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Proanthocyanidins (PACs) containing A-type linkages, like those found in cranberry, have been implicated as beneficial phenolic compounds in human health, but much research is still required to better understand their activity fully. One major impediment to progress has been the existence of effective analytical methods for A-type PAC quantification. This thesis summarizes the development of a high performance liquid chromatography (HPLC) analytical method for the determination PAC A2 in phenolic polymer isolates following acid-catalyzed degradation in the presence of excess phloroglucinol. Isolates from concentrated cranberry juice were extensively characterized and molar extinction coefficients were determined for the terminal A2 unit and extension A2-phlorglucinol adduct,

in order to provide accurate quantitative information. The method was then used to examine several commercially available products, including cranberry juices, cranberry juice cocktails, concentrated white cranberry juice, cranberry fruit, cranberry nutraceuticals, peanuts, and cinnamon spice. Overall, it was found that the method developed here allowed for reproducible quantification of both extension and terminal A2 units in all the products tested. It was found that based on preliminary analysis, on a per serving basis, cranberry juice contains the highest value of A2 in cranberry products. Finally, comparisons between the different products have raised several additional questions regarding A2.

Analysis of Proanthocyanidin A2 and its Presence in Various Commercial Products

by
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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorized release of my thesis to any reader upon request.

Jayma L. Koerner, Author

CONTRIBUTION OF AUTHORS

Dr. James A. Kennedy assisted with writing of all chapters. Dr. Victor Hsu and Dr. Kerry McPhail assisted with NMR data collection for chapter 3. Dr. Jungmin Lee provided preliminary mass spectrometry data for chapter 3. Jeff Morre assisted with mass spectrometry data collection for chapter 3.

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Chapter 1. Introduction

Proanthocyanidins (PACs), also known as condensed tannins, are flavonoid polymers composed of flavan-3-ol monomer subunits. PACs are found in a wide variety of plants and consumables such as fruits, vegetables, legume seeds, cereal grains, some beverages including tea, wine, cocoa, beer and cider (Santos-Buelga and Scalbert 2000) as well as in some spices, like cinnamon, allspice and nutmeg (Schulz and Herrmann 1980). After lignin, PACs are the second most abundant group of natural phenolics (Gu *et al.* 2003a; Prior and Gu 2005). PACs not only undergo the usual phenolic electrophilic aromatic substitution reactions, but they also have the ability to interact with and precipitate proteins and it is believed that it is this ability that is responsible for the astringent mouth-feel of PAC-rich consumables (Santos-Buelga and Scalbert 2000). Because of their phenolic structure, PACs possess a distinct antioxidative potential (Bors *et al.* 2000) and because of this, it is believed PACs provide several health benefits, such as inhibition of cancer cells (Neto 2007; Singh *et al.* 2009a), LDL oxidation, platelet aggregation (Chu and Liu 2005) and urinary tract

infections (Howell *et al.* 1998a), as well as possessing anti-inflammatory properties (Bodet *et al.* 2006).

Chapter 2. Literature review

2.1. Chemical Structure of PACs

The basic flavon-3-ol skeleton consists of two phenolic rings, designated as the "A" and "B" rings, and a heterocyclic ring, designated as the "C" ring (see **Fig. 2.1**) (Santos-Buelga and Scalbert 2000). PACs are often found as homopolymers of a single flavon-3-ol subunit, however, heterogeneous PACs have also been identified in many foods (Prior and Gu 2005). The flavan-3-ol subunits found in PACs differ by the number of hydroxyl units on the aromatic rings and the stereochemistry of the chiral carbons in the heterocycle. The most commonly found monomers are the epimers, (+)-catechin and (-)-epicatichin. Two other less common sets of epimers are gallocatechin/ epigallocatechin, and afzelechin/ epiafzelechin (see **Fig. 2.1**).

2.1.1. Interflavonoid bonds.

In the most common interflavonoid bond, present in B-type PACs monomers are linked via a single bond from the C-4 on the upper unit to either C-6 or C-8 of the lower unit, resulting in a mass loss equal to two hydrogens for each degree of polymerization (see **Fig. 2.2**). A less common linkage, present in A-type PACs, has the same B-type interflavonoid bond, as well as an additional ether bridge from the C-2 on the upper unit to the C-7 hydroxyl of the lower unit

(see **Fig. 2.3**) resulting in a mass loss equal to four hydrogens for each degree of A-type polymerization (Prior and Gu 2005). Other moieties are often found on the flavon-3-ol monomers.

2.1.2. PAC A2

A-type PACs have been indicated as bioactive compounds, potentially playing a significant roll in the health benefits associated with the food products that contain them (see discussion below). A-type PAC compositional variation includes the monomers involved (e.g.: catechin or epicatechin), and the location of the non-ether interflavanoid bond (C-4 on the upper unit to either C-6 or C-8 on the lower unit). A2 is the designation given to a specific dimer made up of (-)-epicatechin monomers linked from the C4 on the upper unit to the C-8 on the lower unit, in addition to the ether linkage (see **Fig. 2.4**). This particular molecule is of interest because it is the only confirmed A-type PAC found in cranberry and has also shown to be active at inhibiting adherence of P-Ffimbriated *Escherichia coli* (Foo *et al.* 2000a; Foo *et al.* 2000b).

A2 was first isolated in crystalline form in the mid 1960s from the seed shells of *Aesculus hippocastanum* (horse chestnut) by Mayer and collaborators (Mayer *et al.* 1966). The researchers gave a couple of proposed structures based on the observation of catechin and epicatechin as hydrolysis products, which was detected by paper chromatography. Then, in the early 1970s, three trimeric PACs based on A2 were isolated and characterized by a team of researchers at the

University of Sheffield, in association with researchers in the I.C.I. Pharmaceuticals Division of Alderley Park. The researchers were able to confirm one of the structures proposed by Mayer *et al.* based on ^{1}H NMR and mass spectrometric analysis of the parent phenolic compound and reaction products, including a diacetate and monoacetate. ^{13}C NMR allowed for the determination of the placement of the ether linkage as being from the C-2 on the upper unit through the C-7 hydroxyl on the lower unit, as opposed to the other proposed structure that had the linkage from the C-4 on the upper unit through the C-7 hydroxyl on the lower unit (Jacques *et al.* 1974).

2.2. PAC Dietary Intake and Health Impacts

It has been suggested that PACs distinct anti-oxidative potential is key in explaining the health benefits associated with PACs (Bors *et al.* 2000), which makes the quantification of dietary intake important. Although PACs are said to account for a major portion of polyphenols consumed in the western diet, until recently there has been no quantitative data available on daily intake. In 2004, Liwei Gu, a Research Assistant Professor at the Arkansas Children's Nutrition Center and colleagues were able to employ a series of methods to successfully quantify PACs (Gu *et al.* 2004) which lead to the compilation of a database for the estimated dietary intake of PAC in the western diet. Gu screened 102 types of foods collected in the United States for types of flavan-3-ols, types of

interflavonoid bonds, and the degree of polymerization. PACs where found in 43 of the 102 types of food tested. Twenty-three out of thirty-two tested fruits where found to contain PACs, which led to the conclusion that fruits are the major source of PACs in the western diet. Staple crops, like corn, rice and wheat do not contain PACs, but they are found in other grains, such as barley and sorghum. PACs were also found in several kinds of nuts, various types of cereals and beans, beverages such as wine, beer and fruit juices, two spices and only one vegetable. Most of the PACs have the common B-type linkage, although a few foods contained the A-type linkage (Prior and Gu 2005).

A-type dimers have been found as the terminal units as well as between the extension units in cranberry and peanuts. Plums have A-type linkage as the terminal unit only. Curry, cinnamon and avocado have A-type linkages found only between the extension units (Prior and Gu 2005). A-type PACs have also been reported to exist in *Aexculus hippocastanum* (Jacques *et al.* 1974), lowbush blueberry (Gu *et al.* 2002), *Prunus armeniaca* (Prasad *et al.* 1998; Prasad 2000), *Prunus prostrata* (Bilia *et al.* 1996), *Adansonia digitata* (Shahat 2006), *Litchi chinensis* (Le Roux *et al.* 1998), *Pavetta owariensis* (Balde *et al.* 1991), *Crataegus sinaica, Parameria laevigata, Lupinus angustifolius, Theobroma cacao,* and *Ecdysanthera utilis* (see Xie and Dixon 2005 for review). Further research needs to be done as it is currently unclear whether or not the location of the A-type PAC in the polymer affects bacterial anti-adhesion activity.

In general, little is known about the metabolism of PACs, however *in vivo* studies have shown that PACs have additional positive health effects. PACs found in cocoa have been shown to have cardio-protective and antioxidant properties. PACs have been shown to possess anti-carcinogenic properties such as preventing UVB-induced carcinomas. Significant reduction in stomach free radical concentrations suggests that PACs help prevent stomach ulcers (see Prior and Gu 2005 for review). PACs also inhibit LDL oxidation and platelet aggregation (Santos-Buelga and Scalbert 2000).

2.3. Cranberry PAC and Health

PACs from cranberry (*Vaccinium macrocarpon*) (C-PACs) are cytotoxic and capable of inhibiting the growth of human cancer cells. In vitro studies have shown C-PAC rich fraction to have anti-proliferation activity on oral, prostate, colon, breast (see Neto 2007 for review), and esophageal cancer cell lines (Kresty *et al.* 2008). C-PACs have also been shown to selectively sensitize platinum-resistant ovarian cancer cells to the chemotherapeutic agent, paraplatin (Singh *et al.* 2009a). C-PACs have recently been reported to prevent pathogens from invading kidney epithelial cells (Tufenkji *et al.* 2010).

A recent characterization of a C-PAC rich fraction was undertaken to identify the active anti-cancer constituents. The C-PAC rich fraction was further subdivided and tested *in vitro* for its ability to inhibit the growth of tumor cell

including lung, cervical, breast, melanoma, colon, prostate, leukemia and Balb/c3T3 fibroblasts. The fraction that was found to have the most growth-inhibiting activity was then subjected to MALDI-TOF MS. This study revealed that the active anti-cancer polymers had a degree of polymerization (DP) ranging from 4 to 12. All of the polymers had at least one A-type bond, with many having up to four A-type bonds (Neto *et al.* 2005).

It has been suggested that the adhesion of the proteinaceous fibers, called fimbriae, found on the cell wall of bacteria, to human or animal mucosal surfaces is the initial event that leads to most bacteria-related infectious diseases (Beachey 1981). This indicates that adhesion of the fimbriae found in bacteria such as *Escherichia coli,* to the epithelial cells in the urinary tract is the initial step in the development of a urinary tract infection (UTI) (Howell *et al.* 2005). Cranberry juice has traditionally been used to treat and prevent UTI. In 1994, a double-blind placebo-controlled trial scientifically demonstrated cranberry juice's effectiveness in the prevention of UTI (Avorn *et al.* 1994). In 1998, C-PACs were one class of compounds identified as exhibiting anti-adhesion activity, thus reducing UTI (Howell *et al.* 1998b). This research led to further specific identification of three A-type proanthocyanidin trimers from cranberry as the lowest molecular weight C-PACs that exhibited the ability to inhibit adherence of *E. coli* in the urinary tract (Foo *et al.* 2000a; Foo *et al.* 2000b).

C-PACs have also been shown to protect against periodontal infections (Labrecque *et al.* 2006). A PAC rich cranberry fraction has been shown to prevent *Porphyromonas gingivalis* from forming a biofilm, which is a major factor in chronic periodontitis (La *et al.* 2009). This same fraction was also shown to have anti-inflammatory properties in that it reduced the production of inflammatory mediators by Lipopolysaccharide-stimulated macrophages (Bodet *et al.* 2006). This fraction has also been shown to significantly protect oral epithelial cells from the toxic effects of bacteria. Further studies are being conducted to see if this fraction can also protect cells against non-bacterial toxic molecules, like nicotine (La *et al.* 2009).

Finally, of the most commonly consumed fruits, cranberry has the highest concentration of phenolic antioxidant compounds (Vinson *et al.* 2001). So in addition to the anti-proliferation and anti-adhesion properties, C-PACs have also been shown to have strong anti-oxidant abilities, including the ability to inhibit LDL oxidation (Singh *et al.* 2009b; Wilson *et al.* 1998).

The relatively rare A-type PACs are of interest because of their potent bacterial anti-adhesion activity. A-type dimers were found as the terminal units as well as between the extension units in C-PACs. Validated analytical methods with good precision and accuracy need to be in place to better understand the potential health benefits associated C-PACs. There are several methods in place to analyze PACs, but analysis of A-type PACs has generally been limited.

2.4. Characterization Methods

Characterizing and quantifying PACs has historically been difficult, largely due to inadequate analytical methods and lack of commercial standards. Characterization difficulties are compounded by fact that PACs are highly unstable because they are susceptible to enzymatic and nonenzymatic oxidation (Prior and Gu 2005), and additionally due to the complex mixtures found in food extracts. Several methods have been developed to analyze PACs, but each method has limitations and not one of them is considered fully satisfactory (Santos-Buelga and Scalbert 2000). Characterization methods include High Performance Liquid Chromatography (HPLC) and Mass Spectrometry (MS), which are sometimes used in combined with characteristic reactions, such as depolymerization and protein precipitation reactions. Following is some background on various methods used to characterize PACs.

2.4.1. Characteristic reactions

2.4.1.1. Colorimetric:

Some methods use reductive phenolic reactions to investigate PACs. A very basic analysis that is performed on phenolic compounds, including PACs, is the Folin-Ciocalteu colorimetric assay (Singleton *et al.* 1999). This assay measures the ability of a sample to reduce the Folin-Ciocalteu reagent, with results generally given in gallic acid equivalents (GAE). Some researchers also use catechin as a standard to give results in catechin equivalents (Vinson *et al.*

2008), which is more useful for PAC research. A major limitation with this method is that the oxidation-reduction reaction is not specific to PACs, but rather can take place with any present reducing agent in complex mixtures (Santos-Buelga and Scalbert 2000). Additionally, this method does not give any insight into the composition of PACs.

2.4.1.2. Protein precipitation:

The complexation of PACs and proteins generally involves hydrogen bonding and hydrophobic interactions. Carbonyl moieties on the proteins are excellent hydrogen bond acceptors for the hydroxyl hydrogens found in PACs. The pyrrolidine moiety on proline residues provides an open face that readily participates in hydrophobic interactions with the aromatic portions of the PACs (Baxter *et al.* 1997; Charlton *et al.* 2002). Additionally, at sufficiently high protein concentrations, PAC's multidentate structure facilitates cross-linking of both proteins and PACs. This produces additional aggregation and precipitation of the complexes (Gawel 1998). Consideration must also be given to the degree of polymerization (DP) of the PACs. Generally, the ability of PACs to bind proteins increases as the DP increases (Gawel 1998). This protein precipitating ability has lead to the development of assays to study PACs. This method, like the colorimetric method, is limited due to other protein-precipitating compounds found in complex mixtures (Santos-Buelga and Scalbert 2000).

