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Abstract approved:

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Polyphenolic compounds found in foods such as fruits, vegetables, legumes, nuts and grains, are an integral part of the human diet. Within polyphenols, proanthocyanidins are referred to as condensed tannins and encompass procyanidins, propelargonidins and prodelphinidins. Among these, procyanidins are the largest group. Recent interest in these compounds is related to their potential health benefits, most notably their antioxidant activity. However, there is considerable interest from scientists and the public about their other health benefits.

The complex structure of procyanidins makes their analysis a challenging task. Procyanidins are composed of isomeric catechin and epicatechin monomeric units with A-type and B-type linkages. B-type procyanidins contain C4 → C6 or C4 → C8 interflavan bonds between subunits, whereas A-type compounds are composed of the same C4 → C6 or C4 → C8 interflavan bond with an additional C2 → O7 bond. Procyanidins can assume a linear formation mainly containing C4

→ C8 B-type linkages or a branched formation consisting of C4 → C6 B-type, A-type or a mix of A- and B- type linkages. These different linkages give rise to different fragmentation patterns during mass spectrometric and tandem mass spectrometric analyses. The most common fragmentation patterns are due to retro-Diels-Alder (RDA), heterocyclic ring fission (HRF), and quinone methide (QM) formation. Current mass spectrometry-based methods are time-consuming and lack specificity. Electrospray ionization is more commonly used given the high molecular mass of procyanidins and ease of ionization, however, issues arise with procyanidins mixtures. These electrospray mass spectra are dominated by lower molecular mass components; the signal intensity diminishes as polymer chain length increases and the formation of multiply charged polymers complicates precursor ion selection for tandem mass spectrometric analysis. Therefore, other methods of analysis need to be explored since procyanidins exist in mixed concentrations of varying degrees of polymerization in different plants.

Procyanidin-rich dietary supplements are marketed as botanical products, most commonly as pine bark extract, grape seed extract, green tea extract, and cranberry dietary supplements. Cranberry dietary supplements have been marketed to combat urinary tract infections. Due to the bitter taste of cranberry fruit, cranberry dietary supplements have become popular alternatives for the prevention of urinary tract infections. Cranberries contain mainly A-type procyanidins which have been reported to inhibit the cellular adhesion of P-fimbriated uropathogenic *Escherichia coli* to uroepithelial cells, which is necessary

for the development of urinary tract infections. Due to the popularity of cranberry dietary supplements, there was a surge in the demand of these cranberry products which resulted in increased pricing. This propelled economic adulteration such that some manufacturers added lower cost procyanidins extracted from sources other than cranberries. The most common cranberry adulterants were peanut skins, grape seed, mulberry fruit, plum, maritime pine bark, black bean skin, and black rice. These adulterants pose a risk to consumers resulting in the loss of money, null perceived health benefits, and exposure to potential allergens.

This dissertation presents the development of rapid and efficient mass spectrometry-based analyses of complex procyanidins leading to the authentication of cranberry botanical dietary supplements. Three chapters of original research are presented in this dissertation. The first chapter is a review of procyanidins with a focus on mass spectrometry-based methods of analysis.

The second chapter utilizes ion mobility, a separation technique in which ions are separated by their drift time in an inert gas under a weak electric field. Conventional chromatographic separation of procyanidins requires minutes, whereas ion mobility separations occur in just milliseconds. Using traveling wave ion mobility (TWIMS) on a Waters Synapt Q-ToF mass spectrometer, ions experience roll-overs due to an opposing drift gas. These roll-overs help separate compounds by size and charge. Smaller compounds will travel more quickly; additionally, multi-charged compounds will travel more quickly than singly charged species. Procyanidins are oligomeric and polymeric, linear or branched, making

them ideal for ion mobility analysis. Ion mobility has the ability to separate procyanidins by degree of polymerization. In addition to speed, time-of-flight mass analyzers provide a large mass range that is suitable for detection of the larger polymeric forms of procyanidins. The method was developed using procyanidin standards and then applied to unknown cranberry fractions. The optimized method demonstrated the rapid separation of procyanidins by varying degree of polymerization as well as varying linkage types. As expected, lower order procyanidins were easily identified by degree of polymerization. Additionally, procyanidins with different linkage types (A-, B- or mixed) were resolved.

The third chapter utilizes matrix-assisted laser/desorption-time-of-flight tandem mass spectrometry (MALDI-ToF/ToF). MALDI-ToF/ToF also avoids the need for time-consuming chromatographic separation while providing rapid analysis. MALDI is an ionization technique which uses laser energy to create ions from large molecules with little fragmentation. The sample is mixed with a matrix, the laser is pulsed, which causes desorption of the sample and matrix as well as ionization by protonation or deprotonation, and the ions are detected in ToF mass spectrometer. The ToF analyzer is suitable for larger molecules such as polymeric procyanidins, additionally, MALDI produces singly charged species which limits interference from multi-charged species and is therefore ideal for complex procyanidins. MALDI-ToF/ToF mass spectra of procyanidins were characterized by the common fragmentation pathways of RDA, HRF, and QM fission. The tandem mass spectra provided structural information about the number of A- and

B-type linkages in each compound as well as the location of the linkages leading to the identification of specific procyanidins.

The fourth chapter utilizes negative electrospray ionization and neutral loss scanning on a triple quadrupole tandem mass spectrometer. The tandem mass spectrometry technique of neutral loss scanning facilitates the identification of precursor ions that fragment to eliminate a stable neutral molecule of structural significance. As previously mentioned, procyanidins have three characteristic fragmentation pathways, which may be utilized for their selective detection. Using neutral loss scanning to follow these characteristic neutral losses, a method was developed to test cranberry botanical dietary supplements for adulteration. To validate this method, cranberry dietary supplements labeled as containing 100% *Viccinium macrocarpon* Aiton (American Cranberry) were purchased as well as with mixed fruit botanicals used as an adulterated reference. The cranberry supplements were confirmed to contain A-type procyanidins whereas the mixed fruit botanicals showed the presence of A- and B-type procyanidins.

The studies described in this dissertation help advance the analysis of complex procyanidins. Procyanidins are found in a variety of plant products and foods making human exposure widespread. The health promoting properties of procyanidins make them attractive to consumers that embrace a healthful lifestyle. The complexity of these compounds makes them difficult to characterize fully. This research will aid in the structural analyses of procyanidins and indirectly advance the study of the therapeutic effects of these powerful phytonutrients.

Investigation of Procyanidins Used in Botanical Dietary Supplements

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

Emily A. Rue, Author

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CONTRIBUTION OF AUTHORS

Michael D. Rush contributed to the experimental design and writing of Chapters 1 and 3. Jan A. Glinski and Vitold B. Glinski contributed to the isolation and elucidation of procyanidins used in Chapters 2, 3, and 4. Alan Wong contributed to the writing of Chapter 3. Paul Kowalski contributed to the experimental design and analysis of Chapter 3.

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CHAPTER 1

PROCYANIDINS: A COMPREHENSIVE REVIEW ENCOMPASSING STRUCTURE ELUCIDATION VIA MASS SPECTROMETRY

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1.1 Abstract

Procyanidins are polyphenols abundant in dietary fruits, vegetables, nuts, legumes, and grains with a variety of chemopreventive biological effects. Rapid structure determination of these compounds is needed, notably for the more complex polymeric procyanidins. We review the recent developments in the structure elucidation of procyanidins with a focus on mass spectrometric approaches, especially liquid-chromatography-tandem mass spectrometry (LC-MS/MS) and matrix-assisted laser desorption ionization (MALDI) MS/MS.

1.2 Introduction

Polyphenols are the largest group of secondary plant metabolites; their structural determination has been an intense area of investigation.[1] Within polyphenols, procyanidins are derived from proanthocyanidins, also known as condensed tannins.[2] Procyanidins and proanthocyanidins naturally occur throughout the plant kingdom; commonly found with varying concentrations in commonly consumed foods such as fruits, vegetables, legumes, grains and nuts[2-4] (Table 1.1), as well as cosmetics[5] and pharmaceuticals[2,4,6] containing plant material.

The study of proanthocyanidins began with Jacques Masquelier in the 1940s with the investigation of the pine park that Native Americans brewed to heal

scurvy.[7] Masquelier identified monomeric proanthocyanidins within the pine bark preparation, noted their safety, and characterized some of their biological activities. Continuing to be used as natural and alternative medicines, products rich in proanthocyanidins entered the natural product market as dietary supplements during the 1980s.[6]

As natural antioxidants, proanthocyanidins are used to stabilize food colors and to prevent rancidity due to oxidation of unsaturated fats[8-10] as well as for chemoprevention of a variety of degenerative diseases.[2,11,12] In addition to antioxidant properties, procyanidins have been reported to exhibit anticancer,[13-15] anti-infectious,[16] anti-inflammatory,[17-19] cardioprotective,[20,21] antimicrobial,[22] antiviral,[23] antimutagenic,[24] wound healing,[25] antihyperglycemic[26] as well as antiallergic activites.[27]

Plants metabolites such as carbohydrates, fats, and proteins can form complexes with procyanidins that interfere with their extraction and isolation.[28] Solvents that have been used are methanol, ethanol, water, dimethylsulfoxide, acetone, and other similar alcohols. Gel permeation chromatography may then be used to obtain fractions enriched in procyanidins.[29] Different approaches to extraction and subsequent chromatographic isolation of these compounds have contributed to the uncertainty regarding procyanidin concentrations in plants and foods.[30]

An area that needs attention is the quantitative analysis of procyanidins. Adamson et al.[31] analyzed procyanidins up to decamers in size using gel

permeation chromatography followed by preparative normal-phase HPLC. Similarly, quantitative analysis of polymeric procyanidins were performed on grape seed extracts with a reverse-phase HPLC method.[32] Sultana et al.[33] looked at three different extraction processes on tea leaves, in which microwave-assisted extraction gave the highest yield, and further analysis was performed using reverse-phase HPLC. Proanthocyanidins from crude plant extracts were quantified with respect to procyanidins and prodelphinidins utilizing a UPLC-MS/MS method.[34]

Although procyanidins levels within plants and foods remain unclear, the application of nuclear magnetic resonance (NMR) and mass spectrometry (MS) has enabled the structure elucidation in these phenolic compounds. To date, the structures of monomeric units catechin and epicatechin as well as some of their lower order oligomers have been established, but the identification of polymeric procyanidins and the extent of their formation in many plants and foods remains limited.[35,36]

1.3 Chemical Structure

Procyanidins are proanthocyanidins (condensed tannins) built from flavan-3-ols (+)-catechin and (-)-epicatechin.[36] Proanthocyanidins are classified based on their monomeric unit linkages and are present in homo- and hetero-polymers. The most common proanthocyanidins are procyanidins (Figure 1.1). Procyanidins are

homo-oligomeric (epi)catechin with two B-ring hydroxyl groups.[37] This review concerns the structure determination of procyanidins and not their related proanthocyanidins.

Procyanidins can be categorized into A-type and B-type depending on the stereo configuration and linkage between monomers. B-type procyanidins are characterized by a single interflavan bond between carbon-4 of the B-ring and either carbon-8 or carbon-6 of the C-ring (Figure 1.1). B-type procyanidins are the most abundant, with procyanidins B1, B2, B3 and B4 occurring most frequently. A-type procyanidins have not only an interflavan bond but also a second ether linkage between the A-ring hydroxyl group and carbon-2 of the A-ring (Figure 1.1).[36] The most common A-type compounds are A1 and A2.

