivities.

Bioavailability of Anthocyanins

Many studies have shown that dietary anthocyanins are bioavailable in animals and humans. Studies using rat, rabbit or pig as animal models reported fast anthocyanin absorption as most of the studies recovered plasma anthocyanins in their original glycoside forms within 0.25 to 2 hours following compound administration [181]. A few studies also detected lower levels of methylated anthocyanins and/or anthocyanin glucuronide as potential metabolites in plasma or urine of rats/pigs following administration of berry fruits or pure compound [182-185].

Human trials confirm the absorption of anthocyanins into human body. Most studies have only detected intact anthocyanin glycosides in human plasma or urine after consumption

of anthocyanin-rich food or wine [181], indicating minimum anthocyanin metabolism in human body. Although anthocyanin absorption rate differs among studies, most of the human trials indicate a very low bioavailability of anthocyanins in human, with the urinary anthocyanin excretion of only 0.004-0.23% of total intake [181]. Three studies indicated human plasma accumulation and urine excretion of cranberry anthocyanins among younger or elder subjects [186-188], major cranberry anthocyanins including galactoside, glucoside and arabinoside of cyaniding/peonidin were all recovered in their intact forms, further suggesting the lack of metabolism of dietary anthocyanins in human body.

Bioavailability of Flavan-3-ols

Flavan-3-ol (PAC) polymers are poorly absorbed into animal or human body. *In vitro* study using human intestinal epithelial Caco-2 cell model reported that only PAC dimers and trimers were able to permeate through the monolayer cells [189]. In fact, no study so far has reported the recovery of PAC polymers with DP $\geq$ 3 in animal or human subjects following PAC administration, suggesting the poor absorption of such large polymeric molecules.

PAC monomers such as catechin, epicatechin, epigallocatechin and epigallocatechin gallate, however, are believed to be absorbed *in vivo*, which is supported by many studies. A maximum level of 257 nmol/L epicatechin was detected in human plasma 2h after consumption of chocolate containing 557 mg PACs [190]. A peak level of 76.7 ± 7.5 nmol/L catechin was recovered in human plasma at an average 1.4 h after ingestion of red wine containing 35mg free catechin [191]. A total of 35 pmol/ml epigallocatechin and

657 pmol/ml epigallocatechin gallate was detected in human plasma at 1.5 h following ingestion of green tea extracts containing 7.5 mg epigallocatechin and 225 mg epigallocatechin gallate [192]. While epigallocatechin gallate is found to be mostly presented in its original free form upon absorption [193-195], metabolites of other monomers have also been detected in human plasma or urine, predominantly in forms of methylated, glucuronidated or sulfated conjugates [196]. Epicatechin-3'-O-glucuronide, 4'-O-methyl-epicatechin-3'-O-glucuronide, and 4'-O-methyl-epicatechin-5 or 7-O-glucuronide were identified in human urine as main metabolites of oral ingested epicatechin [197].

Although an early study reported decomposition of cocoa PAC oligomers (trimer to hexamer) into monomer and dimers in simulated gastric juice [198], later study on human subjects clearly showed that PAC oligomers cannot be degraded and remain stable in human stomach environment [199]. A recent human trial with consumption of PAC mixtures from monomer epicatechin to decamer suggested that all absorbed epicatechin in human plasma or urine came from the ingested epicatechin, not PAC oligomers and polymers [200]. In spite of the poor digestion/absorption of PAC oligomers and polymers in human gastrointestinal tract, studies have shown that PACs can be metabolized into different phenolic acids by colonic bacteria. A 48 h incubation of PAC polymers in human colonic microflora resulted in an almost 100% degradation of PACs into monohydroxylated phenylacetic, phenylpropionic and phenylvaleric acids [201]. An *In vivo* study reported increase of such acids including m-hydroxyphenylpropionic acid, m-hydroxyphenylacetic acid and m-hydroxyphenylacetic acid, together with other phenolic

acids e.g. vanillic acid, ferulic acid and 3,4-dihydroxyphenylacetic acid in human urine after consumption of PAC-rich chocolate.

Bioavailability of Flavonols

Bioavailability of flavonols has been determined by many *in vitro* and *in vivo* studies. Quercetin was detected in human ileostomy effluent and urine after ingestion of fried onion (rich in quercetin glucosides), quercetin rutinoside or quercetin aglycone, with quercetin glucosides exhibited highest absorption rate, followed by aglycone and rutinoside [202]. This study suggests the effect of sugar moieties on flavonols' bioavailability, and similar result was observed by other *in vivo* trials. Average time to reach maximum plasma quercetin concentration was shorter in subjects consumed quercetin aglycone than those consumed rutinoside [203]. While similar bioavailability and pharmacokinetic parameters were observed between subjects consumed onion or quercetin glucoside, delayed absorption and lower plasma quercetin concentration were exhibited on subjects consumed pure quercetin rutinoside or rutinoside-rich tea [204].

Although quercetin and/or other major dietary flavonol aglycones such as myricetin and kaempferol were recovered in human plasma or urine after consumption of various flavonol containing foods or drinks, e.g. onion, apple, cranberry and tea [188, 205, 206], the nature of flavonol glycosides being absorbed and present in human body, is still unclear. Due to the protein-binding activity of flavonols in plasma samples, most of the bioavailability studies introduced acid or enzymatic hydrolysis in their sample extraction, which inevitably resulted in deglycosylation of potentially existing flavonol glycosides into aglycones. With a non-hydrolysis sample extraction method, Sesink et al. [207]

reported quercetin-glucuronides, not glucosides, were presented in human plasma after consumption of quercetin-3-glucoside or quercetin-4'-glucoside. Studies are still limited regard to the bioavailability of other flavonol glycosides, i.e. quercetin-galactoside/arabinoside/rhamnoside, in human body.

Absorbed flavonols can be metabolized into different methylated, glucuronidated or sulfated conjugates in human body. Quercetin-3-glucuronide, 3'-methyl-quercetin-3-glucuronide and quercetin-3'-sulfate were identified major circulating flavonol metabolites in human plasma 1.5 h after consumption of onion [208]. Same metabolites plus two other quercetin conjugates - quercetin diglucuronide and quercetin glucuronide sulphate, were identified in quantifiable amount from human plasma 0.6 -0.8 h after consumption of onion [209]. While onions contain predominantly quercetin glucosides and quercetin diglucosides [61], *in vivo* metabolic status of other dietary flavonol glycosides has yet to be determined.

1.6 Objectives

As described earlier, there has been longstanding interest in dietary flavonoids, especially their various health beneficial potentials. Although previous studies have focused on many important aspects of flavonoid compounds, such as the structure characterization, the distribution and accumulation in different plant and food materials, the *in vitro* and *in vivo* bioactivities and the human absorption and metabolism, due to the limitation on chemical identification, isolation and purification, few studies have attempted to evaluate and compare the variations and variables that influence the quantities, i.e., concentrations, of these bioactive compounds in food sources such as cranberry.

Furthermore, knowledge of bioactivity and bioavailability of individual flavonoid compound is also lacking. Although sharing similar backbone structures, the structural differences among flavonoids, such as the sugar moieties of flavonols and anthocyanins, the degree-of-polymerization and inter-flavan linkage type of flavan-3-ol oligomers and polymers, could all significantly affect compounds' chemical and biological properties.

Therefore, the objectives of this thesis are to add to our understanding of the variation, both genetic and environment, in these constituents in cranberry and identify and characterize the bioactivity and bioavailability of various individual dietary flavonoids, with specific focuses on flavonols and flavan-3-ols from cranberry.

In the next chapters, findings on different aspects of dietary flavonoids are described and discussed. Chapter 2 discusses the production and accumulation of major flavonoids, including anthocyanins, flavonols and flavan-3-ols, as well as organic acids in eight cranberry varieties over fruit development and ripening stages. Chapter 3 describes the effects of flavan-3-ols' structure on their quantification using DMAC colorimetric assay. Chapter 4 focuses on the *in vitro* anti-cancer activities of A-type cranberry flavan-3-ols and B-type cocoa flavan-3-ols are illustrated using two ovarian cancer cell models. Chapter 5 discusses human absorption and metabolism of individual flavonol glycoside and flavan-3-ols from cranberry. The findings discussed in this thesis will contribute to our knowledge on cranberry's nutritional and health beneficial values, especially as it relates to its various flavonoid compounds as secondary metabolites.

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1.8 Tables and Figures

Table 1.1. Summary of chemical analysis methods for anthocyanins, flavonols and flavan-3-ols.

Compound(s)	Method	Mechanism	Advantages and Disadvantages	References
Anthocyanin	HPLC-PDA/MS	LC separation of individual anthocyanins; Determination of compound molecular weight and absorbance spectrum	A: Qualitative & quantitative analysis; Analysis of individual compounds D: Instrumentation; Sample preparation and analysis are time-consuming.	[72]
	NMR Spectroscopy	Magnetic properties of certain atomic nuclei in compound	A: Illustration of structural details D: Instrumentation; Requirement of pre-purified sample; Only for qualitative analysis	[72]
	pH Differential Spectrophotometric Method	Light absorbance of anthocyanins at 520 nm	A: Easy sample preparation and fast analysis D: Only for quantitative analysis; No differentiation of individual compounds	[76,77]
Flavonols	HPLC-PDA/MS	LC separation of individual flavonols; Determination of compound molecular weight and absorbance spectrum	A: Qualitative & quantitative analysis; Analysis of individual compounds D: Instrumentation; Sample preparation and analysis are time-consuming.	[78-84]
	NMR Spectroscopy	Magnetic properties of certain atomic nuclei in compound	A: Illustration of structural details D: Instrumentation; Requirement of pre-purified sample; Only for qualitative analysis	[85]

Compound(s)	Method	Mechanism	Advantages and Disadvantages	References
Flavan-3-ols	HPLC-PDA/FLR	LC separation of individual flavan-3-ols; Determination of compound absorbance and fluorescence properties	A: Qualitative & quantitative analysis; Analysis of individual compounds D: Instrumentation; Sample preparation and analysis are time-consuming. Insufficient separation and low signal intensity for high MW compounds	[90-92]
	LC-ESI/APCI-MS	LC separation of individual anthocyanins; Determination of compound molecular weight and fragmentation pattern	A: Illustration of structural details D: Instrumentation; Sample preparation and analysis are time-consuming; Low sensitivity for high MW (>2000) compounds	[86]
	MALDI-TOF-MS	Determination of compound molecular weight and fragmentation pattern	A: Illustration of structural details; High sensitivity for high MW compounds. D: Instrumentation	[87-89]
	Protein Precipitation Assay	Precipitation of protein by flavan-3-ols	A: Fast analysis; No requirement for advanced instrument. D: Affected by multiple variables, e.g. pH, temperature, protein and flavan-3-ol structures; Only for quantitative analysis; No differentiation of individual compounds	[93-95]
	Methylcellulose Precipitation Assay	Precipitation of flavan-3-ols by methylcellulose	A: Fast analysis; Higher selectivity than protein precipitation. D: Only for quantitative analysis; No differentiation of individual compounds	[96]

Compound(s)	Method	Mechanism	Advantages and Disadvantages	References
Flavan-3-ols	Acid-Butanol Assay	Hydrolysis of flavan-3-ols into anthocyanins, which are measured by spectrophoto metry	A: No chromatograph required D: Only for quantitative analysis; Uncharacterized products; Hydrolysis efficiency affected by flavan-3-ol structure	[97,98]
	Vanillin Assay	Reaction between vanillin and flavan-3-ols yields colored compound with 500 nm absorbance	A: Fast analysis; No chromatograph required D: Only for quantitative analysis; Measurement affected (interference) by anthocyanins/chlorophyll s; Variable molar absorption coefficient (MAC)	[99-101]
	4-dimethylamino-cinnamaldehyde (DMAC) Assay	Reaction between DMAC and flavn-3-ols ds colored compound with 640 nm absorbance	A: Fast analysis; No chromatograph required. No interference of anthocyanins; High selectivity D: No differentiation of individual compounds; Variable MAC, affected by flavan-3-ol structures.	[59,102-106]

Chapter 2

Structural Characterization and Quantification of Flavonoids and Organic Acids over fruit development in American Cranberry (*Vaccinium macrocarpon*) using HPLC and APCI-MS/MS

2.1 Introduction

American cranberry (*Vaccinium macrocarpon*) has long been recognized as one of the leading plant sources of bioactive secondary metabolites with important implications to human health. Among cranberry secondary metabolites, phenolic compounds have received most of the research interests for their high contents in cranberry and well-proven health benefits. Cranberry phenolics are comprised of flavonoids, including flavonols, anthocyanins and flavan-3-ols such as proanthocyanidins, as well as phenolic acids [1]. A study using Folin-Ciocalteu colorimetric method shows that cranberry possesses the highest total phenolic content among a number of analyzed fruits [2]. Besides phenolics, cranberry also contains various non-phenolic organic acids such as quinic, citric and malic acids, and small amount of benzoic and glucuronic acids [3].

Flavonoids have multiple important physiological roles in plants, such as pollinator recruitment, pathogen defense, UV radiation protection and defense against herbivory [4-6]. In cranberry, anthocyanins and flavonols mainly appear as monomeric glycosides conjugated to a number of different sugar moieties [7, 8], although low levels of free aglycone states such as quercetin and myricetin have been reported. Cranberry proanthocyanidins (PACs), on the other hand, are oligomers or polymers of flavan-3-ols. While the most common linkage between PAC building blocks is C-C bond (B-type) [9],

in cranberry and few other plant species, an C-O-C bond also occurs and forms a double linkage (A-type) with the C-C bond [10], making them structurally unique compared with B-type PACs which are found in blueberry, grape ad cocoa. Both flavonols and PACs have shown various bioactivities, such as anti-oxidant, anti-cancer (cytotoxicity to cancer cell lines) and anti-inflammatory activities, as well as cardiovascular health benefits [11-17].

Organic acids are important components in cranberry fruits and contribute to their characteristic flavor. Besides being employed to determine fruit quality such as maturity and flavor [18, 19], they also have been widely used as food additives in juice and beverage products to improve nutrition and preservation stability [20]. Compositions of flavonoids and organic acids in cranberry fruits are influenced by multiple factors such as variety, maturity and growth conditions. Their biosynthesis can be affected by multiple factors, such as precursor availability, enzymatic activity and chemical modifications including methylation, glycosylation and hydroxylation [21]. Although early studies revealed composition patterns of different phytochemicals in cranberry fruit and other species [18, 21-23], diversity of plant materials and analyzed compounds were limited. Comprehensive studies with extensive sampling and analytical approaches are needed for a better understanding of how multiple factors, including genetic background, growth stage and biosynthetic pathway, can affect phytochemical profiles of cranberry.

In the present study, we investigated the concentrations of four major classes of cranberry phytochemicals - anthocyanins, flavonols, PACs and organic acids, during fruit development and ripening in eight cranberry varieties representing diverse genetic backgrounds. HPLC and LC-MS-MS methods have been developed and optimized to

identify individual cranberry phytochemicals and provide accurate quantitative analysis. Total monomeric athocyanins, individual flavonols including four quercetin glycosides and two myricetin glycosides, individual PAC monomer, oligomers and polymers with degree-of-polymerization ranged from 2 to over 11, and four major cranberry organic acids – qunic, malic, citric and benzoic acids, were analyzed. The objectives were to determine: (1) whether the flavonoid and organic acid constituents and their levels were a function of fruit development stages, and (2) is there evidence for genetic variation, (3) determine if certain cultivars are better for certain phytochemical constituents.

2.2 Materials and methods

Plant material

The cranberry varieties used were three selections from native cranberry stands - Early Black (EB) selected in 1852, Howes (HO) selected in 1843 and Ben Lear (BL) selected in 1910; 1st breeding cycle cultivars Stevens (ST) and #35, developed in 1940s; and 2nd breeding cycle cultivars Crimson Queen (CQ), Demoranville (DM) and Mullica Queen (MQ), released post 2000. The variety trial was established at Philip E. Marucci Center for Blueberry and Cranberry Research and Extension in May 2010; plots were arranged in a RCBD with 3 replications. Fruit samples were harvested from each variety at 7-17 day intervals over 8 dates in 2014, initiating at fruit set on July 21 through fruit maturity on October 6. Sampling dates were July 21 (Julian Day (JD) 202), 28 (JD 209); August 7 (JD 219), 18 (JD 230), 28 (JD 240); September 8(JD 251), 19 (JD 262) and October 6 (JD 279). Samples were kept in -20 ºC within 1 hour of collection and remained in -20 ºC until analysis.

Reagents

All solvents, including methanol, acetone, acetonitrile, ethyl acetate, n-hexane and water were purchased from EMD Millipore (Billercia, MA), and were of HPLC grade. Acetic acid was purchased from Avantor Performance Materials (Center Valley, PA), formic acid was purchased from Mallinckrodt Baker (Phillipsburge, NJ) and phosphoric acid was purchased from Amresco (Solon, OH). Sephadex® LH-20 was obtained from GE Healthcare Bio-Science (Piscataway, NJ) and BAKERBOUND® Diol was obtained from Avantor Performance Materials.

The flavonoid standards, including the anthocyanin cyanidin-3-galactoside, and flavonols quercetin-3-galactoside, quercetin-3-glucoside, quercetin-3-rhamnoside and quercetin aglycone were purchased from Indofine Chemical Company (Somerville, NJ). Additional flavonol standards, including myricetin-3-galactoside, myricetin-3-arabinopyranoside, quercetin-3-xylopyranoside, quercetin-3-arabinopyranoside and quercetin-3-arabiniofuranoside were isolated using a semi-prep HPLC system described before [24]. Organic acid standards, including citric acid, malic acid, quinic acid and benzoic acid were obtained from Sigma (St. Louis, MO). Individual PAC standards were isolated using Sephadex® LH-20 [24] and BAKERBOUND® Diol gravity column chromatography. In Diol column, a binary solvent system of solvent A (98% acetonitrile with 2% acetic acid) and solvent B (95% methanol with 3% water and 2% acetic acid) was used, with solvent A gradient gradually reduced from 100% to 75% for PAC isolation and purification.

Extraction of cranberry flavonoids and organic acids

For flavonol and PAC quantification, about 10 g of fruits were weighed and crushed in 40 ml 80% aqueous acetone with 0.1% acetic acid in laboratory blender for 1 minute, followed by 30 min sonication and filtration with filter paper. Liquid extracts were dried in rotary evaporator under high vacuum and 35℃ of water bath and re-dissolved in methanol to a final volume of 6 ml. Samples were filtered through 0.45 μm Spin-X® centrifuge filter tube before HPLC and LC-MS analysis. For flavonoid characterization and isolation, acetone extracts were further partitioned with n-hexane and ethyl acetate and pre-purified in Sephadex® LH-20 column as previously described [24].

For organic acid identification and quantification, about 3 g of fruits were weighed and crushed in 30 ml distilled water in a blender for 1 minutes and heated in 90 ℃ water bath for 10 min. After filtration through filter paper, aqueous extracts were collected for HPLC analysis.

HPLC Apparatus and Conditions

Three HPLC systems were used separately for flavonol, PAC and organic acid identification and quantification:

Cranberry flavonols were analyzed in Dionex UltiMate® 3000 LC system including UltiMate® 3000 RS Pump, Autosampler, Column Compartment and Diode Array Detector. A Gemini® 150 x 4.6 mm 5 μm C18 110 Å LC column was used and flavonols were detected at 366 nm.

Cranberry PACs were analyzed in Waters Alliance® LC system composed of Waters e2695 Separations Module, Waters 2998 PDA Detector and Waters 2475 FLR Detector. A Develosil® 250 x 4.6 mm 100Diol-5, 5μm LC column was used and PACs were

detected in both PDA detector at 280 nm and fluorescence detector with excitation/emission wavelengths at 280/308 nm.

Organic acids were analyzed in a Dionex® HPLC system with AS50 Autosampler, AS50 Thermal Compartment, PDA-100 Detector and GP40 Gradient Pump. A Waters Atlantis® 250 x4.6 mm dC18, 5µm LC column was used and organic acids were detected at 210 to 230 nm in PDA detector.

All solvent systems and elution gradients are summarized in Table 2.1.

MS Spectrometry

An Applied Biosystems API 3000™ triple-quad LC-MS/MS mass spectrometer was used in LC/MS-MS analysis. MS data was obtained under atmospheric pressure chemical ionization (APCI) in negative ion detection mode, with following parameters: Curtain gas: 12 psi, Nebulizer gas: 7 psi, Nebulizer current: -2.0 mA, Entrance potential: -10 V, Focusing potential: -300 V, Declustering potential: -60 V, Collision energy: -50 V, Collision cell exit potential: -5.0 V, Source temperature: 500 ºC. The same LC methods were used for flavonol and PAC separation.

Characterization and quantification of flavonoids and organic acids

Flavonol and organic acid characterization was carried out by comparing their LC retention time, UV spectra and/or MS/MS data with standards or previously published data [25].

PAC characterization was based on comparing their LC retention time, fluorescence spectra and MS data with previously published data [15, 26]. Degree-of-polymerization

(DP) of PACs was determined by matching MW with multiples of monomer's MW, and number of A-type linkage was determined by subtracting the total loss of molecular weight from double linkage formation. HPLC standard curves were prepared using purchased or isolated standards for quantification. Quantification data were based on fresh weight (FW) of cranberry samples.

Quantification of total monomeric anthocyanins

Anthocyanins were quantified using a pH differential spectrophotometric method [27] with slight modification. Cranberry flavonoid extracts (50 to 200 µl) were mixed with pH 1.0 (0.025M potassium chloride) or pH 4.5 (0.4M sodium acetate) buffer (1800 to 1950 µl depending on the volume of added flavonoid extract). Absorbance at 520 and 700 nm were measured by a Thermal Scientific Genesys^{TM} 10S UV-Vis spectrophotometer for both mixtures and the subtracted absorbance $A= (A_{520}-A_{700})pH\ 1.0-(A_{520}-A_{700})pH\ 4.5$ was used for quantification. Cyanidin-3-galactoside was used as the standard.

Statistical analysis

Statistical analysis was performed with SAS 8.0 (SAS Institute, Inc., Cary, NC) and SPSS Statistics 19 (IBM Corporation, Armonk, NY). SAS procedure PROC GLM was used for analysis of variance (ANOVA) and linear/quadratic regression analysis was performed in SPSS Statistics 19.

2.3 Results

Total Monomeric Anthocyanins

Levels of total monomeric anthocyanins in 8 cranberry varieties during fruit development are shown in Figure 2.1. Low concentrations (0.12-0.32 mg/100g fruit) of anthocyanins were detected in all varieties from fruit set (JD 202, July 21). Levels of anthocyanins increased slowly during fruit development and then increased substantially in ripening fruit after September 8 (JD 251). Highest levels of anthocyanins were observed at the last sampling date in all varieties.

In varieties DM, EB, CQ and BL, accumulation rates of total anthocyanins increased significantly after the second sampling date (JD 209), two weeks earlier than other 4 varieties MQ, ST, #35 and HO. Their also exhibited higher anthocyanin levels than other 4 varieties during entire sampling period, with 54-86 mg/100g fruit anthocyanins at the last sampling date (JD 279), compared to 12-38 mg/100g fruit in other 4 varieties. Accumulation of total anthocyanins exhibited strong relationship with JD which accounted for the majority ($R^2 > 95\%$) of the anthocyanin variations for all varieties in quadratic fit.

Flavonol glycosides

HPLC-APCI-MS/MS analysis was used to identify individual flavonols in cranberry fruits. As shown in Figure 2.2 and Table 2.2, 9 flavonols were identified as myricetin-3-galactoside, myricetin-3-arabinofuranoside, quercetin-3-galactoside, quercetin-3-glucoside, quercetin-3-xylopyranoside, quercetin-3-arabinopyranoside, quercetin-arabinofuranoside, quercetin-3-rhamnopyranoside and quercetin aglycone. Despite being identified in Sephadex® LH-20 column purified flavonol fraction, quercetin aglycone

appeared in extremely low levels in cranberry fruit and was not quantified in the current study.

Compositions of individual flavonols are similar in the 8 cranberry varieties across different sampling dates. Quercetin-3-galactoside was the most abundant flavonol glycoside in all varieties, accounted for 31%-46% (w/w) of total quantified flavonols, followed by myricetin-3-galactoside (19%-32%), quercetin-3-arabinofuranoside (7%-17%) and quercerin-3-rhamnopyranoside (7%-14%). Figure 2.3A shows the composition and accumulation of individual flavonol glycosides in variety MQ. Total flavonols differed by 1.4-1.7 fold in most varieties over the sampling period, except DM (2.4-fold) and EB (1.2-fold). Levels of total flavonols exhibited fluctuating patterns across the sampling period. Except BL and EB, 6 varieties showed significant increase in total flavonol concentrations between the last two sampling dates during the final ripening of fruits (Figure 2.3B).

Levels of quercetin-3-galactoside (Q-3-Gal), the most abundant flavonol glycoside, followed a similar pattern with the total flavonol levels. In all varieties, no significant linear/quadratic relationship was evident with JD. Significant differences in Q-3-Gal levels were observed in three sampling dates among varieties: MQ showed higher Q-3-Gal level than other varieties at fruit set (JD 202). BL and ST exhibited higher Q-3-Gal concentrations on August 7 (JD 219) and BL again showed higher Q-3-Gal concentrations than others on September 19 (JD 262). Except for those dates, all 8 varieties had similar levels of Q-3-Gal during sampling period, including the final harvest date (Figure 2.4A).

All varieties showed similar levels of myricetin-3-galactoside (M-3-Gal) at fruit set (JD 202). From July 28 (JD 209) to September 8 (JD 251), during fruit development stages, significant differences on M-3-Gal level were observed: HO and ST exhibited higher M-3-Gal levels than other varieties and BL had relatively less M-3-Gal (Figure 2.4B). Similar with Q-3-Gal, M-3-Gal concentrations exhibited no significant relationship with JD in all varieties.

Quercetin-3-arabinofuranoside (Q-3-A-F), the third most abundant flavonol glycoside exhibited significant quadratic relationship with JD in 6 of the 8 varieties (except CQ and BL), in which JD accounted for 83%-93% of the variation (Table 2.5). All varieties exhibited highest levels of Q-3-A-F in the first 3 sampling dates between late July and early August, during early fruit development, then declined until late September, when Q-3-A-F concentration started to increase again until final harvest (Figure 2.4C). They also exhibited similar levels of Q-3-A-F across most of the sampling period, only CQ and BL showed significant higher Q-3-A-F concentrations than others during early fruit ripening stage on September 8 (JD 251) and September 19 (JD 262), respectively.

Concentration of quercetin-3-rhamnopyranoside (Q-3-R) was significantly different among 8 varieties during sampling period except the final harvest date (JD 279, October 6). ST had highest Q-3-R levels throughout entire sampling period, with an average concentration of 6.03 mg/100g fruit. DM, on the other hand, showed lowest concentrations of Q-3-R during most of the sampling period (Figure 2.4D). Only CQ and BL exhibited a significant relationship (quadratic) between Q-3-R level and JD, in which JD accounted for 88% and 71% of the total Q-3-R variance, respectively (Table 2.5).

Levels of myricetin-3-arabinopyranoside (M-3-A) did not show significant linear/quadratic correlation with JD in all 8 varieties. Significant difference in M-3-A concentrations among varieties was observed from July to August, from fruit set to mid-fruit development stages. All varieties exhibited similar levels of M-3-A for the last two sampling dates representing late fruit maturity. HO had highest M-3-A levels during entire sampling period (Figure 2.4E).

Three varieties MQ, ST and EB showed significant quadratic relationship between their quercetin-3-xylopyranoside (Q-3-X) concentrations and JD. JD accounted for 81%, 78% and 85% of the total variance of Q-3-X levels, respectively (Table 2.5). From July to late September, all varieties showed declined Q-3-X concentration with fluctuations. The level of Q-3-X increased in all varieties except for BL during the last two harvest dates, between September 19 and October 6. BL was the only variety with increased Q-3-X level on September 19 (JD 262), and declined Q-3-X concentrations during the last sampling period (Figure 2.4F).

No variety exhibited a significant linear/quadratic relationship between quercetin-3-arabinopyranoside (Q-3-A-P) concentrations and JD. ST showed relatively higher concentrations of Q-3-A-P from late July to early August, during early fruit development, then followed by reduced Q-3-A-P levels during fruit final development and early ripening stages, until late September. DM, on the other hand, had lowest concentrations of Q-3-A-P during most of the sampling period from July to early September, with Q-3-A-P concentrations less than 1 mg/100g fruit (Figure 2.4G).

As a minor flavonol glycoside quercetin-3-glucoside (Q-3-Glu) accounted for 1.6%-5.0% of total quantified flavonols. All 8 varieties exhibited significant linear and quadratic relationship between Q-3-Glu concentrations and JD, and in quadratic fit, JD accounted for 77%-90% of the total variance (Table 2.5). In general, concentrations of Q-3-Glu increased during fruit development and maturation, from an average 0.8 mg/100g fruit on fruit set (JD 202, July 21) to 1.8 mg/100g fruit on last harvest date at October 6 (JD 279) (Figure 2.4H). Except August 28 (JD 240) and September 8 (JD 251), all varieties exhibited similar levels of Q-3-Glu.

Proanthocyanidins

Figure 2.5 shows HPLC-fluorescence chromatograph of cranberry PACs at excitation/emission of 280/308 nm and Table 2.3 shows the identification of selected PAC peaks. PAC oligomers after DP-7 were identified by comparing their retention times with previously published data [26].

Eight cranberry varieties showed significantly different levels of total PACs. During entire sampling period, HO exhibited highest levels of PACs, with average concentration of 594 mg/100g fruit. CQ and BL showed lowest amount of PACs, with average PAC concentrations of 331 and 337 mg/100g fruit, respectively (Figure 2.6A). Similar with flavonol glycosides, all varieties exhibited similar compositions of individual PACs. PAC polymers with DP greater than 10 (DP-11+) accounted for 45%-55% (w/w) of total PACs, followed by DP-4 (14%-18%), DP-9,10 (10%-12%), DP-6 (7%-8%), DP-3 (6%-8%), DP-7,8 (3%-5%), DP-2 (2%-3%), DP-5 (1%-2%) and epicatechin (<1%). In all varieties, levels of PACs declined during fruit development, and increased after late September

during fruits' final ripening. As a result, significant linear/quadratic relationships were observed between PAC concentrations and JD.

Unlike flavonol glycosides where individual compounds exhibited distinct concentration patterns in same cranberry variety, individual PACs shared same levels of concentration patterns in one variety. HO possessed highest levels of total and individual PACs among varieties. Concentrations of individual PACs in HO continuously declined from fruit set (JD 202, July 21) to early fruit development stage (JD 230, August 18), increased between August 18 (JD 230) and August 28 (JD 240), further declined during ripening (JD 262, September 19), and stayed at similar levels at the later fruit ripening stage (Figure 2.6B). In quadratic regression JD accounted for 89%-94% of the variation in PAC concentrations (Table 2.5).

EB and #35 exhibited similar concentrations of individual PACs during fruit development and ripening, which were higher than ST, MQ, DM, BL and CQ. In EB, except DP-6, concentrations of individual PACs declined by 34%-63% from fruit set (JD 202, July 21) to fruit development (JD 251, September 8), and slightly increased until the last harvest date. DP-6 first increased during early fruit development between July 21 (JD 202) and July 28 (JD 209) and then showed similar concentration pattern (Figure 2.6C). In quadratic relationship JD accounted for 92%-99% of the total variation in PAC concentrations except DP-6 (85%) (Table 2.5). In #35, levels of individual PACs declined gradually by 47%-57% from fruit set (JD 202, July 21) to the final fruit development stage (JD 262, September 19), then slightly increased during the last sampling period (Figure 2.6D). Similar with EB, JD accounted for 94%-99% of the PAC variations in #35 under quadratic fit.

MQ, ST and DM exhibited similar PAC concentrations and concentration patterns. Levels of individual PACs sharply declined during early fruit development between July 28 (JD 209) and August 7 (JD 219) in the 3 varieties, and increased during the last sampling period. Figure 6E shows the PAC accumulation pattern in MQ during fruit development and ripening. In quadratic relationship, JD accounted for 86%-96% of the PAC variations in DM, 71%-88% in MQ (except DP-7, 8) and 75%-88% in CQ (Table 2.5).

BL and CQ contained the lowest levels of total and individual PACs. In BL concentrations of individual PACs showed minor changes during the first (July 21-July 28, JD 202-JD 209) and last (September 19-October 6, JD 262-JD 279) sampling periods, and declined between the two periods (Figure 2.6F). The quadratic relationship with JD accounted for 91%-97% of the PAC variation. CQ showed consistent PAC concentrations from fruit set (JD 202, July 21) to final fruit development stage (September 19, JD 262), followed by slight increase in the last sampling period. JD accounted for 84%-92% of the PAC variations of CQ in quadratic fit.

Organic Acids

Cranberry organic acids, including quinic, malic, citric and benzoic acids were identified and quantified by HPLC-PDA. Figure 2.7 shows the HPLC-PDA chromatograph of cranberry organic acid extract. Table 2.4 shows the identities of assigned peaks.