2.4.1.3. Condensation reaction:

Another method involves a condensation reaction of PACs and aromatic aldehydes, like vanillin or 4-(dimethylamine)-cinnamaldehyde (DMAC), which yields a chromophore ($\lambda_{max,\ PAC\text{-}vanillin}$=500 nm, $\lambda_{max,\ PAC\text{-}DMAC}$=640 nm). This reaction is quite specific for flavanols, however, in the case of vanillin, the condensation reaction can occur at C-6 or C-8 of the A ring of any flanvan-3-ol subunit in the polymer, therefore absorbance cannot be correlated on either a molar or mass basis. The absorbance is additionally influenced by the co-existence of anthocyanidins (ACs) (λ_{max}=515 nm) found in the complex mixture. Unlike vanillin, DMAC only reacts with the terminal unit of the PAC, which results in a molar extinction coefficient that is similar for monomers, oligomers and polymers. Therefore, the DMAC method tends to underestimate the polymeric PACs on a weight basis. Another reported drawback to using DMAC is that DMAC tends to precipitate high DP PACs (Prior and Gu 2005).

2.4.2. Depolymerization methods:

Depolymerization of PACs readily occurs under acidic conditions. ACs are conjugated cations and are products in the depolymerization reaction of PACs under oxidative conditions. One method uses colorimetry to determine the amount of ACs released (Porter *et al.* 1985; Santos-Buelga and Scalbert 2000). This method is specific for PACs because monomeric flavan-3-ols and other

phenolic compounds do not yield ACs. However, a major limitation is that the yield of ACs is low due to various polymer forming side reactions.

Another method uses depolymerization under acidic conditions with the addition of a nucleophilic trapping reagent, like benzyl mercaptan (thiolysis) (Czochanska *et al.* 1979; Matthews *et al.* 1997; Prieur *et al.* 1994; Souquet *et al.* 1996) or phloroglucinol (phloroglucinolysis) (Foo and Porter 1981; Foo *et al.* 1997). The trapping agent limits side reactions and thus improves the desired degradation product yields (Santos-Buelga and Scalbert 2000). In this reaction, the terminal units of B-type PACs are released as a monomeric flavan-3-ol, and the extension units are released as monomeric flavan-3-ol-nucleophile adducts. The additional ether bridge makes A-type PACs less acid labile than B-type PACs. A-type PAC degradation products therefore include A2 dimers and/or A2 dimer-nucleophile adducts. In combination with chromatographic methods, the depolymerization with nucleophilic trapping agent method allows for the determination of the terminal units as well as the extension units (Prior and Gu 2005), and an average DP can be calculated. However, this method does not allow for actual DP quantification or absolute determination of polymer configuration.

2.4.3. Chromatographic methods:

HPLC can be used to separate, obtain structural information, quantify, and to a limited extent, determine DP (Santos-Buelga and Scalbert 2000). Methods employing reverse phase HPLC have been developed for the separation of flavan-3-ol monomers to trimers, as well as their degradation products, and is frequently used in conjunction with the nucleophilic/depolymerization method previously mentioned. Normal phase HPLC methods allow for the detection of intact PACs, however, these methods are limited to DP ≤ 10 (Prior and Gu 2005). Other chromatographic techniques include gel permeation chromatography (GPC) and size exclusion chromatography (SEC). Combining several of these methods has given some insight into the characteristics of PACs, but an analytical method for the accurate and precise quantification of PACs remains an undiscovered "holy grail" of phenolic research.

2.4.4. Mass spectrometry:

Mass spectrometry (MS) has historically been an important tool for the analysis of A-type PACs. Several different methods of MS have been used over the years to learn more about PACs. Methods include fast atom bombardment (FAB), electrospray ionization mass spectrometry (ESI), atomospheric pressure chemical ionization (APCI), atmospheric pressure photo-ionization (APPI) and matrix-assisted laser desorption ionization (MALDI) (Fulcrand *et al.* 2008). Analyzers include time-of-flight (TOF), ion-trap (IT), quadrupole (Q), magnetic

sector (B), and Fourier-transform ion cyclotron resonance (FT-ICR). MALDI and FAB mix the compounds with a matrix, which is then irradiated with a laser beam or bombarded with a fast atom or ion beam, respectively. For ESI, APCI and APPI, liquid samples are directly injected, so these techniques are generally used in conjunction with HPLC, which aids in elucidating structures in complex mixtures. (Fulcrand *et al.* 2008)

In 1982, Hemingway *et al.* were able to elucidate the structure of four PAC trimers by using mass spectrometry in combination with thiolysis and NMR. None of these trimers contained A-type PACs, but the groundwork had been laid for sequencing oligomeric PAC (Hemingway *et al.* 1982). In 1986, the researchers isolated PAC A1, which is a dimer of an epicatechin extention unit with a catechin terminal unit. The dimer was subjected to FAB-MS in the positive ion mode, which resulted in a $[M-H]^+$ at *m/z* 577. There was also a peak at *m/z* 425, which was identified as the Retro-Diels-Alder (RDA) fission ion. A major peak was also found at *m/z* 287, which is the ion of the extension epicatechin monomer (Karchesy *et al.* 1986).

In 1998, LeRoux *et al.* used a combination of thiolysis, NMR, HPLC and ESI-MS to characterize an A-type PAC in Litchi chinensis. Based on the data collected in their experiments, it was determined that Litchi chinensis contains both terminal and extension A2 dimers. Additionally, for the ESI-MS data, they

were able to identify PACs up to DP 22 that had 5 or 6 A-type linkages, but the MS method is mostly qualitative (Le Roux *et al.* 1998).

In 1999, Lazarus *et al.* used an HPLC with online MS method that had previously been used for identification of B-type PACs (Hammerstone *et al.* 1999) to examine usefulness of this method for A-type linkages found in peanuts and cinnamon (*Cinnamomum zeylanicum*). The data showed A-type linkages up through octamers in peanut skins, with oligomers containing more than one A-type linkage. The data also showed A-type linkages in cinnamon up through pentamers. The authors also noted that doubly linked oligomers were more abundant, and eluted earlier than singly linked oligomers of the same DP (Lazarus *et al.* 1999).

In 2003, Gu *et al.* used HPLC ESI-MS/MS on plum and cinnamon PACs. They were able to use ions derived from the quinone methide (QM) cleavage of interflavanoid bonds to determine where A-type linkages are found in the oligomers. In plums, operating in the negative mode, an ion at *m/z* 575.1 is characteristic of a QM cleavage of a single interflavanoid bond, placing the A-type link between the middle and terminal unit of a trimer, although no conjugate ion at *m/z* 287.0 was observed. The QM cleavage ions in cinnamon were at *m/z* 573.3 with the conjugate ion at *m/z* 289.0, indicated that the A-type linkage was between the top and middle monomers, with a single linkage between this extension A-type PAC and the terminal unit. The researchers concluded that there

is no observable QM cleavage of A-type bonds when there are B-type linkages in the same oligomer. Rather, the QM cleavage occurs at the B-type link, leaving an A-type dimer product ion. The researchers also presented a schematic that showed the QM, RDA and heterocyclic ring fission (HRF) fragmentation pathway, giving identification to several previously unidentified product ions (Gu *et al.* 2003a). Then, later in 2003, the same researchers used HPLC ESI-MS/MS as well as thiolysis to further characterize and quantify A-type PACs in cranberry, avocado, plum, peanut, curry and cinnamon. The results showed A-type linkages occur only in the terminal unit of plum, only between extension units in avocado, curry and cinnamon, but in both cranberry and peanut, the A-type link is found in both the terminal unit as well as between extension units (Gu *et al.* 2003b).

MS methods help improve our knowledge of A-type PACs, such as whether an A-type bond makes a terminal dimer or if the A-type bond exists as part of extension units in the polymers. But these methods do not give information on the specific monomers that are involved in the A-type bond. Since product ions for any combination of catechin and epicatechin are the same (*m/z* 575.1 for extension units and *m/z* 577.1 for terminal units operating in positive mode), one cannot distinguish whether it is A1, A2 or some other A-type PAC.

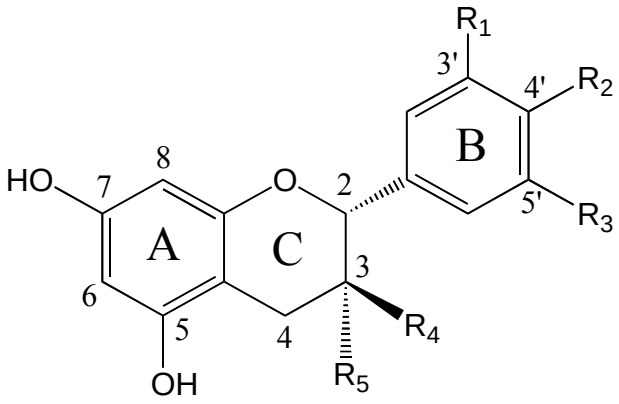
It should also be noted that quantification of PACs is essentially impossible if the PACs are not fully extracted from the material under investigation, regardless of the method used. Several methods of extraction have

been employed with variable results (based on assumed theoretical yields). Acetone with water is generally accepted as the most effective solvent system for PAC extraction. However, the amount of PACs that are not extracted is unpredictable, especially when extraction is being performed on aged or oxidized materials (Santos-Buelga and Scalbert 2000). Additionally, many other compounds are extracted by the acetone/water extraction solvent, so purification of the crude extract is often necessary. SEC gel, like ToyoPearl (Aron and Kennedy 2007; Koerner *et al.* 2009) or Sephadex LH 20 (Foo *et al.* 1996; Gu *et al.* 2003a; Kennedy and Taylor 2003; Li and Deinzer 2007), or solid-phase extraction (SPE) medias (Lazarus *et al.* 1999) are frequently employed to remove lower molecular weight compounds, including organic acids and low molecular weight phenolics.

2.5. Conclusion

PACs are an important class of food polymers as they are abundant in nature and found in many consumables and contribute to the sensory perception of food. Several methods have been developed to characterize PACs, but no single method is considered fully satisfactory for the analysis of PAC A2. Recent improvements in these methods have allowed for the compilation of a database that estimates PAC dietary intake, but specific quantifications of A2 are lacking. Research has shown that PACs have several health benefits, but further research

is needed to fully understand PAC A2's impact on health as well as its metabolic fate. The purpose of the following investigation is to develop an analytical method for the identification of PAC A2, which will allow for the quantification of A2 in various consumables.



Flavan-3-ol	R_1	R_2	R_3	R_4	R_5
(-)-epicatechin	H	OH	OH	H	OH
(+)-catechin	H	OH	OH	OH	H
(-)-epigallocatechin	OH	OH	OH	H	OH
(+)-gallocatechin	OH	OH	OH	OH	H
(-)-epiafzelechin	H	OH	H	H	OH
(+)-afzelechin	H	OH	H	OH	H

Fig. 2.1 Basic flavan-3-ol structure.

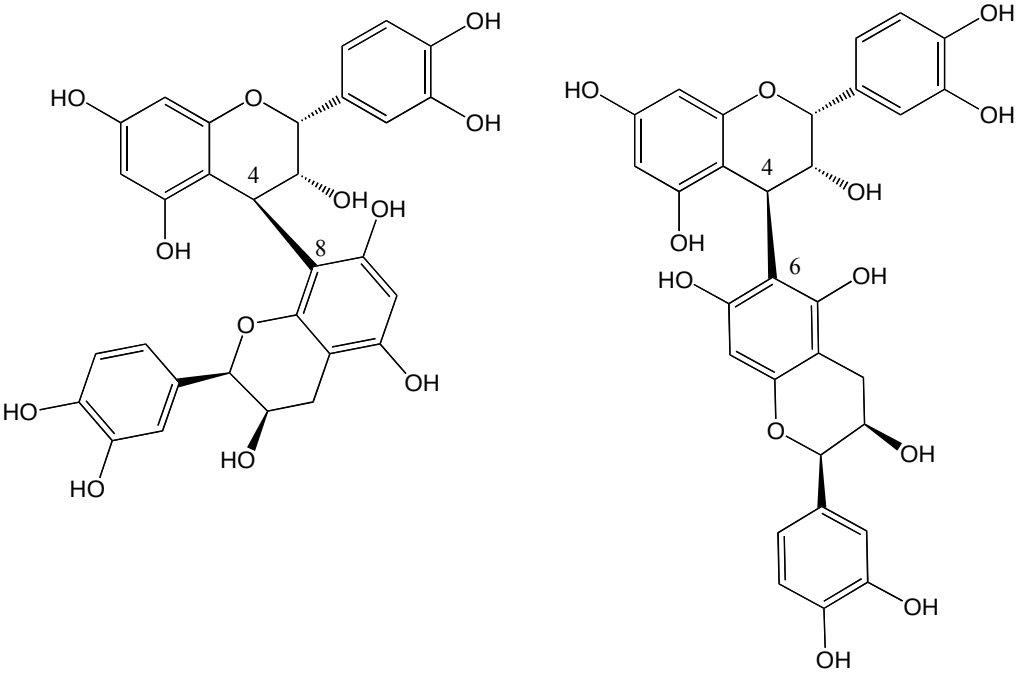


Fig. 2.2 Examples of B-type Proanthocyanidins.

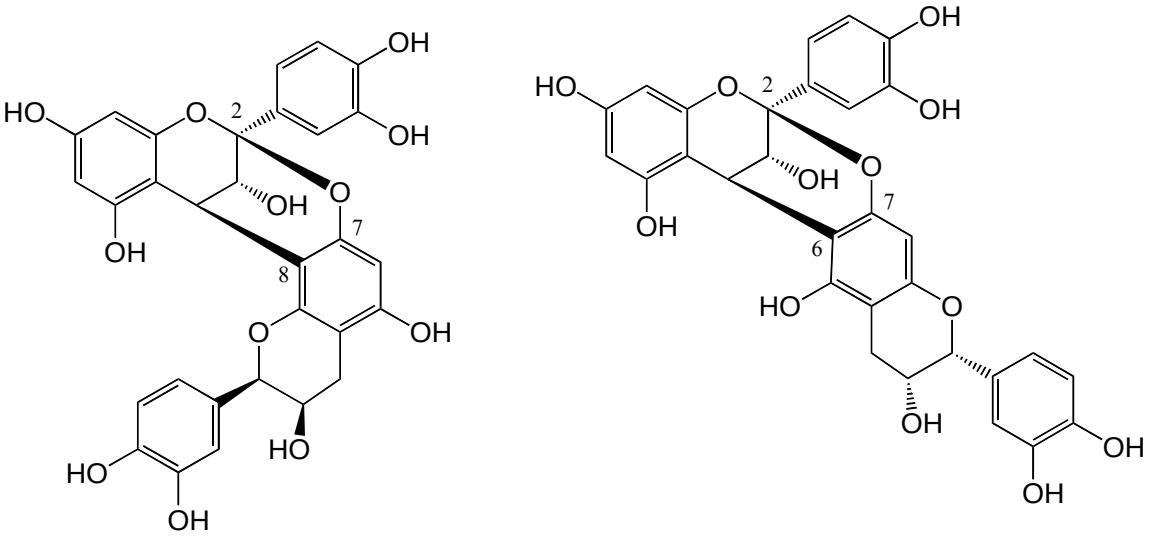


Fig 2.3 Examples of A-type Proanthocyanidins.