Procyanidins can be categorized by their degree of polymerization (DP),[38] monomers form linkages leading to oligomers, further forming polymers. The most common monomeric unit is (-)- epicatechin, with B-type being the most prominent. Procyanidins containing 2-7 monomeric units are defined as oligoprocyanidins.[39]

1.4 Structure Elucidation

1.4.1 Nuclear Magnetic Resonance (NMR)

NMR is a standard spectroscopic approach for the structural elucidation of a wide variety of natural products including procyanidins.[40] The structural

elucidation of procyanidins by NMR has used a combination of homonuclear correlation (COSY) and heteronuclear one-bond (HSQC/HMQC) and multiple bond (HMBC) experiments.[41] Using long range correlation, the flavan junction carbon-4 and carbon-8 or carbon-4 and carbon-6 can be identified.[42]

However, there has been considerable debate over the assignment of some protons and carbons in catechin and epicatechin.[42] The position of these elements are solvent-dependent and are altered when derivatized.[43] It has been suggested that rotation around the interfavan bond and ring interconversion is possible thereby confounding structure determination. To overcome this obstacle, the hydroxyl groups can be acetylated to impede the rotation, or alternatively, the spectra can be measured at varying temperatures with lower temperature slowing rotation and higher temperatures contributing to faster rotation.[44]

Significant limitations of NMR analysis of procyanidins include sample isolation and large sample quantities. Mass spectrometry (MS) is generally carried out in the picogram range, while NMR is less sensitive requiring around 500 ng. Chromatographic separations of procyanidins are typically carried out prior to NMR analysis, but with the quantity requirements, purity problems often arise contributing to multiple interpretations of the data. Compared with MS, NMR is orders of magnitude less sensitive, orders of magnitude slower and to the best of our knowledge, unlike liquid chromatography-mass spectrometry (LC-MS), no LC-NMR analyses of procyanidins have yet been reported. Overcoming limitations of

sample size, purity and speed, mass spectrometry and LC-MS have become effective tools for fast procyanidin structure elucidation.[45]

1.4.2 Liquid chromatography and mass spectrometry (LC-MS)

Introduced in the 1960s, high performance liquid chromatography (HPLC) has become a standard tool for the rapid analysis and purification of nonvolatile compounds including natural products.[46] The recent commercial introduction of ultra-high pressure liquid chromatography (UHPLC) enables even faster and higher resolution separations than HPLC. A variety of stationary phases are available for HPLC and UHPLC, however, separations of polar procyanidins typically utilize reversed phase or normal phase columns.[47] Mobile phases should have low ionic strength and contain only volatile additives when interfaced to mass spectrometry for on-line LC-MS analysis of procyanidins (Figure 1.2). Otherwise, ionization will be suppressed and the mass spectrometer inlet will become fouled by non-volatile deposits from the mobile phase.[48]

As examples of reversed phase separations, Wollgast et al.[49] studied procyanidins in crude chocolate extracts by reversed phase LC-MS, and Calderon et al.[50] studied cocoa proanthocyanidins using C_{18} reversed phase LC-MS/MS and gradient elution from water to acetonitrile. Ortega et al.[51] utilized reversed phase UHPLC-MS/MS to identify and quantify procyanidins up to nonamers in cocoa nib samples. UHPLC separations were carried out using gradient elution from

water/acetic acid to acetonitrile. More recently, Silvan et al.[52] determined phenolic composition in grape seed extract by reverse phase high pressure liquid chromatography, photodiode array, and mass spectrometry (RP-HPLC-PDA-MS) which revealed the 43 individual compounds including seven procyanidin tetramers, seven procyanidin pentamers, and six galloylated procyanidin dimers, trimers, and tetramers.

As an example of normal phase LC-MS, Shoji et al.[53] characterized procyanidins (dimers to octamers) in apple extracts according to the degree of polymerization using a combination of silica column fractionation followed by on-line normal phase LC-MS. The mobile phase consists of a hexane-methanol-ethyl acetate mixture. In another example, Karonen et al.[54] utilized normal phase and reversed phase LC-MS with negative ion electrospray ionization for the identification of procyanidins in pine bark. Sintara et al.[55] used relative response factors to quantify individual oligomers from DP 2-9 in cranberries and cranberry products. In general, proanthocyanidins from monomer to tetramers are optimally separated by reversed phase HPLC while polymers can be separated by their DP more efficiently using normal phase.[56-58]

Several studies of procyanidins have utilized hydrophilic interaction chromatography (HILIC), a type of normal phase separation, in which the analyte is retained by partitioning between an aqueous layer on the hydrophilic stationary phase and the hydrophobic eluent.[59] Oligomeric and polymeric procyanidins from apples to cocoa eluted in order of increasing DP with individual peaks being

obtained up to dodecamers for cocoa and apple extracts and up to tetradecamers for cacao seeds.[60] Karonen et al.[61] used LC-MS with HILIC and high resolution electrospray mass spectrometry to characterize oligomeric and polymeric procyanidins with degrees of polymerization up to 22 units that were obtained from silver birch bark. Bussy et al.[62] used HILIC with fluorescence detection (FLD) for the determination of procyanidins DP 1-7 in cocoa-based products.

Electrospray LC-MS (Figure 1.2) has become the most popular and is the only one to have been applied successfully to the analysis of procyanidins.[40,49] Electrospray was first reported by Dole et al.[63] as a technique to ionize high molecular weight synthetic polymers for mass spectrometric measurement. However, the application of electrospray mass spectrometry to the measurement of biopolymers such as proteins would not be realized for another 20 years,[64] and its application to procyanidins would take even longer.[49] Electrospray is one of the softest ionization techniques, which means that ions will form with the addition of little energy that might contribute to fragmentation in the ion source. Although the formation of molecular ions by the addition or loss of an electron can occur during electrospray, most analytes like procyanidins ionize by losing or gaining a proton to form $[M-H]^-$ or $[M+H]^+$ ions, respectively.[48]

During LC-MS, protonation/deprotonation of analytes containing heteroatoms can be facilitated by adjusting the pH of the mobile phase. As organic acids such as formic acid are often added to reversed phase mobile phases to facilitate HPLC separations,[46] positive ion electrospray mass spectrometry is used

more often than negative ion mode for reserved phase LC-MS. However, electrospray LC-MS is not limited to reversed phase chromatography and may be used with normal phase columns as well.[65]

Once an intact procyanidin ion is formed during electrospray, it can be weighed in the mass spectrometer. The use of high-resolution accurate mass measurement analyzers such as time-of-flight (ToF), ion trap-ToF, quadrupole-ToF, as well as Fourier transform-ion cyclotron resonance and orbitrap mass spectrometers enable the elemental compositions of procyanidins to be determined. Additional structural information may be obtained by fragmenting the procyanidin ions using collision-induced dissociation and then weighing the product ions with high resolution tandem mass spectrometry.[66]

In studies of white birch bark procyanidins with DP up to 22 using negative ion electrospray LC-MS on a ToF analyzer, Karonen et al.[61] found that high-resolution was essential to establish the charge state of each procyanidins based on their isotopic patterns. They also determined that only B-type procyanidins were present. As examples of ultrahigh resolution mass spectrometric analysis of procyanidins, Li et al.[67] utilized FT-ion cyclotron resonance mass spectrometry with electrospray to identify oligomeric procyanidins in *Litchi chinensis*. Rodríguez-Carrasco et al.[68] utilized an Orbitrap high resolution mass spectrometer (UHPLC-Q-Orbitrap- MS/MS) for the analysis of flavanols and procyanidins in cocoa-based products.

Unlike NMR, LC-MS/MS is so rapid that both accurate mass measurement of intact ions and their MS/MS product ions may be completed during single HPLC or UHPLC separations. The recent implementations of rapid polarity switching also enables on-line LC-MS/MS measurements of positive ions and negative ions during the sample chromatographic separation.

Two key components in the mass spectrometric analysis of complex mixtures such as procyanidins include resolution and mass accuracy. Mass accuracy is the degree of conformity of the measured value to the true value. Resolution is the ability to resolve two ions of similar mass-to-charge. Without high resolution accurate mass analysis, accurate charge state determination and accurate quantitation cannot be achieved.[69] The ability to perform structure elucidation of ions using high resolution accurate mass measurement and tandem mass spectrometry has been described by Kind and Fiehn.[40]

Another feature of electrospray that is less common with other ionization techniques is the ability to form not just singly charged ions, but also multiply charged species. Multiple charging is particularly valuable for large molecules, such as high order procyanidins polymers, that might be outside the mass range of a particular mass spectrometer.[48] For example, mass spectrometers measure the mass-to-charge or *m/z* value of an ion, so that if *z*=2, an ion of *m/z* 3000 will appear as *m/z* 1500. Therefore, multiple charging would enable a mass spectrometer with a mass range of just *m/z* 2000 to measure procyanidins with masses of 3000 or more.

As in peptide sequencing using tandem mass spectrometry,[70,71] multiple charging can facilitate the fragmentation of procyanidins into more structurally significant product ions that can be obtained from single charged precursor ions.[37,54,72-74] For example, Wollgast et al.[49] identified procyanidins in cocoa using collision-induced dissociation and tandem mass spectrometry of their doubly-deprotonated, $[M-2H]^{2-}$, pentamers, hexamers, and heptamers.

Positive electrospray mass spectrometry has been used successfully in measuring procyanidins up to pentamers in length, but larger procyanidins do not ionize efficiently in positive mode.[54] Due to the acidity of procyanidins, they are more readily measured as deprotonated molecules using negative ion electrospray mass spectrometry.[75] For example, oligomeric procyanidins have been detected using negative ion electrospray as singly charged deprotonated molecules of *m/z* 577, 865, 1153, 1441, and 1729 for dimeric, trimeric, tetrameric, pentameric, and hexameric procyanidins, respectively.[76,77] Multiply charged molecules formed using negative ion electrospray have been reported for higher order procyanidins such as doubly charged heptamers detected at *m/z* 1009.[75]

As the DP increases, high mass procyanidins tend to form multiply charged species. However, as the DP increases, the ionization efficiency decreases. For example, Karonen et al.[61] reported that singly-charged molecules and fragment ions are abundant for dimers and trimers, doubly-charged species $[M-2H]^{2-}$ are observed for octamers and nonamers, nonamers through octadecamers form triply charged species, $[M-3H]^{3-}$, and only $[M-4H]^{4-}$ are observed for procyanidins with

DP higher than hexadecamers. In studies of apple procyanidins ranging from dimers to octamers, Shoji et al.[53] reported similar results; they observed singly charged species for the lowest mass compounds and doubly and triply charged signals for the higher order procyanidins.

Note that B-type procyanidin oligomers are composed of multiple monomer subunits with interflavanoid C-C linkages that differ by multiples of 288, which corresponds to the mass of the monomeric subunit.[54] As expected, B-type deprotonated procyanidins have been reported to fragment between monomeric subunits forming a series of product ions of $[M-288n-1]^{1-}$.[75] The *m/z* values of deprotonated procyanidin oligomers, their degrees of polymerization (DP), and the masses of their most abundant fragment ions are shown in Table 1.2.

The main fragmentation pathways of procyanidins include quinone methide (QM) cleavage of the interflavanoid bond, as well as heterocyclic ring fission (HRF) and retro Diels-Alder (RDA) fission of the heterocyclic ring system subunits which are distinctive of proanthocyanidins.[75] These pathways can be seen in Figure 1.3 for B-type dimer procyanidins and in Figure 1.4 for an A-type dimer. The key component in understanding A- and B- type is recognizing that A-type dimers have been found to be 2 Da less than those of B-type, this difference accounts for the additional C-O-C linkage.

QM formation will result in fragmentation between two catechin or epicatechin subunits in a procyanidin polymer. During this process, procyanidins will fragment to form one of two different QM ions. In the case of A-type dimers

(Figure 1.4), fragmentation can form monomeric fragment ions of *m/z* 289 and *m/z* 285, and this difference of 4 Da is characteristic of A-type linkages. B-type procyanidin dimers (Figure 1.3), fragment to form monomeric ions of *m/z* 287 or *m/z* 289. Note that this is 2 Da difference between fragment ions distinguishes B-type linkages from A-type. Therefore, QM fragmentation of dimers leads to pairs of product ions differing by 4 Da or 2 Da, which can be used to distinguish between types of procyanidins.[29,75] A-type trimers undergo the QM cleavage producing ions of *m/z* 575 and *m/z* 287. B-type trimers undergo QM cleavage of the upper interflavanoid bond producing ions of *m/z* 287 and 577, whereas cleavage of the lower interflavanoid bond forms ions of *m/z* 289 and 575. For B-type tetrameric and higher order procyanidins, fragment ions for QM cleavage were observed at *m/z* 287, 289, 575, 577, and 865.[54] Chen et al.[78] also described the conversion of B- to A-type trimers by quinone methide reaction mechanisms.

Karonen et al.[54] reported that fragmentation of B-type procyanidin dimers via HRF can take place on either monomeric unit (Figure 1.3). The fragment ion of the dimer at *m/z* 451 indicates B-type (Figure 1.3) whereas a fragment ion at *m/z* 449 indicates an A-type dimer (Figure 1.4), following loss of a phloroglucinol molecule. The B-type procyanidin trimer fragments similarly to that of the dimer, in which a fragment ion of *m/z* 739 is produced.

RDA fragmentation, which was the most common fragmentation pathway of the B-type procyanidin dimer,[79] produces a fragment ion of *m/z* 425 with subsequent water elimination giving rise to an ion of *m/z* 407 (Figure 1.3).

Fragmentation on the upper unit is considered to be energetically more favorable than the lower unit because it produces fragment ions with a larger π - π hyperconjugated system.[72] A-type dimers produce fragment ions of *m/z* 423 (Figure 1.4), and A-type trimers produce fragment ions of *m/z* 711.[29] The ion at *m/z* 713 indicates RDA fragmentation of a B-type trimer.[54]

1.4.3 Matrix-assisted laser desorption ionization (MALDI)

Invented in the 1980s, MALDI is an ionization technique for mass spectrometry that enables the simultaneous desorption and ionization of solid-phase biopolymers.[80,81] Originally used for polymers, MALDI is also suitable for the ionization for procyanidins. As seen in Figure 1.5, the sample is dissolved in a solvent containing a matrix that will absorb the laser light, usually a UV or IR laser. The mixture is dried, loaded onto a MALDI target, and short laser pulses are used to evaporate the matrix which results in desorption and ionization of the associated procyanidin. If using a non-scanning mass spectrometer such as a ToF analyzer, complete mass spectra may be obtained with each pulse of the laser, thereby making MALDI mass spectrometry highly efficient and suitable for small procyanidin samples. Some ToF analyzers are also capable of high resolution and tandem mass spectrometry.