As shown in Figure 2.8A, concentrations of quinic acid gradually declined during fruit development and ripening, from an average 27.7 mg/g at fruit set (JD 202, July 21) to 16.0 mg/g at fully mature fruit (JD 279, October 6). Significant linear/quadratic

correlations were observed between JD and quinic acid concentrations, in quadratic fit, JD accounted for 94%-97% of the quinic acid variation (Table 2.5). Varieties exhibited significantly different levels of quinic acid during sampling period. HO and #35 had the highest quinic acid levels, with average quinic acid levels at 26.1 and 24.6 mg/g fruit. MQ and ST showed average quinic acid levels at 22.0 and 21.2 mg/g fruit, followed by BL (18.4 mg/g), EB (18.0 mg/g) and DM (17.8 mg/g). CQ had lowest quinic acid levels at an average of 17.0 mg/g fruit.

Malic acid concentration increased during cranberry fruit development and ripening in 8 varieties, with average concentration increasing from 6.4 mg/g at fruit set (JD 202, july 21) to 8.3 mg/g at fully mature fruit (JD 279, October 6) (Figure 2.8B). Except for EB, the varieties showed significant linear/quadratic relationships between malic acid concentrations and JD, in quadratic correlation JD accounted for 74%-93% of the total variations (Table 2.5). All varieties had similar levels of malic acid during the final fruit ripening stage between September 19 (JD 262) to October 6 (JD 279). Before September 19 (JD 262), significant differences in malic acid levels were observed, as DM and CQ exhibited highest malic acid concentrations while ST possessed lowest levels of malic acid during entire sampling period.

Concentrations of citric acid fluctuated in most of the varieties over the sampling period (Figure 2.8C). Only MQ and DM showed significant quadratic relationship between their citric acid concentrations and JD, in which JD accounted for 81% of the total variation (Table 2.5). Different varieties exhibited significantly different levels of citric acid during most of the sampling period except August 28 (JD 240). ST, EB and BL showed highest

levels of citric acid during sampling period, with average citric levels at 10.5, 10.1 and 9.3 mg/g fruit, respectively.

Benzoic acid was detected in extremely low concentrations at the first sampling date and increased slowly through July, with concentrations less than 0.01mg/g fruit in all varieties. From August benzoic acid accumulated rapidly in all varieties, along with fruit development, and further substantially increased from middle September to October during fruits' final ripening (Figure 2.8D). Average concentration of benzoic acid on October 6 (JD 279) was 0.08mg/g fruit among 8 varieties. In significant quadratic fit JD accounted for at least 98% of the total benzoic acid variation in 6 out of 8 varieties, except MQ (92%) and BL (88%) (Table 2.5). All 8 varieties did not show significant difference in their benzoic acid concentrations in the final harvest.

2.4 Discussion

Flavonoids are plant secondary metabolites widely spread across different species. They are reported to provide UV protection, play important role in plant sexual reproduction, contribute to plant structure formation and also have anti-herbivory effects [4, 28]. Organic acids in plants are utilized as important intermediates in photosynthesis; they also contribute to plants' reaction with nutrient deficiencies and plant-microbe interaction [29]. Composition and accumulation of both flavonoids and organic acids varies depending on plant species, tissues and growth stages [21, 29-31]. In current study we reported the concentrations and compositions of flavonoids and organic acids, including total anthocyanin, individual flavonols, PACs and organic acids, in cranberry fruit from 8 varieties through fruit development and ripening.

Cranberry fruit development can be visually differentiated by fruit size and color transition from green to red results from anthocyanin accumulation. Levels and accumulation rates of anthocyanins varied across different cranberry varieties during fruit development and ripening. Four early-ripening varieties exhibited not only earlier significant anthocyanin accumulation but also higher anthocyanin levels at the final harvest. This result is consistent with our previous report comparing two varieties ST and BL [21].

Flavonols, e.g. quercetin, and PACs have been studied besides anthocyanins. All 8 varieties showed fluctuated levels of total flavonol glycosides during fruit development and ripening. However, 6 varieties had significantly increased flavonol glycoside levels between the last two sampling dates, being the highest at the later fruit maturity date, except BL (significant decline) and BL (no significant change). Similar accumulation pattern for BL and ST was observed before [21]. Flavonol levels vary in different fruit materials and fruit stages. Quercetin glycosides were reported to decrease by 50% during fruit development in skin of two apple varieties Granny Smith and Splendour, and increased during ripening in Splandour [32]. Flavonol aglycones (based on a quantification method employing hydrolysis) were at lower concentrations in mature fruits of red/white-currants, plums, blueberries and cherries [33]. Similar to cranberry, in grapes total flavonols were found to decline during berry development but increase during ripening [34].

All varieties share similar flavonol glycoside profile during fruit development and ripening. Quercetin-3-galactoside and myricetin-3-galactoside appeared to be the most abundant flavonols in all 8 varieties. Using quercetin-3-galactoside as quantification

standard, we previously determined quercetin-3-arabiniofuranoside as the second most abundant flavonol in the varieties ST and BL [21]. In the current study authentic flavonol glycoside standards were used in their quantification to improve accuracy. Among the 8 varieties, ST exhibited significantly higher concentrations of quercetin-3-rhamnopyranoside and quercetin-3-arabinopyranoside during entire or part of the sampling period; HO showed significant higher levels of myricetin-3-galactoside, myricetin-3-arabinopyranoside and DM exhibited lowest concentrations of quercetin-3-rhamnopyranside and quercetin-3-arabinopyranoside during most of the sampling period. Those results indicate potential metabolic differences among cranberry varieties. As individual flavonol glycosides exhibited differential human health-related bioactivities and bioavailabilities [35, 36], understanding their distinct accumulation patterns in different cranberry varieties could benefit health-related application of cranberry products.

Most of the flavonol glycosides didn't show predictable accumulation patterns during cranberry fruit development and ripening. Accumulation of quercetin-3-arabinofuranoside and quercetin-3-glucoside (Figure 2.4C,H) exhibited significant correlation with JD in most varieties, which suggests a temporal regulation of their biosynthetic pathways in cranberry. Although substrate specificity of flavonoid glycosyltransferases (GTs) has not been thoroughly elucidated, in Arabidopsis rhamnosyltransferase and glucosyltransferase were identified possessing specific substrate selectivity [37]. Thus, it's possible that cranberry expresses specific GTs for biosynthesis of different flavonol glycosides. The distinct accumulation patterns between two quercetin-3-arabinoside isomers also indicate the influence of sugar molecule structure on GTs' selectivity.

Total and individual PACs exhibited similar accumulation pattern in all cranberry varieties: their concentrations first declined during fruit development, and then slightly increased during fruit ripening. A similar pattern for total PACs was observed in ST and BL previously [21]. However, here we reported higher concentrations of total PACs in ST (367-615 mg/100g), BL (248-468 mg/100g) with other varieties than previous study (ST: about 90-220 mg/100g; BL: about 50-190 mg/100g). Colorimetric PAC quantification methods (e.g. vanillin assay used in the early study) cannot distinguish individual PAC oligomers and polymers. Due to the nature of reactions accuracy and selectivity is reduced when analyzing samples with complicated flavonoid profiles [38, 39]. With the HPLC-fluorescence method we were able to quantify individual PAC oligamers separately with high sensitivity (detection limit: 0.038-0.076 ng). Using similar method, Gu et al. reported about 418 mg/100g fruit of total PACs in cranberry [40].

PACs occur in many plant species and play important roles in plant protection against pathogens and herbivores [41, 42]. PAC concentrations were reported to decline during fruit ripening in strawberry, bilberry, grape and apple skin [43-46]. In cranberry we observed an increase in the PAC concentration during the final fruit ripening stage. Individual PAC oligomers exhibited similar accumulation patterns in same variety, suggesting a coordinated regulation of their biosynthetic pathways. Although enzymatic biosynthesis of major PAC building blocks catechin and epicatechin has been extensively studied, mechanisms of PAC oligomer and polymer assembly are yet to be elucidated [47].

In the current study, DP-11+ represented the largest portion of PACs, accounted for 45%-55% (w/w) of total PACs in cranberry. Similarly, in Gu's study [40], DP-11+ (>10 mers)

accounted for an average 55.8% (w/w) of total quantified PACs in cranberry fruit. Relative DP of PACs was found to increase sharply during sorghum seed maturation [48]. It was also speculated that loss of astringency during fruit maturation is connected with increased DP of PACs [49]. However, here we observed consistent compositions of individual PACs during entire fruit development and ripening period for all cranberry varieties. Among 8 varieties, HO possessed highest levels of total and individual PACs during entire sampling period. HO also showed high levels of myricetin-3-galactode and myricetin-3-arabinopyranoside during fruit development and ripening. It is notable that HO had the second lowest level of anthocyanins, suggesting that the variety channeled more resources towards PAC and flavonol production than anthocyanin synthesis.

Similar to many other fruits [18, 50-54], quinic, malic and citric acids are the major organic acids found in cranberry. We observed a decline of quinic acid in all cranberry varieties during fruit development and ripening. Similarly, quinic acid has been found to peak at early development stages and then decline in orange, lime, peach and kiwifruit [50-52] or declined continuously during fruit development and maturation in loquat [53]. As the side product of shikimic acid pathway which primarily contributes to aromatic amino acids biosynthesis, it is suggested that quinic acid metabolism can be involved in aromatic biosynthesis regulation [55]. Thus, decline of quinic acid concentrations in cranberry and other fruits during development and maturation could be result from plants' redistribution of resources toward other shikimic acid pathway products.

We also observed a slight increase of malic acid concentration and fluctuating but consistent levels of citric acid in cranberry during the sampling period (July to early October). Both acids exhibit variable concentration patterns in other fruits species: malic

acid concentrations were reported consistent in orange and lime [50]; declined during late fruit development and increase slightly during fruit ripening in peach [51]; peaked at middle stage of fruit development and declined until fruit ripening in loquat [53] or increased during late fruit development and ripening in pineapple [18]. Levels of citric acid have been found to increase during fruit development in acidic citrus fruits and kiwifruit [50, 52]; increase until late fruit development then decline during fruit maturation in peach and pineapple [18, 51], or peak during middle fruit development stage then declined until fruit maturation in loquat [53].

Levels of benzoic acid in cranberry fruits highly parallel with anthocyanins: at extremely low concentrations during early fruit development stages and increase sharply during late fruit development and ripening. Benzoic acid is an important building block of benzoyl group compounds which further contribute to biosynthesis of numerous plant natural products [56]. Benzoic acid is also highly effective against yeast [57], thus the temporal accumulation of benzoic acid in cranberry fruit could possibly contribute to plant's defense against pathogens.

In summary, the current study revealed that fruit development and ripening process as well as genetic variation (varietal) can all affect cranberry flavonoids and organic acids biosynthesis and composition. As such, phytochemicals carry important function in plants and also possess important human health benefits, further studies will focus on evaluating the effect of different environmental and biological conditions, such as temperature, irrigation, soil pH, light exposure as well as pathogen infection, on the production and accumulation of such phytochemicals in cranberry. Anthocyanins and organic acids are major factors affecting the quality (color, flavor) of cranberry fruits and related products.

Flavonols and PACs are important secondary metabolites with great health benefit potential. Understanding their accumulation details will add insights to the study of their biosynthetic pathways and contribute to improving cranberry breeding and harvest strategy.

2.5 References

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2.6 Tables and Figures

Table 2.1. Solvent systems and elution gradients for HPLC analysis of flavonols, PACs and organic acids in cranberry.

Flavonols				**PACs**				**Organic Acids**			
Time (min)	**Flow (ml/min)**	**A (%)**	**B (%)**	**Time**	**Flow**	**A**	**B**	**Time**	**Flow**	**A**	**B**
0	1	100	0	0	1	100	0	0	0.6	100	0
1	1	85	15	5	1	90	10	11	0.6	100	0
5	1	84	16	7	1	88	12	13	0.6	80	20
10	1	84	16	8	1	88	12	18	0.6	40	60
25	1	83	17	10	1	87	13	20	0.6	20	80
28	1	83	17	15	1	80	20	25	0.6	20	80
30	1	70	30	35	1	60	40	28	0.6	80	20
38	1	55	45	45	1	100	0	30	0.6	100	0
40	1	20	80	50	1	100	0	40	0.6	100	0
43	1	100	0								
50	1	100	0								
Solvent A	0.1% formic acid in water			0.1% acetic acid in acetonitrile				0.5% phosphoric acid in water			
Solvent B	0.1% formic acid in acetonitrile			0.1% acetic acid in 95% methanol/water				0.5% phosphoric acid in acetonitrile			

Table 2.2. Retention time, UV λ_{max}, m/z value of $[M-H]^-$ and fragment ions in LC-MS/MS and peak identities of cranberry flavonols.

Peak	Retention Time (min)	λ_{max} (nm)	$[M-H]^-$ and Fragment Ions in APCI MS-MS (m/z)	Peak Identity
1	10.58	257.2, 355.9	479, 317	myricetin-3-galactoside
2	15.39	260.8, 353.6	449, 317	myricetin-3-arabinofuranoside
3	16.72	254.9, 353.6	463, 301	quercetin-3-galactoside
4	17.93	253.7, 352.4	463, 301	quercetin-3-glucoside
5	20.91	254.9, 352.4	433, 301	quercetin-3-xylopyranoside
6	22.41	257.2, 353.6	433, 301	quercetin-3-arabinopyranoside
7	25.05	254.9, 352.4	433, 301	quercetin-3-arabinofuranoside
8	27.20	254.9, 348.8	449, 301	quercetin-3-rhamnopyranoside
9	36.28	254.9, 365.7	301, 179	quercetin (aglycone)

Table 2.3. Retention time, $\lambda_{excitation}/\lambda_{emission}$, m/z value of $[M-H]^-$ in LC-MS, DPs, and A-type linkage numbers of analyzed cranberry PACs.

Peak	Retention Time (min)	Excitation/ Emission λ_{max} (nm)	$[M-H]^-$ in APCI MS (m/z)	No. of A-type linkage	Degree of Polymerization	Peak Identity
1	11.04	280/308	575.4	1	2	Dimer
2	15.48	280/308	864.0	1	3	Trimer
3	16.71	280/308	864.1	1	3	Trimer
4	20.26	280/308	1149.7	2	4	Tetramer
5	21.20	280/308	1151.7	1	4	Tetramer
6	22.22	280/308	1438.2	2	5	Pentamer
7	23.06	280/308	1440.4	1	5	Pentamer
8	24.23	280/308	1729.4	1	6	Hexamer
9	25.89	280/308	1729.0	1	6	Hexamer
10	27.29	280/308	2014.2	2	7	Heptamer

Table 2.4. Retention times, UV λ_{max} and peak identities of analyzed cranberry organic acids.

Peak Number	Retention Time (min)	λ_{max} (nm)	Peak Identity
1	6.263	214	quinic acid
2	7.140	210	malic acid
3	11.407	210	citric acid
4	21.267	230, 276	benzoic acid

Table 2.5. Relationship between individual flavonols glycoside, PAC and organic acid levels with Julian Day in 8 cranberry varieties. x: Julian Day, y: compound concentration (mg/100g fruit), NA: not available.

A. Mullica Queen:

Compound		R^2		*p* value		Equation
		Linear	Quadratic	Linear	Quadratic	
Flavonols	M-3-Gal	0.046	0.436	0.611	0.238	NA
	M-3-A	0.439	0.749	0.073	0.031	NA
	Q-3-Gal	0.104	0.613	0.435	0.093	NA
	Q-3-Glu	0.901	0.901	<0.001	0.003	$y = -8\times10\text{-}6x^2 + 0.0158x - 2.060$
	Q-3-X	0.322	0.811	0.142	0.016	$y = 0.0005x^2 - 0.256x + 33.011$
	Q-3-A-P	0.002	0.626	0.909	0.085	NA
	Q-3-A-F	0.570	0.929	0.030	0.001	$y = 0.0017x^2 - 0.8632x + 112.11$
	Q-3-R	0.026	0.698	0.703	0.05	NA
PACs	Epicatechin	0.552	0.857	0.035	0.008	$y = 0.0007x^2 - 0.3334x + 44.847$
	DP-2	0.693	0.880	0.010	0.005	$y = 0.0023x^2 - 1.1814x + 163.99$
	DP-3	0.565	0.792	0.031	0.020	$y = 0.0051x^2 - 2.6483x + 367.18$
	DP-4	0.524	0.763	0.042	0.027	$y = 0.0117x^2 - 5.9822x + 827.86$
	DP-5	0.617	0.857	0.021	0.008	$y = 0.0013x^2 - 0.6937x + 95.282$
	DP-6	0.498	0.751	0.051	0.031	$y = 0.0073x^2 - 3.7483x + 508.69$
	DP-7,8	0.563	0.683	0.032	0.052	$y = -0.1241x + 48.61$
	DP-9,10	0.620	0.867	0.020	0.006	$y = 0.0095x^2 - 4.8742x + 666.93$
	DP-11+	0.534	0.709	0.039	0.046	$y = 0.036x^2 - 18.649x + 2606$
Organic acids	Quinic acid	0.793	0.941	0.003	<0.001	$y = 0.003x^2 - 1.5987x + 229.88$
	Malic acid	0.609	0.756	0.022	0.029	$y = 0.0005x^2 - 0.2244x + 30.974$
	Citric acid	0.760	0.805	0.005	0.017	$y = -0.0005x^2 + 0.1956x - 9.627$
	Benzoic acid	0.916	0.936	<0.001	0.001	$y = 7\times10^{-6}x^2 - 0.0021x + 0.1628$

B. Demoranville:

Compound		R^2		*p* value		Equation
		Linear	Quadratic	Linear	Quadratic	
Flavonols	M-3-Gal	0.059	0.076	0.563	0.822	NA
	M-3-A	0.015	0.065	0.774	0.844	NA
	Q-3-Gal	0.002	0.297	0.922	0.414	NA
	Q-3-Glu	0.839	0.856	0.001	0.008	$y = 8\times10^{-5}x^2 - 0.0269x + 2.6711$
	Q-3-X	0.026	0.474	0.701	0.200	NA
	Q-3-A-P	0.118	0.521	0.405	0.109	NA
	Q-3-A-F	0.270	0.743	0.187	0.033	$y = 0.0013x^2 - 0.651x + 83.499$
	Q-3-R	0.223	0.662	0.238	0.066	NA
PACs	Epicatechin	0.715	0.954	0.008	<0.001	$y = 0.0009x^2 - 0.4669x + 62.887$
	DP-2	0.800	0.956	0.003	<0.001	$y = 0.0027x^2 - 1.4055x + 193.37$
	DP-3	0.722	0.930	0.008	0.001	$y = 0.0062x^2 - 3.2421x + 441.02$
	DP-4	0.722	0.936	0.008	0.001	$y = 0.0147x^2 - 7.6499x + 1040.1$
	DP-5	0.640	0.857	0.017	0.008	$y = 0.0014x^2 - 0.7071x + 95.344$
	DP-6	0.677	0.913	0.012	0.002	$y = 0.0076x^2 - 3.9083x + 527.53$
	DP-7,8	0.689	0.896	0.011	0.004	$y = 0.0036x^2 - 1.8696x + 254.05$
	DP-9,10	0.705	0.913	0.009	0.002	$y = 0.0107x^2 - 5.5931x + 757.2$
	DP-11+	0.683	0.881	0.011	0.005	$y = 0.0413x^2 - 21.533x + 2940.2$
Organic acids	Quinic acid	0.838	0.963	0.001	<0.001	$y = 0.0025x^2 - 1.3317x + 192.64$
	Malic acid	0.641	0.928	0.017	0.001	$y = -0.0009x^2 + 0.4536x - 48.89$
	Citric acid	0.388	0.804	0.099	0.017	$y = -0.0006x^2 + 0.2717x - 23.48$
	Benzoic acid	0.976	0.976	<0.001	<0.001	$y = 1\times10^{-7}x^2 + 0.0012x - 0.2453$

C. Crimson Queen:

Compound		R^2		*p* value		Equation
		Linear	Quadratic	Linear	Quadratic	
Flavonols	M-3-Gal	0.118	0.123	0.405	0.720	NA
	M-3-A	0.153	0.184	0.338	0.602	NA
	Q-3-Gal	0.012	0.016	0.795	0.959	NA
	Q-3-Glu	0.769	0.786	0.004	0.021	$y = -1\times10^{-4}x^2 + 0.0621x - 7.7812$
	Q-3-X	0.033	0.065	0.665	0.846	NA
	Q-3-A-P	0.381	0.383	0.103	0.299	NA
	Q-3-A-F	0.301	0.504	0.159	0.173	NA
	Q-3-R	0.728	0.882	0.007	0.005	$y = 0.0006x^2 - 0.2712x + 32.651$
PACs	Epicatechin	0.857	0.957	<0.001	<0.001	$y = 0.0004x^2 - 0.2399x + 34.076$
	DP-2	0.920	0.992	<0.001	<0.001	$y = 0.0011x^2 - 0.5897x + 89.154$
	DP-3	0.878	0.981	<0.001	<0.001	$y = 0.0024x^2 - 1.3018x + 195.19$
	DP-4	0.866	0.965	<0.001	<0.001	$y = 0.0053x^2 - 2.8781x + 435.19$
	DP-5	0.821	0.944	0.002	<0.001	$y = 0.0005x^2 - 0.2815x + 41.539$
	DP-6	0.861	0.961	<0.001	<0.001	$y = 0.0026x^2 - 1.4438x + 218.16$
	DP-7,8	0.843	0.970	0.001	<0.001	$y = 0.0016x^2 - 0.8845x + 128.79$
	DP-9,10	0.878	0.979	<0.001	<0.001	$y = 0.0043x^2 - 2.3356x + 346.85$
	DP-11+	0.858	0.968	<0.001	<0.001	$y = 0.0165x^2 - 8.9199x + 1341.8$
Organic acids	Quinic acid	0.873	0.939	<0.001	<0.001	$y = 0.0015x^2 - 0.8181x + 127.9$
	Malic acid	0.529	0.909	0.041	0.002	$y = -0.0012x^2 + 0.6176x - 68.40$
	Citric acid	0.477	0.478	0.058	0.197	NA
	Benzoic acid	0.962	0.981	<0.001	<0.001	$y = -5\times10^{-6}x^2 + 0.0032x - 0.4465$

D. Stevens:

Compound		R^2		*p* value		Equation
		Linear	Quadratic	Linear	Quadratic	
Flavonols	M-3-Gal	0.034	0.285	0.664	0.432	NA
	M-3-A	0.048	0.286	0.603	0.431	NA
	Q-3-Gal	0.351	0.568	0.121	0.123	NA
	Q-3-Glu	0.899	0.899	<0.001	0.003	$y = -3\times10^{-6}x^2 + 0.0143x - 1.834$
	Q-3-X	0.604	0.777	0.023	0.024	$y = 0.0002x^2 - 0.1154x + 16.227$
	Q-3-A-P	0.147	0.464	0.349	0.212	NA
	Q-3-A-F	0.708	0.826	0.009	0.013	$y = 0.0008x^2 - 0.4365x + 61.38$
	Q-3-R	0.031	0.341	0.679	0.353	NA
PACs	Epicatechin	0.788	0.879	0.020	0.005	$y = 0.0009x^2 - 0.4445x + 59.905$
	DP-2	0.715	0.850	0.008	0.009	$y = 0.0021x^2 - 1.0931x + 155.12$
	DP-3	0.624	0.797	0.020	0.019	$y = 0.0049x^2 - 2.5511x + 357.99$
	DP-4	0.612	0.794	0.022	0.019	$y = 0.0116x^2 - 6.0407x + 846.08$
	DP-5	0.544	0.799	0.037	0.018	$y = 0.0013x^2 - 0.6473x + 88.195$
	DP-6	0.577	0.840	0.029	0.010	$y = 0.0076x^2 - 3.8813x + 524.8$
	DP-7,8	0.510	0.751	0.046	0.031	$y = 0.0039x^2 - 1.9972x + 271.63$
	DP-9,10	0.647	0.827	0.016	0.012	$y = 0.0085x^2 - 4.4512x + 619.72$
	DP-11+	0.594	0.787	0.025	0.021	$y = 0.0355x^2 - 18.404x + 2563.8$
Organic acids	Quinic acid	0.793	0.947	0.003	<0.001	$y = 0.003x^2 - 1.596x + 228.41$
	Malic acid	0.869	0.894	<0.001	0.004	$y = -0.0002x^2 + 0.1396x - 14.05$
	Citric acid	0.416	0.495	0.084	0.181	NA
	Benzoic acid	0.947	0.979	<0.001	<0.001	$y = 9\times10^{-6}x^2 - 0.003x + 0.2648$

E. #35:

Compound		R^2		*p* value		Equation
		Linear	Quadratic	Linear	Quadratic	
Flavonols	M-3-Gal	0.119	0.192	0.402	0.580	NA
	M-3-A	0.016	0.030	0.764	0.926	NA
	Q-3-Gal	0.002	0.069	0.924	0.835	NA
	Q-3-Glu	0.729	0.766	0.007	0.026	$y = -0.0001x^2 + 0.082x - 10.08$
	Q-3-X	0.209	0.353	0.255	0.337	NA
	Q-3-A-P	0.017	0.054	0.762	0.871	NA
	Q-3-A-F	0.670	0.834	0.013	0.011	$y = 0.001x^2 - 0.5205x + 71.765$
	Q-3-R	0.003	0.216	0.899	0.544	NA
PACs	Epicatechin	0.803	0.988	0.003	<0.001	$y = 0.0008x^2 - 0.4235x + 58.249$
	DP-2	0.784	0.970	0.003	<0.001	$y = 0.0031x^2 - 1.6072x + 221.9$
	DP-3	0.740	0.940	0.006	<0.001	$y = 0.0062x^2 - 3.2575x + 454.93$
	DP-4	0.766	0.950	0.004	<0.001	$y = 0.0134x^2 - 7.0169x + 987.67$
	DP-5	0.765	0.970	0.004	<0.001	$y = 0.0015x^2 - 0.795x + 110.27$
	DP-6	0.734	0.950	0.007	<0.001	$y = 0.0075x^2 - 3.8881x + 539$
	DP-7,8	0.922	0.951	0.001	<0.001	$y = 0.0032x^2 - 1.7568x + 255.95$
	DP-9,10	0.771	0.969	0.004	<0.001	$y = 0.0103x^2 - 5.3714x + 746.23$
	DP-11+	0.779	0.959	0.004	<0.001	$y = 0.0398x^2 - 20.924x + 2947.1$
Organic acids	Quinic acid	0.871	0.955	<0.001	<0.001	$y = 0.0023x^2 - 1.2637x + 193.93$
	Malic acid	0.899	0.918	<0.001	0.002	$y = -0.0002x^2 + 0.1411x - 13.01$
	Citric acid	0.355	0.687	0.119	0.055	NA
	Benzoic acid	0.980	0.981	<0.001	<0.001	$y = -2\times10^{-6}x^2 + 0.0019x - 0.3231$

F. Early Black:

Compound		R^2		*p* value		Equation
		Linear	Quadratic	Linear	Quadratic	
Flavonols	M-3-Gal	0.005	0.106	0.874	0.756	NA
	M-3-A	0.256	0.413	0.201	0.264	NA
	Q-3-Gal	0.116	0.528	0.410	0.153	NA
	Q-3-Glu	0.758	0.869	0.005	0.006	$y = -0.0002x^2 + 0.0819x - 9.534$
	Q-3-X	0.456	0.845	0.066	0.009	$y = 0.0002x^2 - 0.1151x + 15.754$
	Q-3-A-P	0.039	0.402	0.638	0.277	NA
	Q-3-A-F	0.723	0.924	0.008	0.002	$y = 0.0008x^2 - 0.4096x + 56.473$
	Q-3-R	0.156	0.243	0.332	0.498	NA
PACs	Epicatechin	0.805	0.971	0.003	<0.001	$y = 0.0009x^2 - 0.4793x + 66.057$
	DP-2	0.853	0.986	0.001	<0.001	$y = 0.0023x^2 - 1.2333x + 175.59$
	DP-3	0.756	0.988	0.005	<0.001	$y = 0.0065x^2 - 3.3565x + 462.93$
	DP-4	0.764	0.986	0.005	<0.001	$y = 0.0143x^2 - 7.4464x + 1033.8$
	DP-5	0.657	0.992	0.015	<0.001	$y = 0.0016x^2 - 0.8355x + 112.38$
	DP-6	0.683	0.847	0.011	0.009	$y = 0.0053x^2 - 2.7835x + 399.37$
	DP-7,8	0.630	0.923	0.019	0.002	$y = 0.0068x^2 - 3.5071x + 463.38$
	DP-9,10	0.786	0.988	0.003	<0.001	$y = 0.0098x^2 - 5.1182x + 714.65$
	DP-11+	0.778	0.966	0.004	<0.001	$y = 0.036x^2 - 18.901x + 2693$
Organic acids	Quinic acid	0.797	0.960	0.003	<0.001	$y = 0.0025x^2 - 1.3034x + 186.87$
	Malic acid	0.451	0.452	0.068	0.222	NA
	Citric acid	0.049	0.286	0.599	0.431	NA
	Benzoic acid	0.978	0.978	<0.001	<0.001	$y = -5\times10^{-7}x^2 + 0.0012x - 0.2236$

G. Ben Lear:

Compound		R^2		*p* value		Equation
		Linear	Quadratic	Linear	Quadratic	
Flavonols	M-3-Gal	0.283	0.283	0.175	0.436	NA
	M-3-A	0.117	0.122	0.406	0.722	NA
	Q-3-Gal	0.025	0.106	0.709	0.757	NA
	Q-3-Glu	0.765	0.779	0.004	0.023	$y = -7\times10^{-5}x^2 + 0.045x - 5.5802$
	Q-3-X	0.004	0.175	0.876	0.619	NA
	Q-3-A-P	0.329	0.336	0.137	0.320	NA
	Q-3-A-F	0.259	0.611	0.198	0.094	NA
	Q-3-R	0.568	0.714	0.031	0.044	$y = 0.0005x^2 - 0.2353x + 28.215$
PACs	Epicatechin	0.831	0.932	0.002	<0.001	$y = 0.0006x^2 - 0.3467x + 48.052$
	DP-2	0.837	0.944	0.001	<0.001	$y = 0.0016x^2 - 0.8808x + 125.33$
	DP-3	0.809	0.936	0.002	0.001	$y = 0.0037x^2 - 1.9993x + 283.15$
	DP-4	0.803	0.935	0.003	0.001	$y = 0.0089x^2 - 4.739x + 671.28$
	DP-5	0.734	0.912	0.007	0.002	$y = 0.0008x^2 - 0.4441x + 61.338$
	DP-6	0.782	0.946	0.004	<0.001	$y = 0.0052x^2 - 2.7706x + 384.8$
	DP-7,8	0.828	0.945	0.002	<0.001	$y = 0.002x^2 - 1.0537x + 151.34$
	DP-9,10	0.818	0.944	0.002	<0.001	$y = 0.0064x^2 - 3.4301x + 486.18$
	DP-11+	0.880	0.969	<0.001	<0.001	$y = 0.0201x^2 - 11.082x + 1655.1$
Organic acids	Quinic acid	0.930	0.971	<0.001	<0.001	$y = 0.0012x^2 - 0.7272x + 120.27$
	Malic acid	0.886	0.910	<0.001	0.002	$y = -0.0002x^2 + 0.1487x - 13.97$
	Citric acid	0.244	0.256	0.213	0.478	NA
	Benzoic acid	0.877	0.881	<0.001	0.005	$y = -4\times10^{-6}x^2 + 0.0033x - 0.5095$

H. Howes:

Compound		R^2		*p* value		Equation
		Linear	Quadratic	Linear	Quadratic	
Flavonols	M-3-Gal	0.203	0.339	0.263	0.356	NA
	M-3-A	0.220	0.314	0.241	0.390	NA
	Q-3-Gal	0.038	0.231	0.644	0.518	NA
	Q-3-Glu	0.806	0.840	0.002	0.010	$y = -0.0002x^2 + 0.1002x - 12.503$
	Q-3-X	0.420	0.657	0.082	0.069	NA
	Q-3-A-P	0.032	0.422	0.674	0.254	NA
	Q-3-A-F	0.739	0.927	0.006	0.001	$y = 0.0009x^2 - 0.4585x + 62.76$
	Q-3-R	0.011	0.802	0.507	0.171	NA
PACs	Epicatechin	0.716	0.889	0.008	0.004	$y = 0.0009x^2 - 0.475x + 66.397$
	DP-2	0.876	0.944	<0.001	<0.001	$y = 0.002x^2 - 1.1089x + 168.66$
	DP-3	0.839	0.908	0.001	0.003	$y = 0.0041x^2 - 2.3044x + 355.6$
	DP-4	0.829	0.897	0.002	0.003	$y = 0.0092x^2 - 5.126x + 792.54$
	DP-5	0.853	0.939	0.001	<0.001	$y = 0.0012x^2 - 0.6489x + 96.913$
	DP-6	0.814	0.914	0.002	0.002	$y = 0.0062x^2 - 3.3791x + 496.72$
	DP-7,8	0.846	0.893	0.001	0.004	$y = 0.0021x^2 - 1.2008x + 191.62$
	DP-9,10	0.811	0.924	0.002	0.002	$y = 0.0089x^2 - 4.7918x + 695.45$
	DP-11+	0.821	0.936	0.002	0.001	$y = 0.0377x^2 - 20.354x + 2956.6$
Organic acids	Quinic acid	0.924	0.959	<0.001	<0.001	$y = 0.0014x^2 - 0.8573x + 147.56$
	Malic acid	0.681	0.738	0.012	0.035	$y = -0.0005x^2 + 0.273x - 29.808$
	Citric acid	0.022	0.475	0.728	0.200	NA
	Benzoic acid	0.960	0.979	<0.001	<0.001	$y = 9\times10^{-6}x^2 - 0.0029x + 0.2184$

Figure 2.1. Accumulation of total monomeric anthocyanins in 8 cranberry varieties during fruit development and ripening.