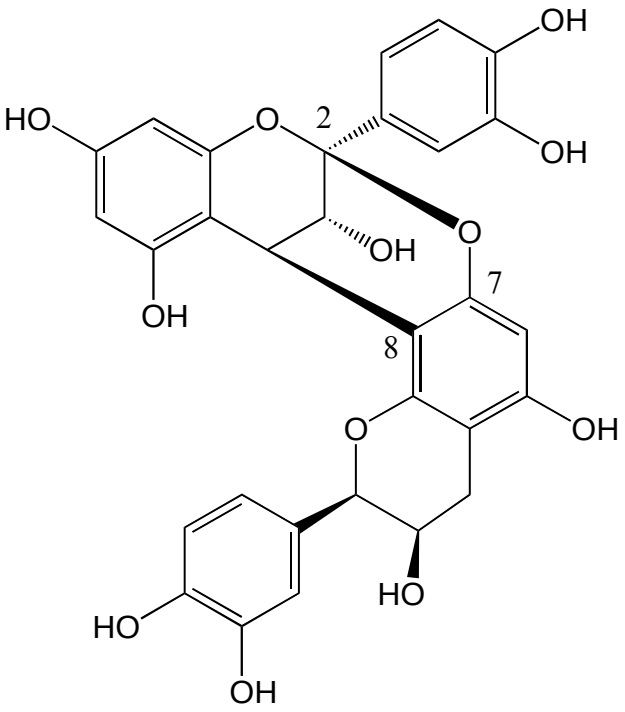


Fig 2.4 Proanthocyanidin A2.

Chapter 3. Determination of proanthocyanidin A2 content in phenolic polymer isolates by reversed-phased high-performance liquid chromatography

(As published by Koerner, J.; Hsu, V.L.; Lee, J.; Kennedy, J.A. (2009) *Journal of Chromatograpy A*, **1216**(9), 1403-1409.)

3.1. Introduction

Proanthocyanidins (PACs), also known as condensed tannins, are polymeric flavan-3-ols. PACs are the second most abundant group of natural phenolics (Prior and Gu 2005) and are found in a wide variety of plants and plant-derived products, including fruits, vegetables, legume seeds, cereal grains and some beverages including tea, wine, cocoa, beer, and cider (Santos-Buelga and Scalbert 2000). PACs have the ability to interact with and precipitate proteins and it is believed that this property gives rise to the astringent mouth-feel of PAC-rich consumables (Santos-Buelga and Scalbert 2000). PACs are also effective antioxidants (Bors *et al.* 2000) and it is believed that PACs provide several health benefits, including prevention of urinary tract infections (UTI) (Howell *et al.* 2005), cancer, cardiovascular disease, and the inhibition of LDL oxidation and platelet aggregation (Chu and Liu 2005; Neto 2007; Santos-Buelga and Scalbert 2000).

In the most common PAC polymerization linkage, known as B-type, monomers are linked via a single bond from the C-4 on the upper unit to either C-

6 or C-8 of the lower unit. Another less common linkage, know as A-type (**Fig. 3.1**), has the same B-type linkage, with an additional ether bridge from the C-2 on the upper unit to the C-7 hydroxyl of the lower unit (Prior and Gu 2005). Studies suggest that A-type PACs may be beneficial in the prevention of UTIs (Foo *et al.* 2000a; Foo *et al.* 2000b). Tools for studying these polymers are vital to improving our understanding of potential health benefits.

Characterizing and quantifying PACs has historically been difficult, largely due to inadequate analytical methods and lack of commercial standards. Characterization difficulties are compounded by the fact that PACs are reactive, being susceptible to acid-catalyzed and oxidation reactions (Prior and Gu 2005), and additionally due to the complex mixtures found in food extracts. Several methods have been developed to analyze PACs, but each method has limitations and not one of them is considered fully satisfactory. Historically, HPLC combined with mass spectrometry has been used to detect the presence of A-type PACs (Gu *et al.* 2003a; Karchesy *et al.* 1986; Yu *et al.* 2006) and quantification has been based upon non A-type flavan-3-ol extinction coefficient information (Gu *et al.* 2003b; Yu *et al.* 2006). However, an HPLC method for the qualitative and quantitative analysis of A2-containing PACs (A-type dimers of (-) - epicatechin monomers) has up-to-now not been presented.

Cranberries contain both extension and terminal A2 PACs (**Fig. 3.1**) (Foo *et al.* 2000a; Foo *et al.* 2000b; Gu *et al.* 2003a), which makes concentrated

cranberry juice (CCJ) ideal for the development of an HPLC method for the analysis of either or both A2 PAC units. Peanuts have also been reported to contain both terminal and extension A-type PACs (Yu *et al.* 2006) therefore a total peanut system (TPS) was used to test the effectiveness of the method presented here. Reported here is the development of a method that maximizes the cleavage of A2-containing PACs into A2 subunits by acid-catalyzed depolymerization in the presence of excess phloroglucinol (phloroglucinolysis), and quantifies the terminal and extension A2 subunits products using measured extinction coefficients of purified and characterized standard material.

3.2. Experimental

3.2.1. Chemicals

Acetone, acetonitrile, diethyl ether, ethyl acetate, glacial acetic acid, high purity hydrochloric acid, trifluoroacetic acid (TFA), and methanol were all HPLC grade and 0.1% aqueous formic acid was LC/MS grade and all were purchased from VWR (Tualatin, OR, USA). Reagent grade phloroglucinol and (-)-epicatechin were purchased from Sigma-Aldrich (St. Louis, MO, USA). L-(+)-ascorbic acid (99.8%) was purchased from J.T. Baker (Phillipsburg, NJ, USA). Ammonium acetate (HPLC grade) was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Anhydrous sodium acetate (ACS grade) was purchased from Mallinckrodt (Paris, KY, USA). Water was purified using a Millipore Milli-Q

system (Bedford, MA, USA). Toyopearl HW 40F chromatography resin was purchased from Supelco (Bellefonte, PA, USA).

3.2.2. Instrumentation

Analytical HPLC with diode array detection (DAD) was performed on an Agilent 1100 series HPLC (Palo Alto, CA, USA) composed of a degasser (G1322A), quaternary pump (QuatPump G1311A), autosampler (G1313A ALS), and column heater (G1316A ColCom) controlled by Agilent's ChemStation for LC Rev. A.08.04 software. Semi-preparative HPLC was performed on the same Agilent system with the addition of a 1400 μL seat capillary and the replacement of the column heater by an Eppendorf CH-430 column heater with TC-50 temperature control unit (Westbury, NY, USA).

Preliminary LC/MS of the CCJ PAC reaction products was performed using an Agilent HP1100 system equipped with a DAD and XCT ion trap (Palo Alto, CA, USA). Final LC/MS of the purified products was performed using Analyst 1.2.1 software to control an Applied Biosystems 4000 Q-Trap (Foster City, CA, USA) with Shimadzu SIL-HTC autosampler (Columbia, MD, USA), Shimadzu LC-20AD pump, and Shimadzu DGU-20A5 prominence degasser. Accurate mass was determined on a Waters Micromass LCT Classic (Milford, MA, USA) using a polypropylene glycol standard.

^{13}C NMR data were acquired on a Bruker DRX 300 MHz (Fremont, CA, USA). ^{1}H NMR, heteronuclear single quantum coherence (HSQC), and

heteronuclear multiple bond correlation (HMBC) data were acquired on a Bruker Magnex Scientific 600MHz cryomagnet. All experiments were internally referenced to acetone, using acetone-d_6 as the solvent purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA).

Molar extinction coefficient determinations in methanol were performed using 282 QS 1.000 cuvettes on a Shimadzu UV-3101 PC UV-VIS-NIR Scanning Spectrophotometer (Columbia, MD, USA).

3.2.3. Sample preparation

3.2.3.1. PAC isolation and purification

CCJ received from Ocean Spray Cranberries, Inc. (Boston, MA, USA) was diluted to 25% v/v concentration with water and applied to a 5 cm x 30 cm Toyopearl HW-40F column pre-equilibrated with 0.05% v/v aqueous TFA. As previously described (Aron and Kennedy 2007; Kennedy and Jones 2001), the loaded column was rinsed with 1.0 L 0.05% v/v aqueous TFA to remove sugars and organic acids, followed by 1.0 L of 1:1 methanol:water containing 0.05% v/v TFA to remove low molecular weight phenolics. Finally 300 mL of 2:1 acetone:water containing 0.05% v/v TFA was used to elute the PAC fraction (Aron and Kennedy 2007; Kennedy and Jones 2001) . The PAC fraction was then lyophilized to a magenta colored powder and stored at -80°C. The yield was approximately 16 g/L CCJ. The solubility of the crude PAC powder was approximately 8 mg/mL in water.

For the TPS, shelled, whole peanuts with skins were purchased from a local market (Corvallis, OR, USA). The peanuts were broken apart and extracted in 2:1 acetone:water under argon and in reduced light. The liquid extract was vacuum filtered and the acetone was removed under reduced pressure at 40 °C. The aqueous solution was then applied to the above column. The fraction elution and additional purification steps were the same as for CCJ (see above) (Aron and Kennedy 2007; Kennedy and Jones 2001). The PAC fraction was lyophilized to a pale yellow powder and stored at -80°C.

3.2.3.2. Phloroglucinolysis of PACs for isolation of A2 and A2-P

Phloroglucinolysis for analytical samples has been previously described (Kennedy and Jones 2001). Large-scale phloroglucinolysis of the CCJ PAC powder was performed for the semi-preparative HPLC purification of compounds of interest. 8 g CCJ PAC was dissolved in 1.6 L of 0.1N methanolic HCl. 80 g of phloroglucinol was added and the reaction flask was placed in a 50 °C water bath and allowed to react for 130 min. Ascorbic acid was not used in order to simplify the purification process, therefore special care was taken to reduce oxidation in the depolymerized PACs by minimizing post-phloroglucinolysis light and heat exposure. The reaction was quenched with 4.93 g ammonium acetate. The methanol was removed under reduced pressure and the products were taken up in Milli-Q water and washed with diethyl ether. The aqueous layer was then extracted with ethyl acetate. The ethyl acetate portions were combined and

concentrated under reduced pressure. A minimal amount of water was added to solubilize the products. The remainder of the ethyl acetate was then evaporated. The solubility of the depolymerized CCJ products in water was approximately 29 mg/mL. The saturated aqueous solution was then injected directly onto the semi-preparative column as discussed below.

3.2.3.3. Semi-preparative HPLC

Peaks of interest were isolated using a LiChrospher 100 RP-18 (10μm) semi-preparative column (250 mm x 10 mm) with a guard column of the same material. The mobile phases were 0.1% v/v aqueous TFA (A) and acetonitrile containing 0.1% v/v TFA (B) with a flow rate of 5 mL/min. The stepwise gradient was as follows: time in min (%B), 0-3 (3%), 3-11 (10%), 11-19 (12%), 19-24 (15%), 24-35 (16.5), and 35-42 (80%). DAD detection was at 280 nm. The peaks of interest were collected and the fractions were lyophilized. Isolates were then subjected to this method two additional times to obtain >99% purity (by HPLC) products. Purified products were lyophilized to white powders and stored at -80° C (isolated approximately 15 mg of each product). Based upon the optimized method presented here, CCJ consisted of approximately 3.35 g/L A2 extension units, and 2.17 g/L A2 terminal units.

3.2.4. Compound characterization

Preliminary HPLC/DAD/ESI-MS/MS identification of CCJ PAC reaction products was performed using previously described HPLC (Kennedy and Taylor

2003) and MS (Lee and Finn 2007) conditions. The mobile phases were 1.0% v/v aqueous acetic acid (A) and acetonitrile containing 1.0% v/v acetic acid (B). The column temperature was 30 °C; flow rate, 3 mL/min; and with a linear gradient as follows: time in min (%B), 0 (3%), 4 (3%), 14 (18%), 16 (80%). The following modifications were made to the previously described conditions (Kennedy and Taylor 2003; Lee and Finn 2007): two serially connected Oynx Monolithic columns (100.0 mm x 4.6 mm), fitted with a guard column (5.0 mm x 4.6 mm) from Phenomenex (Torrance, CA, USA) were used; scan range was *m/z* 50 to 2200; DAD detection at 280, 320, 370, and 520 nm; and a graduated micro-splitter valve (P-470, Upchurch Scientific, Oak Harbor, WA, USA) reduced the flow from the column to the ion trap MS.

Final HPLC/DAD/ESI-MS/MS conditions for the identification of purified A2 and A2-P differed from the preliminary identification as follows: The mobile phases were 0.1% v/v aqueous formic acid (A) and acetonitrile (B) with a flow rate of 0.1 mL/min. The linear gradient was as follows: time in min (%B), 0 (10%), 10 (50%), 12 (20%). The column was a single Zorbax SB-C18 (1 mm x 15 mm, 5 μm) column from Agilent (Santa Clara, CA, USA). Scan range was *m/z* 200 to 1200 for A2-P and 200 to 1000 for A2.

3.2.5. Analytical HPLC

The previously published method for the analytical preparation of PACs (Kennedy and Jones 2001) was optimized for PAC A2 quantification in both CCJ

and TPS. For CCJ, 10 mg crude cranberry PAC was dissolved in 2.0 mL of a 0.1 N methanolic hydrochloric acid solution containing 100 mg/mL phloroglucinol and 10 mg/mL ascorbic acid. The reaction mixture was vortexed to solubilize (~3 seconds) and then allowed to react for 135 min at 50° C. The reaction was quenched with 10.0 mL of 40 mM aqueous sodium acetate. The quenched reaction mixture was centrifuged (RCF=16 128g) to remove gross particulates. The reaction mixture was then subjected to the analytical HPLC method discussed below. The same conditions were used for TPS, except a concentration of 50 mg/mL phloroglucinol was found to be optimal.

The previously published analytical HPLC method (Kennedy and Taylor 2003) was modified by extending the end time to assure removal of all late eluting material. Additionally, a three minute post run was added to equilibrate the columns prior to the next injection. Briefly, two 100 mm x 4.6 mm Chromolith RP-18e columns, purchased from EM Science (Gibbstown, NJ, USA), were connected in series with a guard column of the same material. The mobile phases were 1.0% v/v aqueous acetic acid (A) and acetonitrile containing 1.0% v/v acetic acid (B). The column temperature was 30 °C, flow rate was 3 mL/min, and with a linear gradient as follows: elution time in min (%B), 0 (3%), 4 (3%), 14 (18%), 14.01 (80%), 20 (80%). Eluting peaks were monitored at 280, 320 and 520 nm to identify peaks of interests in the CCJ PAC system. The final method has an

approximate runtime of 25 min, from injection to conclusion of the post run. This same method was applied unmodified to the TPS.