During MALDI, analytes typically form abundant singly-protonated or deprotonated molecules, although molecular ion radicals and multiply charged

species are possible. Analyte ionization during MALDI has been described as a photo-ionization process during which analytes become charged by a proton transfer during collisions with matrix ions.[82] Alternatively, a cluster ionization mechanism has been proposed for MALDI in which preformed analyte ions are released from clusters during evaporation of matrix ions.[83]

The first MALDI MS measurements of procyanidins were reported by Ohnishi-Kameyama et al.[84] using a ToF analyzer. Looking only at monomeric and dimeric procyanidins, DHB, α-cyano-4-hydroxycinnamic acid, sinnapinic acid, and 9-nitroanthracene were effective matrices. DHB is an optimum UV matrix for procyanidin MALDI mass spectrometry.[85] While there might be advantages of using IR lasers for procyanidin analysis, most MALDI MS studies of procyanidins have utilized UV lasers.[86]

Unlike electrospray mass spectrometric studies of procyanidins, which usually utilize negative ion mode, the majority of MALDI MS studies of procyanidins have been carried out using positive ion mode. In positive ion MALDI mass spectra, the ion current is divided among several cationized species including $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$, and sometimes others. Dividing the procyanidin signals in this manner lowers the sensitivity of the analysis and complicates interpretation of the data, especially when measuring mixtures of compounds. To enhance the abundance of particular cationized procyanidins, cationization agents have been added to the MALDI matrix.[87] Addition of sodium chloride, sodium iodide, silver trifluoroacetate, and cesium trifluoroacete have all

been used with varying degrees of success to detect procyanidins as $[M+Na]^+$, $[M+Ag]^+$ or $[M+Cs]^+$ ions.[84,88-91] Unless there is a compelling reason to use positive ion MALDI, the detection of procyanidins can be improved significantly by utilizing negative mode.[92] This is consistent with the electrospray studies discussed above as well as with related desorption ionization studies of procyanidins using fast atom bombardment.[93]

1.4.4 MALDI MS/MS of procyanidins

Although MALDI-ToF/ToF is frequently used for peptide sequencing, few applications have been reported for procyanidin analysis. Previously, the published MALDI MS/MS spectra of procyanidins had reported relatively few product ions and have high background noise levels.[94,95] Because MALDI MS is not compatible with on-line HPLC or UHPLC, samples must be prepared in advance and might degrade before analysis or contain impurities that can suppress ionization. In this respect, MALDI mass spectrometry shares this limitation with NMR analysis of procyanidins. However, an advantage of MALDI analysis is easy interpretation of data including complex mixtures of procyanidins. While it is difficult to interpret DP with NMR, it is easily obtained using MALDI mass spectrometry. More recently, Enotomo et al.[96] utilized negative ion MALDI-MS/MS to localize flavan-3-ols to the outer epidermis of peanut testa.

While it is possible to determine the type of procyanidin using MALDI-ToF mass spectrometry (MS^1),[97,98] definitive typing can also be obtained based on fragmentation patterns during product ion tandem mass spectrometry. A-type procyanidins have different fragmentation patterns than B-type procyanidins that can be used to differentiate unknown procyanidins by the type of linkages between monomeric units. For example, in the MALDI ToF/ToF mass spectrum of a B-type procyanidin trimer (Figure 1.6), 13 fragment ions are recognizable corresponding to the expected fragmentation pathways of procyanidins-quinone methide formation, HRF, and RDA fragmentation.

Fragmentation observed for MALDI is similar to that for electrospray. The advantages of MALDI over electrospray include speed, sensitivity, and the ability to obtain singly charged procyanidin ions for tandem mass spectrometric analysis, especially for the higher DP compounds. With the benefit of multiple charging, procyanidins of higher DP have been reported using electrospray, although product ion tandem mass spectrometric analysis of larger procyanidins have been reported using MALDI ToF/ToF mass spectrometry.[53,54,99,100]

Using MALDI ToF/ToF mass spectrometry for product ion analysis of higher order procyanidins, Mateos-Martin et al.[95] described the fragmentation pathway of a tetrameric proanthocyanidin. In tetrameric form, the proanthocyanidin ions follows the general fragmentation pathway of QM, RDA, and HRF. The tetrameric ion showed a loss of 126 Da during HRF, loss of 152 Da via RDA reaction followed by subsequent loss of water and a loss of 288 Da to form the trimeric

form. The trimeric ion showed the same fragmentation pathways including HRF, RDA reaction and subsequent water elimination as well as QM fragmentation forming the dimeric form. The dimeric ion showed similar fragmentation and to produce two monomeric units. In Figure 1.7, a tetrameric proanthocyanidin, the fragmentation pathways facilitated identification of the mixed type containing two B-type linkages with a lower A-type linkage.[95]

In the negative ion MALDI product ion tandem mass spectrum of Procyanidin C1 in Figure 1.6, the dimer doublet ions of *m/z* 575 and 577 and monomer doublet ions of *m/z* 287 and 289 show the 2 u mass difference that is characteristic of quinone methide formation by B-type procyanidins. In the case of the tandem mass spectrum of Procyanidin A1 in Figure 1.8, the pair of ions of *m/z* 285 and 289 shows a 4 u mass difference indicating the formation of quinone methides by an A-type procyanidin. Figure 1.6 shows a fragment ion at *m/z* 739 corresponding to HRF of the trimeric unit whereas the fragment ion of *m/z* 451 corresponds to HRF of the dimeric form. In Figure 1.8, the fragment ion of *m/z* 449 corresponds to HRF from procyanidin A1 which is a dimer. Figure 1.6 shows fragment ions at *m/z* 713 which corresponds to RDA and a subsequent loss of water at *m/z* 695. Fragment ions of *m/z* 425 and *m/z* 407 correspond to RDA and water loss of the dimeric form.

All of these patterns of fragmentation- QM, HRF, and RDA- are useful in quickly identifying the type of procyanidin. While MALDI-ToF MS analyses of procyanidins has been reported frequently, more attention should be given to the

abundant structural information that may be obtained when using negative ion MALDI-ToF/ToF mass spectrometry.

1.5 Conclusion

Procyanidins, oligomeric compounds composed of catechin and epicatechin monomers, are widespread in foods and can have significant medicinal properties. Although much is already known about their biological activities, research concerning their medicinal benefits is continuing. Essential to this effort is a more thorough understanding of the procyanidin structures (polymer chain length and composition of A-type versus B-type linkages) and content in plants, foods and research materials. The high molecular weight of the longer chain procyanidins has hindered their analysis, but advances in MALDI ToF/ToF mass spectrometry mass range and the ability to form multiply charged ions using electrospray are helping to overcome this limitation. To date, the highest DP for procyanidins yet reported has been 28,[101] but this value is expected to increase.

As the capabilities of mass spectrometers improve, not only with respect to mass range but also improved sensitivity, resolving power, accuracy, and new functionalities, the identification of higher order procyanidins should become routine. An example of an emerging new functionality in the field of biomedical mass spectrometry that might be useful for procyanidin analysis is ion mobility. A fast gas-phase separation technique based on ion size and shape, ion mobility

mass spectrometry is orders of magnitude faster than HPLC and should be useful for procyanidin analysis. Another area that requires additional development is the quantitative analysis of higher order DP procyanidins using mass spectrometry. Altogether, the application of state-of-the-art biomedical mass spectrometry is facilitating the structural analysis of procyanidins, and the quantitative analysis of these important botanical natural products will follow.

1.6 Acknowledgements

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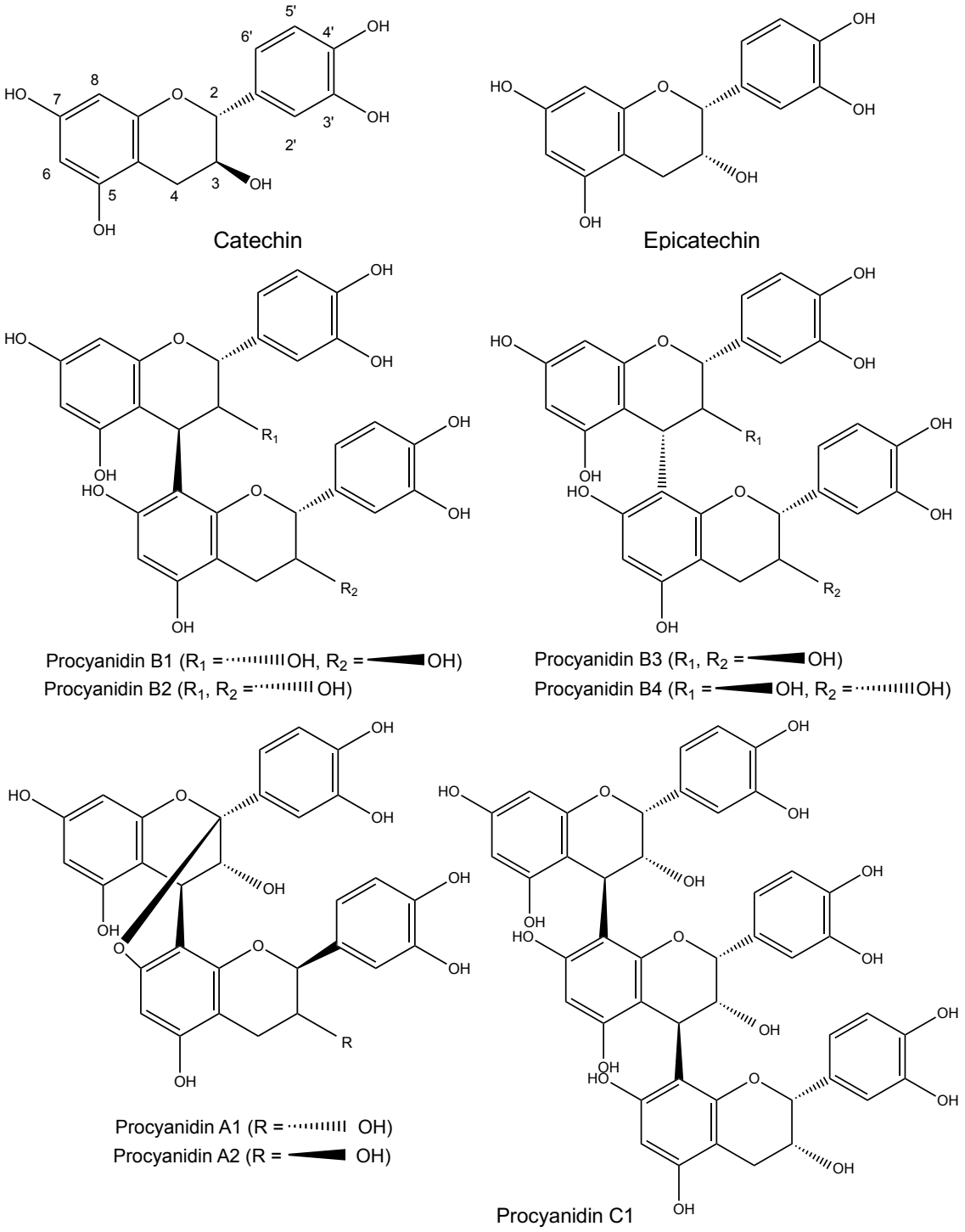

Figure 1.1 Chemical structures of monomeric and polymeric procyanidins.[39, 104]

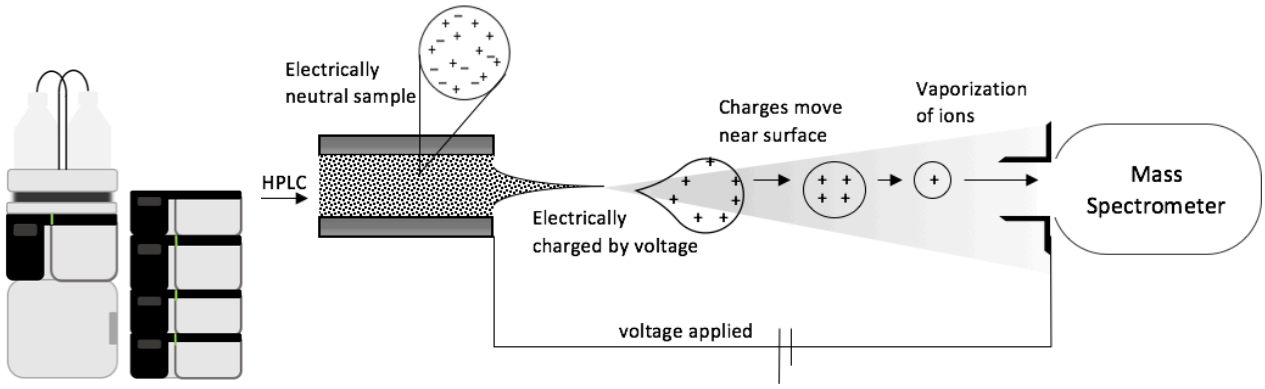

Figure 1.2 Electrospray serves as an atmospheric pressure interface between the HPLC and the mass spectrometer while simultaneously facilitating the formation of gas-phase ions of non-volatile and thermally labile solutes in the mobile phase such as procyanidins.