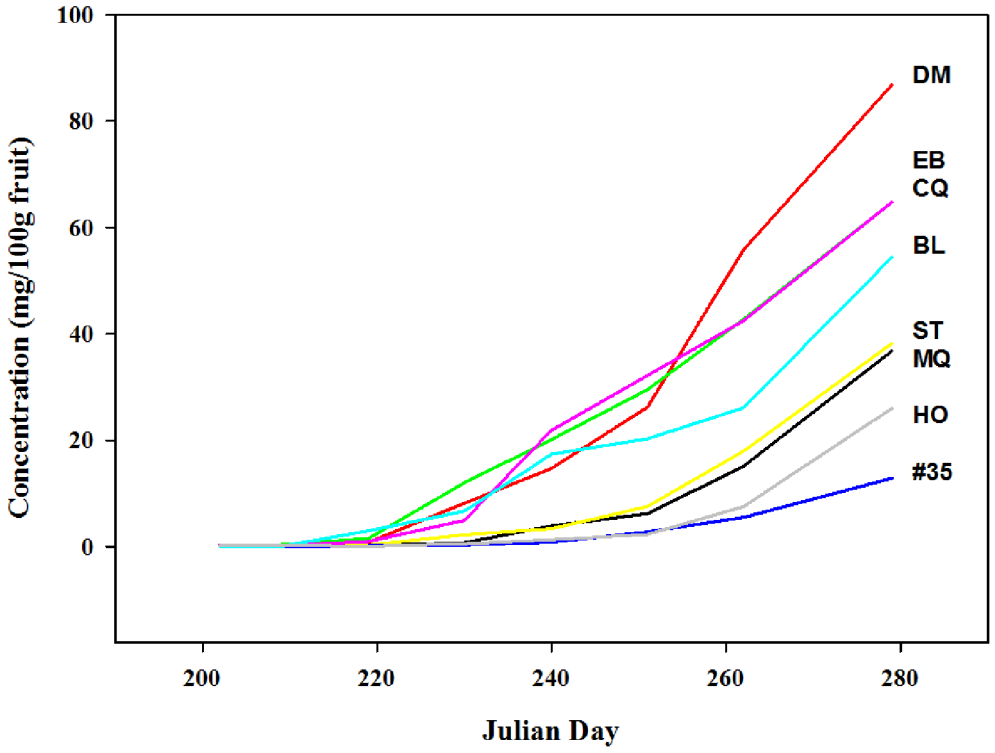


Figure 2.2. HPLC chromatograph of cranberry flavonols purified by Sephadex® LH-20 column. All peaks were detected at UV absorbance of 366 nm. Labels 1-9 on peaks correspond to peaks 1-9 in Table 2.2.

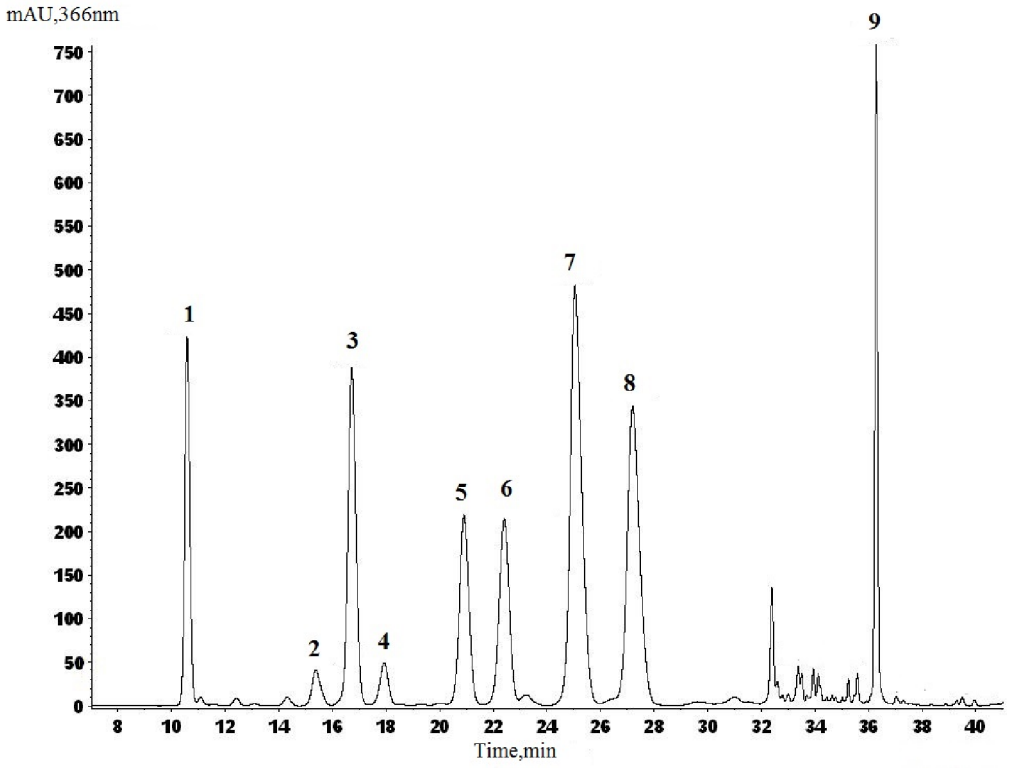


Figure 2.3. Accumulation and composition of cranberry flavonols during fruit development and ripening. (A) Average composition of individual flavonol glycosides in 8 cranberry varieties. (B) Accumulation of total quantified flavonols in 8 cranberry varieties during fruit development and ripening.

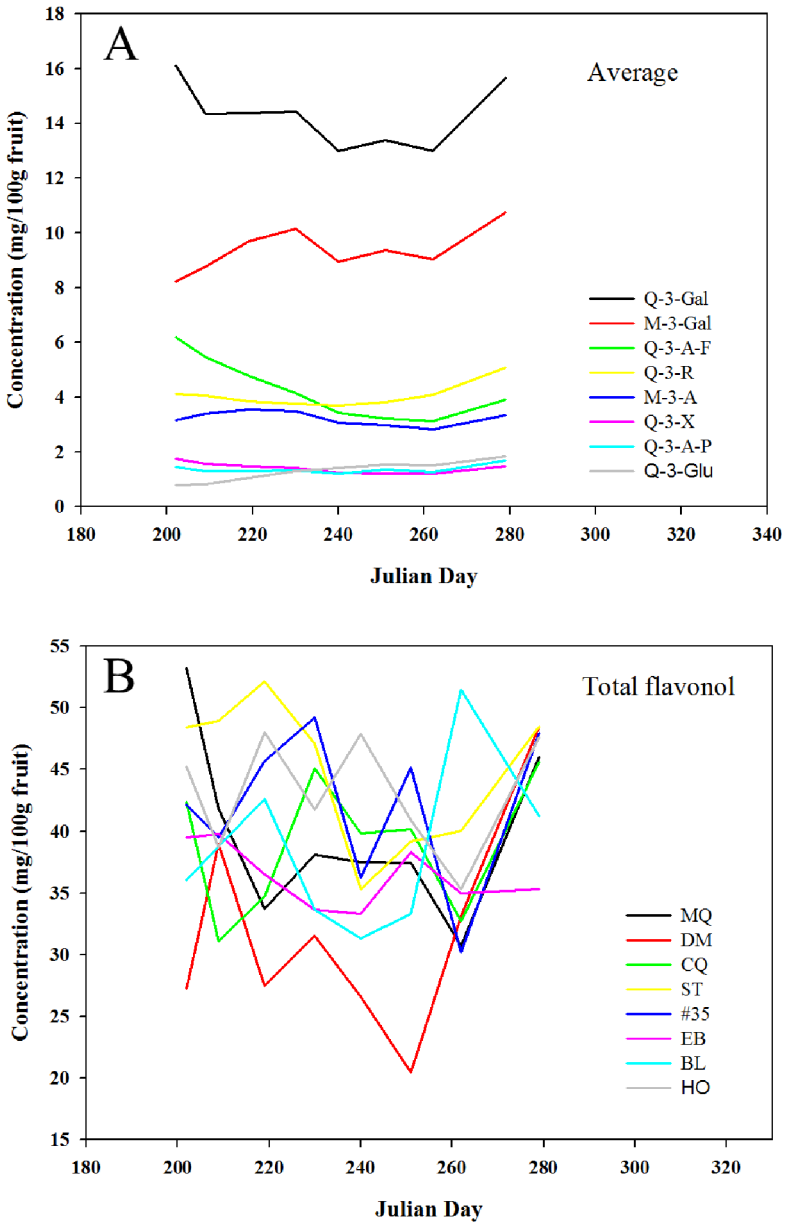
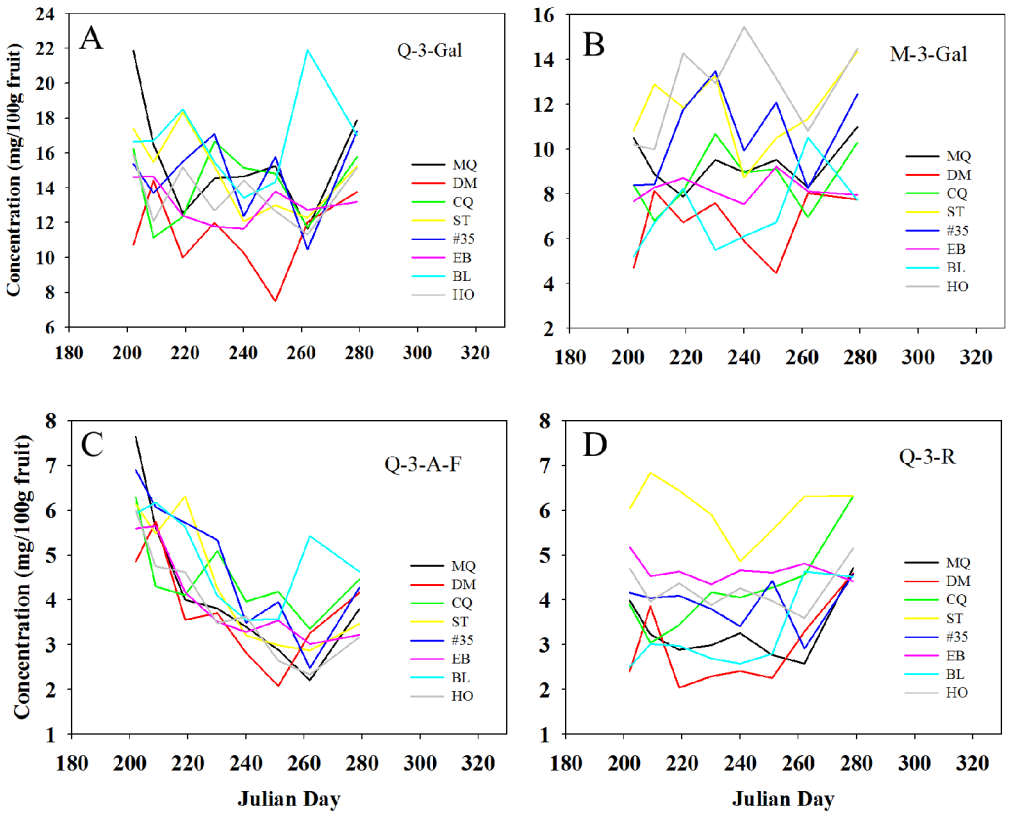


Figure 2.4. Accumulation of individual flavonol glycosides in 8 cranberry varieties during fruit development and ripening. (A) quercetin-3-galactoside; (B) myricetin-3-galactoside; (C) quercetin-3-arabinofuranoside; (D) quercetin-3-rhamnopyranside; (E) myricetin-3-arabinpyranoside; (F) quercetin-3-xylopyranoside; (G) quercetin-3-arabinopyranoside; (H) quercetin-3-glucoside.



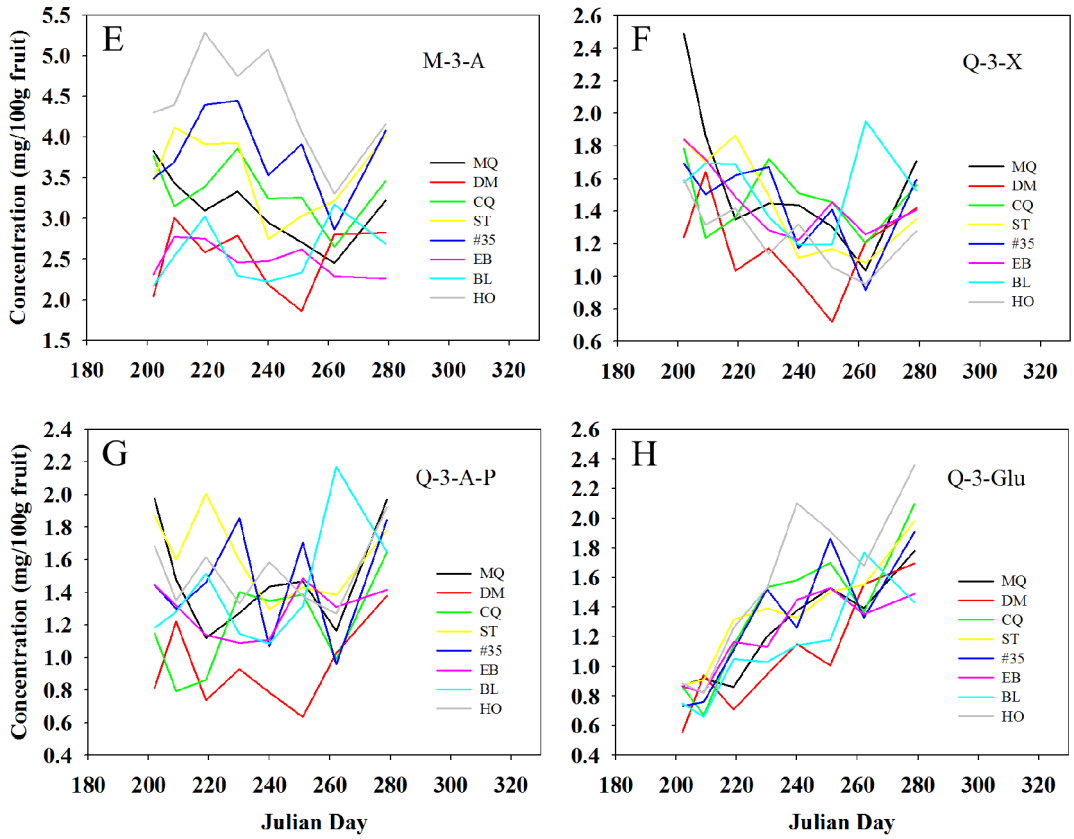

E
M-3-A
F
Q-3-X
G
Q-3-A-P
H
Q-3-Glu
MQ
DM
CQ
ST
#35
EB
BL
HO
Concentration (mg/100g fruit)
Julian Day

Figure 2.5. HPLC chromatogram of cranberry PACs purified by Sephadex® LH-20 column. All peaks were detected at 280/308 nm of excitation/emission in fluorescence. Labels 1-10 on peaks correspond to peaks 1-10 in Table 2.3.

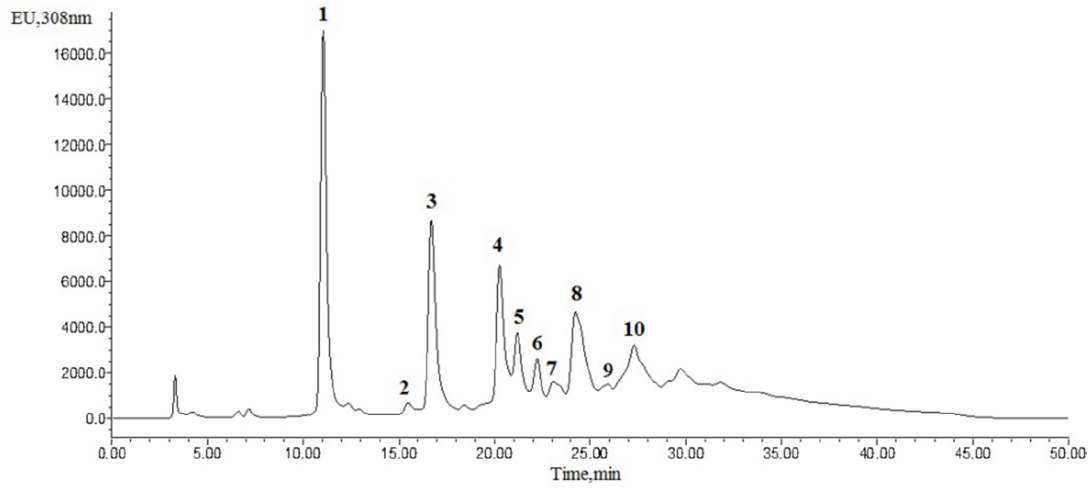


Figure 2.6. Accumulations of PACs during cranberry fruit development and ripening. (A) Accumulation of total PACs during cranberry fruit development and ripening in 8 varieties. (B-F): Accumulation of individual PAC oligomers and polymers during cranberry fruit development and ripening in varieties Howes (B); Early Black (C); #35 (D); Mullica Queen (E) and Ben Lear (F).

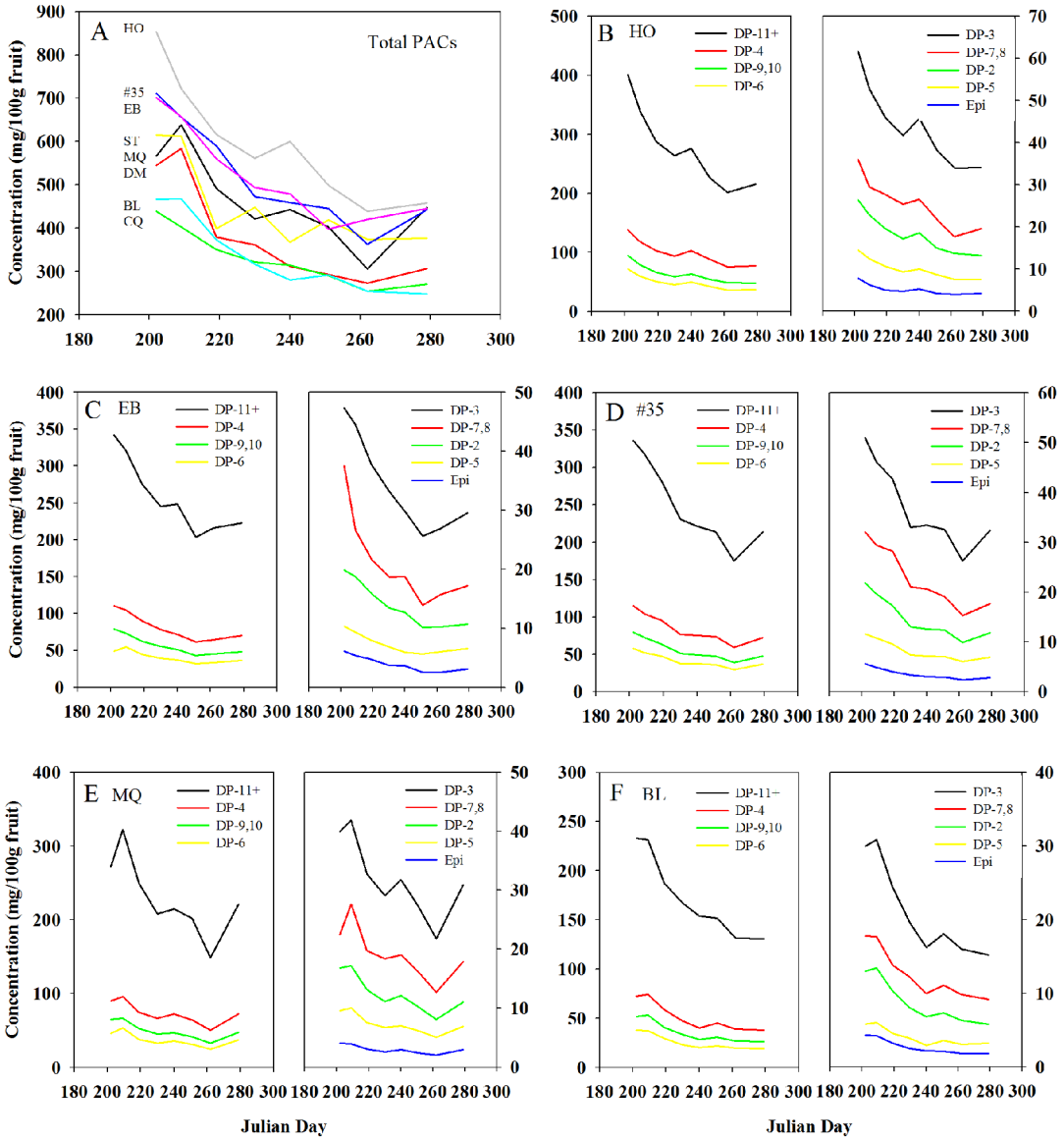


Figure 2.7. HPLC chromatogram of cranberry organic acid at UV absorbance of 210 nm (peaks 1-3) or 230 nm (peak 4). Labels 1,2,3,4 on peaks correspond to peaks 1,2,3,4 in Table 2.4.

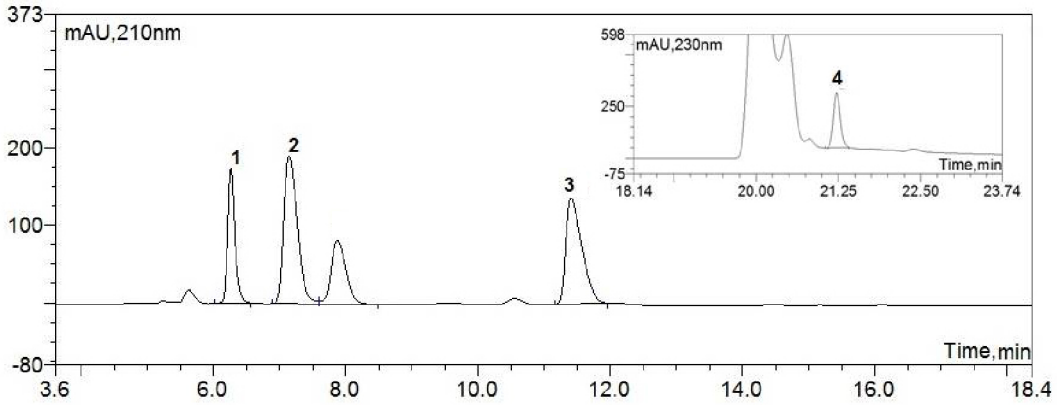
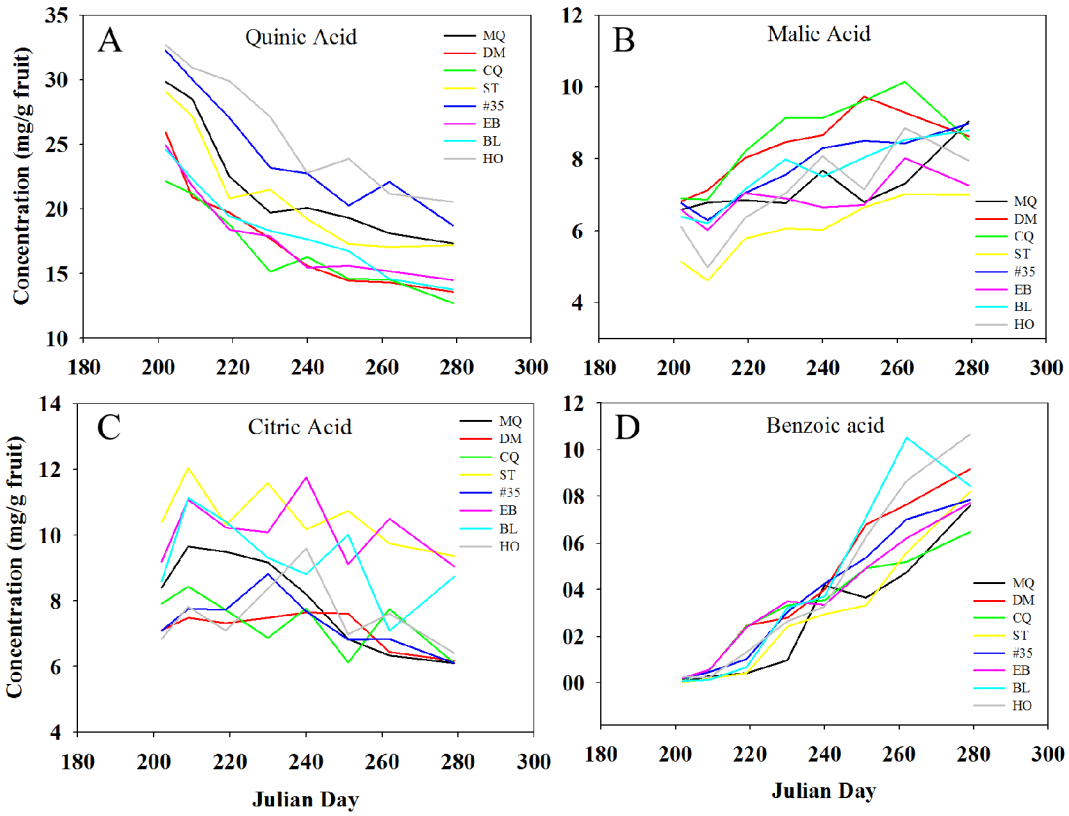


Figure 2.8. Accumulation of quinic acid (A), malic acid (B), citric acid (C) and benzoic acid (D) in 8 cranberry varieties during fruit development and ripening.



Chapter 3

Quantitative Analysis of Proanthocyanidins - Issues with the 4-Dimethylaminocinnamaldehyde (DMAC) Method and Potential Improvement

Wang, Y., A.P. Singh, W.J. Hurst, J.A. Glinski, H. Koo, and N. Vorsa, *Influence of Degree-of-Polymerization and Linkage on the Quantification of Proanthocyanidins using 4-Dimethylaminocinnamaldehyde (DMAC) Assay.* Journal of agricultural and food chemistry, 2016. **64**(11): p. 2190-2199.

3.1 Introduction

Proanthocyanidins (PACs) are oligomeric and polymeric flavan-3-ols, which are members of naturally occurring flavonoid compounds present in plants and plant-derived foods. Dietary sources include fruit, vegetables, cereals and beverages such as wine and tea [1]. PACs are considered to be the most consumed from dietary flavonol class [2] and have been associated with a number of bioactivities beneficial toward human health. PACs have been reported to have anti-oxidant and anti-inflammatory activities which may ameliorate degenerative diseases, anti-cancer properties, as well as improving cardiovascular health [3-9]. PAC oligomers of specific degree-of-polymerization (DP), e.g. DP-4 and 9, from cranberry exhibited high bioactivities against pathogenic oral biofilms and ovarian cancer cell lines [10-13].

The polymeric nature of PACs provides for numerous structural variations, including DP, linkage type and position between constituent units. Catechin and epicatechin (Figure 3.1A, B) are the two most common flavan-3-ol units present in PAC oligomers and polymers. The most common linkage between two linked flavan-3-ol units is a single C-

C bond (B-type), between the C4 of one (referred to as upper) flavan-3-ol unit and the C8 or C6 of the second (lower) unit (Figure 3.1E, F, H). In PACs of cranberry and a few other plant sources, e.g., peanut skin [14, 15] and cinnamon bark [16, 17], in addition to the C-C linkage, there is also an ether linkage between the C2 of upper unit and the oxygen at C7 or C5 of the lower unit forming a double linkage, referred to as A-type, between two subunits of the polymer (Figure 3.1C, D, G, I-M) [1]. In addition, PACs of higher molecular weights, DP > 4, have been identified and isolated from cranberry and cocoa.

Because of their polymeric nature and variations in stereochemistry, the quantification of PACs has been challenging and problematic with sub-optimal analytical methods and lack of available individual PAC standards beyond epicatechin and catechin monomers and certain dimers. PACs are also found to form complexes with other compounds such as anthocyanins during processing of PAC containing fruits [18-20], which further contributes to the intricacy of their quantification analysis. HPLC methods are available for PAC quantification in various plant and food materials [21-26], however, due to utility and reduced costs, previously developed spectrophotometric methods have been continuously optimized to facilitate quantification of PACs in plant tissues, food stuffs and nutraceuticals [27-30].

Two traditionally often used spectrophotometric methods for PAC quantification include the n-butanol hydrochloride assay and the vanillin-acid assay. In n-butanol hydrochloride assay, PACs are first hydrolyzed and converted to anthocyanins, whose absorbance (545-550 nm) is then measured [31, 32]. Incomplete PAC hydrolysis and transformation, side reactions and DP-dependent anthocyanin yield are major disadvantages of the method. In

vanillin-acid assay vanillin aldehyde group reacts with PAC to form a color compound with absorbance at 510 nm. However, in many instances, the presence of anthocyanins, e.g., cranberry, which have similar absorbance spectra (520 nm) may confound the measured absorbance, as well as chlorophyll and ascorbic acid can also affect color development [33, 34]. Moreover, PACs with different DPs have different molar absorption coefficient (MAC) since additional or side reactions appear to take place at sites other than the terminal units [35].

Currently, the 4-dimethylaminocinnamaldehyde (DMAC) assay is favored over the vanillin-acid assay and is being widely used [30, 36-38] for several reasons. Although similar to vanillin-acid assay, DMAC is deemed to have a higher sensitivity [31]. Secondly, light absorbance is measured at 640 nm in the DMAC assay, reducing the likelihood of interference from anthocyanins [34]. However, the postulate that MAC is constant across the various PAC species is the most significant reason. This postulate results from a believe that DMAC reacts selectively with the C8 position of the PAC terminal unit, producing MACs that are constant across structurally diverse PAC oligomers and polymers [31, 37, 39-42]. Based on this postulate, the DMAC assay is thought to be suitable for quantification of PACs among various plant sources and PAC structures. Several DMAC assay protocols have been developed for PAC quantification of different food materials [38, 43, 44].

The premise of 4-dimethylaminocinnamaldehyde's high selectively for the C8 of the terminal unit [39, 40, 42, 45] implies that the various oligomers would have identical or similar MACs [37, 40]. However, the monomers catechin and epicatechin, as well as the procyanidin dimers B1 and B2 did not produce equivalent results in Payne et al.'s study

[30]. Considering the structural variations that exist for PACs across different plant and food stuffs, and that the DMAC assay is being used to compare PACs content across various plant species, organs and foods, the objective of the study, herein, was to verify the validity of the constant MAC in DMAC assay. In this study, we utilized various individual PAC dimers and trimers, as well as a series of oligomeric PAC DPs from cranberry (A-type) and cocoa (B-type), with two often used DMAC assay protocols (methanol vs. ethanol based), to determine the effects of DP, linkage type and position on PACs' reactivity with DMAC reagents, specifically reflected by their MAC in the assay.

3.2 Materials and methods

Chemicals and Reagents

All solvents, including methanol, ethanol, acetone, acetonitrile, ethyl acetate, n-hexane and water were purchased from EMD Millipore (Billerica, MA), and were of HPLC grade. 4-Dimethylaminocinnamaldehyde (DMAC) was purchased from Sigma (St Louis, MO), hydrochloric acid was from Fisher Scientific (Hampton, NH), acetic acid and formic acid were from Avantor Performance Materials (Center Valley, PA). Sephadex® LH-20 gel was purchased from GE Healthcare Bio-Science (Pittsburg, PA) and BAKERBOUND® Diol gel was from Avantor Performance Materials.

Epicatechin, catechin, procyanidin A2, B1 and B2 were purchased from Indofine Chemical Company (Somerville, NJ). Individual cocoa PACs, procyanidin A1, peanut trimers A and C,14 cinnamtannin B-1 and D-1,16 lindetannin17 were kindly provided by Planta Analytica (Danbury, CT). All compounds came as solids with $\geqslant$ 95% purity as assessed by HPLC and LC-MS.

Preparation of DMAC reagent

DMAC reagents were prepared daily before use. Two formulas were used based on previously published studies [40, 44]:

(1) Methanol-based DMAC reagent: 0.1 g DMAC was weighed and added to 25 ml HCI (37%), then brought up to 100 ml volume with methanol.

(2) Ethanol-based DMAC reagent: 0.05 g DMAC was weighed and added to 50 ml acidified ethanol solution. The acidified ethanol solution was prepared by mixing 12.5 ml HCI (37%) with 12.5 ml distilled water and 75 ml 91% ethanol (prepared from HPLC grade ethanol (> 99.9%) with water).