It should be noted that sodium acetate (pH 4.00) and phosphate (pH 2.00) buffered mobile phases were tested in an attempt to reduce peak drifting due to environmental fluctuations, however both buffers resulted in a co-elution of A2 with an as yet unidentified flavonol.

The determination of the molar extinction coefficients allowed for the quantification of A2 and A2-P using the readily available (-)-epicatechin to calibrate the HPLC's DAD. A 100 ppm (-)-epicatechin standard underwent the described HPLC analysis in 20 μL increments from 20-100 μL. A calibration curve was generated by plotting the mass of injected (-)-epicatechin (in ng) verses the integral of the absorbance over time (in pAU). The slope of the regression line was then multiplied by the integral of the A2 or A2-P absorbance over time and the respective corrected relative mass response (**Table 3.1**) to give the mass (in ng) found in the injected volume. For mole quantification, the determination was conducted in a similar fashion using moles of (-)-epicatechin and the relative molar response.

Table 3.1. Retention Properties, Extinction Coefficient, and Response Factors for the Major CCJ and Grape Proanthocyanidin Cleavage Products.

Compound	Retention factor (k)	Extinction coefficient (ε280)[a,b]	Relative molar response[a,c]	Corrected relative mass response[a,c,d]
EGC-P	1.0	1344	0.34	0.32
C-P	2.6	4218	1.06	1.06
EC-P	2.9	4218	1.06	1.06
C	5.8	3988	1.00	1.00
A2-P	7.5	8003	2.01	1.01
ECG-P	7.8	14766	3.70	2.44
EC	8.9	3988	1.00	1.00
ECG	11.8	12611	3.16	2.07
A2	12.6	5857	1.47	0.74

[a] non-A2 values previously published in (Kennedy and Jones 2001)
[b] In methanol.
[c] Relative to EC. [d] Not including the phloroglucinol (G-ring) moiety.

3.3. Results and discussion

3.3.1. Characterization of cleavage products

The analytical HPLC method (Kennedy and Taylor 2003) was directly applied to the depolymerized CCJ PAC mixture. The CCJ PAC mixture was also co-injected with grape seed and skin standards produced in this lab. Comparison of the chromatograms with previously published data (Foo *et al.* 2000a; Foo *et al.* 2000b; Kennedy and Taylor 2003) resulted in a probable identification of peak 1 as epigallocatechin-phloroglucinol adduct (EGC-P), peak 2 as catechin-phloroglucinol adduct (C-P), peak 3 as epicatechin-phloroglucinol adduct (EC-P), peak 5 as catechin (C), and peak 7 as epicatechin (EC) (**Fig. 3.2**). Identification of

these peaks was confirmed by LC/MS. Four new peaks (4, 6, 8 and 9) were targeted as peaks of potential interest. Preliminary LC/MS (M –H) data gave a major ion peak of 987.6 for peak 4, 699.3 for peak 6, 863.6 for peak 8 and 575.4 for peak 9, which were tentatively identified as an A-type trimer-phloroglucinol adduct (A-type trimer-P), A2-phloroglucinol adduct (A2-P), an A-type-trimer (A-type trimer) and A2 respectively. Kinetic studies showed the disappearance of the two potential trimer peaks as the A2 and A2-P peaks increased in area (**Fig. 3.3**), which improves the preliminary identification of the A-type trimer-P and A-type trimer. However, further studies are needed to confirm the trimers identity. The tentatively identified A2-P and A2 peaks were chosen to undergo further characterization.

3.3.2. Isolation and characterization of peaks of interest

The semi-preparative HPLC method described above was used to isolate approximately 15 mg of each peak from CCJ. The purified products then underwent characterization to confirm the preliminary identification.

3.3.2.1. Mass spectrometry

Isolates from the CCJ system first underwent MS analysis. The positive ion mass spectrum of peak 9 gave an *m/z* of 577.4. TOF MS ES+ elemental composition analysis (ECA) gave a mass of 577.1360, which is a difference of 2.4 ppm from the calculated mass of 577.1346 for A2 ($C_{30}H_{25}O_{12}$). Enhanced product ions (EPI) on a triple quadrapole with ion trap gave major (M +H) daughter peaks

at *m/z* 451.1, 437.1, 425.1, 311.1, 299.1, and 287.0. The positive ion mass spectrum of peak 6 gave an *m/z* of 701.2. ECA gave a mass of 701.1529, which is a difference of 3.2 ppm from the calculated mass of 701.1506 for A2-P ($C_{36}H_{29}O_{15}$). EPI gave (M +H) daughter peaks at *m/z* 575.1, 561.1, 549.2, 533.2, 435.2, 423.2, and 287.1. **Fig. 3.4** is a modification of published schematics (Gu *et al.* 2004; Li and Deinzer 2007) showing the combined A2 and A2-P pathways for the daughter ions. These data are consistent with peaks 6 and 9 being A2-P and A2 respectively.

3.3.2.2. Nuclear magnetic resonance (NMR) experiments

Isolates from the CCJ system then underwent NMR analysis. ^{1}H-NMR and ^{13}C-NMR of peak 9 were consistent with previously published reports on A2 (Foo and Porter 1983; Foo *et al.* 2000b; Lou *et al.* 1999; Vivas *et al.* 1996). HSQC and HMBC further confirmed the identification of this peak as A2.

The NMR data of peak 6 was very similar to that of A2, with a couple of expected differences. ^{1}H-NMR of peak 6, when compared to A2, showed a loss of peaks equivalent to two hydrogens at 2.80-2.97 ppm corresponding to the two C-4 hydrogens on the F ring. A new peak integrating to one hydrogen was found at 4.67 ppm. HMBC correlation to C-2 and C-3 of the F-ring, along with HSQC data allowed for the identification of this peak as the C-4 hydrogen on the F-ring. At ambient temperature, the region of 5.90-6.11 ppm integrated to five hydrogens instead of three hydrogens as it did in A2. The splitting patterns and chemical

shifts were similar to those seen in A2, however no peaks were seen to account for the increased integration value, which should have appeared as a singlet integrating to two hydrogens in this region to account for the hydrogens on the phloroglucinol moiety. This is likely due to rapid rotation about the bond between the C-4 on the F-ring and C-1 on the G-ring. In order to limit this rotation, the probe temperature was reduced to 274 K for ^{1}H-NMR, HSQC and HMBC. The reduced temperature ^{1}H-NMR revealed two broad singlets at 6.08 and 5.90 ppm each integrating to one hydrogen. It is expected that further reducing the probe temperature would eliminate rotation at the C-4 F-ring and C-1 G-ring bond, and thus sharpen the phloroglucinol singlets further. Additionally, the ^{13}C-NMR of A2-P contained a multiplet at 95.30-95.70 ppm that could not be deconvoluted, and C-4 on the C-ring was lost in the solvent peak, therefore these assignments were assigned using HSQC and HMBC data. **Table 3.2** shows the full ^{1}H-NMR and ^{13}C-NMR assignments for both A2 and A2-P. **Table 3.3** shows the HMBC correlations. NMR data, when combined with other data, confirmed the identity of peak 6 as A2-P and peak 9 as A2.

Table 3.2. ^{1}H NMR and ^{13}C NMR Assignments

Ring	Carbon number	Chemical shift of carbon		Chemical shift of proton			
		A2-P	A2	A2-P	Splitting (J value Hz)	A2	Splitting (J value Hz)
A	5[a]	151.3	154.5				
	6	95.5	96.7	6.05	d (2.09)	6.04	d (2.42)
	7	156.3	158.2				
	8	97.5	98.3	5.96	d (2.09)	5.97	d (2.42)
B	1	131.6	131.9				
	2	114.9	115.7	7.16	d (1.69)	7.19	d (2.13)
	3	145.5	146.1				
	4	144.7	146.7				
	5	114.8	115.8	6.84	d (8.41)	6.85	d (7.03)
	6	119.0	115.8	7.05	dd (8.13, 1.69)	7.06	dd (8.37, 2.13)
C	2	99.2	100.4				
	3	67.3	66.0	4.42	d (4.52)	4.34	d (3.27)
	4	28.3	29.1	4.34	d (2.83)	4.24	bs
	4α	105.1	104.8				
	8α[a]	153.4	153.1				
D	5	151.4	152.4				
	6	95.4	96.7	6.06	s	6.14	s
	7	156.1	156.6				
	8	106.6	107.5				
E	1	130.6	131.6				
	2	115.9	120.9	7.29	s	7.29	d (2.05)
	3[a]	145.6	146.1				
	4[a]	144.7	146.7				
	5	115.1	120.4	6.82	d (8.08)	6.84	d (7.18)
	6	120.4	116.8	7.00	d (8.13)	7.06	dd (8.20, 2.05)
F	2	78.3	82.2	5.32	s	4.95	s
	3	71.3	67.6	4.10	m	4.15	d (3.32)
	4	37.5	30.8	4.61	s	2.80/2.94	dd (17.36,4.65)
	4α	104.5	102.9				
	8α[a]	151.7	151.0				
G	1[a]	103.5					
	2, 4, 6[a]	156.3					
	3, 5	95.2		6.02	bs		
	3, 5	95.6		5.85	bs		

[a] assigned based on HMBC data and previously published assignments (Lou *et al.* 1999; Vivas *et al.* 1996).

Table 3.3. HMBC Correlations, H → C

Ring	Carbon number	A2-P[a]	A2[a]
A	6	A-5, A-7, C-4α	A-5, A-7, A-8, C-4α
	8	A-6, C-4α	A-6, A-7, C-4α
B	2	B-6, B-3, C-2	B-1, B-4
	5	B-1, B-4	B-1, B-3, B-4
	6	B-4, B-5	B-3, B-5, C-2
C	3		C-2, C-4α, D-8
	4	C-2, C-3, C-4α	C-3
D	6	D-5, F-4α	D-5, D-7, D-8, F-4α
E	2	E-3, E-6, F-2	E-5, F-2
	5	E-1, E-5	E-1, E-3, E-4
	6	E-2, F-2	E-5
F	2	E-1, E-2, E-6, F-3	E-1, E-2, E-6, F-4
	3	F-4α	
	4	D-5, F-2, F-3, F-4α, G-2/G-4	D-5, F-2, F-3, F-4α
G	3, 5	G-1, G-3/G-5, G-2/G-4/G-6	n/a
	3, 5	G-3/G-5	n/a

[a] letter-number indicates ring letter and carbon number.

3.3.2.3. Molar extinction coefficient determination

The molar extinction coefficients were determined in methanol for A2-P and A2 isolated from the CCJ system. Since A2 is a dimer of epicatechin units, and the molar absorptivity at 280 nm (ε_{280}) = 3988 $M^{-1}cm^{-1}$ for epicatechin in methanol (Kennedy and Jones 2001), it was estimated that A2 and A2-P would have $\varepsilon_{280} \approx 8000\ M^{-1}cm^{-1}$. With this assumption, a concentration of 100 μM for A2 and A2-P would produce an absorbance near 0.80. A2 and A2-P were isolated from CCJ in triplicate as described above and dissolved in methanol to the above concentration. It was determined that for A2-P, ε_{280} = 8003 $M^{-1}cm^{-1}$, and for A2,

ε_{280} = 5857 $M^{-1}cm^{-1}$. These data were used to calculate the relative molar response and corrected relative mass response in **Table 3.1**.

3.3.3. Kinetic studies

Previous published kinetic studies of the acid-catalyzed cleavage of PAC using excess phloroglucinol as the trapping reagent found 20 min to be the optimal reaction time to obtain 90% of the maximal PAC products measured in a grape-based system (Kennedy and Jones 2001). It was anticipated that the optimal reaction conditions for A2 and A2-P would differ from B-type PACs, therefore a kinetic studies were performed on CCJ PACs. In order to optimize the reaction time for the two A-type PACs, CCJ PAC concentrations of 2.5 g/L and 5 g/L were tested using the published method (Kennedy and Jones 2001), which called for 50 g/L phloroglucinol. The results showed the A-type PACs reached a maximum considerably later than 20 min (**Fig. 3.5)**. Additionally, it was found that higher phloroglucinol equivalents resulted in an apparent increase in product stability. The large error bars seen in **Fig. 3.5** for both A2 and A2-P at 300 min are due this variation in phloroglucinol equivalents.

To determine the maximum yield and stability of the desired products, phloroglucinol concentrations were varied from 5 g/L to 100 g/L with a constant PAC concentration of 5 g/L. Combining the results of these studies, it was concluded that a concentration of 5 g/L crude CCJ PAC, 100 g/L phloroglucinol and 10 g/L ascorbic acid in 0.1 N methanolic HCl at 50ºC was found to be

optimal for a CCJ system. A reaction time 135 min was optimal to obtain >90% of the maximum of both A2 and A2-P. Therefore this time point was used for quantification of these PACs.

3.3.4. Testing analytical method with TPS

Based on the above work, it was concluded that the analytical method provided single product peaks for both A2 and A2-P in the CCJ system and therefore could be used to quantify these PACs. The method was then applied on another A-type system to determine if terminal and extension A2 PACs were purely eluted in a system known to contain additional A-type PACs. Previously published work provided evidence for six A-type dimers in peanuts (Yu *et al.* 2006). Therefore, TPS was chosen to test the analytical method. An analytical sample of TPS was prepared as described above, which then underwent HPLC analysis. The results showed peaks at both k = 7.5 and 12.6 that correspond to A2-P and A2 respectively in the CCJ system. Co-injection of CCJ and TPS resulted in clean co-elution of A2-P and A2 in CCJ with the k = 7.5 and 12.6 peaks of TPS.

TPS was optimized using the same kinetic studies as CCJ. The TPS studies indicated a reduction in the yield of A2-P (~8% reduction) and A2 (~12% reduction) at higher phloroglucinol equivalents (100 g/L vs. 50 g/L phloroglucinol). Additionally, the stability of A2 and A2-P did not depend on concentration, as was seen in CCJ. Therefore a concentration of 50 g/L

phloroglucinol (Kennedy and Jones 2001) was used for TPS. As seen in the CCJ system, a reaction time 135 min was optimal to obtain >90% of the maximum of both A2 and A2-P. Therefore this time point was used for quantification of these PACs. The CCJ and TPS kinetic studies suggest that reaction conditions should be optimized for any PAC system under investigation to ensure maximum yield of A2 PACs. The TPS study also reveled four additional peaks that presented similar A2/A2-P kinetics, which could potentially correspond to the additional A-type PACs previously reported (Yu *et al.* 2006).