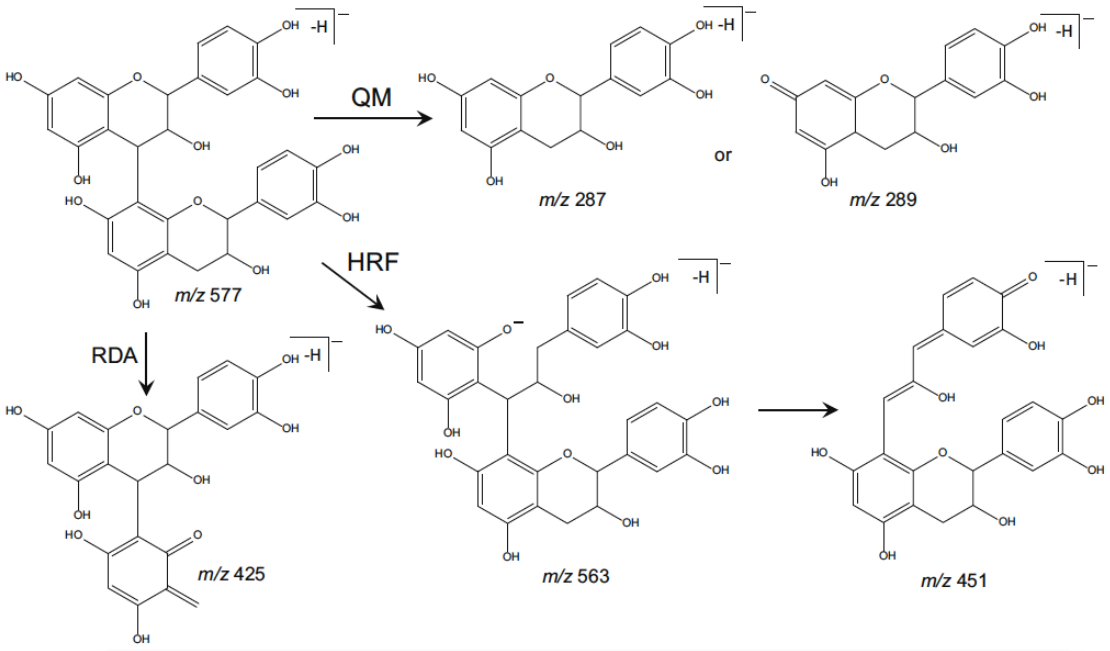

Figure 1.3 Fragmentation pathway of a deprotonated B-type procyanidin dimer during tandem mass spectrometry showing the products formed by quinone methide (QM), heterocyclic ring fission (HRF), and retro-Diels–Alder (RDA) reactions.[75]

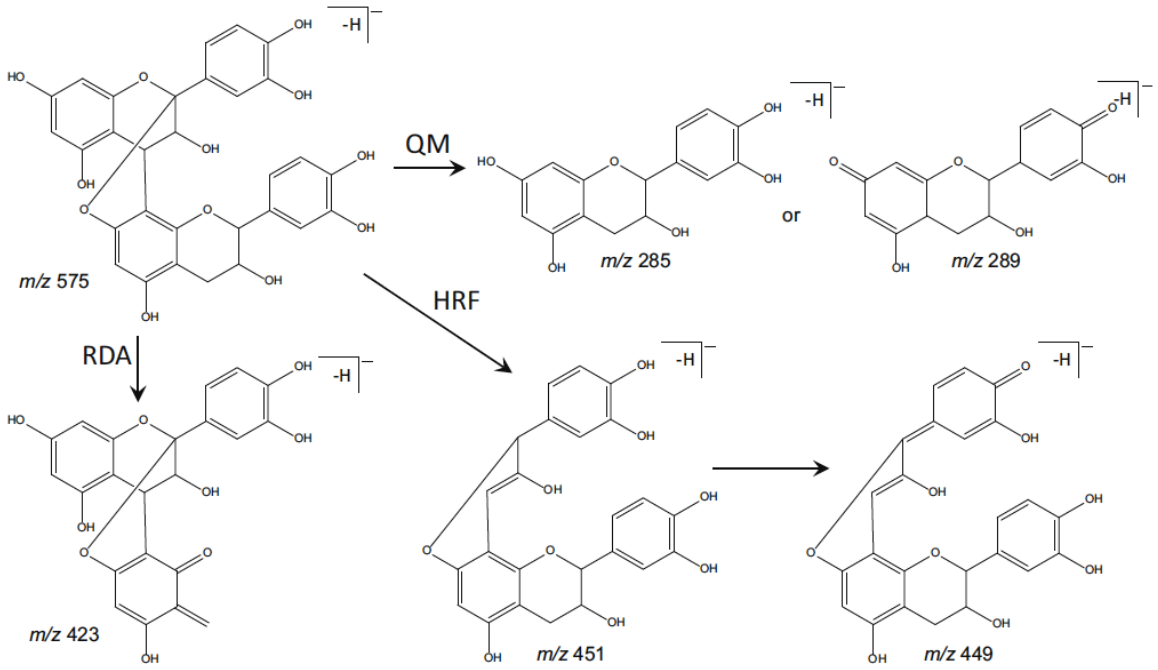

Figure 1.4 Fragmentation pathway of a deprotonated A-type procyanidin dimer during tandem mass spectrometry showing the products formed by quinone methide (QM), heterocyclic ring fission (HRF), and retro-Diels–Alder (RDA) reactions.[29]

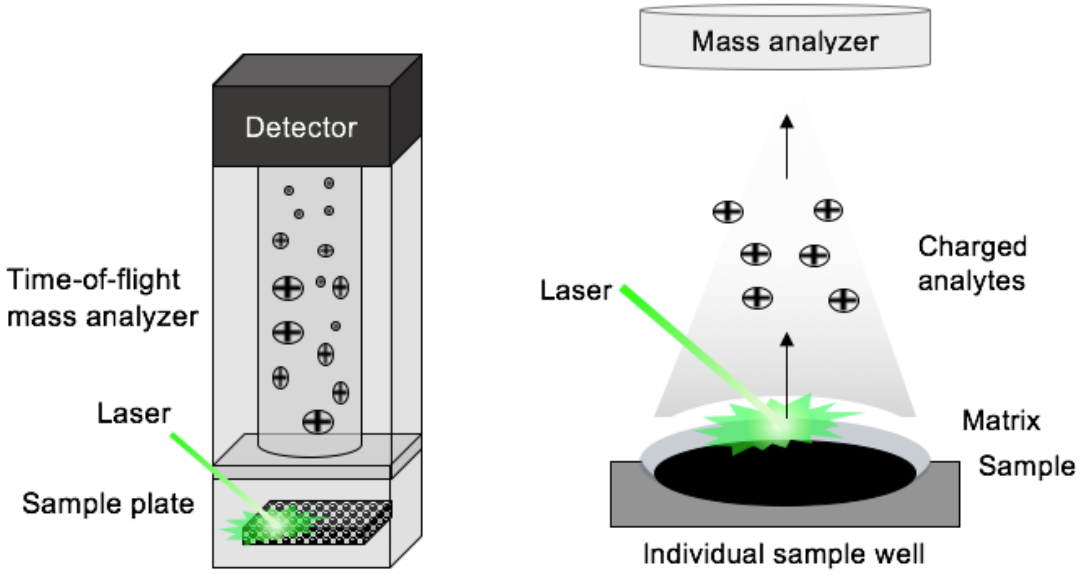

Figure 1.5 Matrix-assisted laser desorption ionization utilizing a laser for desorption of a sample in a matrix material facilitating the protonation/ deprotonation of samples such as procyanidins.

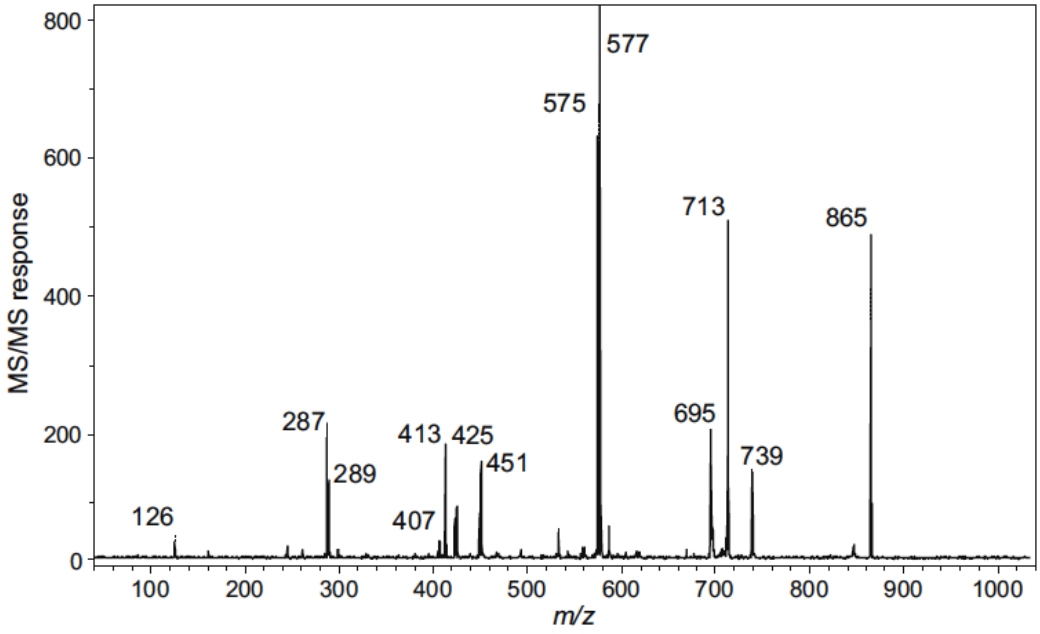

Figure 1.6 Negative ion MALDI-ToF/ToF product ion mass spectrum of deprotonated procyanidin C1. This procyanidin B-type trimer was provided by Jan Glinski of Planta Analytica (New Milford, CT), and the tandem mass spectrum was obtained by Paul Kowalski using a Bruker Daltonics UltrafleXtreme (Billerica, MA) MALDI-ToF/ToF mass spectrometer.

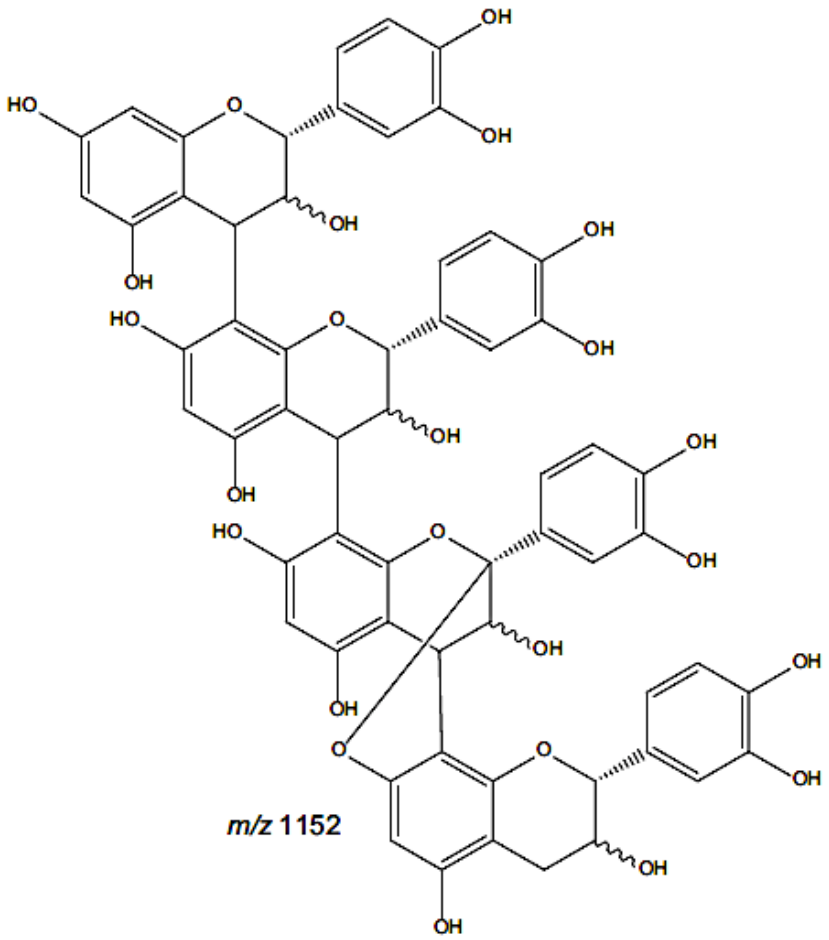

Figure 1.7 Tetramer proanthocyanidin displaying mixed type configuration.[94]

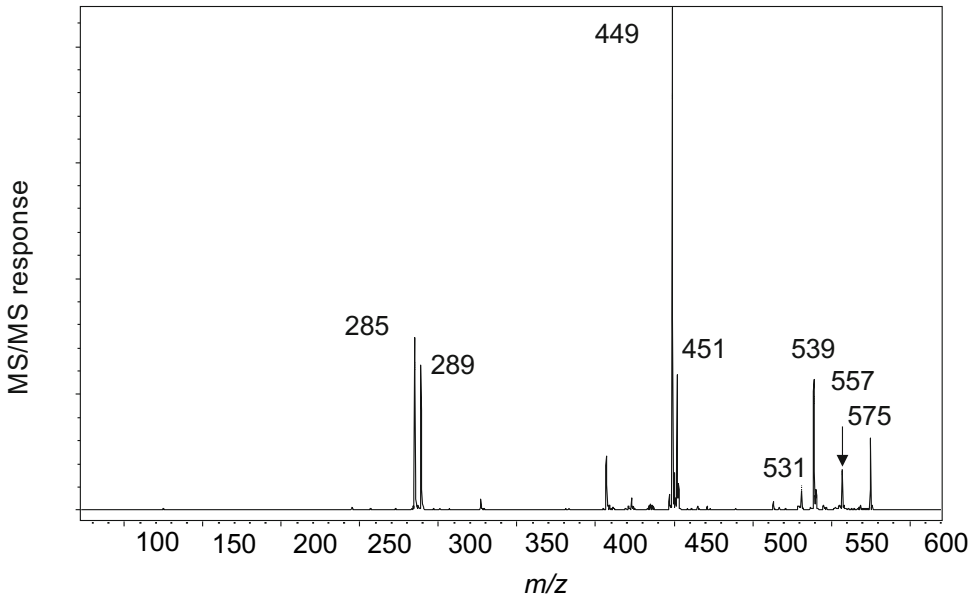

Figure 1.8 Negative ion MALDI-ToF/ToF product ion mass spectrum of deprotonated procyanidin A1. This A-type procyanidin dimer was provided by Jan Glinski (Planta Analytica), and the tandem mass spectrum was obtained by Paul Kowalski (Bruker Daltonics).