DMAC assay and calculation of molar absorption coefficient

DMAC assay was carried out based on the protocols from previously published studies [40, 44] with sight modification:

(1) Methanol-based DMAC reagent:

PAC stock solutions (1 mg/ml) and dilutions were made with 100% methanol. Mixtures of 0.25 ml sample solution with 1.75 ml DMAC reagent or 0.4 ml sample solution with 1.2 ml DMAC reagent were made in cuvette with 1 cm path length. Maximum absorbance (640 nm) of the PAC-DMAC conjugate was recorded for PAC dimers and trimers (see Results) and absorbance after 9 min of reaction is measured for cranberry and cocoa PAC oligomers (see Results). Absorbance is measured in a GenesysTM 10S UV-Vis spectrophotometer (Thermal Scientific, Waltham, MA).

(2) Ethanol-based DMAC reagent:

PAC stock solutions (1 mg/ml) were made with 91% ethanol. A dilution solution contained 80/20 (v/v) ethanol (91%) and water was prepared to make dilutions from stock. Mixtures of 0.25 ml sample solution with 1.75 ml DMAC reagent or 0.4 ml sample solution with 1.2 ml DMAC reagent were made in a cuvette with 1 cm path length and continuously measured for maximum absorbance (640 nm).

(3) Calculation of molar absorption coefficient (MAC):

Mass concentrations (mg/ml) of PACs were converted to molar concentrations (mol/L) based on compounds' molar mass. For each compound, a standard curve with linear regression equation was generated based on its molar concentration against absorbance. MAC of PAC was calculated based on the Beer-Lambert law: $A= \varepsilon * c * l$, where A is the absorbance, ε (L/mol*cm) is the MAC of the compound, c (mol/L) is the compound molar concentration and l (cm) is the path length. In the calculation, the original concentration of PAC solution before being added to DMAC reagent is used.

Isolation of individual cranberry PAC oligomers

Individual cranberry PACs were isolated with a regular Diol gravity column chromatography as previously reported.46 PAC oligomers with degree-of-polymerization (DP) from 2 to 10 (named DP-2 to DP-10, as solids with $\geqslant$ 95% purity assessed by HPLC and LC-MS) were isolated and used in DMAC assay.

LC-MS analysis for PAC compound identity and purity

Identity and purity of PACs were analyzed by a Dionex UltiMate® 3000 LC system coupled with Applied Biosystems API 3000™ triple quad LC-MS/MS mass spectrometer.

For LC separation, a Develosil® 250 x 4.6 mm 100Diol-5, 5µm LC column was used. A binary solvent system with solvent A (0.1% acetic acid in acetonitrile) and solvent B (0.1% acetonitrile in 95% methanol + 5% water) was used with linear gradient of 0% B to 10% B from 0-5 min; 10% B to 12% B from 5-7 min; isocratic elution of 12% B from 7-8 min; linear gradient of 12% B to 13% B from 8-10 min; 13% B to 20% B from 10-15 min; 20% B to 40% B from 15-35 min; 40% B to 0% B from 35-45 min and isocratic elution of 0% B from 45-50 min at a flow rate of 1 ml/min.

MS data were obtained under atmospheric pressure chemical ionization (APCI) in negative ion detection mode, with following parameters: Curtain gas: 12 psi, Nebulizer gas: 7 psi, Nebulizer current: -2.0 mA, Entrance potential: -10 V, Focusing Potential: -300 V, Declustering potential: -60 V, Source temperature: 500 ºC.

Statistical Analysis

Statistical analysis for difference on MAC values of individual PACs was evaluated by Student–Newman–Keuls test using IBM SPSS Statistics 19 (Armonk, NY).

3.3 Results

The reagent solvent influences DMAC absorbance maximum and time to reach maximum absorbance

To compare the effect of DMAC reagent solvent (methanol or ethanol) on PAC-DMAC reaction, we used commercially available PACs monomers and dimers to carry out DMAC assay with different reagent solvents and PAC/DMAC ratios.

As shown in Figure 3.2, for both methanol-DMAC and ethanol-DMAC reagents, the 1:3 ratio (0.4 ml PAC sample + 1.2 ml DMAC) assay showed higher MAC than 1:7 ratio method (0.25 ml PAC sample + 1.75 ml DMAC) for each of the PAC monomers and dimers. Ratios of MAC in 1:3 vs. 1:7 method ranged from 2.2-2.4 for methanol-DMAC reagent and 1.8-2.1 for ethanol-DMAC reagent. Considering the higher ratio of PACs in the 1:3 method (2.3 times compared to 1:7 method), this result indicate that in both 1:7 and 1:3 method, there is excess of DMAC reagent to fully react with all PAC molecules in the assay.

When comparing the MAC of individual PACs in same DMAC assay, significant differences were observed. PAC monomers epicatechin and catechin generally showed higher MAC than dimers, as was first observed by McMurrough and McDowell [40], except procyanidin A2 in two methanol-DMAC assays (Figure 3.2). In methanol-DMAC assays, procyanidin A2 showed slightly higher MAC than the two monomers. The B-type dimers procyanidin B1 and B2 exhibited significantly lower absorption than monomers. In ethanol-DMAC assays, epicatechin exhibited highest MAC value among all the compounds, however in contrast to methanol-DMAC assays, procyanidin A2 showed the lowest MAC with ethanol-DMAC reagent, which was half the value of epicatechin (Figure 3.2).

The reagent solvent influences absorbance dynamics over time as well as the time required of the PAC-DMAC conjugate to reach maximum absorbance. In methanol-DMAC assays, all tested PACs reached maximum absorbance within 2 min after mixing with DMAC (Figure 3.3A, B), whereas in ethanol-DMAC assays only B-type dimers procyanidin B1 and B2 reached maximum absorbance within 5 min. PAC monomers and

A-type dimer procyanidin A2 reached maximum absorbance only after 15-30 min (Figure 3.3 C, D). In methanol-DMAC assay the conjugate absorbance declines shortly after the maximum is reached, whereas the ethanol-DMAC conjugate maintains absorbance at maximal level.

The methanol-DMAC assay with 0.25 ml sample/1.75 ml DMAC reagent ratio was selected for the subsequent studies because it was the original protocol for the DMAC assay [40] and similar methanol-reagents were used by others [42, 43, 45, 46]. It also provides better solubility for the highly polymeric PAC-DMAC complex as no precipitation was observed in our study, in contrast to what Prior and Gu has reported [37]. The methanol-DMAC assay protocol is being used in our lab for routine cranberry PAC quantification and yields consistent results.

PAC dimers and trimers exhibit differential MAC in DMAC assay

Preliminary DMAC method comparison revealed differences in MAC for PAC monomers and dimers. To further determine the variation of MAC of specific PAC molecules we measured the absorbance of PAC dimers and trimers having various structures with the DMAC assay, and calculated corresponding MAC values.

The MACs of various PAC dimers are shown in Figure 3.4A. Three A-type and three B-type dimers, including dimers isolated from cranberry (essentially procyanidin A2) and cocoa (essentially procyanidin B2), exhibited two MAC patterns compared to reference monomer epicatechin. Procyanins A1 and A2 (Figure 3.1C, D), together with the cranberry A-type dimer DP-2 had MAC between 9000 to 12000 L/mol*cm, significantly higher than the three B-type dimers (Figure 3.1E, F), which had MAC values between

4000 to 6000 L/mol*cm. Within the A-type or B-type dimers, procyanidins A2 and B2 had higher MAC values than procyanidins A1 and B1, respectively (Figure 3.4A, Table 3.1).

Seven different PAC trimers were analyzed by DMAC assay to determine their MAC values. As shown in Figure 3.4B, differential MAC values were also observed among tested trimers. Cinnamtannin B-1 (Figure 3.1I) had highest MAC (~17000 L/mol*cm), followed by cinnamtannin D-1 (Figure 3.1J, ~14000), peanut trimer C (Figure 3.1M, ~14000) and lindetannin (Figure 3.1K, ~13000). The B-type trimer cocoa DP-3, aka trimer C1 (Figure 3.1H) had MAC (~9000) significantly lower than the four aforementioned A-type trimers as well as epicatechin (~11000). Two A-type trimers, peanut trimer A (Figure 3.1L) and cranberry DP-3, showed the lowest MAC values (~6000), less than half of other A-type trimers. Thus, the lowest and highest MAC values differed by 3 fold.

These results have revealed again that both PAC dimers and trimers exhibit significantly different MAC in DMAC assay, which results from their different structures.

Relationship between PAC structures and resulting DMAC-conjugate light absorption

Differential MAC values among PAC dimers and trimers clearly indicate that the structure of PAC molecule, especially the inter-flavan linkage greatly affects the absorbance of the resulting PAC DMAC conjugate, and possibly the structure of PAC-DMAC conjugate. The DMAC assay of various PAC dimers and trimers produced PAC-

DMAC conjugates having different absorbance spectra, as also revealed visually by the solution color.

Table 3.1 and Figure 3.5 show the structures, MS spectra, MAC, color and absorbance spectra of PAC-DMAC conjugates of individual dimers. The DMAC reaction of all B-type dimers including procyanidins B1, B2 and cocoa DP-2 exhibited a green hue in contrast to a blue hue, observed with the three A-type dimers. Accordingly, in the absorbance spectra, all examined B-type dimers showed a secondary 440 nm absorbance peak, besides the primary 640 nm peak which is typically measured in DMAC assay. With the presence of double inter-flavan linkage in A-type dimers, only 640 nm absorbance peak appeared for PAC-DMAC conjugates.

Similarly, the B-type trimer from cocoa also showed the secondary 440 nm peak after DMAC reaction, as shown in Figure 3.6 Unlike A-type dimers which all had a single 640 nm absorbance peak, two of the analyzed A-type trimers, namely peanut trimer A and cranberry DP-3, also exhibited the secondary peak at 440 nm. In DMAC assay, these two trimers also exhibited the lowest MAC values among PAC trimers (Figure 3.4B, Table 3.2). Based on previously published study [23], the MS fragmentation pattern for the two trimers revealed that their double inter-flavan linkage was located between the 2nd and 3rd flavan-3-ol units (see Figure 3.1; where the 1st, ‘upper’ unit is defined as having a C4 bond to the C8 (Figure 3.1G) or C6 (Figure 3.1L) in the second unit), whereas other A-type trimers have the double linkage between the 1st (upper terminal) and 2nd units (Figure 3.1 I-K, M, Table 3.2 Note: the 3rd ‘lower terminal’ unit is defined as having either the C8 (e.g. Figure 3.1J, K) or C6 (e.g. Figure 3.1M) involved in a C-C bond to the 2nd unit). Together with PAC dimers, these findings indicate that for PAC molecules, the

absence of double linkage between the 1st and 2nd flavan-3-ol units results in an additional 440 nm absorbance peak in the DMAC assay. Similar results have also been observed in cocoa and cranberry PACs with DP > 3 (data not shown).

Relationship of MAC and DP for cranberry or cocoa PAC oligomers

Based on the observation that the DMAC assay exhibited differential absorbance for dimers and trimers we compared the molar absorbance of PACs on a DP series, from DP-2 to DP-10, of isolated cranberry and cocoa PAC oligomers as they represent A-type (cranberry) and B-type (cocoa) PACs respectively.

The cranberry versus cocoa PAC oligomer series exhibited different relationships with MAC (Figure 3.7). For cranberry PACs, representing A-type, DP-2 had MAC (~9000 L/mol*cm) similar to that of epicatechin (~9500). From DP-3 to DP-4, MAC declined to less than half value of epicatechin, and maintained at similar level through DP-7, increased to about 9000 at DP-10. DP-6 exhibited a slightly elevated MAC (~6200) over either DP-5 or 7. MAC differed by more than 2 fold between lowest (DP-4) and highest (DP-10) MAC values in cranberry PACs.

DPs of B-type cocoa PACs exhibited a different MAC relationship than that from cranberry PACs (Figure 3.7B). As mentioned earlier, MAC dropped significantly from monomer control epicatechin (~9300 L/mol*cm) to cocoa DP-2 (~4000), then increased linearly ($y=1029.8x+2494.5$, $R^2=0.949$) until DP-9 (~12000), with no further increase at DP-10. The data from both cocoa and cranberry PACs indicate that the resulting absorbance (MAC) of the PAC-DMAC conjugates varied significantly across various

natural PAC oligomers. MAC differed up to 3 fold between lowest (DP-2) and highest (DP-9) values with cocoa PACs.

Consistent with previous observations, B-type dimers and trimers which include cocoa DP-2 and DP-3 exhibited a secondary absorbance at 440 nm in DMAC assay. Moreover, for cocoa PAC oligomers of DP > 3, we also observed absorbance at both 640 nm and 440 nm after DMAC reaction. Interestingly, compared with the primary absorbance at 640nm, the secondary absorbance at 440 nm was reduced as the level of polymerization increased. As shown in Table 3.3, while all tested cocoa PACs exhibited the secondary absorbance at 440 nm, the overall ratio of A640/A440 increased by 1.6 fold from 1.3 for cocoa DP-2 to 2.0 for cocoa DP-10.

3.4 Discussion

PACs occur widely in plants and common foods as families of structurally variable chains. Most plants and food plant-derived products contain solely B-type PACs, such as those from cocoa, sorghum, apple and grape. Other species, including cranberry, plum, peanut and cinnamon synthesize A-type PACs [34, 37]. With the growing evidence of PACs' benefits to human health, the quantification of PACs in plant and food materials is critical for a better understanding of their nutritional and possible therapeutic values. Although HPLC-UV/Fluorescence/MS analysis provides certain detailed information regarding PACs' structure, such as their DP and inter-flavan linkage type, complicated sample preparation and purification, lengthy analysis time and high costs makes it less favorable for routine spectrophotometric analysis methods.

DMAC assay as a well-accepted spectrophotometric method for PAC quantification has many advantages compared with other analysis, due to its high sensitivity and selectivity, and reduced interference by anthocyanins [31]. There is a general acceptance of the basic premise that the DMAC reagent reacts only with the C8 carbon of the A-ring of the PAC terminal units [39]. In consequence, a major assumption is that PAC molecules with different DPs should produce similar MAC values [37]. Therefore, DMAC assay, under such an assumption should provide accurate PAC quantitative data on molar concentration basis of PACs of various DP and structures. However, based on the results derived from our study, this assumption is not accurate.

Furthermore, we discovered that the solvent system used in the DMAC assay has profound impact on the resulting conjugate absorbance and the MAC values among the various PAC monomers and oligomers. A-type dimeric procyanidin A2 exhibited a MAC more than twice that with B-type dimers with methanol-based DMAC reagent but showed the lowest MAC in ethanol-based reagent (contains less HCI). These results indicate that both solvent environment (methanol/ethanol) and acidity of DMAC reagent influence the reaction, making a comparison of the results derived from different solvent systems unreliable and misleading. Reaction dynamics also differed between the two DMAC reagents. Compared with the ethanol-DMAC, methanol-DMAC reagent required significantly less time to reach maximum absorbance with PAC compounds, and less time was required for complete reaction in 1:7 sample/DMAC ratio (contains more HCI in reaction system) than 1:3 ratio. Wallace and Giusti reported 15 min reaction time for maximum absorbance of catechin in methanol-DMAC reagent with H+ concentration less than 20% of the current study (1:7 sample/DMAC ratio) [45]. Using ethanol-based

reagent with the same DMAC concentration (0.1g/100 ml) and a sample/DMAC ratio of 1:5, Payne et al. also observed reduced reaction rates with lower HCI concentrations [30]. For DMAC reaction, a strong acid condition is required to protonate aldehyde (DMAC) and form electrophilic carbonations [31], enabling a more rapid reaction.

Structure complexity is one of the major confounding factors in PAC quantification. In a sample consisting of various oligomers and polymers, PACs can have molecular weights from 300 for monomers to well over 9000 and multiple inter-flavan linkage types including second linkage locations [1, 24]. To determine the influence of PAC structure on PAC-DMAC reaction, it is critical to utilize individual PACs with known structures. In this study, we focused on two types of structure variations in PACs: (a) inter-flavan linkage type and position and (b) degree-of-polymerization.

The significantly lower MACs of B-type dimers as compared to those of monomers and A-type dimers observed in this study were similar to results reported by Payne et al. using ethanol-DMAC reagent where two B-type dimers exhibited lesser MAC values (about 2/3) than monomers [30]. Cranberry and cocoa dimers, essentially represented by procyanidin A2 and B2 respectively, exhibited reduced MACs compared to the reference standards, probably due to impurities and/or residual solvent during isolation. Absorbance profiles of PAC-DMAC conjugates were also different between the two types of dimers as B-type dimers showed an additional absorbance peak at 440 nm, reflected by their green color in DMAC assay. These findings suggest a difference in chromophores of the DMAC-PAC conjugates between A-type and B-type dimers. The B-type dimers could either possess multiple available positions to react with DMAC besides the terminal unit and generate additional chromophore(s), or have different chromophore

electron resonance structure due to lack of ether linkage, in spite of sharing the same active DMAC reaction site with A-type dimers. To our knowledge this observation has not been reported in previous studies.

Two A-type trimers - peanut trimer A and cranberry DP-3 with double linkage between 2nd and 3rd units (1st unit has C4 bond to either C6 or C8 of 2nd unit) exhibited the lowest MACs among analyzed trimers. Similar with B-type dimers, the two A-type trimers together with cocoa B-type trimer (trimer C1) exhibited two absorbance peaks at 440 and 640 nm. These results indicate that not only the presence of double inter-flavan linkage, but also its position has an impact on resulting absorbance of the PAC-DMAC reaction. The absence of double linkage between 1st and 2nd flavan-3-ol units in a PAC molecule resulted in a low 640 nm absorbance in DMAC reaction, and created a secondary absorbance peak at 440 nm, as has been shown in PAC dimers (B-type) and trimers (B-type and A-type with single C-C linkage between 1st and 2nd units).

The DP in both A-type and B-type PACs results in variable MAC values in DMAC assay. Interestingly, MAC of cocoa PACs increased in a linear manner with their DP, and finally reached similar levels with monomer epicatechin. Moreover, all B-type cocoa PACs exhibited a secondary 440 nm absorbance peak that declined in intensity when DP increased. In the characterization of PAC structure, analysis of mass spectrum data is helpful in revealing structural details. Regular ESI/APCI-MS analysis resulted in difficulties when analyzing PAC polymers with high (DP > 6) DPs due to their high molecular weight (> 2000). Specific ionization-detection techniques such as Matrix-Assisted Laser Desorption/Ionization - Time-of-Flight (MALDI-TOF) mass spectrometry is well suited for the analysis of large DPs [3, 9, 47]. The positive correlation between

A640/A440 ratio and DP of B-type cocoa PACs in DMAC assay observed in the current study can be potentially used for estimation of polymerization level of B-type PACs.

DMAC assay has been widely used for PAC quantification in various plants and food stuffs [30, 36-39, 41, 43, 44, 46]. Although reagent formulas, standards and assay protocols vary across different studies, DMAC has been considered as a highly specific reagent, which only reacts with PAC's terminal unit [31, 37, 39, 42]. Contrary to these assumptions, here we report that PAC molecules with different linkage type (A-type vs. B-type) and position or DPs (monomers vs. oligomers/polymers) exhibited differential absorbance response with DMAC reagent, which significantly impacted their MAC in DMAC assay. As a result, we documented that the DMAC assay is less accurate than previously reported. We have shown that monomers exhibited higher MAC than oligomers with low DPs in both A-type (cranberry) and B-type (cocoa) PACs. Thus, using PAC monomer as quantification standard, PAC contents in natural materials can be underestimated on both mass and molar basis. Using DMAC to compare PAC contents from different plant and/or food materials can lead to the estimation errors resulting from their different PAC linkage types and/or proportions (e.g. cranberry vs. cocoa), even with unified standard. Furthermore, our findings suggest that DMAC would be particularly useful in relative evaluations of PACs of fractions extracted under the same extraction protocols from the same source materials. In addition, DMAC reaction is helpful in identifying location of the A-type linkage.

Potential improvement of DMAC assay measurement can be achieved by pre-analysis of PAC constituents in plant materials and optimization of quantification standard. The relatively high reaction specificity between DMAC reagent and PACs still provides

advantage for DMAC assay to be properly utilized in PAC quantification. Instead of using single PAC standard for quantification of samples with complex PAC profiles, efforts should be made to acquire highly purified PAC fractions from relevant materials, which can be utilized as quantification standards for DMAC assay of samples from same material. Although DMAC does offers an expedient method of quantifying PACs of identical molecular weight and structure, standards of pertinent oligomers should be used as reference standards to obtain the most accurate estimate of quantification. As specific PAC oligomers exhibited promising health beneficial activities, e.g., cranberry DP-4 and DP-9 identified to be most active against dental caries pathogens or ovarian cancer cells [10, 13], effort should also be made to determine the MACs of the compounds of interest for quantitative analysis.

In summary, the study reports differential absorbance responses of individual PACs with DMAC. The PAC-DMAC MAC is affected by both DMAC reagent environment and PAC structural variation. Therefore, the prior assumption and understanding of the reaction mechanism and absorbance response need to be re-evaluated. The variation in MAC as well as the absorbance maxima also suggests the formation of the PAC-DMAC conjugates of different structures, resulting in formation of the chromophores in accordance with the structure of a specific PAC. The secondary absorbance peak (440 nm) observed in the study and its connection with inter-flavan double linkage position and PAC polymerization also indicate a potential usefulness of utilizing DMAC reaction in PAC qualitative studies. Further studies will aim at illustrating the reaction mechanism of DMAC reagent with different type of PAC molecules, such as the structures of PAC-

DMAC conjugates with different absorbance profile, and using that information to optimize DMAC reaction condition and increase the accuracy and reliability of the assay.

3.5 References

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3.6 Tables and Figures

Table 3.1. MS spectra, structure and MAC values of PAC dimers in DMAC assay. 0.25 ml PAC was reacted with 1.75 ml methanol-DMAC reagent. Epicatechin was measured as reference compound. Experiments were performed in triplicate.

compound	$[M-H]^-$ and fragment ions in APCI MS-MS (m/z)	double linkage	MAC (L/mol*cm) average ± std
procyanidin A1	575.5, 449.0	yes	10146 ± 173
procyanidin A2	575.7, 449.4	yes	11722 ± 366
procyanidin B1	577.7, 289.2	no	4493 ± 88
procyanidin B2	577.6, 289.3	no	5245 ± 267
cranberry DP-2	575.7, 449.4	yes	10204 ± 231
cocoa DP-2	577.7, 289.2	no	4242 ± 67
epicatechin	289.1, 245.2	none	9859 ± 235

Table 3.2. MS spectra, structure and MAC values of PAC trimers in DMAC assay. 0.25 ml PAC was reacted with 1.75 ml methanol-DMAC reagent. Epicatechin was measured as reference compound. Experiments were performed in triplicate.

compound	$[M-H]^-$ and fragment ions in APCI MS-MS (m/z)	double linkage (number, location)	MAC (L/mol*cm) average ± std
peanut trimer A	863.7, 575.7	1, 2→3	6312 ± 86
peanut trimer C	863.6, 573.4, 289.1	1, 1→2	14786 ± 0.002
cinnamtanin B-1	863.7, 573.3, 289.3	1, 1→2	17812 ± 312
cinnamtanin D-1	863.7, 573.4, 289.2	1, 1→2	14642 ± 218
lindetannin	863.6, 573.4, 289.1	1, 1→2	13633 ± 349
cranberry DP-3	863.6, 575.6	1, 2→3	6024 ± 50
cocoa DP-3	865.7, 577.7, 289.2	0	9533 ± 654
epicatechin	289.1, 245.2	none	10923 ± 296

Table 3.3. Absorbance at 640 nm and 440 nm of individual cocoa PAC oligomers in DMAC assay. 0.25 ml PAC was mixed with 1.75 ml methanol-DMAC reagent.

compound (200 µg/ml)	**A_{640}**	**A_{440}**	**A_{640}/A_{440}**
cocoa DP-2	1.0093	0.78156	1.29
cocoa DP-3	1.2915	0.78001	1.66
cocoa DP-4	1.1345	0.66581	1.70
cocoa DP-5	0.89695	0.51738	1.73
cocoa DP-6	0.82073	0.45779	1.79
cocoa DP-7	0.73321	0.39901	1.84
cocoa DP-8	0.72168	0.38058	1.90
cocoa DP-9	0.66158	0.34396	1.92
cocoa DP-10	0.73795	0.36789	2.00

Figure 3.1. Chemical structures of (+)-catechin (A), (+)-epicatechin (B), procyanidin A1 (C), procyanidin A2 (D), procyanidin B1 (E), procyanidin B2 (F), A-type procyanidin trimer (G), procyanidin C1 (H), cinnamtannin B-1 (I), cinnamtannin D-1 (J), lindetannin (K), peanut trimer A (L) and peanut trimer C (M). EC: epicatechin, C: catechin.

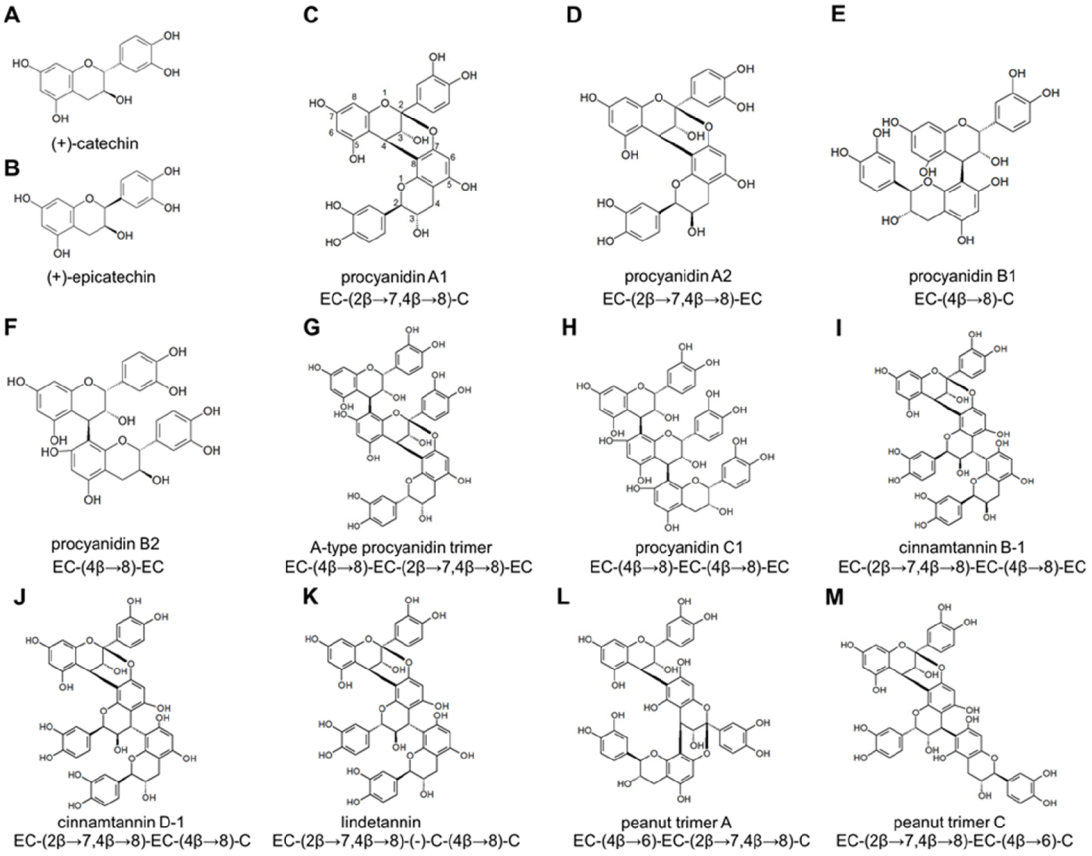


Figure 3.2. MAC values of individual PAC monomers and dimers in two different DMAC reagent formulas (Methanol vs. Ethanol). Two sample/DMAC ratios 1:7 (A)/1:3 (B) were used. Experiments were performed in triplicate.*In same DMAC assay method, MAC values with different letters have significant difference.

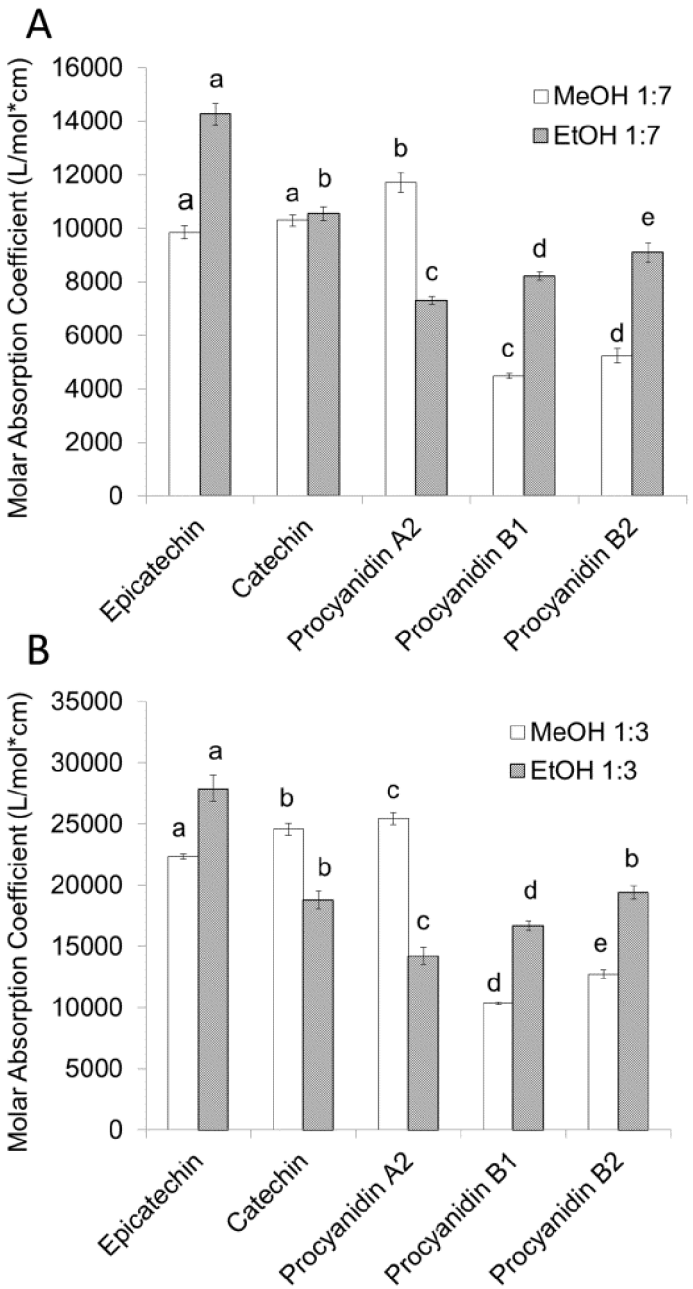


Figure 3.3. Absorbance (640 nm) of PAC-DMAC conjugates over time. Individual PAC monomers and dimers were reacted with DMAC reagent under different conditions: (A) methanol-DMAC, 0.25 ml PAC + 1.75 ml DMAC; (B) methanol- DMAC, 0.4 ml PAC + 1.2 ml DMAC; (C) ethanol-DMAC, 0.25 ml PAC + 1.75 ml DMAC; (D) ethanol-DMAC, 0.4 ml PAC + 1.2 ml DMAC.