Approximately 2 mg of the k = 7.5 peak was isolated from the TPS. There was an insufficient amount of the k = 12.6 peak for isolation. The other four potential A-type peaks, while present in significant quantities, were not isolated or characterized at this time. ^{1}H NMR was performed on the peak at k = 7.5 isolated from the TPS and the results showed a pure product that was consistent with the CCJ data, allowing for the confirmation of this peak as A2-P in TPS.

3.3.5. Quantitative application of A2 method

The kinetically optimized analytical methods were performed in triplicate to quantify the A2-P and A2 in CCJ and TPS (**Table 3.4**). As previously mentioned, the identity of the peak at k = 12.6 in TPS was not confirmed as being A2, so the quantity listed in **Table 3.4** is tentative.

Table 3.4. Quantity of A2-P and A2 in CCJ and TPS (n=9).

System	Mass A2-P (mg/g)[a,b]	% A2-P[b,c]	Mass A2 (mg/g)[a]	% A2[c]
CCJ	209.4 ±4.9	21%	135.7 ±4.7	14%
TPS	404.9 ±14.4	40%	29.1 ±0.9	3%

[a] Mass of A2 per gram of crude PAC powder.
[b] Not including phloroglucinol (G-ring) moiety.
[c] Percent A2 found in crude PAC powder (w/w).

3.4. Conclusion

Methods for the identification, isolation and purification of two A-type PACs were presented here. Isolates were extensively characterized and molar extinction coefficients were determined for the terminal A2 dimer and phloroglucinol adduct of the A2 extension dimer. Kinetic studies were conducted and reaction conditions were optimized for the A2 units in both CCJ and TPS. The end result is an analytical method that can be used for the quantification of terminal and extension A2 dimers in CCJ and TPS.

Using this analytical method to study A-type PACs, and comparing the kinetics of the reaction with previous studies (Kennedy and Taylor 2003), suggests that reaction kinetics need to be optimized for each PAC under investigation. Given the variation in PAC subunit composition, interflavonoid bond location and molecular mass distribution, this conclusion is somewhat expected.

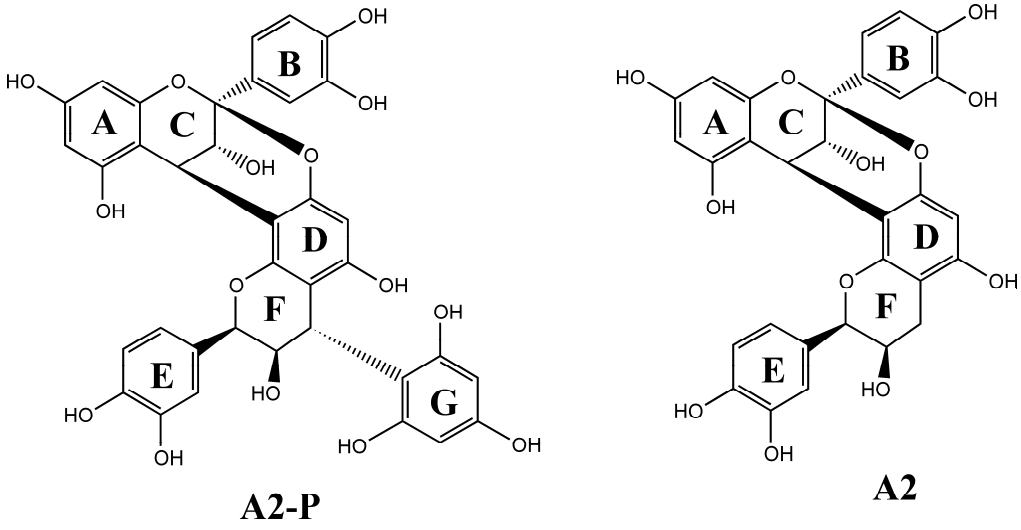


Fig. 3.1 Structure of A2-P and A2

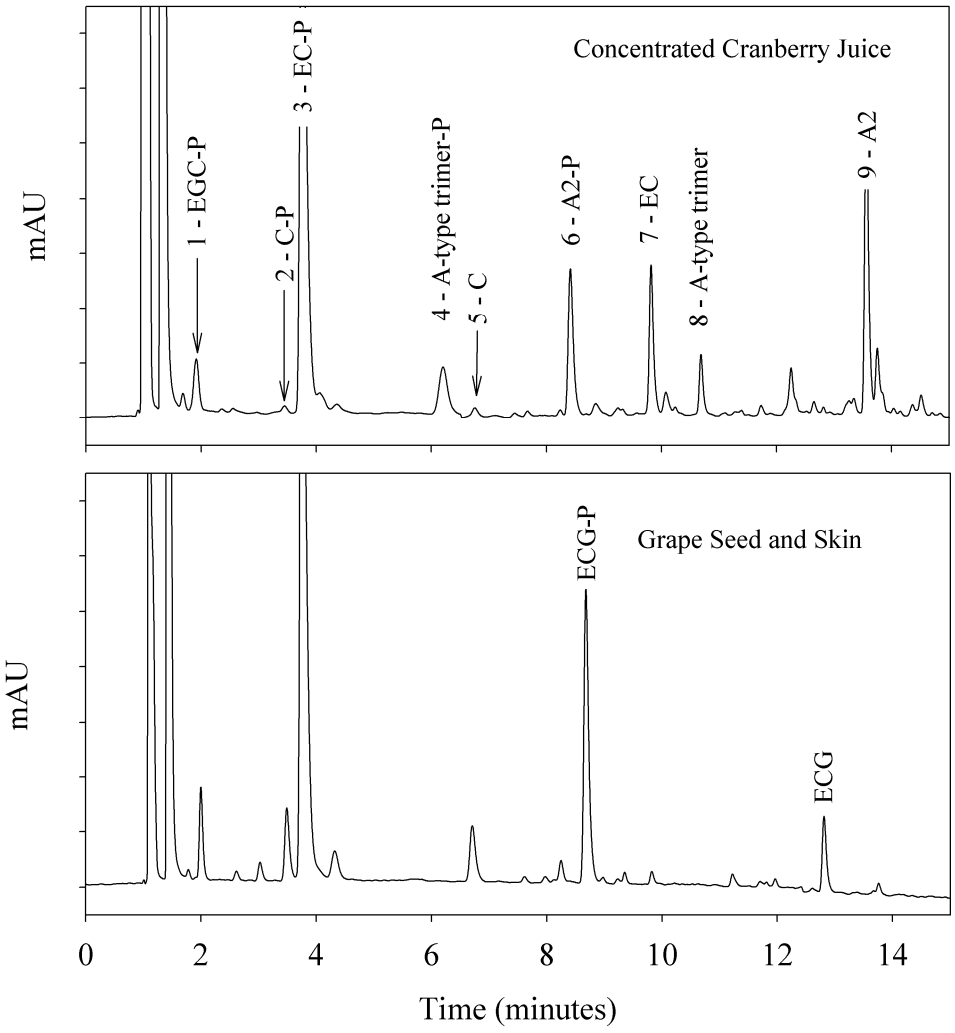


Fig. 3.2 Analytical HPLC Chromatograms (280 nm) for CCJ and Grape seed and skin at 20 min reaction time. -P = phloroglucinol adduct, EGC = epigallocatechin, C = catechin, E = epicatechin, ECG = epicatechin gallate. Peaks 4 and 8 are tentatively identified as A-type trimers.

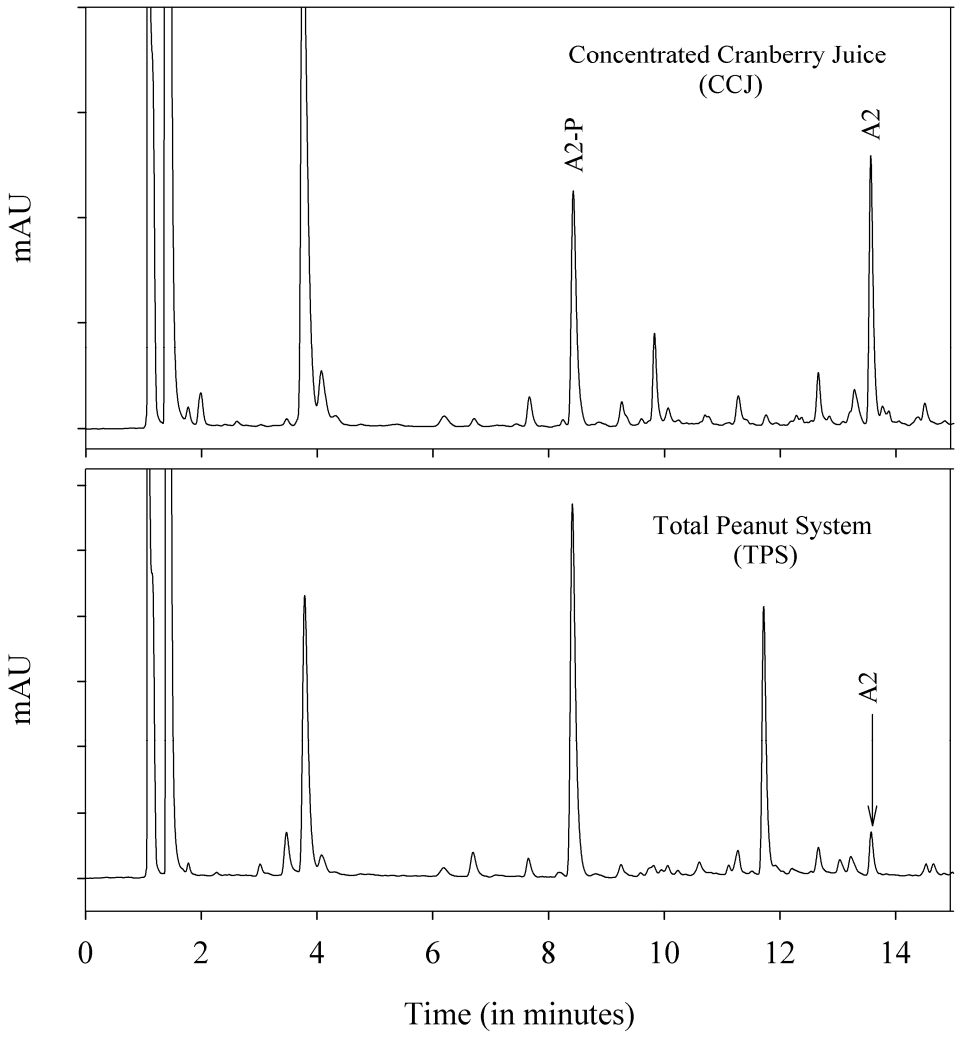


Fig. 3.3 Analytical HPLC Chromatograms (280 nm) for CCJ and TPS at 135 min reaction time. -P = phloroglucinol adduct.

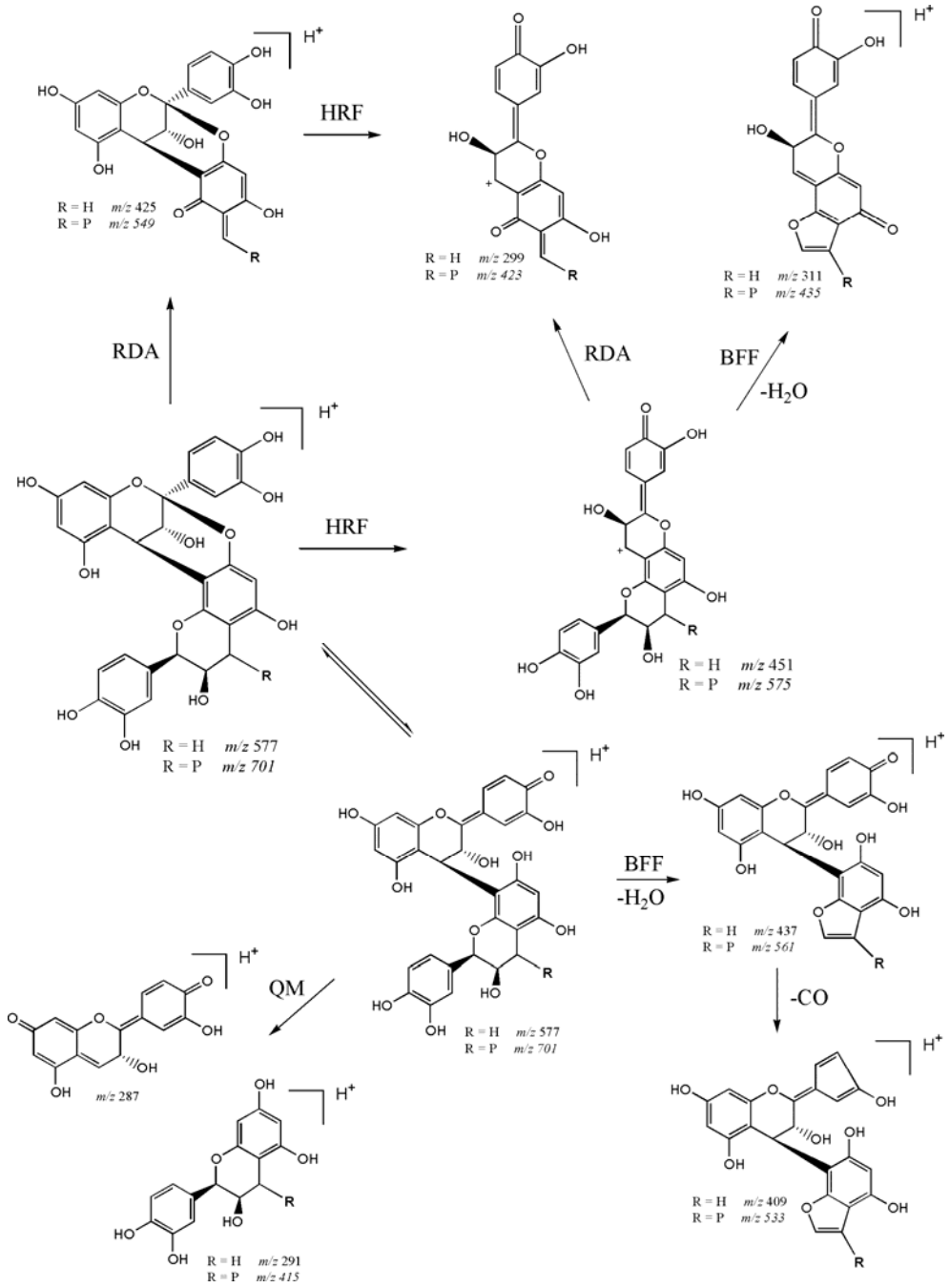


Fig. 3.4 Fragmentation pathways for A2 and A2-P.
P = phloroglucinol, BFF = benzofuran forming, HRF = heterocyclic ring fission, QM = quinone methide fission, RDA = retro-Diels-Alder. Modified from (Gu *et al.* 2004; Li and Deinzer 2007).