Table 1.1 Commonly consumed food that have been found to contain procyanidins.[3, 102, 103]

Fruits		
Apple	Grape	Quinces
Apricot	Kiwi fruit	Raspberry
Avocado	Lingonberry	Red currant
Banana	Lychee	Rhubarb
Bilberry	Mango	Rose hip
Black currant	Marionberry	Rowanberry
Blackberry	Nectarine	Saskatoon berry
Blueberry	Orange	Sea buckthorn
Cherry	Peach	Strawberry
Chokeberry	Pear	Sweet rowanberry
Cloudberry	Persimmons	Tangerine
Cranberry	Pineapple	Tomatoes
Crowberry	Plum	Watermelon
Dates, deglet noor	Pomegranate	Whortleberry
Gooseberry		
Vegetables		
Carrots	Indian squash	Pepper
Eggplant	Lettuce	Potato
Figs	Onion	Zucchini
Nuts, Legumes, Grains, Miscellaneous		
Almonds	Chickpeas	Sorghum
Cashews	Faba beans	Wheat flours
Hazelnut	French beans	Cinnamon
Peanuts	Kidney beans	Dark/ milk chocolate
Pecan	Lentils	Hops
Pistachio	Pinto beans	Tea beverage
Walnuts	White beans	Red wine
Black beans	Barley flour	White wine
Black eye peas	Buckwheat grits/ flour	Rose wine
Cacao beans	Rice	Sherry wine

Table 1.2 Deprotonated molecules and characteristic fragment ions reported for procyanidins using negative ion electrospray.[29,54, 57, 67, 75]

Degree of polymerization	$[M - H]^-$	Characteristic fragments	Type
1	289	245, 205	(+)-catechin, (−)-epicatechin
2	577	559, 451, 425, 407, 289,287	B
	575	449, 423, 289, 285	A
3	865	739, 713, 695, 577, 575, 451, 407, 289, 286	B
	863	711, 693, 575, 573, 559, 451, 449, 423, 411, 289	A
4	1153	1027, 1001, 983, 865, 863, 739, 577, 576*, 575, 289, 287	B
	1151	863, 861, 711, 577, 573, 451, 449	A
5	1441	1315, 1289, 1271, 1153, 1151, 1027, 865, 863, 720*, 575	B
	1439		A
6	1730	1603, 1577, 1559, 1441, 1315, 1153, 1135, 1027, 865*, 863, 739, 575	Mixed
7	2017	1729, 1441, 1151, 1009*, 863, 673*, 575	Mixed
8	1152*, 769*		
9	1297*, 865*		
10	1441*, 960*		

* Multi charged species

CHAPTER 2

ION MOBILITY-MASS SPECTROMETRY FOR THE SEPARATION AND ANALYSIS OF PROCYANIDINS

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2.1 Abstract

Procyanidins are polymeric flavan-3-ones occurring in many plants with antioxidant and other beneficial bioactivities. Composed of catechin and epicatechin monomeric units connected by single carbon-carbon B-type linkages or A-type linkages containing both carbon-carbon and carbon-oxygen-carbon bonds. Their polymeric structure makes analysis of procyanidin mixtures always difficult. Even evaluation of procyanidins according to degree of polymerization (DP) using HPLC is time consuming and at best has resolved polymeric families up to DP-17. To expedite studies of procyanidins, the utility of positive ion electrospray ion mobility-mass spectrometry (IM-MS) was investigated for the rapid separation and characterization of procyanidins in mixtures. Applying IM-MS to analyze structurally defined standards containing up to 5 subunits, procyanidins could be resolved in less than 6 ms not only by degree of polymerization but also by linkage type. A-type procyanidins could be resolved from B-type and both could be at least partially resolved from mixed-type procyanidins of the same DP. IM-MS separated higher order procyanidins with DP of at least 24 from extracts of cranberry. As DP increased, the abundances of multiply charged procyanidins also increased. During IM-MS of ions of similar *m/z*, the ion drift times decreased inversely with increasing charge state. Therefore, IM-MS was shown to separate mixtures of procyanidins containing at least 24 interconnected subunits in less

than 16 ms, not only according to DP, but also according to linkage type between subunits, and charge state.

2.2 Introduction

Procyanidins, a subgroup of the proanthocyanidins which constitute the second largest group of natural products after lignans,[1] occur naturally in plant-based foods such as fruits, vegetables, nuts and grains.[2-4] In addition to antioxidant activity, procyanidins have been reported to have antiviral, antibacterial, anti-inflammatory, and anticarcinogenic therapeutic benefits.[5-11] Composed of catechin and epicatechin monomeric units, procyanidins are polymeric flavan-3-ols with two linkage types, the common B-type and the less common A-type, between monomeric units (Figure 2.1). While B-type linkages consist of a single carbon-carbon bond, the A-type linkage consists of a carbon-carbon bond in addition to a carbon-oxygen bond between adjacent subunits.

Mixed-type procyanidins contain combinations of B-type and A-type linkages between monomeric units.[12,13] Furthermore, the polymeric chains of procyanidins are usually linear but can be branched. The incorporation of A-type linkages can add rigidity and stability to the compound.[14] Due to the complexity of these molecules and their occurrence in mixtures, analysis can be challenging and separation using chromatographic approaches is nearly always time consuming.

Current approaches for procyanidin analysis include several modalities of liquid chromatography for separation followed by electrospray or MALDI mass spectrometry and NMR for structure elucidation.[15-22] The primary limitations of these methods have been the time required for chromatographic isolation of procyanidins as well as the large amount of material required for NMR compared with mass spectrometry. Taking advantage of the selectivity, speed and sensitivity of mass spectrometry, we investigated the feasibility of using ion mobility spectroscopy instead of liquid chromatography for faster procyanidin separation coupled on-line with electrospray mass spectrometric analysis.

During ion mobility, separation of ions moving through an inert gas in a weak electric field depends on their mass, charge and shape.[23-25] Separations can be achieved in milliseconds with ions of small collision cross sections traveling faster than larger ions and multiply charged ions travelling faster than ions of lower charge states. Different ion mobility techniques are available,[26-28] and traveling wave ion mobility was utilized during this investigation.[29,30] In addition to speed, another advantage of ion mobility is the ability to separate and distinguish isomers with similar mass spectra,[31,32] which can be useful in studies of polymeric procyanidins. Rapid analysis and high sensitivity can make IM-MS ideal for the analysis of procyanidins.

2.3 Experimental

2.3.1 Materials

Procyanidin standards, which had been purified from cocoa, cinnamon, peanut skins, cranberries, and crab apples were obtained from Planta Analytica (New Milford, CT). The chemical structures of procyanidin standards were determined using mass spectrometry and NMR. Procyanidin mixtures, which had been partially purified from cranberry, were also obtained from Planta Analytica. All solvents were HPLC-grade and were purchased from Thermo Fisher (Pittsburgh, PA).

2.3.2 Instrumentation

Positive ion electrospray ion mobility mass spectrometry was carried out using a Waters (Milford, MA) Synapt G1 quadrupole time-of-flight mass spectrometer equipped with travelling wave ion mobility. Individual procyanidins or mixtures were infused into the ion source in methanol/water (50:50, v/v) containing 0.1% formic acid using a syringe pump at a flow rate of 10 μL/min. Instrument parameters were as follows: capillary voltage, 3.6 kV; sample cone voltage, 26 V; extraction cone voltage, 4 V; desolvation gas (nitrogen) flow, 500 L/h; trap collision

energy, 6 V; transfer collision energy, 4 V; trap gas (argon) flow, 5 mL/min; source temperature, 120 ºC; and desolvation temperature, 200 ºC. The traveling wave velocity was held constant at 300 m/s while the wave height was increased from 10 – 30V. The IM gas was nitrogen at a flow rate of 20 mL/min. Data were acquired using Waters MassLynx software (version 4.1) and DriftScope software (version 2.1).

2.4 Results

Positive ion electrospray IM-MS separation of procyanidins based on degree of polymerization was investigated using equimolar mixtures of linear B-type procyanidins (Figure 2.2). Epicatechin dimers, trimers, tetramers, and pentamers were each separated by up to a millisecond, while the drift time of the monomer was only slightly less than that of the dimer. Specifically, the drift times of monomeric epicatchin and dimeric procyanidin B1 were 1.10 ms and 1.13 ms, respectively, while the drift times of trimeric procyanidin C1, cocoa tetramer D, and cocoa pentamer E were 1.71 ms, 2.62 ms and 3.60 ms, respectively. Although the epicatechin monomer and B-type procyanidin dimer B1 were only partially resolved by drift time using these IM-MS conditions optimized for higher order procyanidins, they could be distinguished easily by mass. The most abundant signals for all procyanidins during positive ion electrospray corresponded to their sodium adducts, $[M+Na]^+$, of *m/z* 313 (epicatechin), *m/z* 601 (procyanidin B1), *m/z*

889 (procyanidin C1), *m/z* 1177 (cocoa tetramer D), and *m/z* 1465 (cocoa pentamer E).

To assess IM-MS separation of procyanidins of different linkage types as well as different degrees of polymerization, a mixture of six compounds including peanut and cocoa dimers, trimers, and tetramers were analyzed (Figure 2.3). These compounds included A-type (procyanidin A1 and peanut trimer B), B-type (dimeric procyanidin B1, trimeric procyanidin C1, and cocoa tetramer D), and mixed A and B-type linkages (linear peanut tetramer E containing two A-type bonds and one B-type linkage) (Figure 2.1). All 6 procyanidins were resolved in less than 6 ms. B-type procyanidin dimers, trimers and tetramers were each separated by more than 1 ms, while A-type and B-type procyanidins of equal degrees of polymerization were partially resolved. For example, the B-type procyanidin cocoa trimer C1 had a shorter drift time (2.9 ms) than did the A-type peanut trimer B (3.4 ms), and the linear B-type procyanidin cocoa tetramer D had a shorter drift time (4.1 ms) than did the mixed type peanut tetramer E (4.6 ms). The drift times of the linear B-type procyanidins were always smaller than the linear A-type and mixed type procyanidins of identical degree of polymerization (Figure 2.3). The extra bond between subunits of A-type procyanidins increased their rigidity compared with B-type procyanidins and thereby increased their collision-cross section and ion mobility drift time.

One branched procyanidin, parameritannin A1, containing 4 epicatechin subunits and one A-type linkage (Figure 2.1), was evaluated using IM-MS in a

mixture of linear B-type and mixed-type procyanidin tetramers (data not shown). The drift time of branched parameritannin was ~0.1 ms less than that of the linear B-type cocoa tetramer D, which was 0.5 ms less that A-type peanut tetramer E (Figure 2.3). Although the compact branched structure of parameritannin decreased its drift time compared with linear procyanidins of comparable mass, the presence of one A-type linkage added rigidity to the structure resulting in a drift time only slightly shorter than the linear B-type cocoa tetramer D.

As an application of IM-MS to a complex botanical extract, a procyanidin-rich fraction of a cranberry extract was analyzed, and a two-dimensional heat map plot of *m/z* value vs drift time is shown in Figure 2.4. Singly-charged procyanidins were detected with degrees of polymerization up to 8 with drift times from 2 to 16 ms. Examination of the corresponding positive ion electrospray mass spectra indicated that the procyanidins in this fraction were predominantly mixed-type and contained only one A-type linkage per molecule (Figure 2.5). For example, the singly-charged procyanidin at a drift time of 12.6 ms was detected as a sodium adduct of *m/z* 1751 corresponding to a hexamer containing 4 B-type single bonds and 1 A-type linkage (Figure 2.5B).