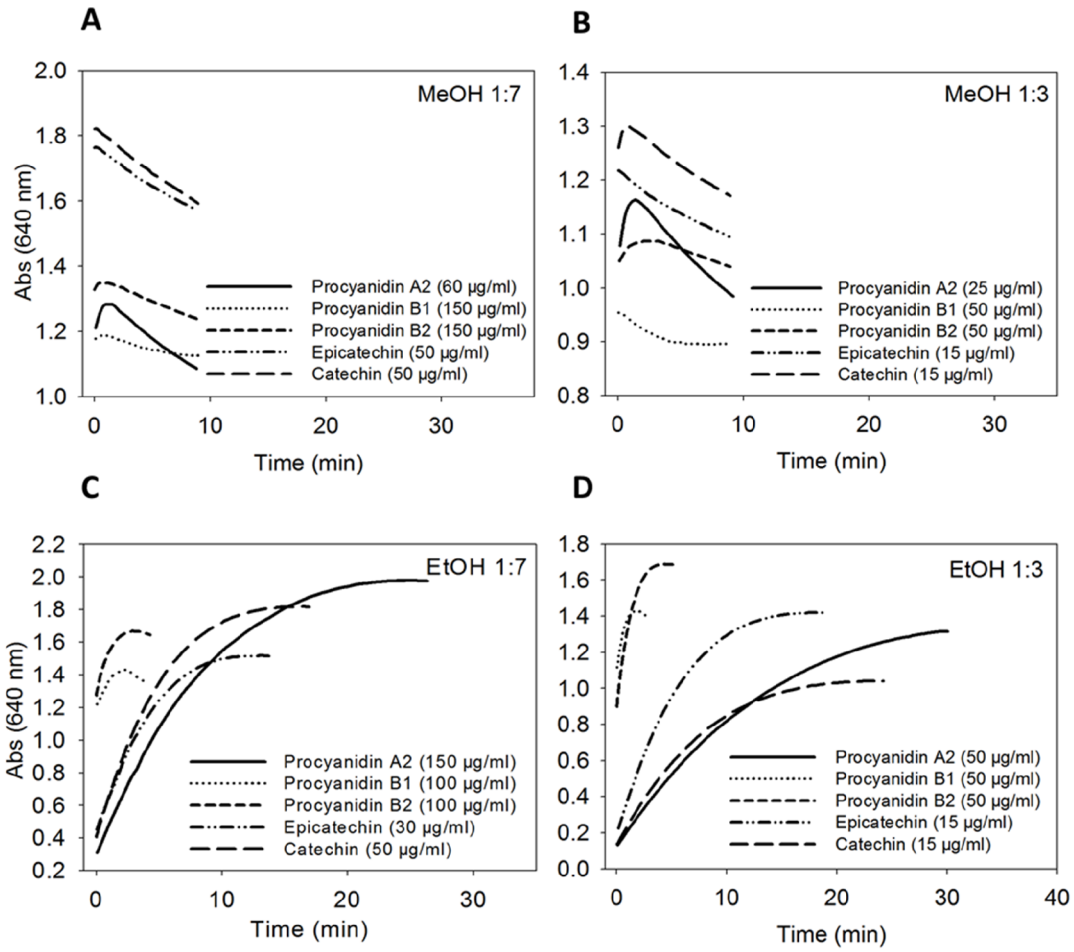


Figure 3.4. MAC of individual PAC dimers (A) and trimers (B) after DMAC reaction. 0.25 ml PAC was mixed with 1.75 ml methanol-DMAC reagent. Epicatechin was measured as reference compound. Experiments were performed in triplicate. *MAC values with different letters have significant difference.

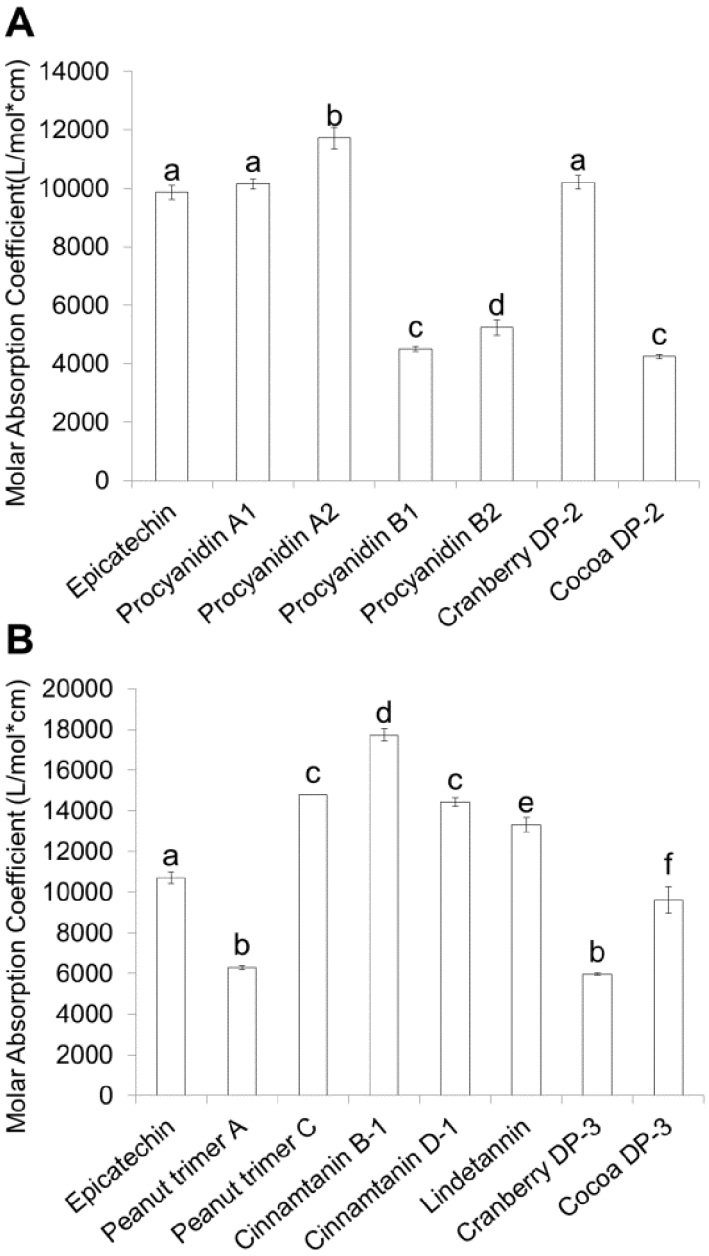


Figure 3.5. Color and visible absorbance spectra of PAC dimers in DMAC assay. 0.25 ml PAC was reacted with 1.75 ml methanol-DMAC reagent. Three A-type dimers procyanidin A1, B1 and cranberry DP-2 plus three B-type dimers procyanidin B1, B2 and cocoa DP-2 were analyzed.

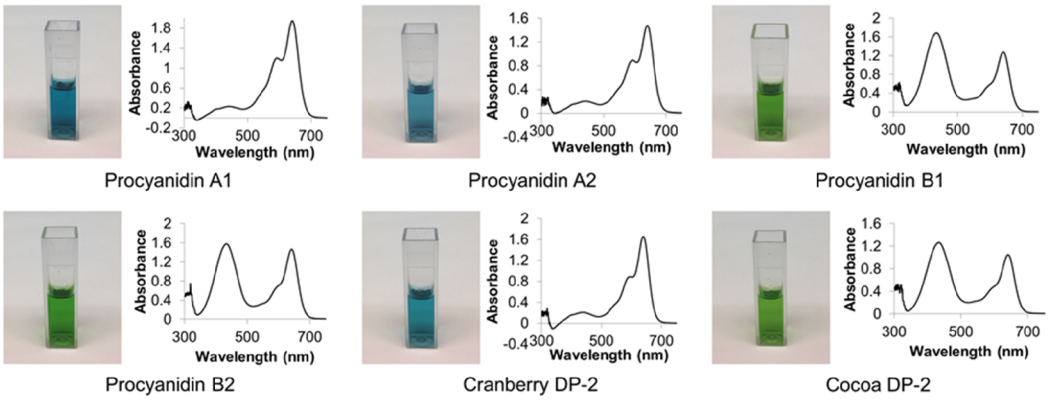


Figure 3.6. Color and visible absorbance spectra of PAC trimers in DMAC assay. 0.25 ml PAC was reacted with 1.75 ml methanol-DMAC reagent. Six A-type trimers peanut trimer A, C; cinnamtannin B-1, D-1; lindetannin and cranberry DP-3 plus one B-type trimer cocoa DP-3 were analyzed.

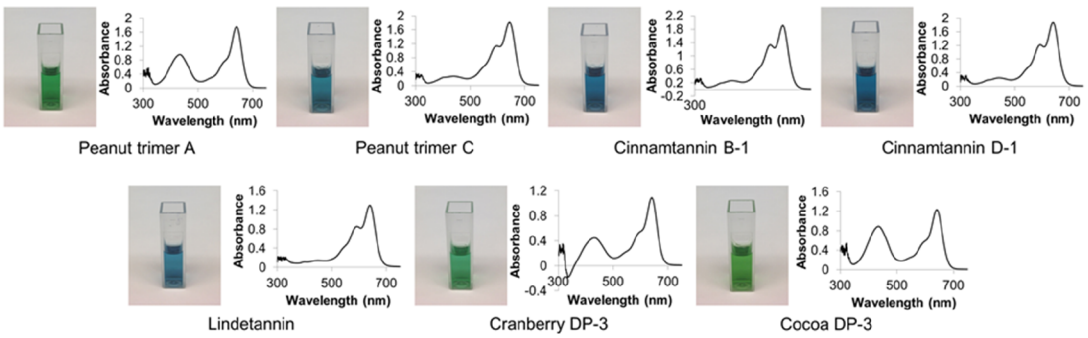
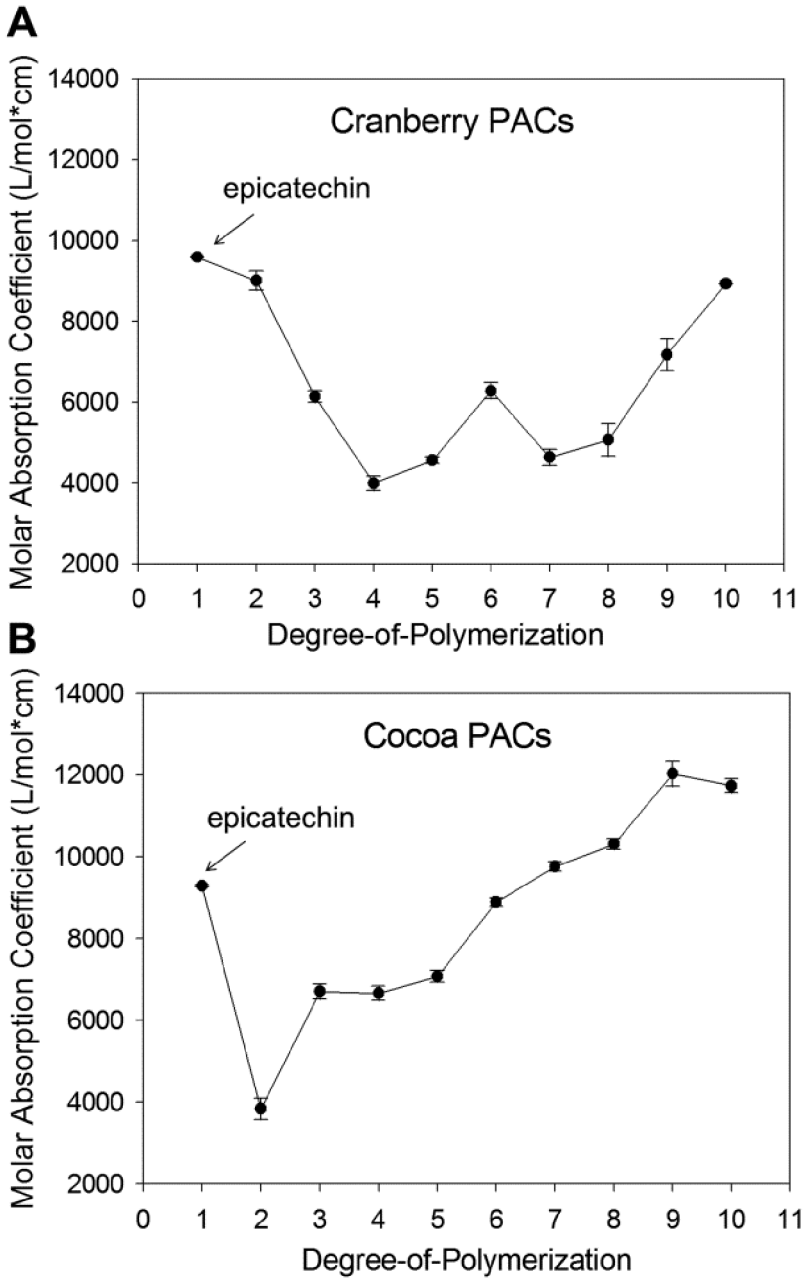


Figure 3.7. MAC values of individual cranberry (A) and cocoa (B) PAC oligomers in DMAC assay. 0.25 ml PAC was mixed with 1.75 ml methanol-DMAC reagent and measured for 640 nm absorbance after 9 min. Epicatechin was measured as reference compound. Experiments were performed in triplicate.



Chapter 4

In Vitro Anti-ovarian Cancer Properties of Flavonols and Proanthocyanidins from Cranberry and Cocoa

Wang, Y., A. Han, E. Chen, R.K. Singh, C.O. Chichester, R.G. Moore, A.P. Singh, and N. Vorsa, *The cranberry flavonoids PAC DP-9 and quercetin aglycone induce cytotoxicity and cell cycle arrest and increase cisplatin sensitivity in ovarian cancer cells.* International journal of oncology, 2015. **46**(5): p. 1924-1934.

4.1 Introduction

Epithelial ovarian cancer (EOC) is an asymptotic lethal disease that is frequently diagnosed at late stages in women [1]. Initial responsiveness to chemotherapy and surgery is often thwarted by inherent or acquired resistance, resulting in early recurrence and premature death [2]. Approximately 22,000 women will be diagnosed with EOC this year in the US alone, and more than 14,000 will succumb to their disease [3]. Because first-line platinum-taxane and second-line dox-topotecan therapies often fail, third-line chemotherapy options are urgently needed.

American cranberry (*Vaccinium macrocarpon*) has received attention in our laboratories and elsewhere because of the potential for cranberry A-type proanthocynidines (PACs) and flavonols to treat upper urinary tract infections and cancer [4-6]. However, the isolation of pure PACs and flavonol constituents has remained an unmet challenge that frequently impedes broad-spectrum screening for biological activity. Semi-pure cranberry PACs have consistently exhibited potent anti-proliferative activity against various cancer cells *in vitro* [6-8]. We had previously developed an iterative but efficient HPLC and

mass spectrometry-based approach to generate high-purity polymeric PAC fractions from cranberries [9]. Purified PACs had exhibited cytotoxic effects against a panel of ovarian cancer and neuroblastoma cells in our laboratories [9-11]. PACs exerted cytotoxic effects in ovarian cancer or neuroblastoma cells via cell cycle arrest, production of lethal levels of intracellular reactive oxygen species (ROS), and induction of pro-apoptotic signal transductions at low microgram concentrations [10, 11]. Further optimization of the purification and a detailed investigation of the mechanism of anti-proliferative action have been pursued in our laboratories since purified PACs became accessible.

In this study, we further elaborate analytical methodology to isolate and purify individual flavonols and A-type PACs of cranberry for broad-spectrum biological activity screening studies. To determine the effect of linkage type on PACs' anti-cancer activity, we also utilize individual B-type PACs isolated from cocoa. We describe the two most active leads in cranberry, A-type PAC DP-9 and quercetin aglycone, as well as B-type DP-8 in cocoa, in SKOV-3 and OVCAR-8 ovarian cancer cells, and we characterize their anti-proliferative efficacy and mechanism of cell cycle arrest, induction of apoptotic activities, and inhibition of oncogenes and DNA repair machinery. The multifaceted anti-proliferative properties exerted by these flavonoids highlight their potential for treatment of ovarian cancer.

4.2 Materials and methods

Plant material

Cranberry fruits of cultivar "Stevens" were harvested from the Philip E. Marucci Center for Blueberry and Cranberry Research and Extension and kept frozen at -20 ºC before use.

Reagents and LC-MS instrumentation

All solvents were purchased from EMD Millipore (Billercia, MA). Sephadex® LH-20 was obtained from GE Healthcare Bio-Science (Piscataway, NJ), and BAKERBOUND® Diol was obtained from Avantor Performance Materials (Center Valley, PA). Individual B-type PACs from cocoa were kindly provided by Planta Analytica (Danbury, CT). LC-MS spectra were obtained with a Dionex UltiMate® 3000 LC system including the UltiMate 3000 RS Pump, UltiMate 3000 RS Autosampler, UltiMate 3000 RS Column Compartment and UltiMate 3000 RS Diode Array Detector coupled with Applied Biosystems API 3000™ triple quad LC-MS/MS mass spectrometer. Previously described HPLC methods for flavonol and PAC identification [12, 13] were modified slightly for LC-MS analysis. Structure and purity of flavonols and PACs were determined by HPLC-PDA/Fluorescence and/or LC-MS.

Extraction and isolation of individual cranberry flavonols and PACs

Crude cranberry flavonoids were extracted and further separated in a Sephadex® LH-20 column as previously described [14]. Individual cranberry flavonols were isolated using a semi-preparative HPLC system as described previously [14]. Individual PACs were isolated with a regular Diol gravity column chromatography as previously reported [9]. Eight flavonols were isolated and characterized as myricetin-3-galactoside, quercetin-3-galactoside, quercetin-3-glucoside, quercetin-3-xylopyranoside, quercetin-3-arabinopyranoside, quercetin-3-arabinofuranoside, quercetin-3-rhamnopyranoside and quercetin aglycone. Eleven cranberry A-type PACs from dimer to polymer 12 (named as

PAC DP-2 to PAC DP-12) were isolated and characterized. Purity of all isolated cranberry flavonoids was > 95% (w/w) based on HPLC and LC-MS analysis.

Cell lines and cell culture

SKOV-3 and OVCAR-8 cells (ovarian epithelial adenocarcinoma) were purchased from ATCC (Manassas, VA). Cells were cultured with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 µg/ml streptomycin and 100 µg/ml penicillin in an incubator at 37 ºC, 5% CO_2 and 95% humidity. For all assays, cells were allowed to attach for 24 hours prior to treatment.

Cell viability assay

Cells (5000/well) were seeded in 96-well flat bottom plates (USA Scientific, Orlando, FL) and treated with various concentrations of flavonoids. Cell viability was determined by CellTiter 96 Aqueous One Solution Assay (Promega, Madison, WI) following manufacturer's protocol. Experiments were performed in triplicate; data are expressed as mean of triplicate measurements (X ±SD) in percentage of untreated cells (100%). SPSS Statistics 19 (IBM Corporation, Armonk, NY) was used to perform ANOVA with linear regression between cell viability and compound concentration, calculate IC_{50} value of each cranberry flavonoid, and conduct Student's T-tests and calculate *p*-values based on mean cell viability for each treatment condition.

DNA fragmentation analysis

DNA fragmentation as a hallmark of apoptosis was studied using the Roche *in situ* Cell Death Detection Kit (Branford, CT) (TUNEL assay). SKOV-3 and OVCAR-8 cells

(2×10^4/well) were seeded in Lab-Tek 8-well chamber glass slides (Nalge Nunc., Naperville, IL) and treated with 50μM PAC DP-9, 25μM quercetin aglycone, or DMSO vehicle control for 12 hours. Cells were fixed with 10% neutral buffered formalin, and stained according to the manufacturer's protocol. Slides were then cover-slipped with Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA). Images were acquired on a Nikon E800 upright microscope using SPOT Advanced Software (SPOT Imaging Solutions, Sterling Heights, MA).

Western blot analysis

Cells (3×10^6/dish) were seeded into 100 mm^2 tissue culture dishes and treated with individual cranberry flavonoids. Cells were then lysed with cell lysis buffer (Cell Signaling Technology, Inc., Danvers, MA) supplemented with phenylmethylsulfonyl fluoride (5μl/ml). Protein concentrations of cell lysates were determined with Pierce BCA protein assay kit (Pierce Technology, Rockford, IL). Gel electrophoresis was performed in NuPAGE Gel system (Invitrogen™) per the manufacturer's instructions. Separated proteins were transferred onto a nitrocellulose membrane, which was then blocked with 5% non-fat milk in PBS-Tween buffer and probed against various primary antibodies (cleaved caspase-3 no. 9664, cleaved PARP no. 5625, EGFR no. 4267, phospho-EGFR no. 3777, phospho-c-Raf no. 9427, phospho-ERK1/2 no. 4370, phospho-p53 no. 9286, p18 INK4C no. 2896, p21 Waf1/Cip1 no. 2947, p27 Kip1 no. 3686, CDK2 no.2546, Cyclin D1 no. 2926, Cyclin D3 no. 2936, β-Actin no. 8457, β-Tubulin no. 2128; Cell Signaling Technology, Danvers, MA). Protein bands were visualized using horseradish peroxidase conjugated anti-rabbit or anti-mouse secondary antibody (Cell

Signaling Technology) and Pierce ECL Western Blotting Substrate, and documented by Bio-Rad Gel Doc system (BioRad, Hercules, CA).

Immunofluorescence microscopy analysis

SKOV-3 and OVCAR-8 cells (2×10^4/well) were seeded in Lab-Tek 8-well chamber glass slides and treated with individual cranberry flavonoids. Cells were fixed with 10% neutral buffered formalin, washed 3 times with PBS-Tween 0.1% and blocked with 5% horse serum (Vector Laboratories) in PBS-Tween for 30 min. Blocked cells were incubated with various primary antibodies (EGFR no. 4267, p21 Waf1/Cip1 no. 2947, phospho-ERK1/2 no. 4370, DNA-PK no. 4602; Cell Signaling Technology and MEK no. sc-166197, phospho-Histone H3 no. sc-12927, Cyclin D1 no. sc-246; Santa Cruz Biotechnology, Dallas, TX) at 4 ºC overnight. Probed proteins were visualized with DyLight 488/594 (Thermo Scientific, Waltham, MA) or Alexa Fluor® 594 (Cell Signaling Technology) secondary antibodies, and cell nuclei were stained with Vectorshield® mounting medium with DAPI (Vector Laboratories). Images were acquired on Olympus FSX100® (for EGFR) or Nikon E800 upright (for other proteins) microscope system.

Co-immunoprecipitation analysis

SKOV-3 and OVCAR-8 cells were cultured in 100 mm^2 tissue culture dishes to 80% confluency. Cells were lysed and protein concentration was quantified as described earlier. Lysate containing 500 µg protein was incubated with target antibody or control IgG antibody for 4 hours with rotation at 4 ºC. 75 µl of Protein G Sepharose (50% slurry, GE Healthcare Life Sciences, Piscataway, NJ) was added to the lysate and incubated at 4

ºC overnight. After incubation, beads were washed with 500 µl lysis buffer 3 times and re-suspended in 40 µl Laemmli buffer (2x, Bio-Rad), vortexed, and heated to 95 ºC for 5 min. Suspension was centrifuged to collect supernatant for western blot analysis.

Cell cycle analysis

SKOV-3 and OVCAR-8 cells (3×10^5/well) were seeded in 6-well plates and treated with a series of concentrations of individual flavonoids (0-100 µg/ml, 24-48 h). After trypsinization, cells were collected and fixed in ice-cold 70% ethanol and stained with solution containing propidium iodide (0.1mg/ml), sodium citrate (2mg/ml) and Triton X-100 (1µl/ml). Cell counting data were acquired in an Accuri® C6 Flow Cytometer and analyzed with ModFit LT software (Verity Software House, Inc., Topsham, ME).

Determination of DNA-bonded cisplatin

SKOV-3 and OVCAR-8 cells (3×10^6/dish) were seeded into 100 mm^2 tissue culture dishes and treated by cisplatin with the presence or absence of cocoa B-type PAC DP-8. After treatment, cellular DNA was extracted by an UltraClean® Tissue & Cells DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA) following manufacture's instruction. DNA concentration in extracts was determined by a Nanodrop 2000 microvolume UV-Vis spectrophotometer (Thermal Scientific, Wilmington, DE). Cisplatin concentration in extracts was determined by inductively coupled plasma mass spectrometry (ICP-MS) and normalized based on sample DNA concentration.

4.3 Results

Cytotoxicity of individual cranberry flavonoids against ovarian cancer cell lines

Individual cranberry flavonols and PACs exhibited different levels of cytotoxicity against SKOV-3 and OVCAR-8 cell-lines (Figure 4.2). Quercetin-3-xylopyranoside did not show cytotoxicity against SKOV-3 and OVCAR-8 cells, while myriceitn-3-galactoside and quercetin-3-arabinopyranoside were cytotoxic to OVCAR-8 cells (IC_{50} = 130 and 212 µg/ml) but non-toxic to SKOV-3 cells at treatment concentrations. Compared to quercetin glycosides, quercetin aglycone exhibited higher cytotoxicity against the two cancer cell lines (IC_{50} = 83 and 61 µg/ml for SKOV-3 and OVCAR-8 cells, respectively).

Cranberry PAC DP-3, DP-7, and DP-10 exhibited less cytotoxicity against both SKOV-3 and OVCAR-8 cells than other PACs. PAC DP-5 and DP-12 were more effective against SKOV-3 cells (IC_{50} = 126 µg/ml and 162 µg/ml for DP-5 and DP-12, respectively) than OVCAR-8 cells. Compared to other cytotoxic PAC molecules, PAC DP-9 exhibited the highest activity against SKOV-3 cells (IC_{50} = 82 µg/ml) and relatively high cytotoxicity against OVCAR-8 cells (IC_{50} = 138 µg/ml). Based on these results, quercetin aglycone and A-type PAC DP-9 (Figure 4.1) were selected as lead candidates for further studies in SKOV-3 and OVCAR-8 cells. Cytotoxicity values for individual cranberry flavonoids and statistical analysis including *p*-values and correlation coefficients are provided in Table 4.1.

Quercetin aglycone and A-type PAC DP-9 induced apoptosis and increased cisplatin sensitivity in SKOV-3 and OVCAR-8 cells

To examine whether apoptosis was induced in ovarian cancer cells upon treatment with cranberry flavonoids, western blot and DNA fragmentation analysis (TUNEL assay) were carried. As shown in Figure 4.3A, quercetin aglycone induced caspase-3 activation in

both SKOV-3 and OVCAR-8 cells, and A-type PAC DP-9 led to caspase-3 and cleaved-PARP expression specifically in SKOV-3 cells.

TUNEL assay was performed to detect apoptosis-induced DNA fragmentation. Quercetin aglycone and A-type PAC DP-9 treated cells showed apoptosis-induced DNA fragmentation, which was detected in both SKOV-3 and OVCAR-8 cells after 12 h of treatment (Figure 4.3B). Both western blot and DNA fragmentation analysis confirmed apoptosis induction in ovarian cancer cells exposed to these two cranberry flavonoids.

To investigate whether sublethal concentrations of quercetin aglycone and A-type PAC DP-9 sensitized ovarian cancer cells to cisplatin, quercetin aglycone or A-type PAC DP-9 pretreated ovarian cancer cells were exposed to cisplatin, and cell viability was analyzed. As shown in Figure 4.4, while sublethal concentrations of quercetin aglycone or A-type PAC DP-9 alone did not reduce SKOV-3 and OVCAR-8 cell viability after 24 h, their pretreatment significantly reduced viability of cisplatin-treated SKOV-3 and OVCAR-8 cells, with quercetin aglycone acting more strongly than A-type PAC DP-9. Thus, quercetin aglycone and A-type PAC DP-9 at very low concentrations sensitize ovarian cancer cells' response to cisplatin, resulting in enhanced cisplatin cytotoxicity.

A-type PAC DP-9 down-regulated expression and activation of epidermal growth factor receptor (EGFR) in SKOV-3 cells and induced EGFR nuclear translocation

Elevated EGFR expression results in poor prognosis in lung, breast and ovarian cancer [15]. EGFR expression in quercetin aglycone or PAC DP-9 treated ovarian cancer cells was analyzed by immunoblotting and immunofluorescence microscopy. To eliminate the effect of medium-contained growth factors on cellular EGFR regulation, ovarian cancer

cells were serum-deprived for 4 h prior to treatment, and low concentrations (5-40 μg/ml) of cranberry flavonoids were applied with serum-free medium to avoid massive cell death. After 12 h of treatment, quercetin aglycone did not affect phosphorylated-EGFR levels in both SKOV-3 and OVCAR-8 cells, but A-type PAC DP-9 induced a dose-dependent down-regulation of both phosphorylated-EGFR and total EGFR in SKOV-3 cells (Figure 4.5A). As shown in Figure 4.5B, EGFR was expressed predominantly at the cell membrane in the untreated controls, and exhibited nuclear translocation within 3 h of treatment with A-type PAC DP-9 in a dose-dependent manner such that lower concentrations (12.5-25 μg/ml) induced EGFR peri-nuclear localization in SKOV-3 cells, and higher concentrations (50-200 μg/ml) of A-type PAC DP-9 mediated EGFR nuclear translocation.

Quercetin aglycone and A-type PAC DP-9 down-regulated pro-survival MAP kinase proteins in ovarian cancer cells

We examined changes in MAP kinase signaling regulation exerted by quercetin aglycone and A-type PAC DP-9 in ovarian cancer cells. Expression of MEK (Figure 4.6A) and phospho-ERK1/2 (Figure 4.6B) was down-regulated after 12 h of treatment with quercetin aglycone or A-type PAC DP-9, indicating an inhibition of pro-survival MAPK-ERK signal transduction by cranberry flavonoids. Similarly, treatment with quercetin aglycone and A-type PAC DP-9 led to rapid, significant down-regulation of phospho-p42/22 MAPK and phospho-c-Raf in OVCAR-8 cells within 6 h (Figure 4.7A), demonstrating their inhibitory effect on the activated pro-survival MAPK pathway.

Quercetin aglycone and A-type PAC DP-9 affected cell cycle progression of ovarian cancer cells

Immunofluorescence microscopy analysis of quercetin aglycone and A-type PAC DP-9 treatment revealed down-regulation of cyclin D1 and up-regulation of p21 in SKOV-3 and OVCAR-8 cells (Figure 4.6D,F). Phospho-histone H3 (Figure 4.6C) and DNA-dependent protein kinase (DNA-PK, Figure 4.6E), proteins often over-expressed in ovarian cancer cells, were also down-regulated in SKOV-3 and OVCAR-8 cells upon treatment with cranberry flavonoids. Based on immunoblot study, phospho-p53 and p21 expression were first down-regulated after 6 h of treatment with quercetin aglycone in OVCAR-8 cells and then exhibited sustained up-regulation (Figure 4.7A). Three other cell cycle regulators, p18, p27, and CDK2 were also up-regulated within 6 to 24 h of treatment with quercetin aglycone. Independent of p53 expression, SKOV-3 cells exhibited rapid up-regulation of cell cycle inhibitor p21 after 6 h of treatment of quercetin aglycone, together with up-regulation of p18, p27, and CDK2 within 24 h of treatment. Similarly, A-type PAC DP-9 induced up-regulation of p21 in both SKOV-3 and OVCAR-8 cells within 6 to 24 h treatment regardless of p53 expression level. CDK2 expression was also up-regulated in SKOV-3 and OVCAR-8 cells after 24 h of A-type PAC DP-9 treatment.

Because immunofluorescence microscopy and western blot analysis showed inhibition of SKOV-3 cellular EGFR expression by A-type PAC DP-9 (Figure 4.5), co-immunoprecipitation (Co-IP) was utilized to examine potential protein-protein interactions involved with EGFR and cell cycle regulatory factors, including p18, p21, p27, CDK2, cyclin D1, and cyclin D3. While most of the probed proteins did not show a

positive signal, CDK2 signal was detected in both SKOV-3 and OVCAR-8 EGFR Co-IP samples. As shown in Figure 4.7B, CDK2 was recovered in both positive controls and two Co-IP samples as well as in the OVCAR-8 negative control, indicating a false positive signal in the OVCAR-8 Co-IP sample. The absence of CDK2 signal in SKOV-3 negative control confirmed direct interaction between CDK2 and EGFR in SKOV-3 cells.

Both western blot and Co-IP studies indicate a direct effect of cranberry flavonoids on ovarian cancer cell progression. Subpopulations of propidium iodide-stained SKOV-3 and OVCAR-8 ovarian cancer cells treated with cranberry flavonoids were analyzed by flow cytometry. As illustrated in Figure 4.7C,D, quercetin aglycone or A-type PAC DP-9 treatment for 24 h in SKOV-3 cells led to G2/M-phase arrest dose-dependently. The G2/M subpopulation increased from 10.81% to 16.52% for SKOV-3 cells treated with 50 µg/ml quercetin aglycone and further increased to 32.35% after treatment with 100 µg/ml quercetin aglycone. Similarly, 50 and 100 µg/ml A-type PAC DP-9 caused an increase in the SKOV-3 cell G2/M subpopulation to 17.69% and 29.63% respectively. After 48 h of treatment in SKOV-3 cells, A-type PAC DP-9 exhibited similar dose-dependent G2/M-phase arrest, whereas 50 and 100 µg/ml quercetin aglycone led to an increase in S subpopulation from 22.01% to 45.54% and 50.35% respectively, with no significant change in G2/M subpopulation. This suggests that quercetin aglycone caused S/G2-phase arrest in SKOV-3 cells after 48 h of treatment.

Quercetin aglycone treatment for 24 and 48 h in OVCAR-8 cells caused G1/S-phase arrest. At 50 µg/ml, quercetin aglycone led to retention of 75.69% and 86.12% of cells in G0/G1 phase after 24 h and 48 h of treatment, respectively, and the subpopulation of S-phase cells decreased from 38.67% to 12.61% after 24 h and from 26.35% to 6.45% after

48 h (Figure 4.7D). A-type PAC DP-9 showed a similar effect in OVCAR-8 cells, thus confirming that G1/S-phase arrest was caused by the two compounds in OVCAR-8 ovarian cancer cells.

Cytotoxicity of B-type cocoa PACs against ovarian cancer cells

Similar with cranberry flavonoids, individual cocoa B-type PACs also exhibited differential cytotoxicity against SKOV-3 and OVCAR-8 cells. In SKOV-3 cells B-type PACs with higher molecular weight (DP ≥ 6) showed stronger cytotoxicity than low molecular weight oligomers (Figure 4.8A). Among them, B-type PAC DP-7 and DP-8 exhibited highest cytotoxicity with IC_{50} at 31.8 and 22.4 μg/ml respectively. Consistent with A-type PACs, B-type PACs also exhibited stronger cytotoxicity against SKOV-3 cells than OVCAR-8 cells. Cytotoxicity of PAC oligomers increases with DP from DP-3 to DP-8, then decreases at DP-9 and DP-10 (Figure 4.8B). B-type DP-7 and DP-8 again showed highest activity among tested compounds, with IC_{50} at 84.1 and 91.0 μg/ml. B-type DP-8 was selected in further studies for its high cytotoxicity against two cancer cell lines.