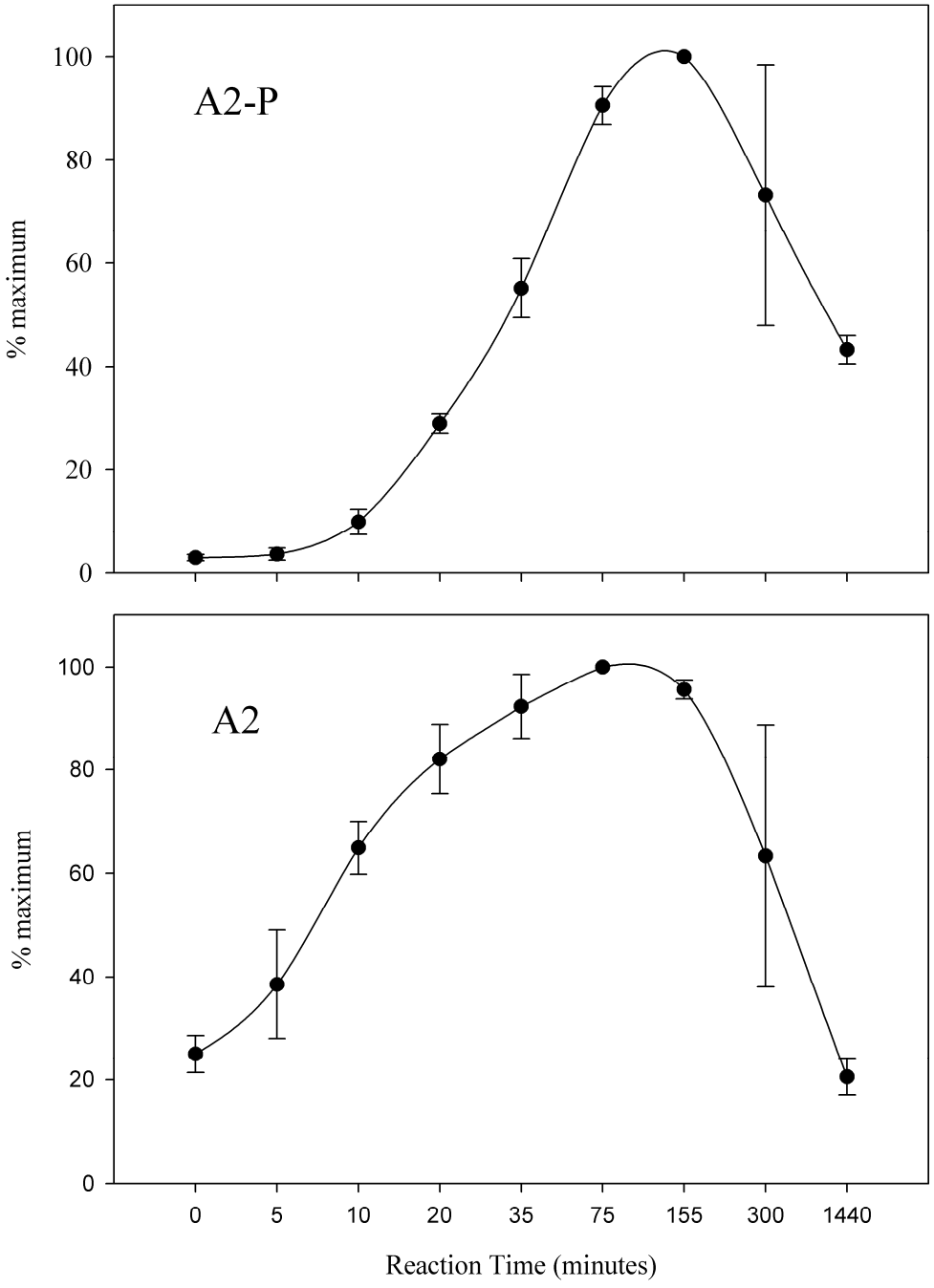


Fig. 3.5 Kinetics of A2-P and A2. n=4, error bars are standard deviation

Chapter 4. Presence of proanthocyanidin A2 in various commercial products

4.1. Introduction

The phloroglucinolysis and HPLC method for the quantification of A-type proanthocyanidins (A-PAC) developed in Chapter 3 (Koerner *et al.* 2009) was used to compare different products to identify differences in concentrations of A-PACs.

As was previously shown, concentrated cranberry juice (CCJ) has a high concentration of A-PACs, therefore a comparison of various brands and types of cranberry juice was performed, which included various cranberry juice cocktails (CJC), various pure cranberry juice (PCJ), and a white cranberry juice (WCJ). Whole, frozen cranberry that had not undergone any other processing was also examined. Both red and yellow bell varieties were tested.

Many alternative and healthfood stores offer a variety of nutraceutical supplements which contain cranberry products and claim to prevent or even cure urinary tract infections. It has been reported that A-PAC is believed to be partially responsible for anti-adhesion of bacteria in the bladder (Foo *et al.* 2000a; Foo *et al.* 2000b; Howell *et al.* 2005) that results in bladder infections (Beachey 1981). Therefore, three of these dietary supplements were tested for presence and concentrations of A-PACs.

Cinnamon has previously been shown to contain high levels of PACs, including A-PACs (Gu *et al.* 2003a; Gu *et al.* 2003b). Since several different species of cinnamon exist in various quality grades, a selection of several different cinnamon brands, species and products were also tested. This test included a few Saigon cinnamon brands, organic bulk cinnamon, an herbal extract of *Cinnamomum aromaticum*, and an herbal dietary supplement of *Cinnamomum verum*.

4.2. Experimental

4.2.1. Chemicals

Acetone, acetonitrile, glacial acetic acid, high purity hydrochloric acid, trifluoroacetic acid (TFA), and methanol were all HPLC grade and all were purchased from VWR (Tualatin, OR, USA). Reagent grade phloroglucinol and (-)-epicatechin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anhydrous sodium acetate (ACS grade) was purchased from Mallinckrodt (Paris, KY, USA). Water was purified using a Millipore Milli-Q system (Bedford, MA, USA). Toyopearl HW 40C chromatography resin was purchased from Supelco (Bellefonte, PA, USA).

4.2.2. Instrumentation

Analytical HPLC with diode array detection (DAD) was performed on an Agilent 1100 series HPLC (Palo Alto, CA, USA) composed of a degasser (G1322A), quaternary pump (QuatPump G1311A), autosampler (G1313A ALS), and column heater (G1316A ColCom) controlled by Agilent's ChemStation for LC Rev. A.08.04 software.

4.2.3. Sample preparation

4.2.3.1. Sample information: Acquisition and identification

For the cranberry juice CJC comparison, all of the following were purchased from local markets in Corvallis, OR. Ocean Spray Cranberry Juice Cocktail (27% juice) (OS-CJC) contained water, cranberry juice concentrated, cane or beet sugar, and ascorbic acid. Albertson's cranberry juice cocktail (27% juice) (Al-CJC) contained filtered water, cranberry juice from concentrated and cranberry juice, high fructose corn syrup, elderberry concentrate (for color) and ascorbic acid. Albertson's "Light" Cranberry Juice Cocktail (27% juice) (Al-LCJC) contained filtered water, cranberry juice from concentrate and cranberry juice, fructose, pectin, natural flavors, sodium citrate, acesulfame potassium and sucralose. RW Knudsen Just Cranberry juice (100% juice) (RWK-PCJ) contained filtered water sufficient to reconstitute cranberry juice concentrate. L & A All Cranberry juice (100% juice) (LA-PCJ) contained filtered water and cranberry juice concentrate.

Two lots of concentrated white cranberry juice (W-CCJ) were provided by Ocean Spray Cranberries, Inc. (Boston, MA, USA). The processing pull dates were 2/25/09 (W-CCJ 2/25/09) and 3/2/09 (W-CCJ 3/2/09). It should be noted that the W-CCJ is made from the same cranberry variety that the regular red concentrate is made, however the berries are harvested before the berries turn red.

Fresh Ocean Spray whole red cranberry fruit was purchased from a local market (Corvallis, OR, USA) (OS-WRC) and immediately frozen to -80 °C until extracted. Two lots of frozen Yellow Bell cranberries (YBC) from two different years of harvest (YB-2006 and YB-2007) were obtained from Ocean Spray Cranberries, Inc. (Boston, MA, USA) and stored at -80 °C until tested.

For the cranberry nutraceutical comparison, the products were all purchased from The First Alternative Co-Operative Market (Corvallis, OR). New Chapter Urinary Tract Take Care (NCUTTC), calls itself a full spectrum cranberry extract, and reports that a single capsule contains 140 mg cranberry fruit extract (*Vaccinium macrocarpon*) and 10 mg cinnamon bark extract (*Cinnamomum cassia*). Other ingredients are maltodextrin, cyclodextrin, silicon dioxide, modified starch (maize <non-GMO>), magnesium stearate and tricalcium phosphate. SoLaray CranActin Urinary Tract (CAUT) reports "Guaranteed bacterial Antiadherence Activity" with one capsule containing 400 mg cranberry extract (as CranActin® Cranberry AF™ Extract) (*Vaccinium macrocarpon*). Other ingredients are magnesium hydroxide, cellulose,

magnesium oxide, magnesium stearate, silica, vegetable juice concentrate, maltodextrin and calcium phosphate. Oregon's Wild Harvest Freeze-dried Cranberry (*Vaccinium macrocarpon*) (OWH-FDC) has 400 mg of Cranberry fruit-freeze-dried in a polysaccharide capsule.

For the cinnamon comparison, all of the following products were purchased from Albertson's supermarket (Corvallis, OR). McCormick Gourmet Collection pre-ground Saigon Cinnamon (MC-GSC), McCormick Ground Cinnamon (MC-GC), Spice Islands Ground Saigon Cinnamon (SI-GSC), Albertson's Ground Cinnamon (A-GC), and McCormick Cinnamon Grinder (MC-CGr). The following products were purchase from The First Alternative Co-Operative Market (Corvallis, OR): bulk Organic cinnamon – ground (Org-GC), Herb Pharm liquid herbal extract (*Cinnamomum aromaticum)* (HP-LHE), and Oregon's Wild Harvest True Cinnamon (*Cinnamomum verum)* (OWH-TC).

4.2.3.2. PAC isolation and purification

Aliquots of OS-CJC, A-CJC, AL-CJC, RWK-PCJ and LA-PCJ were centrifuged at 3000g to remove gross particulates and then 7 mL of each sample was loaded onto a Bio-Rad (Hercules, CA, USA) 9 cm poly-prep chromatography column packed with 6.5 mL Toyopearl HW-40C and pre-equilibrated with 0.05% v/v aqueous TFA. The loaded column was rinsed with 15 mL 0.05% v/v aqueous TFA to remove sugars and organic acids, followed by 15 mL of 1:1 methanol:water containing 0.05% v/v TFA to remove low molecular weight

phenolics. Finally 10 mL of 2:1 acetone:water containing 0.05% v/v TFA was used to elute the PAC fractions. The PAC fractions were then lyophilized and stored at -80°C until used for further analysis.

25 mL of W-CCJ 2/25/09 and W-CCJ 3/2/09 were each diluted to 25% v/v concentration with water and individually applied to a 5 cm x 15 cm Toyopearl HW-40C column pre-equilibrated with 0.05% v/v aqueous TFA at ambient temperature. The loaded column was rinsed with 250 mL 0.05% v/v aqueous TFA to remove sugars and organic acids, followed by 250 mL of 1:1 methanol:water containing 0.05% v/v TFA to remove low molecular weight phenolics. Finally 200 mL of 2:1 acetone:water containing 0.05% v/v TFA was used to elute the PAC fraction. The PAC fraction was then lyophilized and stored at -80 °C. The yield was 5.86 g/L for the W-CCJ 2/25/09 and 6.65 g/L for the W-CCJ 3/2/09.

Cranberry fruit was thawed and 50 g of whole berries were accurately measured for extraction. The berries were extracted three times at ambient temperature by crushing with a mortar and pestle in approximately 1 ml 2:1 acetone:water per gram of fruit, with the supernatant being collected each time. The supernatant and pulp was then combined in and Erlenmeyer flask and brought up to approximately 4 mL per gram of fruit and place on a shaker table at 150 RPM for 24 hours. The mixture was passed through a Whatman filter paper to remove pulp and large particulates. The acetone was then removed from the filtrate under reduced pressure, after which the aqueous portion was loaded onto

the 5 cm x 15 cm Toyopearl column as previously described for W-CCJ, with the following change: The 1:1 methanol:water was not used in order to determine if A2 monomers are present. The acetone from the PACs fractions was removed under reduced pressure on the centrovap, and the remaining aqueous solution was lyophilized to a magenta powder for the red cranberries and a yellow powder for the Yellow Bell cranberries. The yield for both Yellow Bell samples was approximately 3.20 mg/g of whole fruit.

For the cinnamon extractions, 100 mg of cinnamon was accurately weighed and extracted overnight in 2:1 acetone:water under argon, covered with aluminum foil and room lights off. The liquid extract was vacuum filtered and the acetone was removed under reduced pressure at 40 °C. No additional purification steps were necessary (see discussion). The PAC fraction was lyophilized stored as a powder at -80 °C.

4.2.3.3. Phloroglucinolysis of PACs

A 200 mg phloroglucinol and 20 mg ascorbic acid per mL of 0.2 N methanolic HCl phloroglucinol solution was made as a reaction solution (PG-solution). PAC solutions were made by dissolving PAC powders in methanol, with a final concentration of ~10 mg/mL. For A2 monomers and other monomeric PACs: 100 µL of the PAC solution was added to 100 µL methanol and 1 mL 40 mM aqueous sodium acetate, and the solution was then subjected to HPLC analysis. For A2 and A2-P quantification: 100 µL of the PAC solution was added

to 100 µL of the PG-solution. The reaction mixture was vortexed to solubilize (~3 seconds) and then allowed to react for 135 minutes at 50 °C. The reaction was stopped by addition of 1 mL of 40 mM aqueous sodium acetate. The reaction solution was centrifuged for 10 minutes to remove gross particulates, after which the solution was subjected to HPLC analysis.

4.2.4. Analytical HPLC

The previously published analytical HPLC method for A2 quantification (Koerner *et al.* 2009) was used unmodified and applied to all tested products. Briefly, two 100 mm x 4.6 mm Chromolith RP-18e columns, purchased from EM Science (Gibbstown, NJ, USA), were connected in series with a guard column of the same material. The mobile phases were 1.0% v/v aqueous acetic acid (A) and acetonitrile containing 1.0% v/v acetic acid (B). The column temperature was 30 °C, flow rate was 3 mL/min, and with a linear gradient as follows: elution time in min (%B), 0 (3%), 4 (3%), 14 (18%), 14.01 (80%), 20 (80%). Eluting peaks were monitored at 280, 320 and 520 nm to identify peaks of interests in the PAC systems. The final method has an approximate runtime of 25 min, from injection to conclusion of the post run.