Plotting *m/z* 1751 vs drift time revealed 3 distinct peaks at 5.6, 7.5 and 12.6 ms (Figure 2.5A). The peak with a drift time of 12.6 ms corresponded to a singly-charged mixed-type procyanidin hexamer. Based on differences of 0.5 Da (Figure 2.5C) and 0.3 Da (Figure 2.5D) between adjacent ions, the ions of *m/z* 1751 at drift times of 7.5 ms and 5.6 ms corresponded to doubly-charged and triply-

charged procyanidins with degrees of polymerization of 12 and 24, respectively. Although singly charged procyanidins were not observed for molecules with degrees of polymerization >8, electrospray formed doubly and triply-charged ions of higher degrees of polymerization up to at least 24 in this cranberry preparation. Furthermore, singly, doubly and triply-charged procyanidins of the same nominal mass were completely resolved from each other during ion mobility. Groups of singly, doubly and triply-charged procyanidins are indicated in the 2-D plot in Figure 2.4.

2.5 Conclusion

Procyanidin standards were used to demonstrate that IM-MS can resolve procyanidins by degree of polymerization as well as by linkage type. This is more recently demonstrated by Li et al.[33] use of ion mobility to develop a modified data filtering strategy for targeted characterization of procyanidins DP 1-15 from grape seed extracts. Furthermore, IM-MS may be used to separate and characterize procyanidins in milliseconds compared with minutes for LC-MS. In general, the higher the degree of polymerization of the procyanidin, the longer is the drift time; and B-type procyanidins have shorter drift times than do A-type procyanidins of comparable degrees of polymerization. During electrospray, higher order procyanidins form multiply charged ions, and IM-MS drift times become shorter as charge state increases. The use of high-resolution mass spectrometry facilitates

the determination of charge states of procyanidin ions during electrospray IM-MS analysis of complex mixtures.

2.6 Acknowledgement

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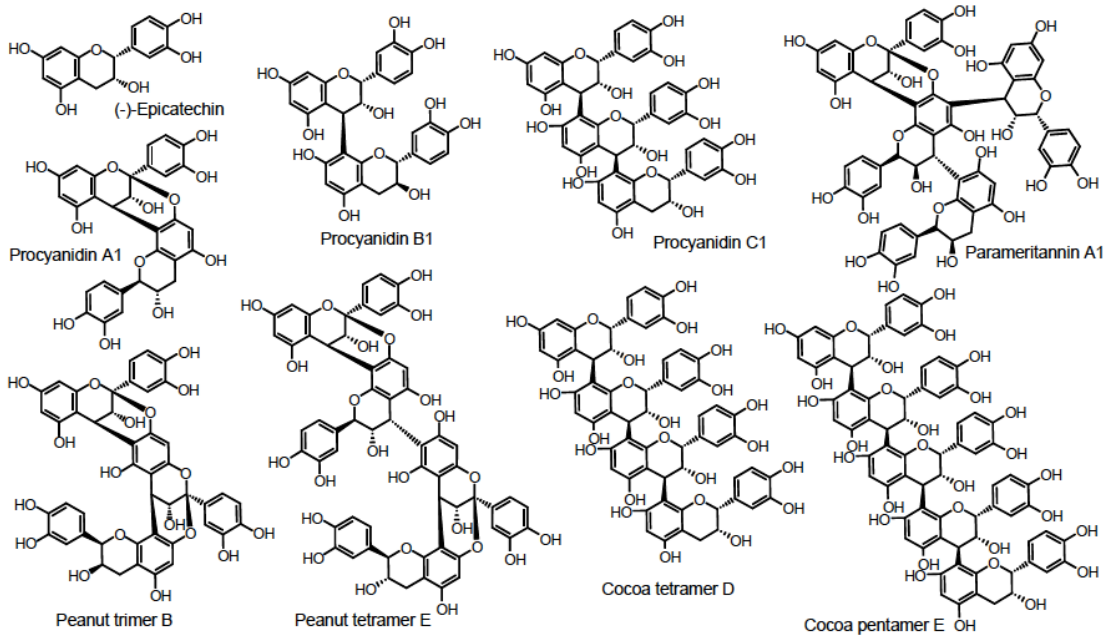

Figure 2.1 Chemical structures of procyanidin standards analyzed using IM-MS.

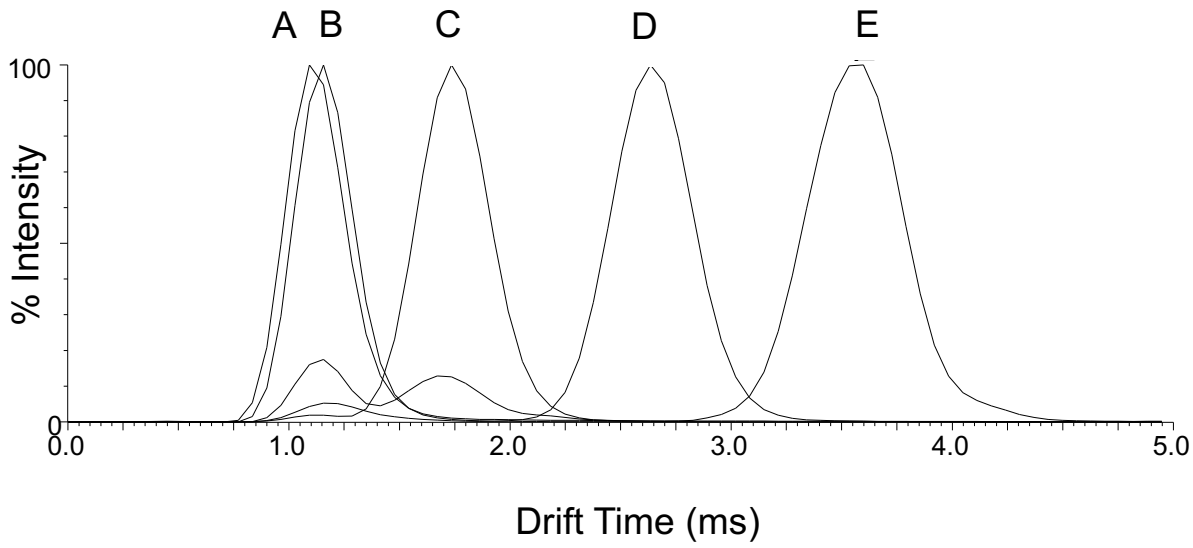

Figure 2.2 Positive ion electrospray ion mobility drift time distribution based on degree of polymerization for a mixture of B-type procyanidins. A) epicatechin (monomer); B) procyanidin B1 (dimer); C) procyanidin C1 (trimer); D) cocoa tetramer D; and E) cocoa pentamer E. Note that these procyanidins were detected as sodium adducts, $[M+Na]^+$.

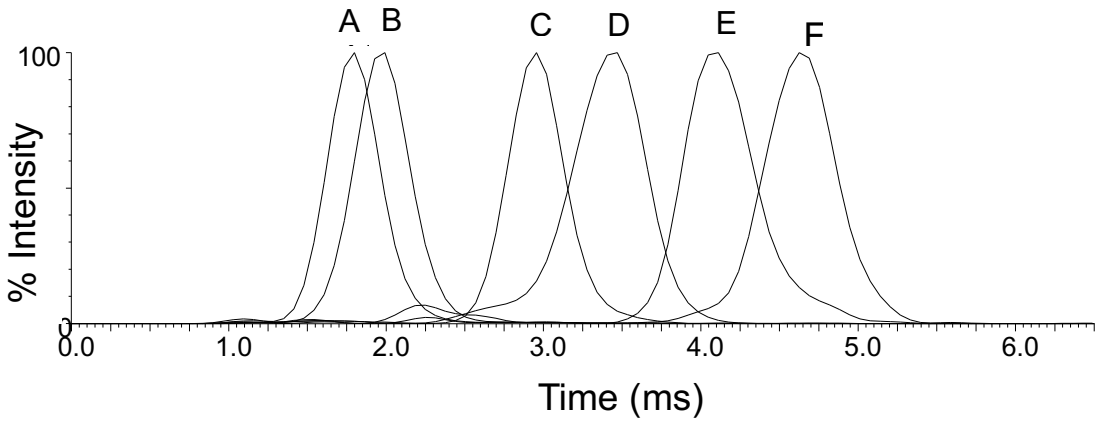

Figure 2.3 Positive ion electrospray IM-MS drift time distributions of A-type and B-type procyanidins by degree of polymerization as well as by linkage type. A) procyanidin B1 (B-type dimer), *m/z* 601; B) procyanidin A1 (A-type dimer), *m/z* 599; C) procyanidin C1 (B-type trimer), *m/z* 889; D) peanut trimer B (A-type), *m/z* 885; E) cocoa tetramer D (B-type), *m/z* 1177; and F) peanut tetramer E (mixed type), *m/z* 1173. Each procyanidin signal is shown as the singly-charged sodium adduct.

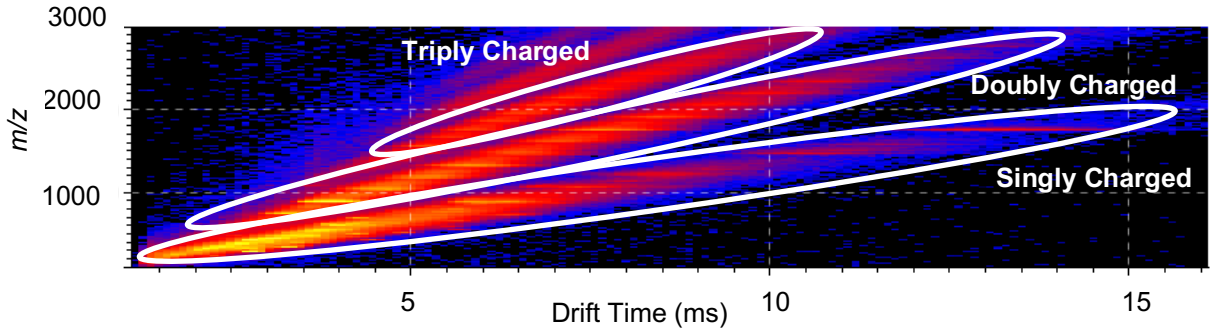

Figure 2.4 Positive ion electrospray IM-MS analysis of a cranberry extract plotted using DriftScope showing *m/z* value (y) vs drift time (x). Groups of abundant ions are indicated that were separated by charge state, $[M+Na]^{+}$, $[M+2Na]^{2+}$ and $[M+3Na]^{3+}$.

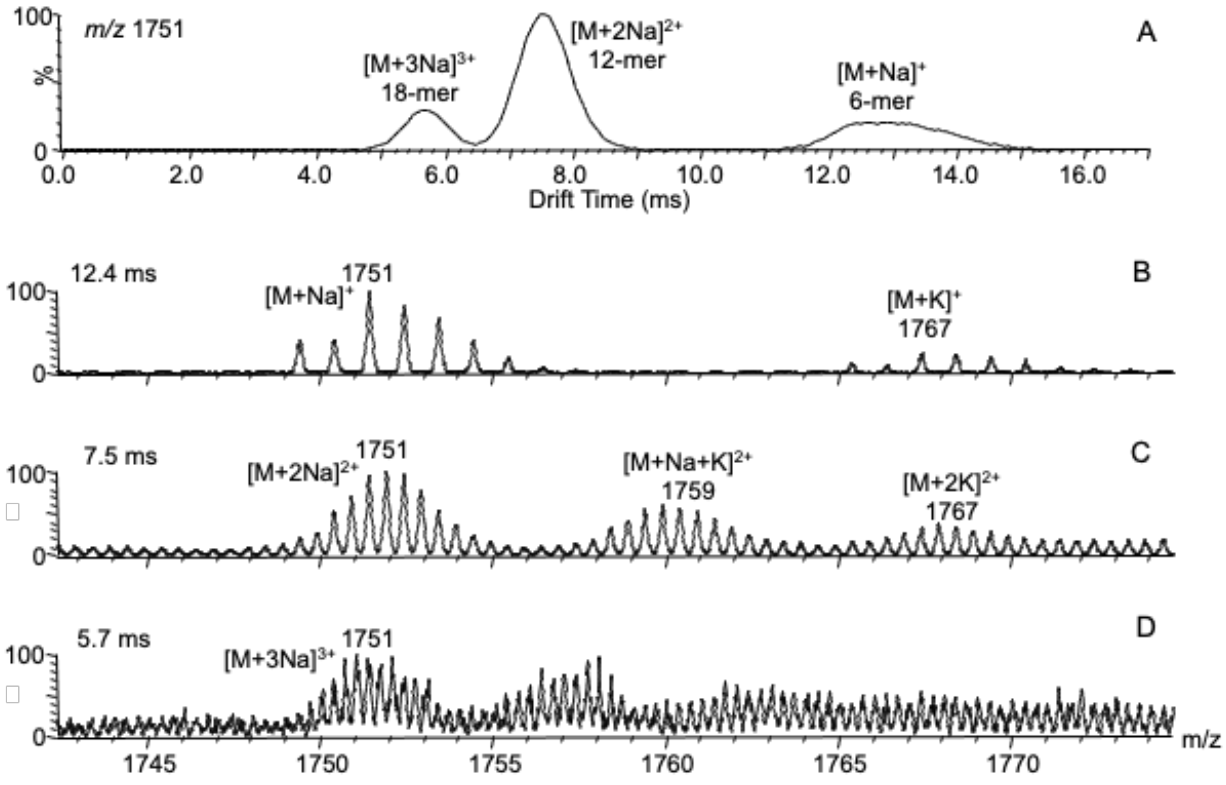

Figure 2.5 Computer-reconstructed selected ion drift time profile and corresponding mass spectra from the IM-MS analysis of the cranberry extract shown in Figure 4. A) Drift time profile for ions of nominal mass *m/z* 1751; B) mass spectrum of a singly-charged procyanidin hexamer obtained at a drift time of 12.6 ms; C) doubly-charged procyanidin 12-mer at a drift time of 7.5 ms; and D) triply-charged procyanidin 18-mer detected at a drift time of 5.6 ms.