Sublethal concentrations of B-type PAC DP-8 also sensitized ovarian cancer cells to cisplatin. As shown in Figure 4.9A, while 5 μM or 2.5 μM B-type PAC DP-8 alone did not reduce the cell viability of SKOV-3 or OVCAR-8 cells within 24 h's treatment, its pretreatment significantly reduced cell viability of cisplatin-treated SKOV-3 or OVCAR-8 cells. This result again suggests the PACs' capability to sensitize ovarian cancer cells into platinum based drug. Following such result, levels of DNA-bonded cisplatin were determined using ICP-MS in cisplatin treated or cisplatin and B-type PAC DP-8 co-

treated SKOV-3 and OVCAR-8 cells. Inconsistent with cell viability assay, co-treatment of B-type DP-8 did not up-regulate the formation of DNA-cisplatin adduct (Figure 4.9B). On the contrary, DNA-bonded cisplatin levels were non-significantly reduced upon co-treatment with different concentrations of B-type PAC DP-8.

While lower concentrations (6.25-12.5 μg/ml) of B-type PAC DP-8 did not affect cell cycle progression in SKOV-3 cells, treatment of 25 μg/ml B-type PAC DP-8 induced G2/M-phase arrest. As shown in Figure 4.10, subpopulation of G2/M-phase cells increased from 14.42% to 32.37% after 24 h of treatment. Cell cycle progression of OVCAR-8 cells was not affected by different concentrations (12.5-50 μg/ml) of B-type PAC DP-8, as ratio of G0/G1, S and G2/M-phase cells remained similar across different treatments (Figure 4.10B).

4.4 Discussion

Cranberry phenolic extracts have shown *in vitro* anti-cancer and chemo-preventive properties in different cancer cell lines [5-11]. Research on individual phenolic compounds of cranberry has been limited by the difficulty of compound isolation due to complex structural variations, including degree-of-polymerization, linkage type, position of the double linkage between constituent units, and attachment with sugar moieties [9, 12]. Serial application of Dionex UltiMate® 3000 LC system consisting of UltiMate 3000 RS Pump, UltiMate 3000 RS Autosampler, UltiMate 3000 RS Column Compartment, and UltiMate 3000 RS Diode Array Detector coupled with Applied Biosystems API 3000™ triple quad LC-MS/MS mass spectrometer has allowed the reproducible isolation and characterization of individual A-type PACs with up to the 10th degree of

polymerization in >95% purity in our laboratories. So far, we have successfully isolated 19 high-purity individual cranberry flavonoids, characterized their structures by LC-APCI-MS, and used them as primary materials to determine bioactivity.

SKOV-3 and OVCAR-8 ovarian cell lines, which possess several key oncogenic hallmarks such as p53 mutation, EGFR over-expression, and cisplatin resistance [16-18], were employed as an *in vitro* cell culture model to determine the mechanism of action of individual flavonoids. Individual A-type PACs DP-2 to DP-4 failed to show strong cytotoxicity against SKOV-3 cells, and high molecular weight PAC polymers generally exhibited stronger cytotoxicity similar to what we showed earlier [9]. Quercetin aglycone emerged as the most cytotoxic of the tested cranberry flavonols. Shen *et al.* showed that quercetin aglycone exhibited significantly higher cytotoxicity against human promyelocytic leukemia cells HL-60 compared to its rutinose and rhamnose glycosides [19]. Murota *et al.* also reported that quercetin aglycone was much more efficiently absorbed by Caco-2 colorectal adenocarcinoma cells than its glycosides [20]. Compared with A-type PACs, B-type cocoa PACs exhibited considerable higher cytotoxicity against the two ovarian cancer cell lines. Interestingly, the two PAC molecules with similar scope of molecular weight (DP) – A-type DP-9 and B-type DP-8, were identified as the most active compounds within their groups. These results indicate both DP and inter-flavan linkage type have profound influences on the bioactivity of PAC oligomers and polymers.

The high cytotoxicity against ovarian cancer cells of the three flavonoid compounds - A-type PAC DP-9 (cranberry), B-type PAC DP-8 (cocoa) and quercetin aglycone (cranberry), as revealed by cell viability assay, suggests a promising pharmacological

potential in ovarian cancer therapy. Compared with the two PAC polymers (molecular weight: 2300-2600), quercetin aglycone is significantly smaller (molecular weight: 302) with a simple monomer structure. Their differences in molecular size and structure provide different bioactivity mechanisms against ovarian cancer cells. To further illustrate their mechanisms of action, we focused on these three compounds in the subsequent studies.

Sublethal concentrations of quercetin aglycone, A-type PAC DP-9 and B-type PAC DP-8 all significantly increased the efficacy of cisplatin against ovarian cancer cells. Platinum-based drugs such as cisplatin, carboplatin and oxaliplatin are commonly used as chemotherapy agents for treatment of testicular and other cancer types including ovarian cancer. Activity of platinum complexes to inhibit cancer growth is due to a number of their properties, including cross-membrane transportation, intracellular accumulation and coordination into DNA (DNA platination) [21], which results in inhibition of DNA replication and RNA transcription as well as cell cycle arrest and induction of apoptosis. As to cisplatin, approximately 1% of total intracellular cisplatin incorporates with genomic DNA to form a variety of intra- and inter-strand DNA-cisplatin adducts [22]. Although studies indicate DNA platination as the main factor contributing cisplatin's toxicity [21, 23], in the current study, however, we failed to observe any increase of DNA platination in cisplatin and B-type PAC DP-8 co-treated SKOV-3 and OVCAR-8 cells. This result suggests that different mechanism(s) other than DNA platination accounted for the increased cytotoxicity of cisplatin against PAC treated ovarian cancer cells. Because developed resistance to platinum-based drugs is one of the major causes of mortality in ovarian cancer [2], the observation that cranberry and cocoa flavonoids can

sensitize drug-resistant ovarian cancer cells to cisplatin provides an opportunity to improve the efficacy of platinum chemotherapies and reduce side effects associated with cisplatin.

EGFR, a receptor tyrosine kinase, is involved in many signaling pathways that modulate cell survival, proliferation and apoptosis. Aberrant activation of EGFR has been shown to play a critical role in cancer cell survival and development [18, 24]. B-type PACs isolated from grape seeds have been shown to target and down-regulate EGFR expression in human head and neck squamous cell carcinoma (HNSCC) cells and inhibited their invasiveness [25]. Our study showed that treatment with A-type PAC DP-9 decreased expression and activation and induced nuclear translocation of EGFR in SKOV-3 cells dose dependently. Association of EGFR with DNA-PK, which is involved in DNA repair, has been previously reported [26, 27], and its nuclear translocation has been confirmed to modulate DNA repair caused by cisplatin or radiation in mouse fibroblast cell lines [28]. Although EGFR nuclear translocation can be expected to activate DNA-PK as a counter measure to DNA damage due to quercetin and A-type PAC-9 treatment, immunofluorescence microscopy analysis revealed down-regulation of DNA-PK in ovarian cancer cells exposed to quercetin aglycone or A-type PAC DP-9. The DNA-PK-mediated DNA repair that is induced by exposure to cisplatin in cancer cells is believed to be an important factor in reducing the efficacy of platinum-based chemotherapy [29, 30]. Therefore, the inhibition of DNA-PK expression in quercetin aglycone or A-type PAC DP-9-treated ovarian cancer cells may partially account for the increased efficacy of cisplatin in ovarian cancer cells following pre-treatment of the two flavonoids.

EGFR also regulates the extracellular signal-regulated kinase ERK-MAPK pathway to maintain normal cell growth, proliferation and differentiation [31]. Aberrant activation of the EGFR-Ras-Raf-MEK-ERK cascade is believed to contribute to cancer development and progression [32]. Both quercetin aglycone and A-type PAC DP-9 down-regulated activated Raf, ERK1/2 and MEK in OVCAR-8 cells, suggesting the ERK-MAPK pathway may be one of the anti-proliferative mechanisms. The Raf-MEK-ERK pathway is also known to control cell cycle progression through induction of key cell cycle regulatory factors such as cyclins, cyclin-dependent kinases (CDKs), and p21 [33]. Phosphorylation of histone H3, which is believed to play an important role in cell division and oncogene induction, was shown to be stimulated by Ras-MAPK signaling pathway [34, 35]. In our study, we report different levels of up-regulation of CDK inhibitors p18, p27, and most significantly, p21 after treatment with quercetin aglycone or A-type PAC DP-9, as well as down-regulation of cyclin D1 and phospho-histone H3, indicating their effects on cell cycle regulation. Expression of p21 can be induced by either p53-dependent or independent pathways [36, 37]. In SKOV-3 cells, where the p53 gene is mutated and loses its expression, A-type PAC DP-9 induced p21 up-regulation inversely correlated with the expression of ERK1/2, suggesting that PAC DP-9 modulated a p53-independent ERK pathway that mediates p21 regulation similar to a published mechanism in rhabdosarcoma cells [37].

Up-regulation of CDK inhibitors and down-regulation of cyclin D1 and phospho-histone H3 induced by the two cranberry flavonoids reflected the ability of quercetin aglycone and PAC DP-9 to cause cell cycle arrest in SKOV-3 and OVCAR-8 cells. At concentrations lower than IC_{50}, both quercetin aglycone and A-type PAC DP-9 induced

G1/S phase cell cycle arrest in OVCAR-8 cells, consistent with the up-regulation of cellular p21 that has been shown to inhibit CDK regulation in G1/S phase progression [38]. On the other hand, SKOV-3 cells showed cell cycle arrest at G2/M phase within 24 h of treatment with quercetin aglycone, A-type PAC DP-9 or B-type DP-8, similar as shown previously [10]. Interestingly, CDK2, which is inhibited by p21 and facilitates G1/S phase transition [38], was up-regulated after treatment with quercetin aglycone or A-type PAC DP-9 in both SKOV-3 and OVCAR-8 cells. We confirmed direct protein-protein interaction between EGFR and CDK2 in SKOV-3 cells through Co-IP, suggesting that CDK-2 overexpression could be induced by nuclearly localized EGFR to facilitate DNA repair and synthesis after exposure to quercetin aglycone or A-type PAC DP-9. These observations suggest that cell division of the two ovarian cancer cell lines was regulated through different mechanisms by cranberry flavonoids. Targeting cell cycle checkpoints has been proposed as a promising approach to cancer treatment [39].

In conclusion, our study suggests that certain cranberry and cocoa flavonoids possess cytotoxic properties against ovarian cancer cells. The integration of such flavonol and PAC compounds in ovarian cancer chemotherapy may provide for improved outcomes. Their capabilities to induce cellular apoptotic events including cell cycle arrest and suppress DNA repair pathways highlight their potential as dietarily available therapeutic agents. Further studies will focus on the *in vivo* anti-cancer activities of these bioactive cranberry and cocoa flavonoid compounds, with the aim of closely evaluating their pharmacological potentials in cancer therapy and underlying action mechanisms.

4.5 References

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4.6 Tables and Figures

Table 4.1. Correlation coefficients (r), *p*-values, and IC_{50} values of individual cranberry PACs and flavonols against SKOV-3 and OVCAR-8 cells. Linear regression was conducted for each cranberry flavonoid between cell viability and compound concentration (untransformed and logarithm-transformed). IC_{50} value was calculated based on best-fit regression model.

Compound	SKOV-3			OVCAR-8		
	Correlation Coefficient	*p* value	IC_{50} (μg/ml)	Correlation Coefficient	*p* value	IC_{50} (μg/ml)
DP-2	-0.917	0.0000263	194.8	-0.919	0.0000239	143.1
DP-3	-0.804	0.00163	210.8	-0.513	0.0882	ND
DP-4	-0.923	0.0000185	200.0	-0.936	0.00000741	164.1
DP-5	-0.983	1.15×10^{-8}	126.3	-0.738	0.00616	209.9
DP-6	-0.929	0.0000129	201.1	-0.9	0.0000674	152.6
DP-7	-0.821	0.00106	288.7	-0.736	0.00631	211.2
DP-8	-0.944	0.00000397	137.9	-0.945	0.00000356	125.7
DP-9	-0.939	0.00000584	81.9	-0.923	0.0000189	137.8
DP-10	-0.494	0.103	ND	-0.565	0.0558	ND
DP-11	-0.966	3.53×10^{-7}	165.9	-0.931	0.0000107	146.9
DP-12	-0.824	0.000978	162.6	-0.791	0.00216	248.5
M-Gal	0.331	0.293	ND	-0.78	0.00275	130.7
Q-Gal	-0.833	0.000757	164.8	-0.896	0.000082	132.7
Q-Glu	-0.899	0.0000684	160.6	-0.929	0.000013	160.6
Q-A-P	-0.665	0.0182	410.2	-0.777	0.00293	212.7
Q-A-F	-0.791	0.00217	173.5	-0.908	0.0000435	171.2
Q-Xylo	0.095	0.77	ND	-0.266	0.404	ND
Q-Rhamno	-0.879	0.000167	207.4	-0.945	0.00000369	163.4
Q-Aglycone	-0.982	1.45×10^{-8}	83.2	-0.966	3.48×10^{-7}	61.1

Figure 4.1. Structures of cranberry flavonoids quercetin aglycone and PAC DP-9. A: HPLC chromatograph of quercetin aglycone; B: HPLC chromatograph of PAC DP-9; C: MS spectrum of quercetin aglycone (m/z=301); D: Structures of quercetin algycone and PAC DP-9.

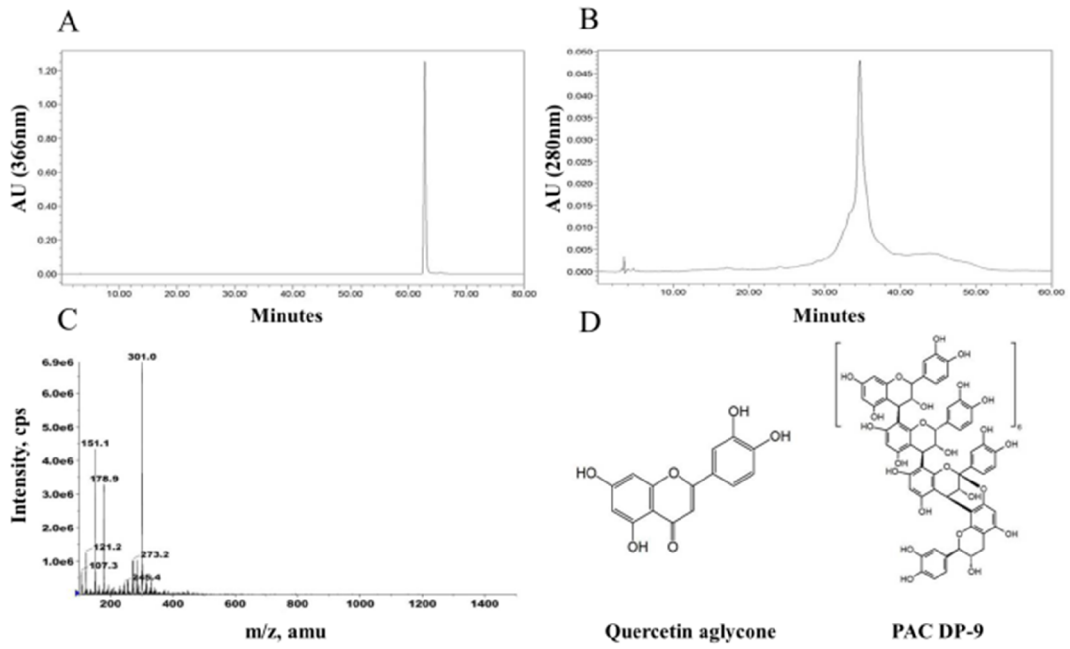
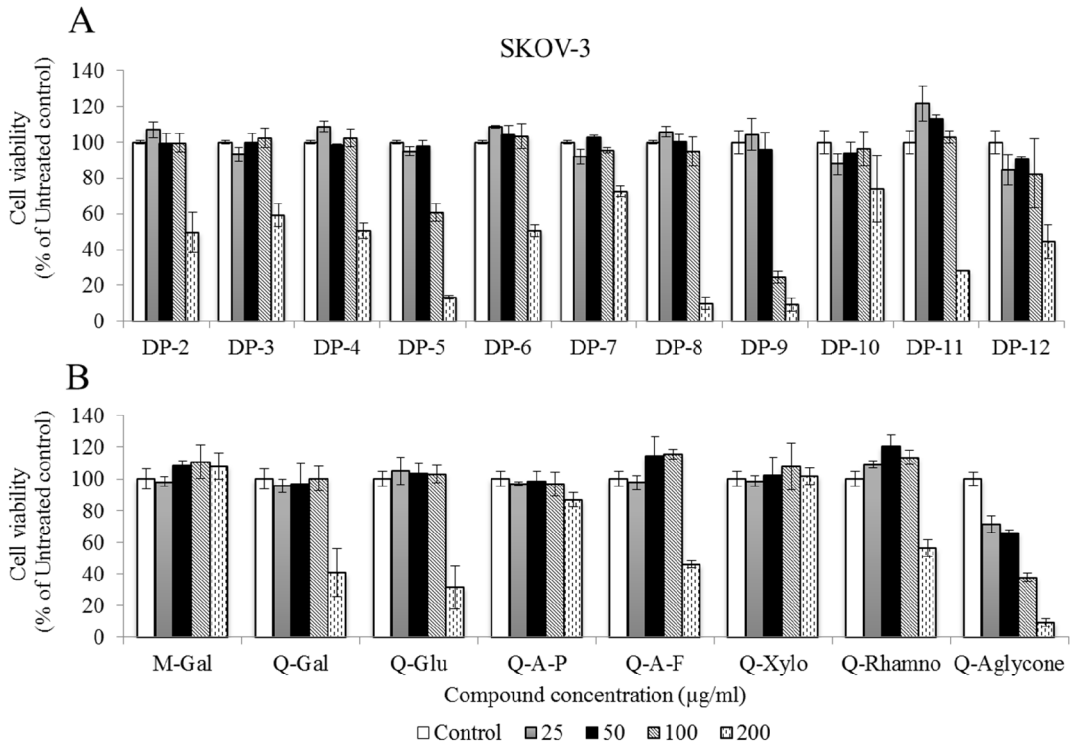


Figure 4.2. Cytotoxicity of individual cranberry flavonols and PACs in SKOV-3 and OVCAR-8 ovarian cancer cells. Cytotoxicity was determined by MTS cell viability assay; cells were treated with DMSO vehicle or different concentrations of cranberry flavonoids (25-200 µg/ml) for 72 h. Experiments were performed in triplicate; data are expressed as mean ± SD in percent of cell viability of untreated cells (100%).



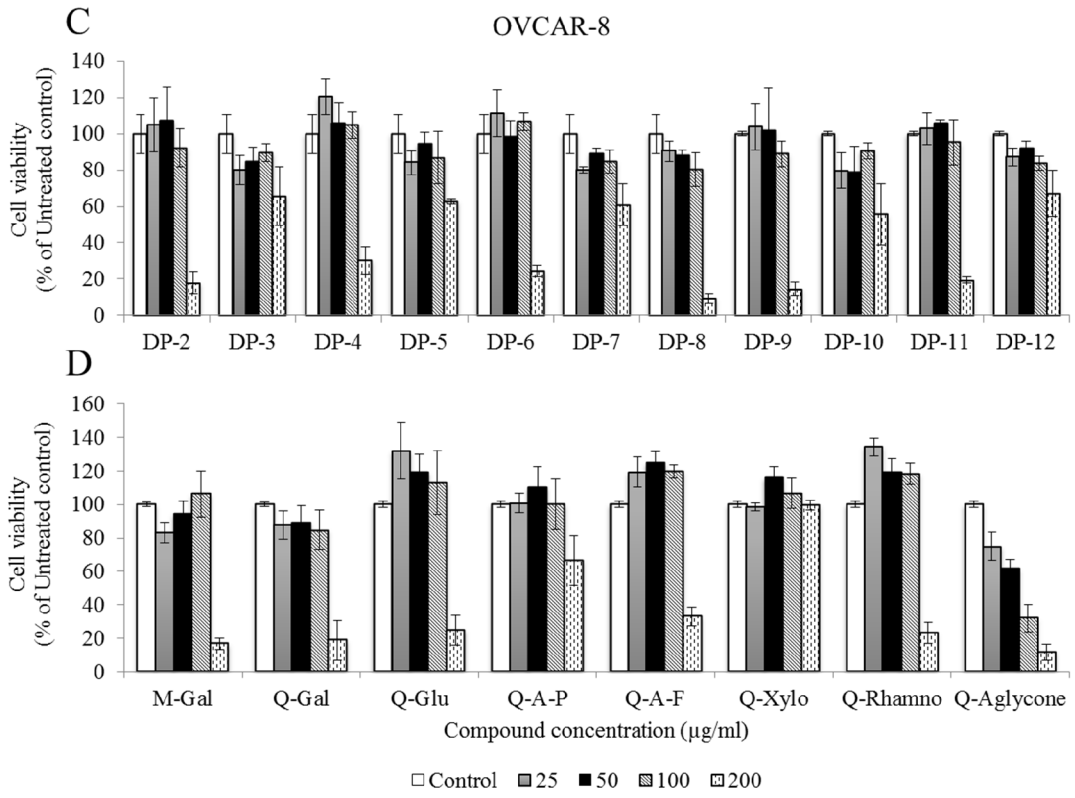
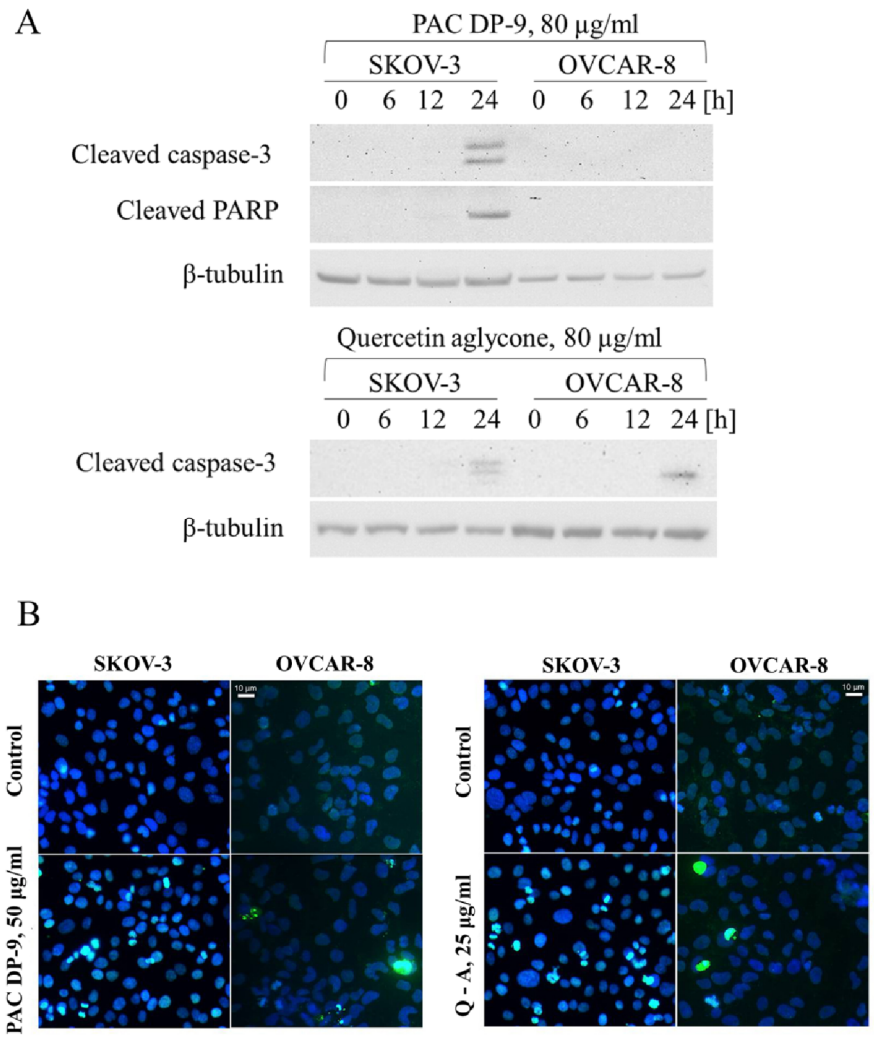

C
OVCAR-8
Cell viability
(% of Untreated control)
140
120
100
80
60
40
20
0
DP-2
DP-3
DP-4
DP-5
DP-6
DP-7
DP-8
DP-9
DP-10
DP-11
DP-12
D
Cell viability
(% of Untreated control)
160
140
120
100
80
60
40
20
0
M-Gal
Q-Gal
Q-Glu
Q-A-P
Q-A-F
Q-Xylo
Q-Rhamno
Q-Aglycone
Compound concentration (µg/ml)
Control
25
50
100
200

Figure 4.3. Induction of apoptosis by quercetin aglycone and PAC DP-9 in SKOV-3 and OVCAR-8 cells. A: Expression of cleaved caspase-3 and cleaved PARP after treatment with PAC DP-9 and quercetin aglycone. Ovarian cancer cells were treated with PAC DP-9 or quercetin aglycone (80 µg/ml) for 0-24 h. Actin or tubulin were probed as internal loading controls; B: TUNEL assay in PAC DP-9 and quercetin aglycone (Q-A)-treated SKOV-3 and OVCAR-8 cells. Cells were treated with PAC DP-9 (50 µg/ml), quercetin aglycone (25 µg/ml) or DMSO vehicle for 12 h. DNA strand breaks due to apoptosis were detected and labelled by fluorescein-labelled nucleotides (green).



Figuer 4.4. Cell viability of cranberry flavonoid and cisplatin-treated SKOV-3 (A) and OVCAR-8 (B) cells. Cells were pre-treated with either DMSO vehicle, PAC DP-9 (25 µg/ml), or quercetin aglycone (10 µg/ml) for 6 hours in complete DMEM media, followed by cisplatin treatment alone or cisplatin + PAC DP-9/quercetin aglycone. Cells were incubated overnight for 12 hours and analyzed via MTS for viability.

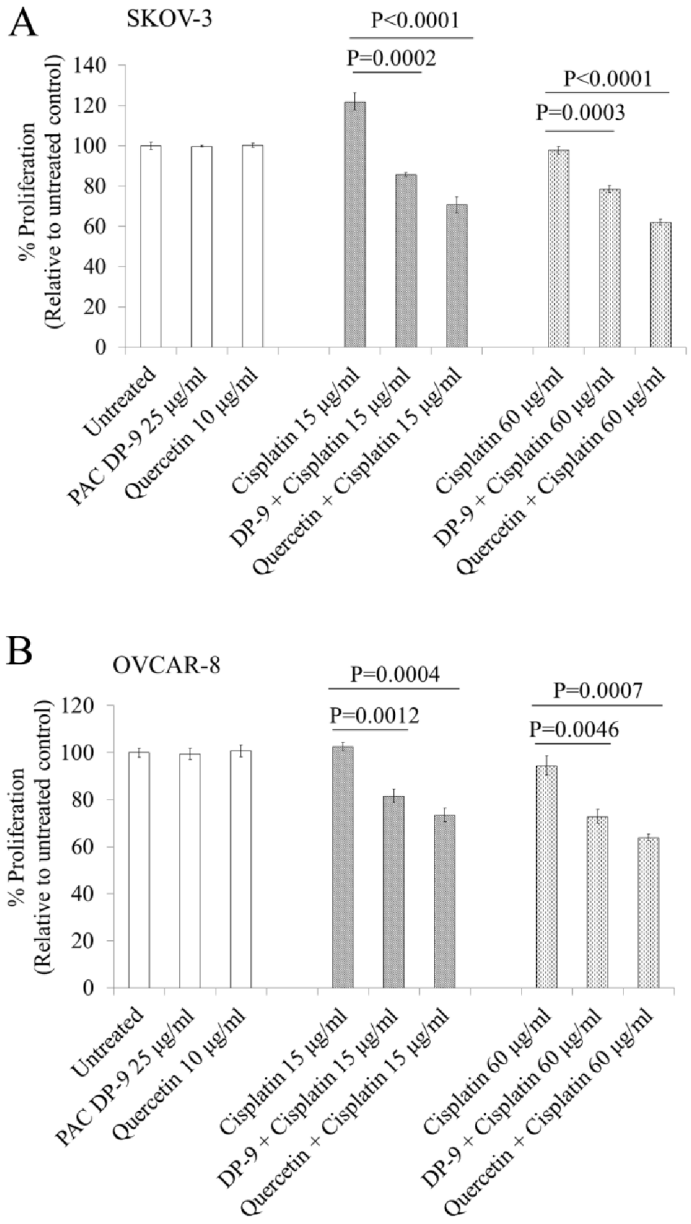


Figure 4.5. Effects of quercetin aglycone and PAC DP-9 on expression, activation and localization of EGFR in SKOV-3 and OVCAR-8 cells. A: Expression of total and activated EGFR on cranberry flavonoid treated SKOV-3 and OVCAR-8 cells. Cells were serum-deprived for 4 h and then treated with 5, 20 or 40 µg/ml quercetin aglycone or PAC DP-9 in serum-free DMEM medium for 12 h. Actin was probed as internal loading control. B: Effects of PAC DP-9 on EGFR expression and localization in SKOV-3 cells. Cells were treated with different concentrations (12.5-200 µg/ml) of PAC DP-9 for 3 h before fluorescent microscopic analysis. EGFR was visualized by Alexa Fluor® 594 secondary antibody (red), and cell nuclei were stained with DAPI.

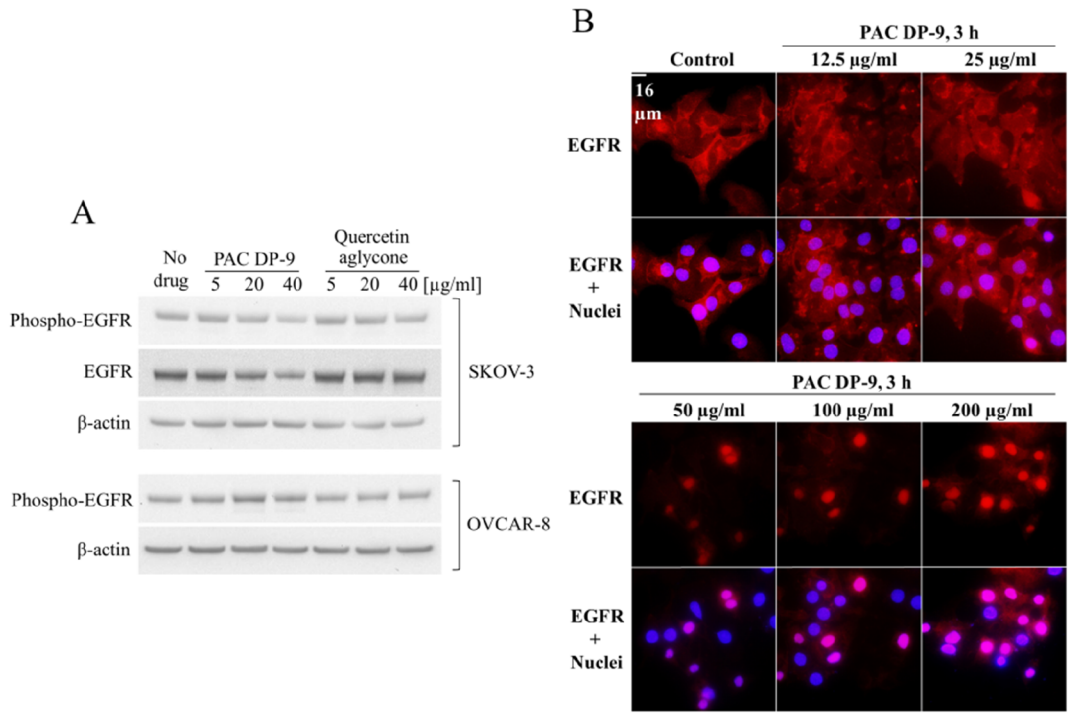


Figure 4.6. Effect of qercetin aglycone and PAC DP-9 on cellular expression of MEK (A), phospho-ERK1/2 (B), phospho-histone H3 (C), cyclin D1 (D), DNA-PK (E) and p21 (F) in SKOV-3 and OVCAR-8 cells. Cells were treated with either DMSO vehicle, PAC DP-9 (80 µg/ml), or quercetin aglycone (80 µg/ml) for 12 hours and then immunostained for target proteins. MEK, cyclin D1 and p21 were probed by DyLight 488 secondary antibody (green); phospho-ERK1/2, phospho-histone H3 and DNA-PK were probed by DyLight 594 secondary antibody (red).