A calibration curve was generated, plotting the mass of injected (-)-epicatechin (in µg) verses the integral of the absorbance over time (in PAU). The slope of the regression line was then multiplied by the integral of the A2 or A2-P absorbance over time and the respective corrected relative mass response (see

Table 3.1) to give the mass (in µg) found in the injected volume. For mole quantification, the determination was conducted in a similar fashion using moles of (-)-epicatechin and the relative molar response (see **Table 3.1)**.

4.2.5. Statistical analysis

All data given as means ± standard deviation. Statistical analysis was carried out using Student's t-test (significance set at p-value < 0.01) on R 2.9.2 GUI 1.29 Tiger build 32-bit (5464) (R Foundation for Statistical Computing).

4.3. Results and discussion

4.3.1. Commercial cranberry juice

Of the CJCs tested, OS-CJC had the highest and the Al-CJC had the lowest concentrations of both A2 and A2-P (see **Table 4.1**), all three of which had 27% cranberry juice. There was no difference between the A2-P concentrations found in OS-CJC and Al-LCJC, but the A2 concentration was different. There was no difference in either the A2 or A2-P concentrations between Al-LCJC and Al-CJC. There was a higher concentration of both A2 and A2-P found between the two 100% pure cranberry juices, as well as between these juices and all the juice cocktails (see **Fig. 4.1**).

Table 4.1. A2 and A2-P concentrations in cranberry juice and juice cocktails.

Juice Brand [a]	A2-P [b,c] (mg/100mL juice)	A2 [c] (mg/100mL juice)
OS-CJC	4.31 (±0.15)	2.64 (±0.05)
Al-CJC	3.70 (±0.08)	2.15 (±0.28)
Al-LCJC	4.01 (±0.17)	2.27 (±0.08)
LA-PCJ	8.72 (±0.34)	5.35 (±0.15)
RWK-PCJ	12.98 (±0.35)	6.68 (±0.15)

[a] Ocean Spray Cranberry Juice Cocktail (OS-CJC), Alberson's Cranberry Juice Cocktail (Al-CJC), Alberson's Light Cranberry Juice Cocktail (Al-LCJC), L & A All Cranberry Juice (LA-PCJ), R W Knudsen Just Cranberry Juice (RWK-PCJ).
[b] mass does not include the phloroglucinol moiety.
[c] number in parenthesis is standard deviation, n = 4.

It is reasonable to assume that the cranberry juice is the only contributor of A2 and A2-P in the commercial blends, as none of the other ingredients have been shown to contain A-PACs. Therefore, if in theory we were to calculate the concentration of A2 and A2-P in the commercial blends as if they were made from 100% cranberry juice (dividing the mass of the compound of interest by the percent cranberry juice in the cocktail, or 0.27, or 27%), we find that there is a considerably higher concentration of A2 and A2-P in the commercial blends when compared to the 100% pure, unfiltered cranberry juices (see **Fig. 4.2**). This could be due to a solubility limit of the A-PACs, or it could also be due to other compounds in the juice blends that aid in solubilizing the A-PACs in the cocktails. As expected, the PCJs both had overall higher concentrations of A2

and A2-P when compared to the CJCs, but both also had differences for both compounds when compared to each other.

4.3.2. Concentrated white cranberry juice

There were soluble solids differences in the concentration levels of the various concentrated juices as determined by °Brix readings. Therefore, and in order to normalize for comparison, A2 concentrations were taken back to the crude PAC powder that was isolated by using the SEC column as opposed to volumes of juice concentrate. As was stated earlier, there was a higher concentration of crude PACs in the W-CCJ 3/2/09 sample at 6.65 g/L concentrate, than was found in the W-CCJ 2/25/09 sample having 5.86 g/L concentrate. This is approximately a 13% difference in yield for the two samples that were processed a little less than a week apart. Additionally, there was a 13% higher concentration of both A2 and A2-P (see **Table 4.2**) found in the juice concentrate processed at a later date (W-CCJ 3/2/09) when compared to the earlier processed juice concentrate (W-CCJ 2/25/09). So not only was there a higher yield of crude PACs in the W-CCJ 3/2/09, but there was also a higher percentage of A-PACs. The higher yield of crude PACs could potentially be attributed to a difference in the concentration level of the juice. However, the 13% higher yield of A-PACs per the same mass of powder indicates that gram for gram, there are more A-PACs in the juice that was processed at the later date. This seems to indicate a potential time dependence on A-PAC concentrations.

Table 4.2. A2 and A2-P concentrations in concentrated cranberry juice.

Concentrate [a]	A2-P [b,c] (mg/g PAC crude powder)	A2 [b,c] (mg/g PAC crude powder)
CCJ (optimized)	209.4 (±4.9) [d]	135.7 (±4.7) [d]
W-CCJ-2/25/09	74.3 (±1.0)	24.2 (±0.4)
W-CCJ-3/02/09	99.7 (±1.0)	32.0 (±0.3)

[a] Ocean Spray concentrated cranberry juice (CCJ), Ocean Spray Concentrated white cranberry juice, batch date 2/25/09 (W-CCJ-2/25/09), Ocean Spray Concentrated white cranberry juice, batch date 3/2/09 (W-CCJ-3/2/09)
[b] mass does not include the phloroglucinol moiety.
[c] number in parenthesis is standard deviation, n = 9.
[d] As published in Koerner *et al*. 2009.

Comparing the W-CCJs to CCJ, there was over twice the concentration of A2-P and over four times the concentration of A2 in crude CCJ PAC powder compared to the crude W-CCJ crude powders (see **Table 4.2**). Considering these juice concentrates are made from the same variety of cranberry, but only differ in harvest time, this seems to further support the idea that concentrations of A2 may depend on time-related factors such as harvest maturity, processing time, and shelf life. This is further discussed (see section 4.3.5.2) below.

4.3.3. Cranberry fruit

YBC are a variety of cranberry that is considered a true "white" cranberry because this fruit never turns red as is seen in the mass-marketed red cranberry fruit (RCF). Based on the analysis done here, it was determined that the YBC variety contains both terminal and extension A2 units. This is the first known

identification of these PACs in YBC. Overall, there was a significantly higher percentage of extension A2 units found in RCF than were found in YBC (see **Table 4.3**). The amount of terminal A2 was a little more complicated. The highest amount of A2 per gram of fruit was found in YB-2006. But the YB-2007 had a statistically significant lower amount of A2 than both YB-2006 and OS-WRC. However, since these are different species of cranberry, it is expected that differences would be observed.

Table 4.3. A2 and A2-P concentrations in whole cranberry fruit.

Fruit [a]	A2-P [b,c] (mg/100g fruit)	A2 [c] (mg/100g fruit)
OS-WRC	46.5 (±0.5)	19.1 (±0.4)
YB-2006	42.1 (±0.5)	22.9 (±0.7)
YB-2007	34.2 (±0.3)	17.2 (±0.2)

[a] Ocean Spray whole red cranberry fruit (OS-WRC), Ocean Spray yellow bell cranberry from 2006 harvest (YB-2006), Ocean Spray yellow bell cranberry fruit from 2007 harvest (YB-2007)
[b] mass does not include the phloroglucinol moiety.
[c] number in parenthesis is standard deviation, n = 9.

Ignoring the OS-WRC and looking just at the YBC, a 12-13% higher concentration of A-PACs was found in the YB-2006 fruit compared to YB-2007. If we look at the ratio of A2-P to A2 (see **Fig. 4.3**), we see that there is also a statistically significant difference between the two YBC, with the YB-2007 having a slightly higher A2-P to A2 ratio. As well, there is a significant difference between both YBC and the OS-WCF. Previous studies have shown that

climatological factors can affect the concentrations of PACs in other fruits, such as wine grapes (Jones and Davis 2000). The only information known for the YBC is that they were grown and harvested in Wisconsin in 2006 and 2007, therefore exact climate and weather data was not obtainable for the area where the YBC were grown. Cranberries in North America tend to bloom in early July with fruit set later in that month. According to Ocean Spray's website (http://www.oceanspray.com.au/ about-cranberry-harvest.asp), cranberry fruit is harvested from mid-September through mid-November. Based on graphs obtained from the National Weather Service for the Twin Cities, MN, Weather (NWS 2010), which is in the same region of the country as Wisconsin, there was more fluctuation in temperatures as well as lower temperatures seen during the approximate time of fruit set through harvest in 2007 when compared to the same time period in 2006. It is therefore reasonable to believe that temperature as well as other weather and climate variables could account for some of the differences seen in the two different year's harvest of YBC. Additionally, differences in fruit size, surface area of skin, amount of seeds and fruit maturity at harvest could also account for observed differences.

4.3.4. Cranberry nutraceuticals

The cranberry nutraceuticals had very low concentrations of A-PACs. Values given in **Table 4.4** for the recommended daily dose, which is 2 capsules for NCUTTC, 4 capsules for CAUT, and 3 capsules for OWH-FDC. To better

understand this data, the nutraceuticals were compared to a 100 mL (approximately 3.4 fluid ounces) of CJC. Both the NCUTTC and CAUT had very low concentrations of A2 and A2-P. In order to get the same concentration of these compounds as is found in 100 mL of OS-CJC, one would have to take 6 to 8 times the recommended daily dose. The OWH-FDC was a little better, in that taking twice the recommended daily dose would give you more A2-P and a little less that the same amount of A2 as you would get from drinking 100 mL of OS-CJC.

Table 4.4. A2 and A2-P concentrations in cranberry nutraceuticals.

Brand [a]	A2-P [b,c] (mg/daily dose)	A2 [c] (mg/daily dose)
NCUTTC	0.70 (±0.02)	0.38 (±0.01)
CAUT	0.53 (±0.01)	0.43 (±0.01)
OWH-FDC	2.89 (±0.10)	1.04 (±0.07)

[a] New Chapter Urinary Tract Take Care (NCUTTC), CranActin Urinary Tract (CAUT), Oregon's Wild Harvest Freeze-dried Cranberry (OWH-FDC)
[b] mass does not include the phloroglucinol moiety.
[c] number in parenthesis is standard deviation, n = 6.

4.3.5. Comparisons of A2 across cranberry products.

Based on current research, it is unclear whether terminal, extension A2, or both are significant contributors to the prevention of UTI and other health benefits associated with A2. In order to get a different perspective on how the various cranberry products compare, an investigation into the ratio of A2-P to A2 was conducted. By looking at this ratio, it is possible to determine if there are significant differences in the composition of PACs relative to A2, and to speculate on what these differences could indicate.

4.3.5.1. Whole cranberry fruit and cranberry juices

The ratio of A2-P to A2 is significantly higher in the fresh and freeze-dried RCF compared to all the different red cranberry juices (see **Fig. 4.4**). This could indicate that there is a higher proportion of A2-P found in the seeds, which would have been extracted during the analytical process. Since the seeds from the juice were removed before the analysis was conducted, there would have been little contribution from the seeds to the overall PAC count. Therefore, it is possible to conclude that much of the extension A2 is lost in the pulp after the fruit has been juiced. If it is found that extension A2 is a significant contributor to the prevention of UTI, then further investigation into value added products from the pulp would be well advised.

4.3.5.2. White versus red concentrated cranberry juice.

The ratio of A2-P to A2 is nearly twice as high in both of the W-CCJ when compared to the CCJ (see **Fig. 4.5**). Since these products are made from the same fruit but differ only in harvest time, this indicates a relationship between the age of the fruit and the proportion of extension and terminal A2 units. Since the numbers reported here are in units of mg/g crude PAC powder, then gram per gram, there is a much higher percentage of A2-P in the young fruit juice concentrate than is found in the mature fruit juice. As was indicated by the comparison of whole fruit to fruit juices above, the additional A2-P units could be coming from the seeds of the younger fruit. It is possible that this could be due to a difference in maturity of the seeds. Immature seeds may have not yet formed a hard seed coat and therefore PACs from the seeds of young fruit could be more easily extracted during the juicing process than PACs from seeds with a hard seed coat seen in mature fruit.

Looking at the actual concentrations of A2 in crude fruit juice powders, there is a higher concentration of A2-P and A2 in the crude powder of CCJ when compared to the W-CCJ. According to recent studies on the changes in flavonoid composition during fruit development and maturation, the relative proportion of PACs is highest early on, and then decreases as the fruit matures and ripens (Vvedenskaya and Vorsa 2004). But this study only gives data in relative proportions of PACs compared to anthocyanins and other flavonoids. It is

possible that extension A2 PACs may be preferentially oxidized over terminal A2 during fruit maturation; however since changes in absolute PAC concentrations are not known, it is difficult to conclude how the A-PACs are changing in relation to the overall PAC concentrations. In order to understand all of these observations seen as differences between younger versus mature fruit juice would require a detailed investigation into the composition PACs found in the seeds versus the skins, as well as a temporal study that accurately quantifies the A-PACs, total PACs and total flavonoids.

4.3.6. Extension A2 in cinnamon species

Concentrations in the Saigon cinnamon samples were very low, and should likely be considered below the quantifiable threshold. The numbers are included here to signify that some A2-P was seen, but is so low as to be considered insignificant (see **Table 4.5**). Based on these data, if A2-P is shown to be responsible for health benefits, then Saigon cinnamon would be a poor choice for dietary intake of this compound.

Table 4.5. A2-P concentrations in cinnamon.

Brand [a]	A2-P [b,c] (mg/g spice)
MC-GSC	0.76 (±0.00)
SI-GSC	2.10 (±0.05)
MC-GC	31.11 (±0.51)
A-GC	31.59 (±1.20)
MC-CGr	26.73 (±0.35)
Org-GC	39.69 (±0.95)
OWH-TC	17.76 (±0.23)
HP-LHE	nd

[a] McCormick gourmet collection pre-ground Saigon cinnamon (MC-GSC), Spice Islands ground Saigon cinnamon (SI-GSC), McCormick ground cinnamon (MC-GC), Albertson's ground cinnamon (A-GC), McCormick cinnamon grinder (MC-CGr), Organic bulk ground cinnamon (Org-GC), Oregon's Wild Harvest True Cinnamon (OWH-TC), Herb Pharm liquid herbal extract (HP-LHE).
[b] mass does not include the phloroglucinol moiety.
[c] number in parenthesis is standard deviation, n = 3.

It was not possible to determine the species for MC-GC, A-GC, MC-CGr and Org-GC, generally for proprietary reasons. However, all of these spices had about the same concentrations of A2-P so they are being discussed as an A2-P containing group (Cin-A2-P) (see **Fig. 4.6**). The MC-CGr was notably lower than the others in this group. The cinnamon in the grinder was rather large pieces of cinnamon bark that was only ground to a coarse powder. So the difference in the concentration between it and the others in this group is likely due to the cinnamon not being ground into as fine as a powder as the commercially ground products, thus less surface area in the MC-CGr was exposed to the extraction solvents.

There was no difference between the store-brand A-CG and the national brand MC-GC. However, the organic Org-GC had a higher concentration of A2-P than all brands tested, and therefore would be deemed the best source for extraction of A2-P of the products tested. Finally, the OWH-TC had the least amount of A2-P of the products that contained the compound. Considering the differences, it appears that OWH-TC may not be the same species as the Cin-A2-P group, but it should be considered a significant source of A2 extension units. On the other had, the HP-LHE had no detectable A2-P, so no heath benefits uniquely attributed to A-PACs would be gained from using this product.