CHAPTER 3

RAPID DETERMINATION OF PROCYANIDINS USING MALDI-TOF/TOF MASS SPECTROMETRY

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3.1 Abstract

Although procyanidins constitute a unique class of polymeric plant secondary metabolites with a variety of biological properties including potent antioxidant activity, structure determination has been challenging and structures of many complex procyanidins remain uncertain. To expedite the characterization of procyanidins, negative ion matrix-assisted laser desorption ionization high-energy collision-induced dissociation tandem time-of-flight (MALDI-ToF/ToF) mass spectra of 20 isolated procyanidins containing catechin and epicatechin subunits with degrees of polymerization up to five were obtained and evaluated. Structurally significant fragmentation pathways of singly charged, deprotonated molecules were identified representing quinone methide, heterocyclic ring fission and retro-Diels-Alder fragmentation. The interpretation of the tandem mass spectra for sequencing A-type, B-type, mixed-type, linear, and branched procyanidins is explained using specific examples of each.

3.2 Introduction

Also known as condensed tannins, proanthocyanidins occur in a wide variety of botanicals and contribute astringent flavor to foods.[1] Different plant species produce distinct mixtures of gallotannins, ellagitannins and condensed tannins.[2,3] Based on the hydroxylation patterns of their monomeric flavanol units

and the linkages between these units in oligomers/polymers, proanthocyanidins can be divided into three types, one of the most common being procyanidins.[4] The most widely studied procyanidins are dimeric procyanidin B and trimeric procyanidin C variants, both of which contain exclusively (+)-catechin and/or (-)-epicatechin monomeric units. Categorized into A-type and B-type, depending on linkages between the monomeric subunits (Figure 3.1), B-type procyanidins are characterized by a single carbon-carbon interflavan bond between the C-ring of one subunit and the A-ring of the next, whereas A-type procyanidins have not only a carbon-carbon interflavan bond but also a carbon-oxygen linkage (Figure 3.1).

Procyanidins have mixed stereochemistry and varying degrees of polymerization which can produce different biological activities.[5] For example, procyanidins are potent antioxidants and have been reported to demonstrate antibacterial, antiviral, anti-carcinogenic, anti-inflammatory, as well as vasodilatory activities.[6] It has been hypothesized that the free radical scavenging properties of procyanidins provide therapeutic benefits in preventing or treating cardiovascular disease, cancer, and urinary tract infections.[7]

Due to their complexity, the analysis of procyanidin mixtures is by no means straightforward. Various analytical techniques have been used for the analysis of procyanidins including nuclear magnetic resonance (NMR), [6] circular dichroism,[6] fast atom bombardment tandem mass spectrometry spectrometry,[8] thermospray liquid chromatography mass spectrometry,[9] electrospray liquid chromatography mass spectrometry,[10-14] and matrix-assisted laser desorption ionization (MALDI)

mass spectrometry.[15-21] The structural elucidation of procyanidins using NMR is limited by the large amount of material that must be isolated prior to analysis compared with mass spectrometry, which has been used to identify procyanidins in mixtures at levels that are orders of magnitude lower than those required for NMR.[13]

During positive ion electrospray MS/MS of procyanidins, structurally significant fragment ions have been reported that form through retro-Diels-Alder (RDA) reaction, heterocyclic ring fission (HRF), benzofuran forming (BFF) reaction, and quinone methide (QM) fission.[13,14] Despite the potential for electrospray to ionize high molecular mass procyanidins, the electrospray mass spectra of tannin mixtures are dominated by the lower molecular mass components, signal intensities diminish as polymer chain length increases, and the formation of multiply-charged oligomers complicates precursor ion selection for tandem mass spectrometric analysis.[13]

The ease of sample preparation, tolerance toward contaminants, simultaneous determination of mixtures, and rapid analysis of both low and high mass compounds help make MALDI-TOF mass spectrometry ideal for the analysis of procyanidins.[15-21] MALDI also forms predominantly singly-charged ions thereby simplifying data interpretation and facilitating the selection of precursor ions for MS/MS analysis.[22] Although MALDI-ToF MS/MS should be ideal for procyanidin characterization and identification, few studies of procyanidins using MALDI have used tandem mass spectrometry and, among these, even fewer have used

negative ion mode. Instead, most MALDI-ToF mass spectrometric studies of procyanidins have used positive ion analysis to measure adducts with sodium, potassium, silver, or cesium.[13-21] Here, we show how negative ion MALDI forms abundant deprotonated molecules of procyanidins, elimates the need for alkali metal cationization, and in combination with tandem mass spectrometry, forms a variety of structurally significant fragment ions. Overall, negative ion MALDI-ToF/ToF mass spectrometry provides simple determination of the degree of polymerization as well as the type of linkage between subunits for the rapid characterization of procyanidins from natural sources.

3.3 Experimental

3.3.1 Materials

Procyanidins, which had been purified from cocoa, peanut skins, cinnamon, and crab apples, were obtained from Planta Analytica (New Milford, CT). The structures of all 20 of these procyanidins had been determined using mass spectrometry and NMR. The MALDI matrix, 2,5-dihydroxybenzoic acid (DHB), was purchased from Sigma-Aldrich (St. Louis, MO). All solvents were HPLC-grade and were purchased from Thermo Fisher (Pittsburgh, PA).

3.3.2 Instrumentation

The DHB matrix was prepared at 15 mg/mL in 50% methanol (aqueous) and 0.1% trifluoroacetic acid. Each procyanidin solution (in 50% aqueous methanol) was mixed with the matrix solution (1:9; v:v), and 1 µL was deposited onto a ground stainless steel 384-density MALDI plate and allowed to air dry. Negative ion MALDI mass spectra and tandem mass spectra were acquired using a Bruker Daltonics (Billerica, MA) ultrafleXtremeToF/ToF mass spectrometer in reflector mode. For single-stage mass spectrometry, the ion source 1 voltage was -20 kV, the ion source 2 voltage was -17.85 kV, the lens voltage was -7.5 kV, the pulsed ion extraction delay was 150 ns, the reflector voltage 1 was -20.8 kV, and reflector voltage 2 was -10.8 kV. The UV laser power was set to 10% above the ionization threshold, and the number of accumulated laser shots ranged from 1000 to 7000 depending on signal strength at a frequency of 2000 Hz.

During MS/MS, collision-induced dissociation was carried out using argon at 9.0 x 10^{-6} Torr, the ion source 1 voltage was -7.5 kV, ion source 2 voltage was -6.75 kV, the lens voltage was -3.5 kV, the pulsed ion extraction delay was 200 ns, the reflector 1 voltage was -29.5 kV, and the reflector 2 voltage was -14 kV. The laser power for MS/MS was set 25% above the ionization threshold, the number of accumulated laser shots ranged from 1500 to 4000 depending on signal strength of fragment ions, and the UV laser was operated at a frequency of 1000 Hz.

3.4 Results

3.4.1 Negative ion fragmentation pathways of procyanidins

Negative ion MALDI ToF mass spectrometric analysis produced abundant deprotonated molecules of all 20 procyanidins (Table 3.1; Figure 3.2). The number of catechin and epicatechin subunits (degree of polymerization) in each molecule ranged from two to five based on the measured masses of the deprotonated molecules. Negative ion MALDI was selected over positive ion mode because the signal was concentrated into one abundant deprotonated molecule instead of being distributed among mixtures of $[M+H]^+$, $[M+Na]^+$, and $[M+K]^+$ adducts, which have been reported for not only positive ion MALDI[21] but also for positive ion fast atom bombardment[8] and electrospray.[12] In addition, no doping with cations of sodium, potassium, or cesium was necessary, as had been reported for positive MALDI mass spectrometry of procyandins.[22,23]

Using high-energy collision-induced dissociation with negative ion tandem mass spectrometry, cleavages between monomeric subunits formed three types of class-characteristic and structurally significant product ions consisting of quinone methide (QM) (Figure 3.1), heterocyclic ring fission (HRF) (Figure 3.3), and retro-Diels-Alder (RDA) (Figure 3.4) fragment ions. These three procyanidin fragmentation pathways have also been reported to occur during positive ion electrospray tandem mass spectrometry of procyanidins.[13,14] It should be noted

that benzofuran formation, which occurs during positive ion MS/MS with collision-induced dissociation, was not observed using negative ion MALDI ToF/ToF mass spectrometry. These new data are significant because reports of MALDI ToF/ToF analysis of procyanidins are rare in the literature, and also because most previous papers utilized only positive ion mode. For example, Saldanha *et al.*[24] used positive ion MALDI-ToF/ToF tandem mass spectrometry to characterize procyanidins up to six flavan-3-ol units in length. Sodium ions were added to enhance cationization, and although RDA fragmentation was observed, no HRF or QM fragment ions were described.

Every procyanidin tested formed fragment ions via the QM pathway that facilitated the identification of A-type and B-type linkages (Table 3.1). QM fragmentation cleaves both of the inter-subunit bonds of A-type procyanidins and the single bond between subunits in B-type procyanidins, so that the resulting fragment ions may be used to indicate the number of A-type and B-type linkages between monomeric subunits (Figure 3.1). During QM fragmentation, a diquinone will form in A-type procyanidins generating two possible product ions (Figure 3.1). B-type procyanidins form a single quinone resulting in two possible product ions, but the QM product ion containing the quinone will differ from the corresponding A-type diquinone ion by 2 mass units (Figure 3.1). Examples of QM fragmentation include the pairs of ions of *m/z* 285/575 and *m/z* 571/289 for the A-type procyanidin peanut trimer B (Figure 3.2A) and *m/z* 287/577 and *m/z* 575/289 for the B-type procyanidin C1 (Figure 3.2B). Mixed-type procyanidins such as the

trimer cinnamtannin B1 form combinations of QM fragment ions corresponding to cleavages of both A-type and B-type linkages (Table 3.1).

A second structurally significant fragmentation pathway for deprotonated procyanidins is heterocyclic ring fission (HRF), which results in the elimination of 1,3,5-trihydroxybenzene, $[M-H-126]^-$, from both A-type and B-type compounds (Figure 3.3). Examples of HRF fragmentation include the ions of *m/z* 739, *m/z* 451 and *m/z* 413 in the product ion tandem mass spectra of procyanidin C1 (B-type), *m/z* 735, *m/z* 449 and *m/z* 411 for the peanut trimer B (A-type), and *m/z* 737, *m/z* 451, and *m/z* 411 for the mixed-type procyanidin cinnamtannin B1 (Figure 3.2). In some cases, deprotonated 1,3,5-trihydroxybenzene was detected at *m/z* 125 (Figure 3.2). Additional examples of HRF fragmentation are summarized in Table 3.1.

Retro-Diels-Alder (RDA) reactions are a third structurally significant procyanidin fragmentation pathway occurring during negative ion MALDI tandem mass spectrometry (Figure 3.4). For these B-type procyanidins composed of catechin and epicatechin subunits, RDA fragmentation was characterized by elimination of hydroxyvinyl benzenediol, $[M-H-152]^-$, as well as loss of an additional molecule of water, $[M-H-152-18]^-$ (Table 3.1 and Figure 3.4). A pair of RDA fragment ions 18 u apart were observed for each B-type linkage in the procyanidin. For example, procyanidin C1, which contained two B-type linkages, formed ions of *m/z* 713/695 through RDA fragmentation and *m/z* 425/407 by RDA fragmentation of the QM product ion of *m/z* 577 (Figure 3.2B). Instead of forming

pairs of RDA fragment ions 18 mass units apart, A-type procyanidins formed single fragment ions, $[M-H-168]^-$ (Figure 3.4). For example, the A-type procyanidin dimers procyanidin A1 and procyanidin A2 formed a single RDA fragment ion of *m/z* 407, and the A-type peanut trimer B formed the single RDA fragment ion of *m/z* 693. Mixed-type procyanidins. RDA fragmentation of mixed-type procyanidins formed pairs of RDA ions for each B-type linkage but no RDA fragment ions for A-type linkages. As an example, mixed-type cinnamtannin B1 eliminated hydroxyvinyl benzenediol to form an abundant ion of *m/z* 711 plus a less abundant ion of *m/z* 693 corresponding to an additional loss of water (Figure 3.2 and Table 3.1).