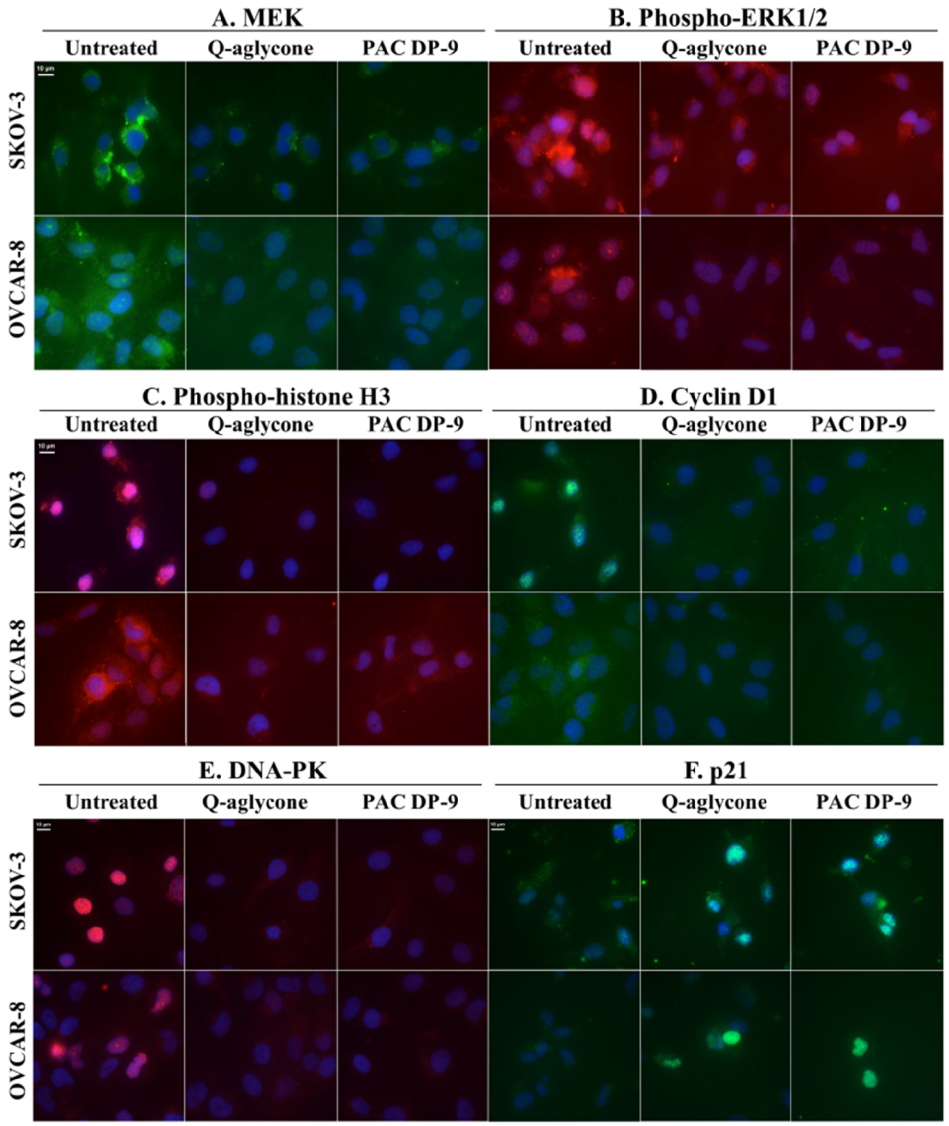
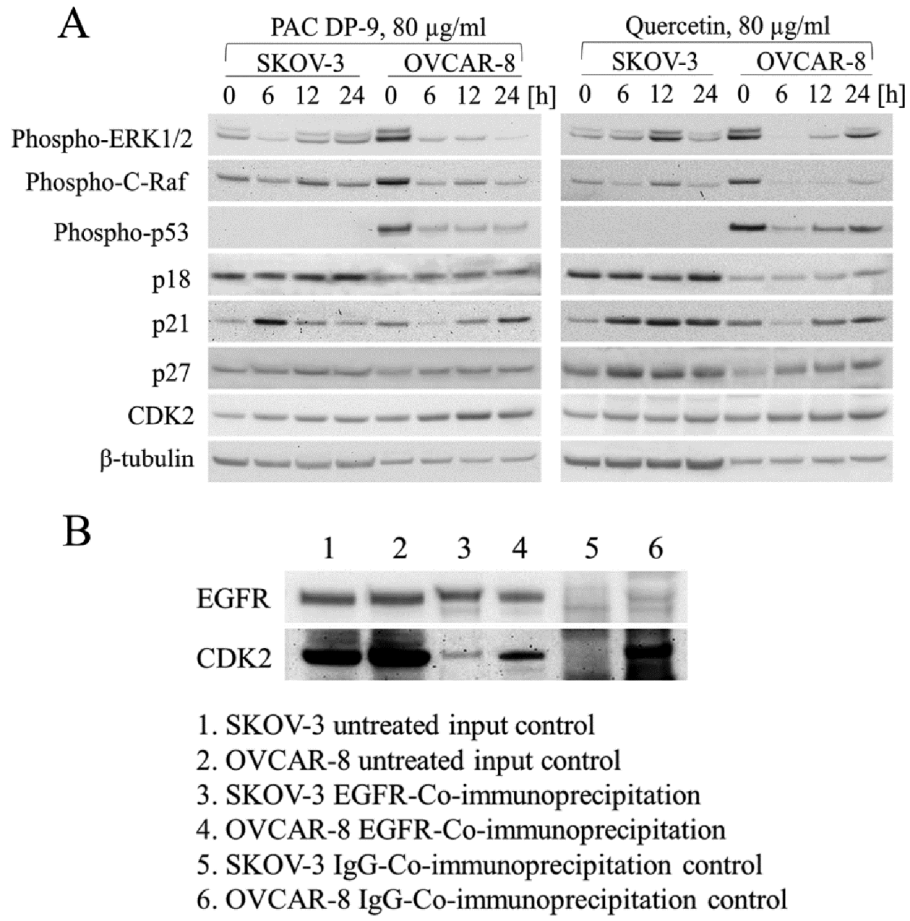
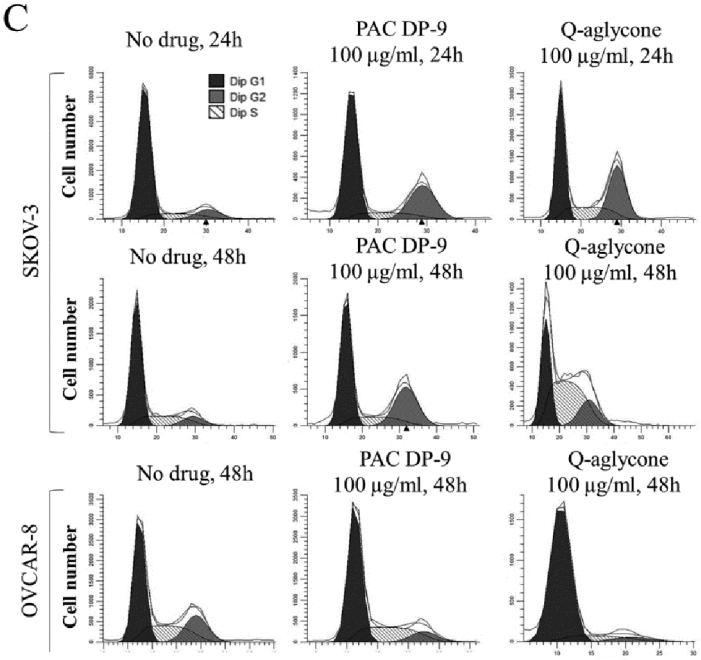


Figure 4.7. Effect of PAC DP-9 and quercetin aglycone on cell cycle regulation in SKOV-3 and OVCAR-8 cells. A: Western blot analysis of ERK-MAPK signal components and cell cycle regulatory factors in SKOV-3 and OVCAR-8 cells after treatment with quercetin aglycone or PAC DP-9 (80 µg/ml) for 0-24 h. Tubulin was probed as an internal loading control; B: EGFR co-immunoprecipitation on SKOV-3 and OVCAR-8 cell lysates. C, D: Determination of cell cycle progression by FACS analysis in SKOV-3 and OVCAR-8 cells treated with 50 or 100 µg/ml quercetin aglycone or PAC DP-9 for 24-48 h. Data are presented as relative fluorescence intensity of G0/G1, S and G2/M phase subpopulations in 2-D charts (C) or tables (D).



C



D

SKOV-3

Incubation time [h]	Compound	Concentration [µg/ml]	G0/G1 [%]	S [%]	G2/M [%]
24	No drug	0	76.7	12.49	10.81
	Quercetin aglycone	50	64.24	19.24	16.52
		100	52.48	15.17	32.35
	PAC DP-9	50	70.43	11.88	17.69
		100	58.58	11.79	29.63
48	No drug	0	68.05	22.01	9.94
	Quercetin aglycone	50	43.61	45.54	10.85
		100	33.3	50.35	16.36
	PAC DP-9	50	64.24	19.24	16.52
		100	52.48	15.17	32.35

OVCAR-8

Incubation time [h]	Compound	Concentration [µg/ml]	G0/G1 [%]	S [%]	G2/M [%]
24	No drug	0	33.39	38.67	27.94
	Quercetin aglycone	50	75.69	12.61	11.7
		100	70.26	15.5	14.24
	PAC DP-9	50	56.03	33.6	10.37
		100	61.09	30.47	8.44
48	No drug	0	52.19	26.35	21.46
	Quercetin aglycone	50	86.12	6.45	7.43
		100	83.9	10.8	5.3
	PAC DP-9	50	47.55	34.18	18.28
		100	63.44	27.36	9.2

Figure 4.8. Cytotoxicity of individual cocoa B-type PACs in SKOV-3 and OVCAR-8 ovarian cancer cells. Cytotoxicity was determined by MTS cell viability assay; cells were treated with DMSO vehicle or different concentrations of cranberry flavonoids (25-200 µg/ml) for 72 h. Experiments were performed in triplicate; data are expressed as mean ± SD in percent of cell viability of untreated cells (100%).

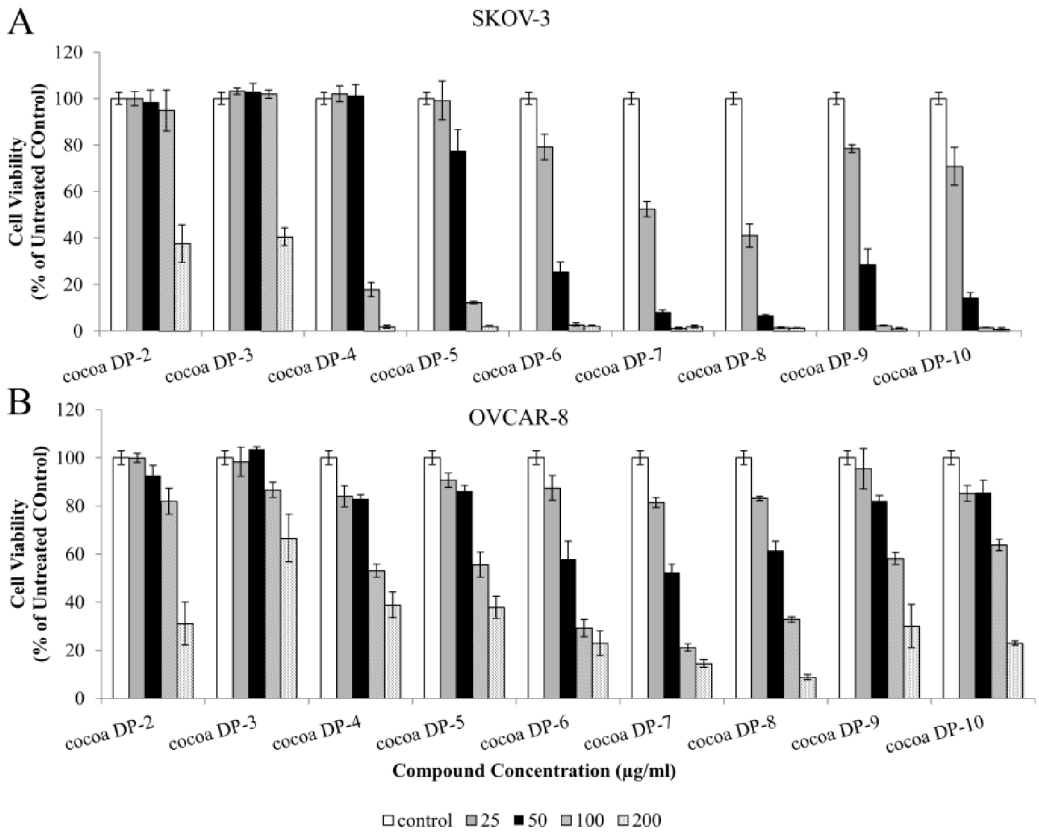


Figure 4.9. Sensitization to cisplatin by cocoa B-type PAC DP-8 in SKOV-3 and OVCAR-8 cells. A: Cell viability of cocoa B-type PAC DP-8 and cisplatin-treated SKOV-3 and OVCAR-8 cells. Cells were pre-treated with either DMSO vehicle or B-type PAC DP-8 (2.5-10 μM) for 6 hours, followed by cisplatin alone or cisplatin + PAC DP-8 treatment for 18 hours. B: Levels of DNA bonded cisplatin in SKOV-3 and OVCAR-8 cells. Cells were pre-treated with either DMSO vehicle or B-type PAC DP-8 (10 μM or 20 μM) for 6 hours, followed by cisplatin treatment alone or cisplatin + PAC DP-8 treatment for 18 hours. *Significant difference at α=0.05 significant level determined by Student's t-test.

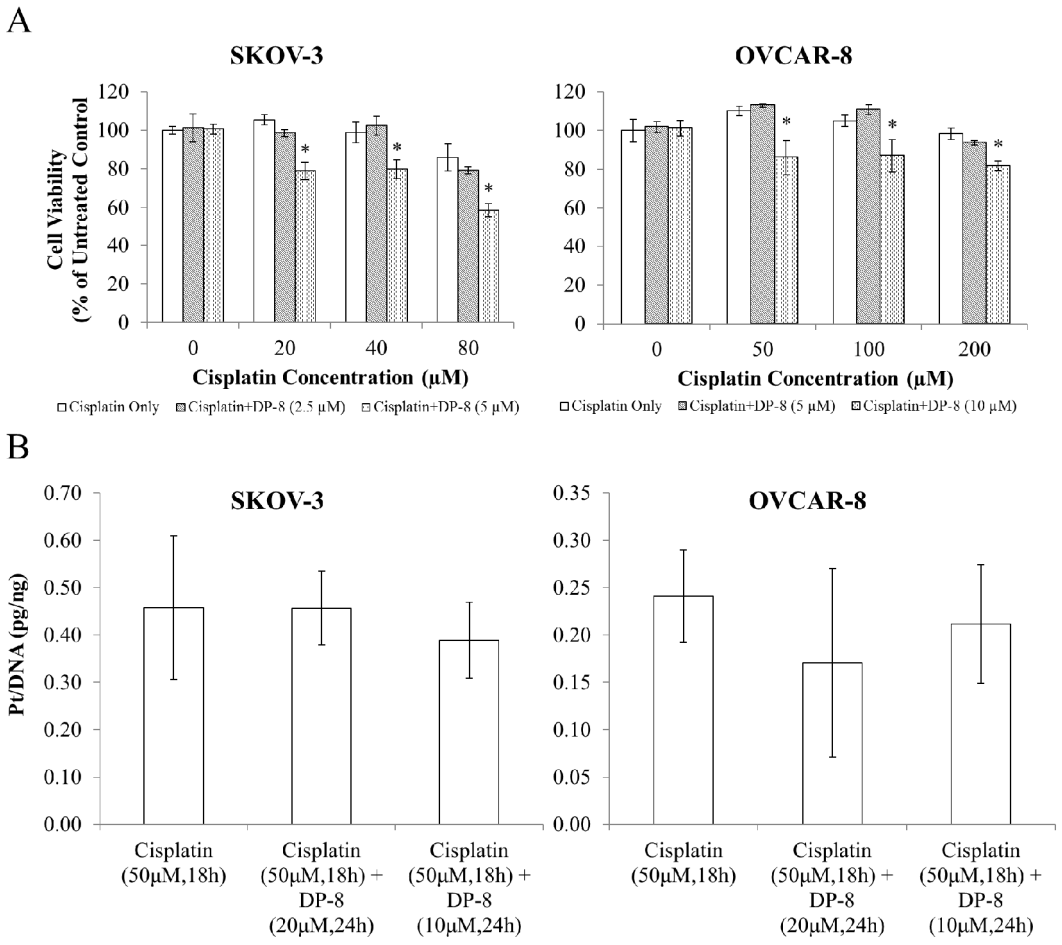
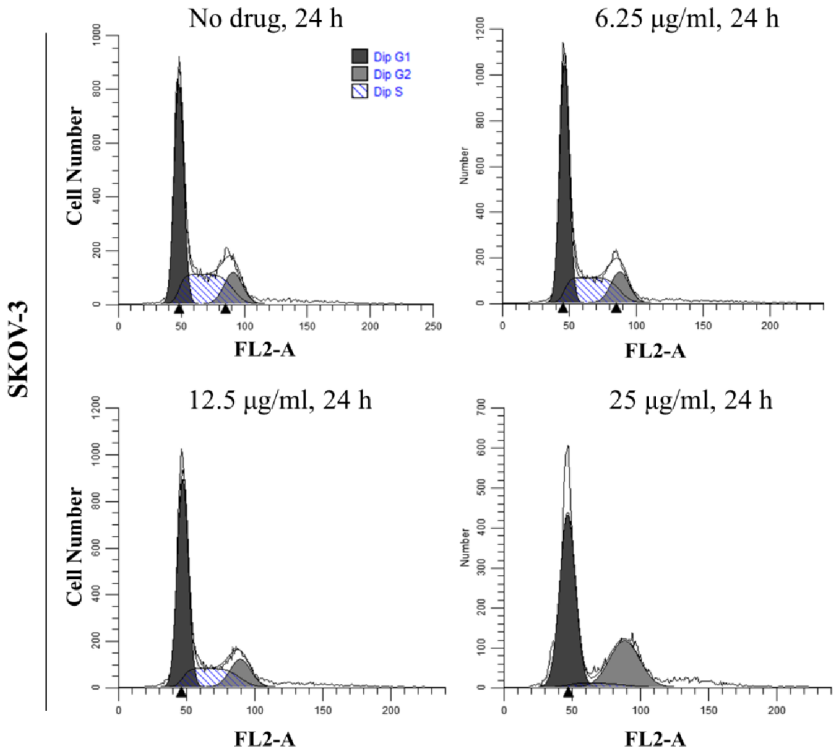


Figure 4.10. Determination of cell cycle progression by FACS analysis in SKOV-3 and OVCAR-8 cells treated with 0-50 µg/ml B-type PAC DP-8 for 24h. Data are presented as relative fluorescence intensity of G0/G1, S and G2/M phase subpopulations in 2-D charts (A) or tables (B).

A



B

Cell line	Concentration [µg/ml]	G0/G1 [%]	S [%]	G2/M [%]
SKOV-3	0	53.52	32.06	14.42
	6.25	58.18	27.77	14.05
	12.5	61.7	22.8	15.5
	25	63.59	4.03	32.37
OVCAR-8	0	46.87	31.97	21.16
	12.5	47.66	28.32	24.02
	25	41.96	30.02	28.02
	50	45.85	28.57	25.58

Chapter 5

Human Absorbance and Urinary Excretion of Cranberry Flavonols and Proanthocyanidins

5.1 Introduction

Flavonoids are polyphenolic secondary metabolites which occur widely in different plants and plant derived foods, such as vegetables, fruits, grains, wine and tea [1]. They are associated with health-beneficial properties including antioxidant, anti-inflammatory and anti-cancer activities [2]. Epidemiologic studies strongly suggest an inverse correlation between dietary flavonoids intake and risk of cardiovascular disease [3].

American cranberry (*Vaccinium macrocarpon*) is rich in polyphenols, in particular flavonoids, including anthocyanins, phenolic acids, flavonols and flavan-3-ols. Cranberry fruit has been shown to possess the highest content of phenols among 20 most consumed fruits in American diet [4]. Cranberry juice (CBJ) consumption has been shown to prevent or reduce urinary tract infection (UTI) [5], and cranberry flavan-3-ols (proanthocyanidins) were suggested to inhibit the adherence of uropathogenic P-Fimbriated *Escherichia coli*, one of the proposed mechanisms by which CBJ consumption reduced UTI pathogenicity [6]. There are many other health-beneficial purported properties of cranberry and its constituents, such as anti-oxidant [7], anti-cancer [8, 9] and anti-oral bacterium activities [10] as well as protection against coronary heart disease (CHD) [11-13] have been shown by in vivo and in vitro studies that cranberry phenolics play an important role in health promotion.

The potential benefits of cranberry phenolics largely depend on their human bioavailability, which can differ greatly between individual compounds [14]. Anthocyanins have been recovered in human plasma and urine at picogram levels following cranberry juice ingestions [15, 16]. Several cranberry phenolic acids were recovered using GC-MS in acid-hydrolyzed human plasma and urine after ingestion of different cranberry products [17].

Flavonols and flavan-3-ols have received much research interest due to their putative beneficial health properties. Despite their high content in some foods, flavan-3-ols are poorly absorbed as intact flavan-3-ols with degree-of-polymerization (DP) > 3 were not found to be permeable to the human Caco-2 intestinal cell line [18]. Evidence for absorption of flavan-3-ols is conflicting with only a few *in vivo* studies reporting minimal absorbance of flavan-3-ol dimers in human and rat [19, 20], whereas in other studies no dimer was detected [21, 22].

Flavonols occur mostly as glycosylated conjugates in plants and in plant derived products that humans consume [23]. While quercetin and myricetin are the two primary aglycone flavonols recovered from human plasma and urine, with sample hydrolysis, after consumption of flavonol-containing foods or drinks [20, 24], flavonol glycoside bioavailability is still poorly understood in part because sample processing methods often utilize enzymes, acid hydrolysis, heating, bases, etc. prior to detection leads to deglycosylation. Using a rat intestinal perfusion model Arts, Sesink, Faassen-Peters and Hollman [25] reported uptake of only quercetin-glucosides, where there was no uptake of quercetin-galactoside, rhamnoside and arabinoside. However, we demonstrated the presence of quercetin-3-galactoside in human urine following ingestion of sweetened

dried cranberries in a recent pilot study [26]. Benefits to human health will be a function of the actual compounds absorbed. Since glycosylated flavonols are very difficult to detect and quantify in animal tissues, many bioavailability studies utilize sample preparation that results in deglycosylated flavonols. However, the bioactivity, metabolism and clearance will likely be mediated by the flavonol, e.g. quercetin and myricetin, conjugates present *in vivo*. Bioactivity of flavonol aglycones versus the conjugated derivatives often differ [9, 27]. Thus, there is a dearth of information on the bioavailability of the various parent flavonol glycosides, their derived circulating flavonol constituents, e.g., potential metabolites, in humans from cranberry and other food products. Since cranberry flavonols consist of largely glycosides, an improved understanding of flavonol glycoside bioavailability is needed to investigate how cranberry product ingestion leads to health benefits for UTIs, CHD and cancer. The objective of the present study investigated human urinary excretion of flavano-3-ols and flavonol glycosides following ingestion of reduced acid CBJ. A protocol with no preparative hydrolysis or enzymolysis was used to avoid flavonol deglycosylation and degradation. A sensitive UHPLC-ESI-MS-MS method was developed to maximize the sensitivity and selectivity of simultaneous determination of flavan-3-ols and flavonol glycosides in urine and cranberry juice.

5.2 Materials and methods

Study design

Following approval by the Winona State University Human Subjects Institutional Review Board (IRB), ten women (19.5 ± 1.1 years; 22.5 ± 2.0 body mass index) were recruited

for this randomized single-cross over study. Exclusions included serious medical conditions (i.e. diabetes), non-smoking status, alcohol consumption of two or fewer drinks or beers/week, and no history of urinary tract infections in last 3 months. Participants were asked to refrain from taking medications (i.e. birth control pill), vitamins or minerals until after the last daily urine sample had been collected. Participants were required to abstain from consuming alcohol, chocolate, and fruits containing phenolic compounds 24 hours before each laboratory visit, and refrain from exercise before presentation to the laboratory.

Participants were required to maintain a special diet for 14 hours before each laboratory visit that included no onions, garlic, chocolate, green vegetables, fruits, fruit juices, pasta sauces, wine, beer or alcoholic beverages in order to limit sources of flavonoids. Nothing except water could be consumed for 9 hours prior to laboratory arrival (fasting condition; 6am) or during the 6 hour study period in the lab. An hour before bedtime, participants ingested 500 ml volume of water, when they woke up to come to the study the next morning they were instructed to urinate and drink an additional 500 ml of water prior to arrival at the laboratory. Water consumption and urination were intended to flush remaining flavonoids form the urinary tract prior to laboratory presentation and facilitate timed urine collections in the laboratory. Upon arrival participants completed a 15 minutes pre-study period for interviews, weight measurements, etc., prior to collecting their own mid-stream urine sample (baseline time-0 minutes). IRB approval required that participants had access to the bathroom for non-timed urinations and access to drinking water as needed during each 6 hour study period, although these activities were rarely utilized.

Cranberries of the low titratable acidity Demoranville cultivar were cultivated in Burlington Co., New Jersey and harvested in September of 2014 washed, dried, and frozen at -70 oC. Cranberries (24kg) were thawed for 24 hours in a cold room at 6oC, prior to being thawed at room temperature for an additional 2 hours. The berries were then pressed with 5.5 tons pressure and filtered through an X3 fiber mesh to yield a first-press CBJ. The supernatant was pasteurized for 60 seconds at 70 o C, and stored in vacuum sealed jars prior to ingestion in the study within 8 days. Additional CBJ aliquots were frozen at -70 o C prior to chemical analysis as describe later in this manuscript.

Dietary interventions consisted of a 27% v/v Demoranville CBJ sweetened with sucrose (74 Calories/240 ml serving) stored on ice and consumed within 2 hours of preparation. The isocaloric control beverage consisted of 240 ml of sucrose (74 Cal/240 ml) in water. For the first laboratory visit, half of the participants were randomized to receive CBJ and the other half received the isocaloric control beverage and two days later participant received the other dietary interventions in single cross-over fashion. One participant receiving CBJ at their second laboratory visit received a second CBJ intervention two days later.

Upon arrival in the laboratory, participants reviewed a video outline how to collect a mid-stream urine sample upon arrival in the laboratory. A sterile mid-stream urine sample (20-40ml) was self-collected by participants at laboratory presentation (0-minutes) after which the dietary intervention (CBJ or control) was consumed. Additional mid-stream urine samples were collected 90, 225 and 360 minutes following ingestion of the dietary intervention. Urine samples were collected within 2.0 ± 2.0 minutes of target collection

times. Collected urine samples were divided into 4ml aliquots and frozen at -70 °C until analysis as described below.

Analysis of flavonol glycosides and flavan-3-ols in cranberry juice and urine

Quercetin-3-galactoside, quercetin-3-rhamnoside, galangin, epicatechin and procyanidin A2 were purchased from Indofine Chemical Company (Somerville, NJ). Creatinine was purchased from Sigma (St. Louis, MO). All solvents, including methanol, acetonitrile, ethyl acetate, and water were purchased from EMD Millipore (Billercia, MA), and were of HPLC grade. Formic acid was purchased from Mallinckrodt Baker (Phillipsburge, NJ).

Cranberry juice (100%) was thawed and diluted 10 fold with HPLC water. After dilution, sample was filtered through 0.45 µm Spin-X® centrifuge filter tube and 5 µl samples were injected for LC-MS analysis.

Urine samples (4ml) were spiked with 100 ng galangin as internal standard. Galangin was selected due to its similar structure with quercetin and its absence in CBJ. Samples were extracted with 4ml ethyl acetate by vortexing for 5 min and the mixture was then centrifuged at 2000 rpm for 5 min. After centrifugation, supernatant was collected and extraction was repeated 2 more times. The combined ethyl acetate fractions were dried under N_2 gas, reconstituted in 200 µl methanol with brief vortex and sonication. Methanol samples were filtered through 0.45 µm Spin-X® centrifuge filter tube and 10 µl samples were injected for LC-MS analysis.

All samples were analyzed by a Dionex UltiMate® 3000 UHPLC system coupled with an Applied Biosystems API 3000™ triple-quad LC-MS/MS mass spectrometer. The LC system consists of UltiMate 3000 RS Pump, UltiMate 3000 RS Autosampler, UltiMate

3000 RS Column Compartment and UltiMate 3000 RS Diode Array Detector. A VARIAN Polaris 3 C18-A 50×2.0 mm LC column was used for analysis. A binary solvent system with solvent A: 0.1% formic acid in water and solvent B: 0.1% formic acid in acetonitrile was used with linear gradient of 0% B to 10% B from 0-1 min; 10% B to 20% B from 1-3 min; isocratic elution of 20% B from 3-6 min; linear gradient of 20% B to 25% B from 6-8 min; 25% B to 40% B from 8-10 min; 40% B to 70% B from 10-13 min; 70% B to 0% B from 13-16 min and isocratic elution of 0% B from 16-20 min at a flow rate of 0.2 ml/min.

MS conditions were optimized with quercetin-3-galactoside standard for maximum sensitivity. Analysis data was obtained under Electrospray Ionization (ESI) in negative ion detection mode, with following parameters: Curtain gas: 12 psi, Nebulizer gas: 12 psi, Collision gas: 10 psi, Ionspray voltage: - 4200 V, Source temperature: 475 ºC, Entrance potential: -10 V, Focusing Potential: -200 V, Declustering potential: -50 V, Collision energy: -40 V, Collision cell exit potential: -12 V.

Identification of flavonols, possible flavonol metabolites and flavan-3-ols was conducted in ESI-MS-MS Product Ion mode and based on comparison of molecular weight, product ion and retention time with those of available standards and/or previously reported data [28]. Flavonols, flavan-3-ols and internal standard were quantified in ESI-MS-MS Multiple Reaction Monitoring (MRM) mode at dwell time of 100 ms for each compound. Concentrations were acquired by calculating peak area corresponding to relevant standard curves obtained using known concentrations of available standards. In urine samples compound concentrations were corrected by the recovery rate of internal standard

galangin and normalized with urine creatinine level to compensate difference on urine volume among subjects.

Determination of urine creatinine

Urine creatinine was quantified by a previously published HPLC method [29] with slight modifications. Before analysis, urine samples were diluted 10 fold with water and filtered through 0.45 μm Spin-X® centrifuge filter tube. Diluted samples were analyzed in a Waters HPLC system composed of Waters 600 Controller, Waters In-Line Degasser, Waters 717plus Autosampler and Waters 996 PDA Detector. A Luna® 5 μ C18 (2) 250 × 4.60 mm column was used with 10 mM potassium dihydrogen phosphate as mobile phase at a flow rate of 1 ml/min. Creatinine was detected at 230 nm and quantified by comparison with standard curve.

Statistical analysis

Flavanol glycosides (pg/mg creatinine) are expressed as least squares means ± standard error. SPSS software (IBM Cooperation, Armonk, NY) was used to calculate predicted peak urine concentrations (C_{max}) and times (T_{max}). The trapezoidal method was used to calculate area under the curve (AUC) values. A repeated measures analysis of variance was used to identify significant differences within flavonol glycosides. Significant differences among least squares means ($P < 0.05$) were determined using the Tukey-Kramer adjustment.

5.3 Results

Flavonols and flavan-3-ols in cranberry juice

The study identified and quantified six major flavonol glycosides and two flavan-3-ols (monomer and dimer) in low acidity Demoranville CBJ ingested in this study using UHPLC-ESI-MS-MS. Figure 5.1 shows the ion chromatogram of cranberry juice. The six flavonol glycosides identified were myricetin-3-galactoside (M-3-gal), myricetin-3-arabinoside (M-3-arab), quercetin-3-galactoside (Q-3-gal), quercetin-3-xyloside (Q-3-xylo), quercetin-3-arabinoside (Q-3-arab) and quercetin-3-rhamnoside (Q-3-rham) (Table 5.1). Q-3-gal (1.8 mg/240 ml) was the most plentiful flavonol, and accounted for 51.6% of total flavonols. Following Q-3-gal are Q-3-arab (0.5 mg/240ml, 14.9%), M-3-gal (0.5 mg/240ml, 12.9%), Q-3-rham (0.3 mg/240ml, 8.7%), M-3-arab (0.2 mg/240ml, 6.6%) and Q-3-xylo (0.2 mg/240ml, 5.4%).

The two flavan-3-ols were epicatechin and proanthocyanidin (PAC) dimer based on comparing their parent/product ions, retention times with those of standards. Epicatechin had a concentration of 1.4 mg/240 ml juice. PAC dimer with A-type double linkage, as suggested by its molecular weight, was 0.8 mg/240 ml.