It should be noted that cinnamon extracts were tested to determine if purification beyond extraction was necessary. There was negligible mass loss from the sample after passing it through a Toyopearl HW-40C column, which indicated that little or no compounds were removed during the process. HPLC of unpurified cinnamon extract was compared to purified extracts and there were no differences observed. However, there is a compound, likely an organic acid, which elutes around the time that the A2 terminal unit elutes that could potentially interfere with A2 quantification. This unknown compound is removed during the column purification, but since there was no terminal A2 detected in any of the cinnamon samples, the unknown compound was not considered to be of concern. Based on this evidence, it was decided that no additional purification was necessary for the cinnamon samples.

4.4. Conclusion

It has been demonstrated here that comparisons can be made between various products based on their A-PAC composition. These comparisons brought up additional questions that call for further research, which is the subject of the next chapter.

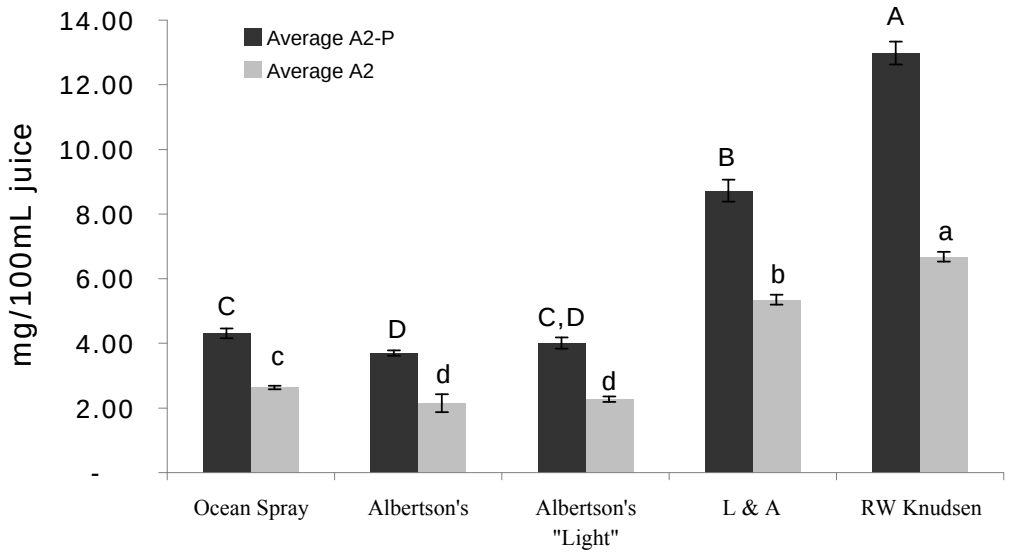


Fig. 4.1 Bar graph of concentrations of A2 and A2-P in cranberry juice cocktails and pure cranberry juice.
Values sharing the same letter are not significantly different. (p-value > 0.01, n = 4, ±sd).

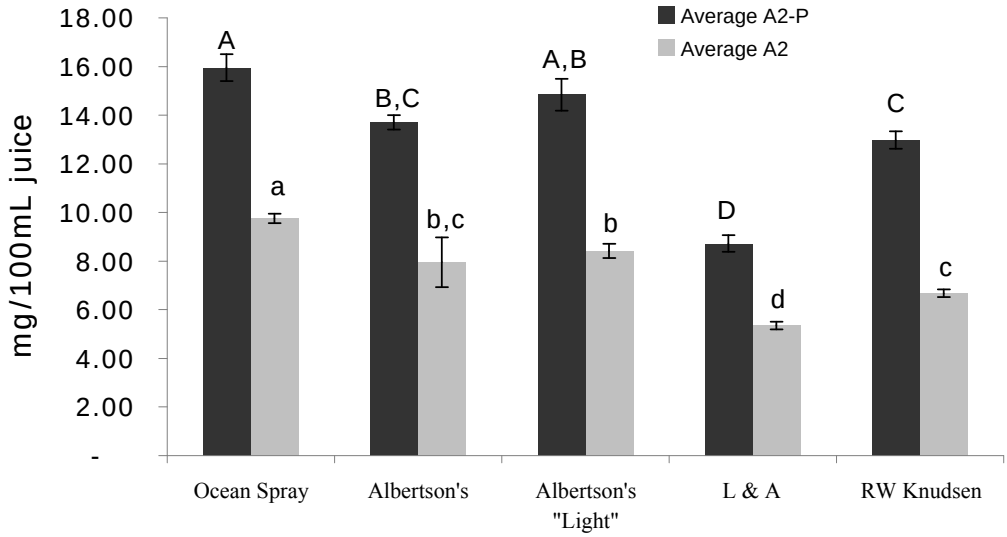


Fig. 4.2 Bar graph of concentrations of A2 and A2-P in theoretical 100% cranberry juice cocktails and pure cranberry juice.
Values sharing the same letter are not significantly different. (p-value > 0.01, n = 4, ±sd).

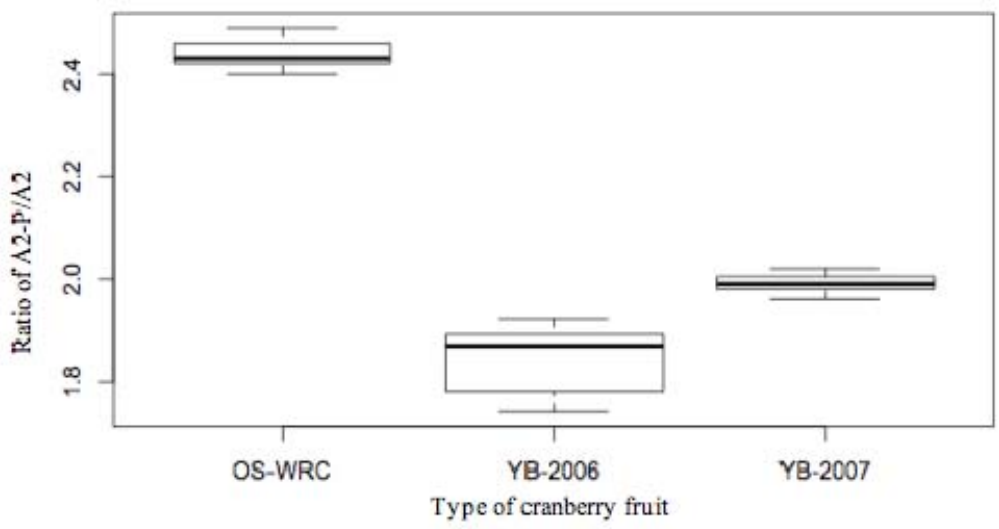


Fig. 4.3 Boxplot showing ratio of A2-P to A2 in cranberry fruit. n = 9

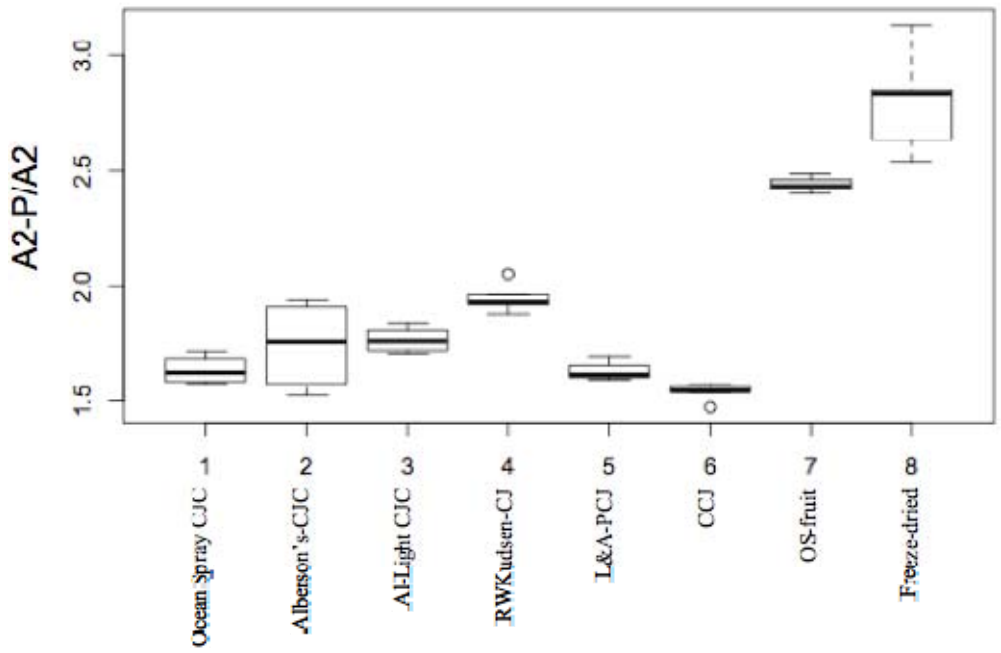


Fig. 4.4 Boxplot showing ratio of A2-P to A2 in red cranberry fruit and juice.

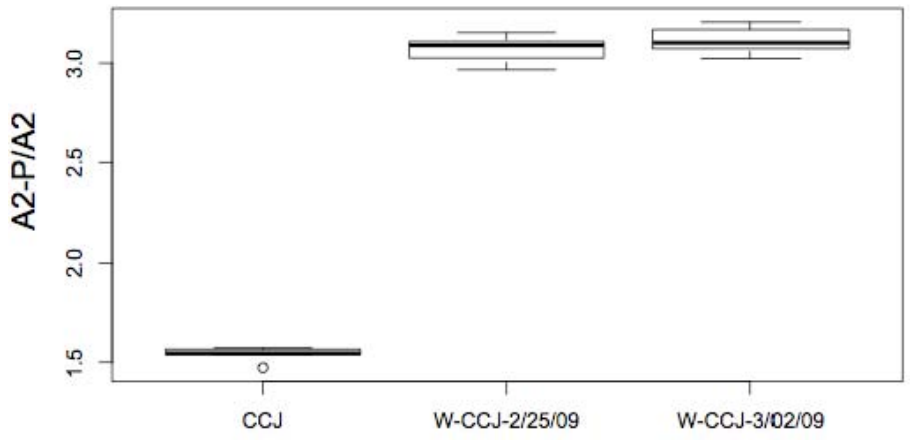


Fig. 4.5 Boxplot showing ratio of A2-P to A2 in concentrated cranberry juice.

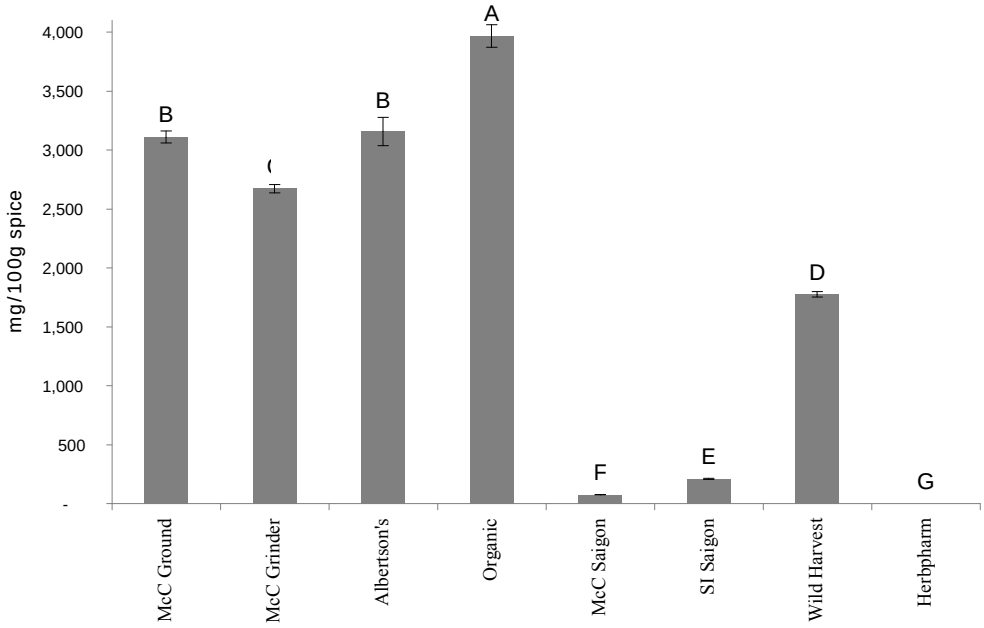


Fig. 4.6 Bar graph of concentrations of A2-P in cinnamon.
Values sharing the same letter are not significantly different. (p-value > 0.01, n = 4, ±sd).

Chapter 5. Concluding remarks

Proanthocyanidins (PACs) are ubiquitous in nature and have been associated with many health benefits, including inhibition of cancer cells (Neto 2007; Singh *et al.* 2009a), LDL oxidation, and platelet aggregation (Chu and Liu 2005), as well as possessing anti-inflammatory properties (Bodet *et al.* 2006). PACs containing A-type linkages, like those found in cranberry, have been implicated as the active compounds in the prevention of urinary tract infections (UTI) (Foo *et al.* 2000a; Foo *et al.* 2000b), but much research is still necessary to fully understand the bioactivity of A-type PACs and their health benefits, such as their role in UTI prevention.

For instance, it is unclear to what level the extension and/or terminal A2 PACs are bioactive. Is there a dependence on the ratio of the two A2 PACs, or is it symbiotic, requiring both units need to be present? There is also the question of when the A2 PACs are formed, and if they have the same activity in younger fruits, like early harvested cranberry used in making white cranberry juice, as they do in mature fruit.

On a fundamental level, research still needs to be performed to understand how A2 is formed. Is it in response to environmental factors? Do changes in

light exposure, water, CO_2 concentration or temperature affect the production of A2 or the ratio of extension to terminal A2? Is A2 synthesized as a protection against certain microbes? Do different soil types impact A2 production?

Another aspect of A-type PACs that needs to be researched is how A-type PACs change the conformation of the PACs. What role does having a higher degree of A-type linkages in the polymer play in the bioactivity? Is it the conformation of the polymer that allows the A-type PAC to survive digestion and be intact and active when it reaches the urinary tract?

Once researchers have a better understanding of A2 synthesis and bioactivity, there will be a high demand for analysis of consumable products. As was shown in Chapter 4, major differences exist between products, but for now, it is not known what these differences indicate. Food scientists will need to look at concentration differences between fresh and processed products. What happens to A2 during certain processes, like pasteurization, cooking, canning, and drying? How does the method of extraction for nutraceuticals affect the active A2 concentration? If it is determined that extension and terminal A2 need to be present in a certain ratio for highest bioactivity, what processes can be performed to enhance products and make them the most healthful for consumers?

The purpose of this thesis was to present a method that can be used for analysis of A2 to aid researchers in understanding the questions asked above.

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