Characterization of flavonols in human urine

Urine extraction samples from both CBJ and isocaloric control groups were analyzed by UHPLC-ESI-MS-MS in Product Ion and MRM modes to identify and quantify potential flavonols and flavan-3-ols absorbed from CBJ. In the control group urine Q-3-gal was detected at a non-quantifiable concentration (<2.5 pg) in one subject of the 0-minute samples but not at 90 minutes and is assumed to reflect carry over from the meals the day before laboratory presentation. No detectable flavonols or flavan-3-ols (including epicatechin and A-type PAC dimers) (<0.5 pg) were observed in the remaining subject

samples from control group between 0-360 minutes. Similarly, in the experimental group, no compounds were detected in 0-minute samples, indicating effective dietary restriction from potential dietary flavonoid contaminant compounds and a successful washout promoted by water consumption at prior evening and when participants awoke and urination prior to lab arrival.

UHPLC-ESI-MS-MS chromatograms of typical urine samples after CBJ ingestion demonstrate the presence of flavonol glycosides (Figure 5.2). Although cranberry flavan-3-ols epicatechin and PAC dimer were presented in the juice, neither was detected in the experimental group. Five flavonol glycosides, including M-3-gal, M-3-arab, Q-3-gal, Q-3-arab and Q-3-rham, were identified in urine samples collected between 90 to 360 minutes after ingestion of CBJ. Q-3-xylo was either absent or presented only at traceable levels in analyzed samples and not quantified in the present report.

Five flavonol glycosides in urine were quantified by ESI-MS-MS in MRM mode and normalized with urine creatinine (Figure 5.3). The most prevalent flavonol glycoside observed in the CBJ and urine was Q-3-gal. The peak concentrations of Q-3-arab, M-3-gal, Q-3-rham and M-3-arab were half to one sixth of that observed for Q-3-gal. Individual subjects exhibited similar excretion patterns for each flavonol glycoside, however with significant inter-individual variability creating large standard errors (Table 5.2).

Q-3-gal had calculated Cmax of 1315 pg/mg creatinine, significantly higher than other four glycosides. It also exhibited highest AUC_{0-360} among detected glycosides, significantly higher than that of Q-3-arab, M-3-arab and M-3-gal (Table 5.2). Most

subjects (8 out of 10) had the highest urine Q-3-gal levels at 90 min post-ingestion, indicating Q-3-gals is rapidly absorbed and cleared in humans. Although urine Q-3-gal remained detectable in 8 subjects at 360 min post-ingestion, its average urine level (74 pg/mg creatinine) was significantly lower than that (1298 pg/mg creatinine) at 90 min post- ingestion.

Q-3-rham had second highest Cmax and AUC_{0-360} among different flavonol glycosides, with the latter significantly higher than that of Q-3-arab, M-3-arab and M-3-gal. Average urine Q-3-rham level slightly reduced from 90 min to 225 min, and significantly declined at 360 min post-ingestion. Tmax of Q-3-rham (151 min) significantly longer than that of M-3-arab and M-3-gal, suggests its slower absorbance and/or clearance in human compared to the two myricetin glycosides.

Although having a higher ingestion amount than Q-3-rham (528 vs.307 μg/ 240 ml CBJ), Q-3-arab showed both lower C_{max} and AUC_{0-360} than the latter. It also exhibited delayed urine clearance compared with other compounds, as only half of the subjects had detectable levels (>0.5 pg) of Q-3-arab in their urine 90 min after CBJ ingestion. Accordingly, urine levels of Q-3-ara at 225 and 360 min post-ingestion were significantly higher than that at 90 min, resulted in a T_{max} of 237 min, significantly longer than the other four flavonol glycosides.

The two myricetin glycosides M-3-arab and M-3-gal showed similar excretion patterns. With Tmax at 104 and 90 min respectively, they exhibited the fastest urine clearance among detected flavonol glycosides. Interestingly, while ingested CBJ contained M-3-

arab only half as much as M-3-gal (233 vs. 458 μg/ 240ml), the former had both higher C_{max} and AUC_{0-360} in urine samples.

To evaluate the repeatability of flavonol glycosides absorption and elimination, preliminary repeated cranberry juice ingestion trial was conducted on one subject following a 48 h washout period. While the time-concentrations differed slightly, the subject exhibited similar urinary excretion patterns for the individual flavonol glycosides between the two trials (Figure 5.4). Urinary levels of M-3-gal, M-3-arab, Q-3-gal and Q-3-rham all peaked 90 minutes post-CBJ ingestion during the two trials, and excretion of Q-3-arab reached its maximum levels after 225 minutes. Peak concentrations of urinary M-3-gal decreased by 26% between first and second trials, and increased by 32% to 103% for other four flavonol glycosides.

5.4 Discussion

Flavonoids are common phenolic secondary plant metabolites distributed across a wide range of plant species and their related products. Estimated dietary flavonoid intake, varies across different studies, ranging from ~20 mg/day to over 1 g/day in US and other countries [30-32], indicates considerable dietary flavonoid consumption. Among different flavonoid sub-groups, flavonols and flavan-3-ols have been most extensively studied as to their potential benefit to human health. Bioavailability of cranberry flavan-3-ols appears to be inconsistent across different studies. As in this study, Zhang and Zuo [33] failed to detect monomers epicatechin and catechin in human plasma after CBJ ingestion. Others only recovered catechin after acid hydrolysis of human plasma following consumption of cranberry products [17]. Following same acid hydrolysis protocol, we

also did not detect the flavan-3-ol monomer nor dimer in urine samples (unpublished observations). A recent study [20] reported nanogram levels of epicatechin, catechin and dimer A2 in enzyme hydrolyzed plasma or urine of health older adults (50-70 y) after CBJ consumption. In contrast the current study did not detect free flavan-3-ol monomer and dimer in unhydrolyzed urine samples after CBJ ingestion, possibly due to the different sample preparation procedures.

Unlike flavan-3-ols, which primarily form oligomers and polymers in plants, flavonols are mainly found in glycosylated forms with various sugar moieties [34]. As such their absorption, distribution, metabolism and excretion (ADME) properties will most likely be a function of their conjugated forms. Determination of flavonol glycoside in biological samples has been problematic due to multiple reasons. Paganga and Rice-Evans [35] in an early study detected high levels of a number of flavonol glycosides by HPLC-PDA in human plasma, however many have questioned the results due to the similar retention times and absorbance spectra between flavonol glycosides and their metabolites. Furthermore, many studies have used enzymolysis or acid hydrolysis steps in sample extraction, which results in deglycosylation of flavonol glycosides into aglycones [20]. Others indicated that flavonol glycosides were hydrolyzed by intestinal glycosidase before absorption in the intestine [36, 37], hence it is suggested by multiple studies that quercetin glycosides are not absorbed intact into human body [38, 39]. Our prior results demonstrate that quercetin-3-galactoside is present in human blood plasma following consumption of CBJ [26]. Following CBJ ingestion, quercetin-3-galactoside reaches peak plasma concentrations of 10-15 ng/ml plasma at between 60 and 90 minutes post-ingestion, suggesting the bioavailability of the intact flavonol glycoside, although it could

be possible that glycosidic linkages are added back to aglycones in the liver or other organs, this seems highly unlikely and to our knowledge not an activity observed in any previous studies. With an ethyl acetate-only extraction method to acquire underivatized urine extracts and an optimized UHPLC-ESI-MS-MS analysis method, herein we provided direct evidence for multiple intact flavonol glycosides in human urine. To our knowledge this has not been previously reported.

In the present study, five of the six major flavonol glycosides in CBJ were shown to be excreted in urine as quercetin-3-galactoside, quercetin-3-rhamnoside, quercetin-3-arabinoside, myricetin-3-arabinoside and myricetin-3-galactoside. High inter-individual variability was observed, possibly due to specific polymorphisms on intestinal enzyme, transporter or microbiota activities. Quercetin-3-galactoside as the most abundant flavonol in cranberry also exhibited highest concentrations in urine across most subjects. Interestingly, while ingested CBJ contained more myricetin-3-galactoside than myricetin-3-arabinoside, the later showed higher excretion level in urine. Similarly, quercetin-3-rhamnoside exhibited higher urine concentrations than quercetin-3-arabinoside although the later was more abundant in cranberry. So far no evidence exits which suggests possible conversion between different flavonol glycosides *in vivo*, thus these results indicate differential absorbance efficiencies and mechanisms of flavonol glycosides in human body. Although data on flavonol glycosides bioavailability is limited, studies have reported differential absorbance and excretion levels of individual anthocyanin glycosides in human after CBJ ingestion [15, 16].

Delayed urine clearance was observed for quercetin-3-arabinoside compared with other flavonol glycosides. Hollman et al. [31] reported reduced and slower plasma absorbance

of quercetin-rutinoside than quercetin-glucoside and suggested different absorbance sites of the two compounds in humans. Thus it is possible that quercetin-3-arabinoside is absorbed by a different mechanism of the gastrointestinal tract in contrast to other flavonol glycosides, as indicated by its slower urinary clearance. Those results suggest both aglycone and sugar moiety structures are determinants of the absorption of dietary flavonols in human.

Quercetin-glucuronide, quercetin-sulfate, 3-methylquercetin-glucuronide and other glucuronide forms of quercetin are reported main quercetin metabolites in human plasma and urine following onion consumption [40]. We screened for those compounds based on their reported parent/product ion pairs but did not detect corresponding peaks in urine samples. Besides the possibility of insufficient sample extraction and compound instability, different flavonol profiles between onion and cranberry should be taken into consideration since the former is rich in quercetin-glucosides which are extremely low in cranberry [28]. In a study involving the distribution of flavonol glycosides in mice following intraperitoneal injection, the flavonol glycosides were distributed through most tissues, with quercetin-3-galactoside, quercetin-3-arabnoside and quercetin-3-rhamnoside minimally metabolized compared with quercetin-3-glucoside [41]. Similarly, Arts, Sesink, Faassen-Peters and Hollman [25] using a rat intestinal perfusion model also reported significant greater accumulation of quercetin aglycone and conjugated quercetin metabolites in treatment with two quercetin-glucosides compared with quercetin-3-galactoside, quercetin-3-arabinoside and quercetin-3-rhamnoside. Hence the high levels of flavonol galactoside, arabinoside and rhamnoside of cranberry parent flavonols and the

lack of quercetin metabolites in human urine after CBJ ingestion suggests minimal metabolism.

In addition to urine clearance, biliary excretion of drugs or dietary compounds and their potential metabolites also significantly affects their bioavailability and pharmacological potential, as compounds excreted into bile can be reabsorbed at the gastrointestinal tract in a certain degree [42]. Dietary flavonoid genistin was found to be rapidly absorbed and excreted into bile in conjugated glucuronide forms in rats. Recovery rates of genistin in 4 h period were 27% and 70-75% of total administered does via duodenal and ileal infusions, respectively, indicates high absorbance efficiency and enterohepatic circulation rate of the flavonoid compound [43]. High biliary excretion levels of quercetin metabolites were also found in rats after administration of quercetin-3-glucosides, with 24 h recovery rate at 20.4% compared to 11.9 % of the urine clearance [44]. Since we observed different patterns of absorbance, urine clearance and metabolism of cranberry flavonol glycosides compared to quercetin-glucosides, evaluating biliary excretion of different cranberry flavonol glycosides would be desired.

Although similar urinary clearance patterns of individual glycosides among the 10 subjects and the one subject among two time points suggests consistent flavonol absorption and clearance mechanisms in human body, the variability in urine flavonol clearance among subjects and prior unpublished observations suggest that flavonol glycoside uptake in the gut is variable (i.e. ± 20-50%) and influenced by possible inherent variability among the subjects of the study. Genetic variation in human multidrug resistance (MDR) proteins has been revealed previously [45]. MDR proteins in normal human tissues are believed to function as active transporters of multiple non-toxic

substrates [46]. Understanding the basis of the differences in human flavonol glycoside bioavailability (i.e. interaction with prior diet, endocrine status, gut flora, etc) should be the focus of future studies. Interestingly, except myricetin-3-galactoside, the other 4 flavonol glycosides exhibited higher urine levels following the second CBJ ingestion trial, suggesting it would be worth to evaluate the effect of repeated flavonol intake on their bioavailability.

In conclusion, the present study has identified and quantified five flavonol glycosides in the urine of healthy female adults after CBJ ingestion. These results demonstrate that the major cranberry flavonol glycosides can be absorbed into human circulatory system and excreted through urine in intact forms, which may contribute to the health benefits of cranberry, such as its effect against UTI. The pharmacokinetic information provided by the study will also facilitate other research on bioavailability, absorption mechanism or clearance efficiency of various dietary flavonoids. Further studies will focus on the proper extraction, characterization and quantification of potential cranberry flavonol glycosides and/or metabolites in human plasma samples. Efforts will also be made to evaluate the biliary excretion pattern of such cranberry flavonol glycosides, to expand our understanding of their bioavailability and health benefit potentials on human.

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5.6 Tables and Figures

Table 5.1. Retention times, parent and product ions, identities and concentrations of flavonols and flavan-3-ols in cranberry juice.

Peak No.	Retention time (min)	Parent ion (m/z)	Product ion (m/z)	Peak identity	*Compound concentration (µg/ml)
1	8.07	289	123	Epicatechin	5.97
2	8.85	479	316	M-3-gal	1.91
3	9.78	449	316	M-3-arab	0.97
4	10.24	463	300	Q-3-gal	7.63
5	11.08	433	300	Q-3-xylo	0.80
6	11.48	433	300	Q-3-arab	2.20
7	11.91	447	300	Q-3-rham	1.28
NS	10.26	575	289	PAC dimer	3.32

*concentrations in 27% cranberry juice consumed by volunteers.

NS: not shown; M: myricetin; Q: quercetin; gal: galactoside; arab: arabinoside; xyl: xyloside; rham: rhamnoside; PAC: proanthocyanidin

Table 5.2. Flavonol glycoside concentrations (pg/mg creatinine), calculated peak concentration (C_{max}) and time (T_{max}) and area under the urine concentration curve from 0 to 360 min (AUC_{0-360}) in human urine following consumption of 27% CBJ. Measurable flavonols were not observed in 0-minute urine. Statistical significance between flavonols within each time indicated by different numbers. Statistical significance within flavonols across time indicated by different letters. Statistical significance between flavonols within T_{max}, C_{max} and AUC_{0-360} indicated by different symbols (#, @, ©). (n=10; Data expressed as least square means ± standard error).

Compound	**Time post-ingestion (min)**			**T_{max}**	**C_{max}**	**AUC_{0-360}**
	90	**225**	**360**	**(min)**	**(*pg/mg)**	**(pg h/mg)**
Q-3-gal	1298 ± 421	302 ± 85	74 ± 59	115 ± 16	1315 ± 416	3196 ± 990
	1; A	1; A,B	1; B,C	#, @	@	©
Q-3-rham	597 ± 206	444 ± 142	122 ± 66	151 ± 21	671 ± 193	2255 ±749
	1; A	1; A	1; B	@	#	@,©
Q-3-arab	36 ± 20	337 ± 92	226 ± 96	237 ± 12	343 ± 91	1080 ± 345
	2; A	1; B	1; B	©	#	#,@
M-3-arab	270 ± 102	56 ± 23	19 ± 15	104 ± 14	300 ± 108	718 ± 270
	1,2; A	2; B	2; B	#	#	#
M-3-gal	169 ± 55	8 ± 5	5 ± 5	90 ± 0	169 ± 55	340 ± 111
	1,2; A	2; B	2; B	#	#	#

*pg/ mg creatinine

M: myricetin; Q: quercetin; gal: galactoside; arab: arabinoside; rham: rhamnoside

Figure 5.1. Extracted-ion Chromatogram (XIC) of cranberry juice analyzed by UHPLC-ESI-MS-MS in MRM mode with negative ionization. Labels 1-7 on peaks correspond to peaks 1-7 in Table 5.1.

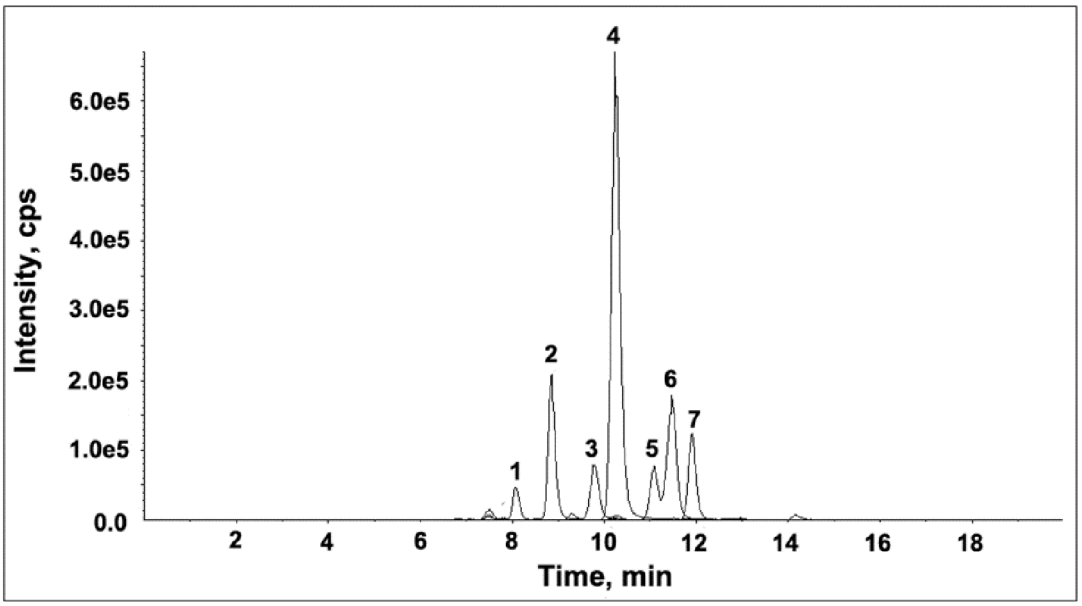


Figure 5.2. Identification of flavonol glycosides in human urine. A-C: Extracted-ion Chromatograms (XIC) of urine samples collected at 90 min (A), 225 min (B), and 360 min (C) after CBJ ingestion analyzed by UHPLC-ESI-MS-MS in MRM mode. Q: quercetin; M: myricetin; gal: galactoside; arab: arabinoside; rham: rhamnoside. D-H: Product ion chromatograms of urine flavonol glycosides extracted at m/z of 479 (D), 449 (E), 463 (F), 443 (G) and 447 (H). Rt: retention time.

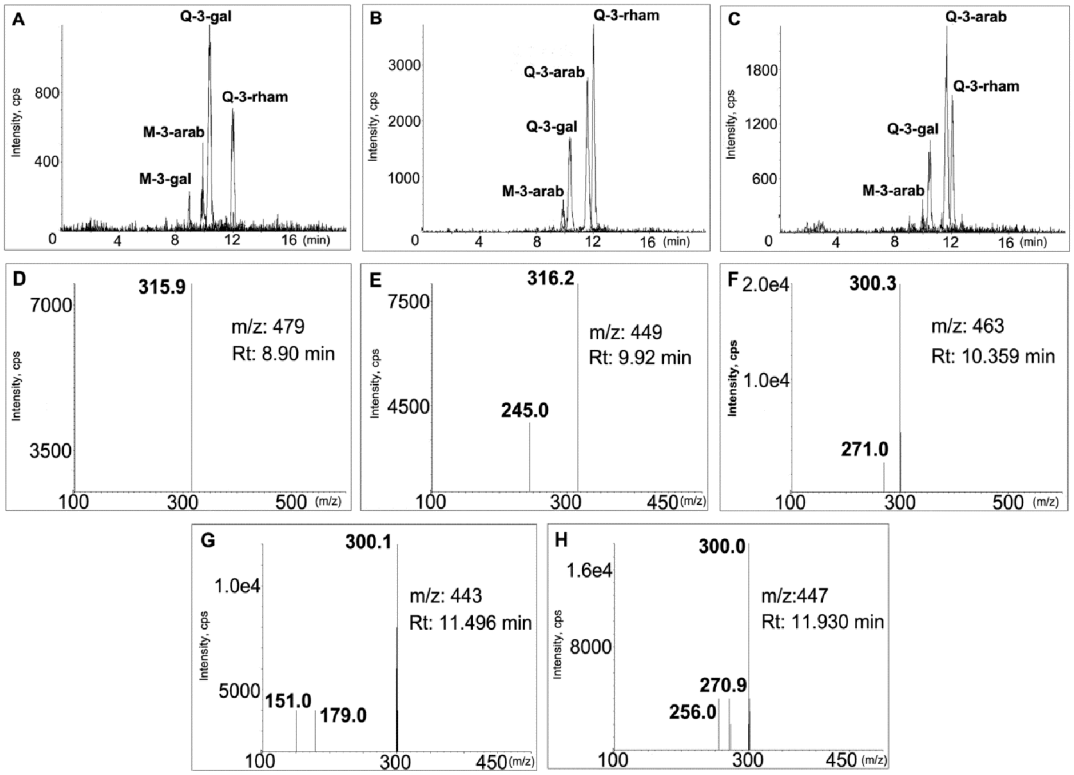


Figure 5.3. Human urine concentrations of flavonol glycosides for 6 hours following cranberry juice ingestion. Values are means, n=10.

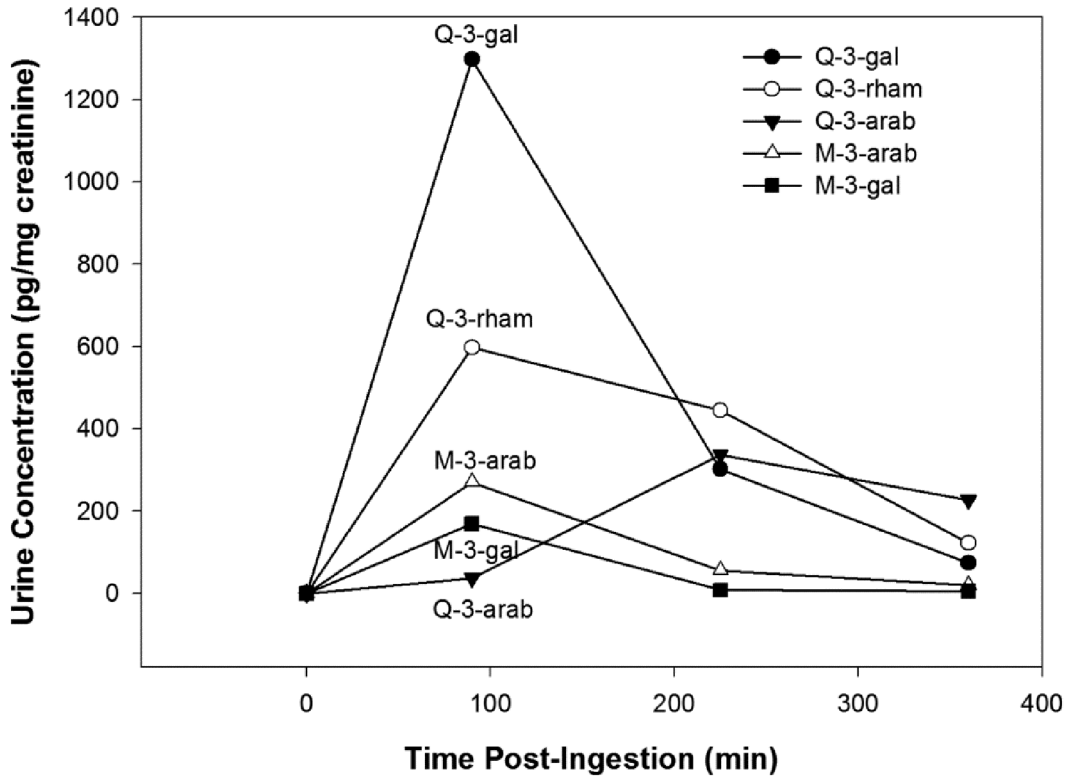
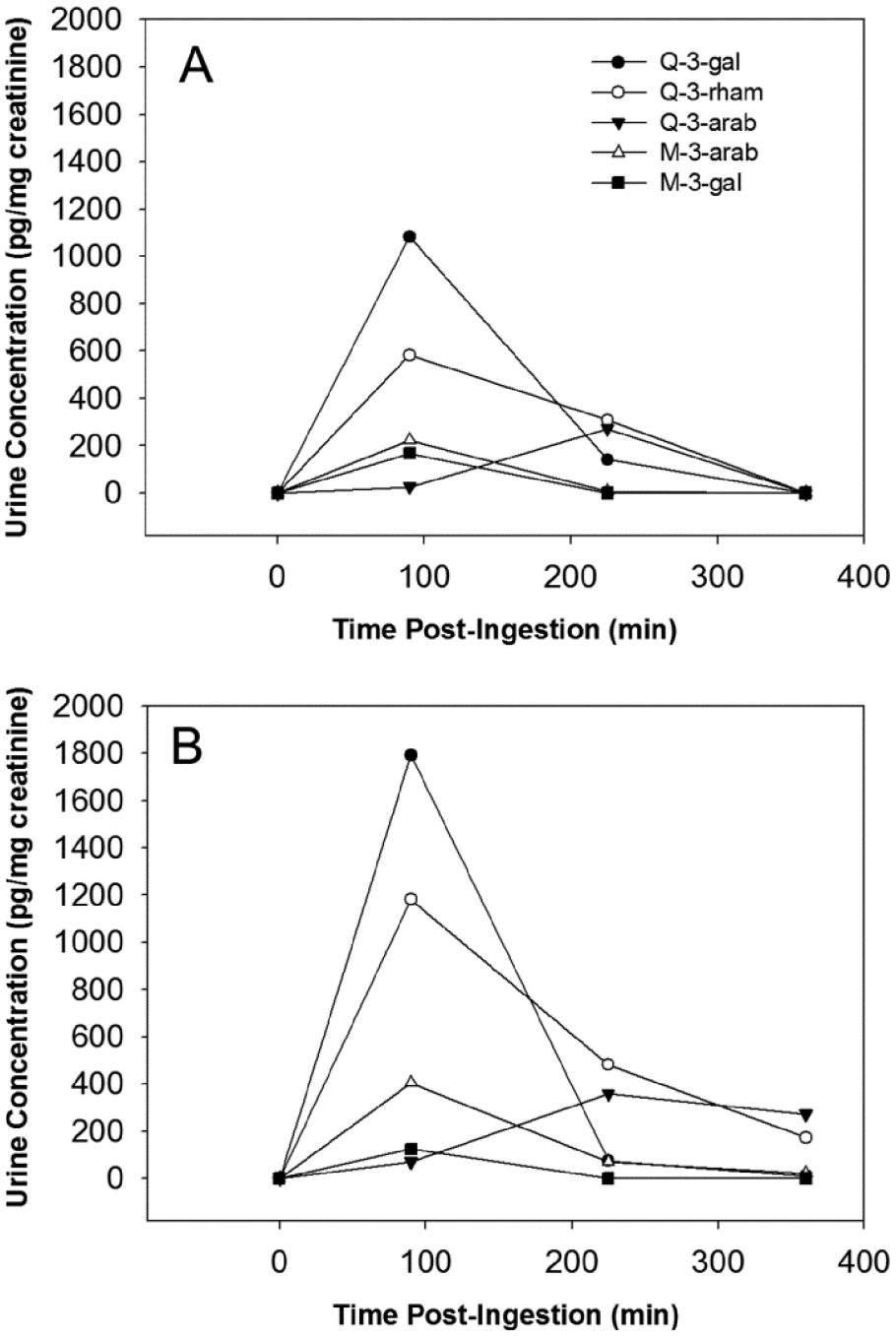


Figure 5.4. Urine concentrations of flavonol glycosides in same subject after cranberry ingestion on first exposure (A), and second CBJ bioavailability trial after a two day washout (B).



Chapter 6

Conclusions

Cranberry is well-known for its high levels of various phytochemicals and their potential human health benefits. Among different cranberry phytochemicals, the flavonoids as secondary metabolite have been shown to have a wide distribution across different plant species and food products and exhibit various beneficial bioactivities for humans. The three major cranberry flavonoid subgroups, anthocyanins, flavonols and PACs, share the common flavonoid backbone structure with two phenyl rings and one non-phenyl heterocyclic ring; however, they exhibit significant structural variations. While anthocyanins and flavonols mostly occur as conjugated glycosides in cranberry and other plant species, PACs are characterized by their polymeric structure with variation in both monomer building unit and inter-flavan linkage. Such structural variation among the different flavonoid subgroups, or within a flavonoid class, not only indicate diverse biosynthetic steps and varied biological functions in plants, but also result in variable bioactivities, bioavailability, and metabolism of individual flavonoid compounds in humans.

Understanding the occurrence of different flavonoids during fruit development and ripening is not only important for understanding their biosynthetic pathways and function in the plant, but also critical for plant breeding and fruit harvest, particularly for targeting both quality and quantity of beneficial flavonoid compounds in the raw cranberry. Modern analytical techniques, such as HPLC and MS spectrometry, have been proved to be powerful in both qualitative and quantitative analysis of natural flavonoids. Through

the combination of regular spectrophotometry and HPLC analysis, total anthocyanins, individual flavonols and PACs as well as different organic acids, were analyzed for eight different cranberry varieties during their fruit development and ripening season. While anthocyanin concentrations sharply increased during the fruit ripening stages, levels of individual and total PACs decreased during cranberry development and early ripening, and increased again when fruits underwent maturation. Harvesting in later stages of the harvest window would maximize proanthocyanidin content in food products such as juices and sweetened-dried-cranberries. Genetic variation was also apparent in the production and accumulation of anthocyanins and PACs, indicated by different concentrations between cranberry varieties. Concentrations of most individual flavonols, on the other hand, were not significantly affected by either fruit development nor ripening or genetic variation; except for quercetin-3-arabinofuranoside and quercetin-3-glucosides similar and consistent level of six flavonols were observed among eight cranberry varieties.

Quantitative analysis of different flavonoids, as shown by the above study, plays an important role in plant breeding, fruit harvest planning and determination of food quality and nutritional values. In spite of the accurate and informative quantitative analysis provided by HPLC and MS spectrometry, regular spectrophotometric methods have always been preferred in certain situations where a fast, affordable and general quantification is needed. The DMAC reaction is currently the preferred method of quantifying PACs by the food and supplement industries. In quantification of polymeric PACs, the 4-dimethylaminocinnamaldehyde (DMAC) assay, which yields colored reactant with 640 nm absorbance, has been favored. Evaluation of DMAC assay using

different individual PAC compounds with structural variation, however, suggests questionable accuracy of this spectrophotometric method in many situations. The molar absorption coefficients (MACs) at 640 nm were not consistent among different PAC dimers and trimers with different inter-flavan linkage type and position. Specifically, PAC molecules with single linkage (B-type) between their first and second building units exhibited significant lower MAC values, accompanied by an extra absorbance peak at 440 nm after reaction. Different PAC oligomers and polymers, with similar inter-flavan linkage but different degree-of-polymerization (DP), also exhibited inconsistent MAC values in DMAC assay. These observations highlight the importance of re-evaluating the DMAC assay mechanism, its accuracy and its use in comparing food products for PAC content.

Flavonoids, particularly flavonols and PACs, have been intensively studied for their various beneficial properties, such as anti-oxidant, anti-inflammation and anti-cancer activities. Individual cranberry flavonols and PACs were isolated and structurally characterized. Evaluation using two classical ovarian cancer cell lines revealed their differential *in vitro* anti-ovarian cancer properties. Quercetin aglycone was most active among flavonols to inhibit cancer cell growth suggesting that glycosylation of flavonols reduces their cytotoxicity against ovarian cancer cells. PAC polymers with high DP exhibited higher cytotoxicity than other oligomers with low DP (e.g. dimer, trimer and tetramer). Although having large structural variations, the two most active flavonol and PAC compounds, quercetin aglycone and PAC DP-9, exhibited many similar bioactivities against ovarian cancer cells including inducing apoptosis, causing cell cycle arrest and sensitizing cancer cells against cisplatin. However, differential activities were also

observed; most notable was the expression inhibition of the cancer cell epidermal growth factor receptor (EGFR) that was only induced by PAC DP-9. In general, the above observations not only provide evidence for the *in vitro* anti-ovarian cancer activities of different cranberry flavonoid compounds, but also emphasize the important relationship between flavonoid compounds' structure and their bioactivity.

Although *in vitro* studies of flavonoids' bioactivities suggest their promising potential to be utilized in drug discovery and development against different diseases, the health benefits of dietary flavonoids are largely determined by their bioavailability in human body. During the course of the research, the recovery of intact cranberry flavonol glycosides following juice consumption provided direct evidence for the human absorption of different cranberry flavonol glycosides. The absence of flavonol aglycones and potential metabolites indicated minimal deglycosylation and metabolism of the cranberry flavonol glycosides in human body. Consistent with flavonols' differential cytotoxicity against ovarian cancer cells, the bioavailability of cranberry flavonol glycosides is also determined by their structures, indicated by the differential pharmacokinetics of individual flavonol glycosides recovered in human urine samples. Significant inter-individual differences were also observed among subjects with regard to urine clearance levels of cranberry flavonol glycosides; thus the importance of human body functions, e.g. variations of the expression of compound transporters for the absorption and clearance efficiency of dietary flavonols, need to be recognized.

In summary, the main purpose of the dissertation research was to provide comprehensive qualitative and quantitative evaluations of different major flavonoid compounds in

cranberry, as well as to provide new evidence of the important anti-cancer property and human absorption and clearance of individual cranberry flavonoids.