Combinations of three fragmentation pathways (QM, RDA and HRF) contributed to the formation of the product ion of *m/z* 299. Observed in abundance for most mixed-type procyanidins (Table 3.1), the ion of *m/z* 299 was formed by a QM A-type elimination of 286 u, RDA B-type loss of 152 u, and HRF elimination of 126 u. Examples included cinnamtannin B1 (Figure 3.2), cinnamtannin D1, lindetannin, aesculitannin B, parameritannin A1, cassiatannin A, peanut trimer A, and peanut trimer C. The product ion of *m/z* 299 was below the limit of detection for the A-type and B-type procyanidins, and for the mixed-type procyanidins peanut trimers D, E and F.

3.4.2 Sequencing procyanidins using negative ion MALDI ToF/ToF

Based on the mass of the singly-charged deprotonated procyanidin formed during negative ion MALDI mass spectrometry, the total number of catechin and epicatechin subunits and the number of A-type and B-type linkages plus branching may be established. Next, determining the A-type or B-type bonding between procyanidin subunits may be accomplished by inspecting the QM fragment ions. Confirmatory data may be obtained by inspecting the HRF and RDA fragmentation of A-type and B-type procyanidins, which also show significant differences as noted above. By combining all this information, A-type, B-type, mixed-type, and branched procyanidins may be sequenced.

Sequencing linear procyanidins with only A-type or B-type linkages is straightforward, mixed-type linear procyanidins are more complicated, branched procyanidins are even more challenging, and sequencing mixed-type branched procyanidins are the most challenging. Beginning with a mixed-type linear example, the negative ion MALDI ToF/ToF mass spectrum of peanut tetramer E formed a deprotonated molecule of *m/z* 1149.217, which (based on mass alone) corresponds to four catechin/epicatechin subunits connected by 2 A-type and 1 B-type linkages. The RDA fragment ion of *m/z* 997 and its corresponding ion formed by an additional loss of water (*m/z* 979) confirm that peanut tetramer E contains one B-type linkage. The HRF fragment ion of *m/z* 411 indicates that the first linkage (connecting the middle A-ring of the first subunit to the C-ring of the

second subunit) is A-type. Based on a deprotonated molecule of *m/z* 1149 and the QM series of fragment ions of *m/z* 863, *m/z* 575 and *m/z* 289, the initial linkage is confirmed to be A-type (ΔM 286), the middle linkage is B-type (ΔM 288), and the terminal linkage is A-type (ΔM 286). The complementary QM fragment ion series of *m/z* 285, *m/z* 573 and *m/z* 859 confirm that the initial linkage is A-type, the middle linkage is B-type (ΔM 288), and the terminal linkage is A-type (ΔM 286). Additional product ions in the negative ion MALDI tandem mass spectrum of peanut tetramer E included *m/z* 449/447 and *m/z* 737, which were formed by a combination of HRF and QM fragmentation.

As a more complicated example, the deprotonated, mixed-type branched procyanidin parameritannin A1 (structure in Figure 3.5), was measured at *m/z* 1151.235, which for a linear procyanidin would correspond to four catechin/epicatechin subunits and perhaps two A-type and one B-type linkage. However, the observation of two pairs of RDA fragment ions of *m/z* 999/981 and *m/z* 711/693 indicates the presence of two B-type linkages instead of just one. Therefore, this procyanidin must be branched to account for the missing two hydrogen atoms and contains two B-type linkages and one A-type linkage. The HRF ion of *m/z* 1025 is consistent with a mixed procyanidin containing one A-linkage and two B-linkages, and the HRF fragment ion of *m/z* 451 indicates that the bottom linkage is B-type. The most abundant QM ion of *m/z* 863 (ΔM 288) is consistent with loss of a B-type fragment. However, the next most abundant QM ion of *m/z* 573 is too low in mass to be a typical A-type or B-type fragment ion, and

no ions of *m/z* 575 or *m/z* 577 were detected. Therefore, the second catechin/epicatechin subunit is in the middle and serves as a branch point (Figure 3.5).

3.5 Conclusion

Requiring no chromatographic separation step, MALDI ToF/ToF mass spectrometry is inherently fast and provides structural information for procyanidins that includes not only the number of A-type and B-type linkages but also their locations in the oligomer. Negative ion MALDI procyanidin ToF and ToF/ToF mass spectra are easier to interpret than positive ion mass spectra, due to the signals being concentrated in the deprotonated molecules instead of distributed among several cationized species,[13,14] and because only QM, RDA and HRF pathways but no benzofuran forming product ion species are formed. Negative ion MALDI ToF/ToF mass spectrometry facilitates the rapid and nearly complete structure determination of oligomeric procyanidins and need only be complemented by specific NMR experiments to determine stereochemistry and confirm structure.

3.6 Acknowledgement

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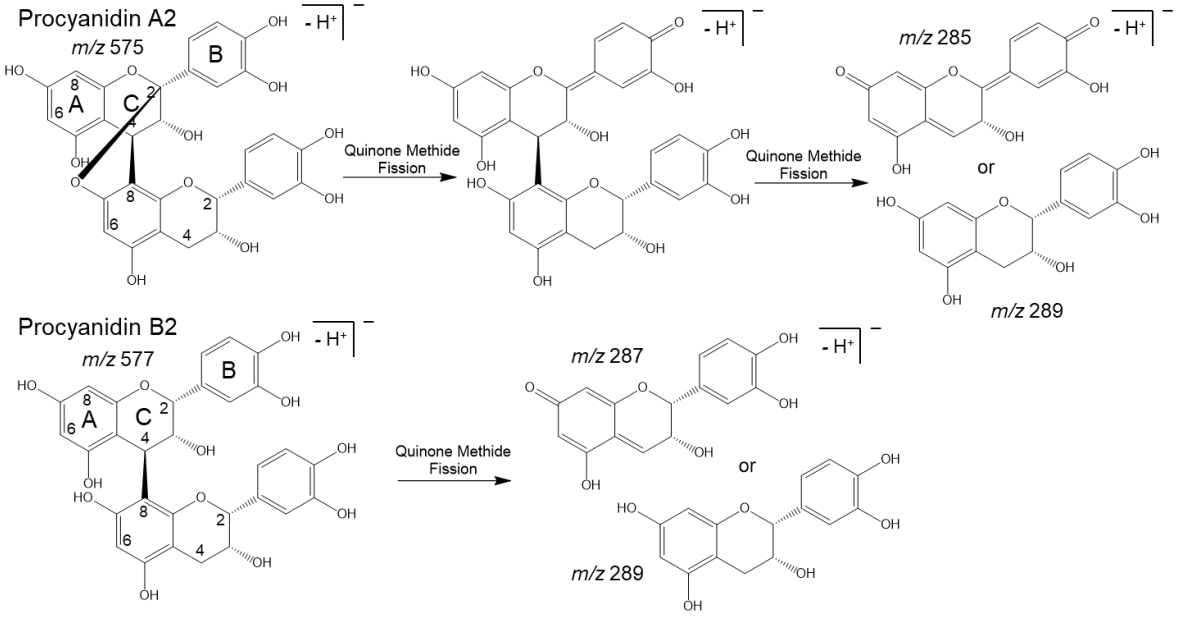

Figure 3.1 Quinone methide fragmentation of procyanidin dimers with A-type (procyanidin A2) and B-type (procyanidin B2) linkages.

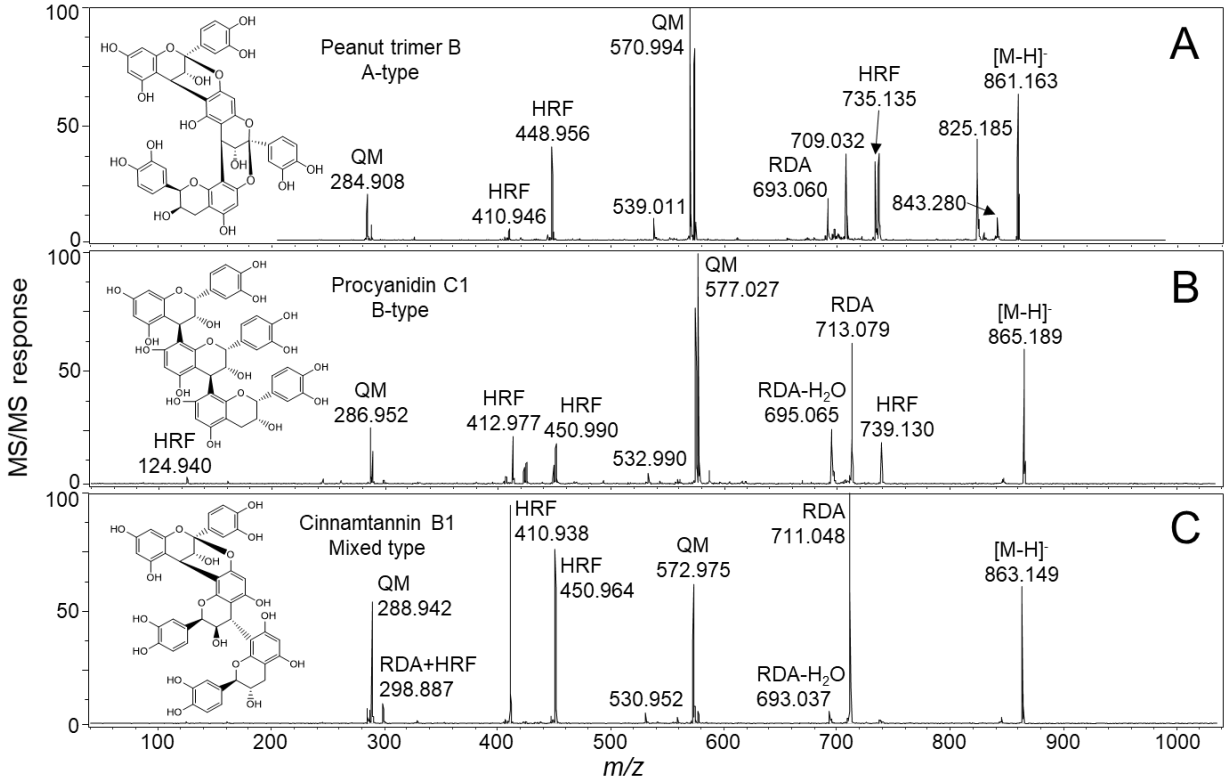

Figure 3.2 Comparison of negative ion MALDI-ToF/ToF mass spectra of procyanidin trimers with different types of bonding between the catechin/epicatechin subunits. A) A-type procyanidin trimer with two bonds between monomeric subunits. B) B-type procyanidin trimer with one bond between the subunits; and C) mixed type procyanidin trimer with both A-type and B-type bonds between subunits.

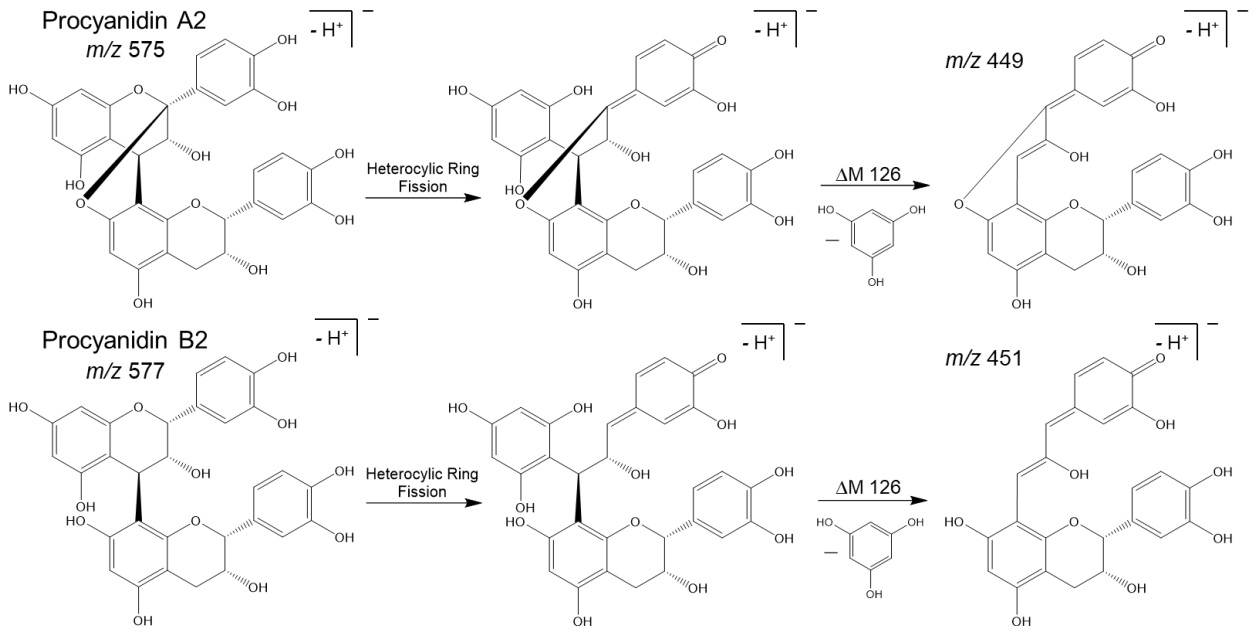

Figure 3.3 Heterocyclic ring fission fragmentation of procyanidins results in elimination of 126 u from compounds containing A-type (procyanidin A2) and/or B-type (procyanidin B2) linkages.

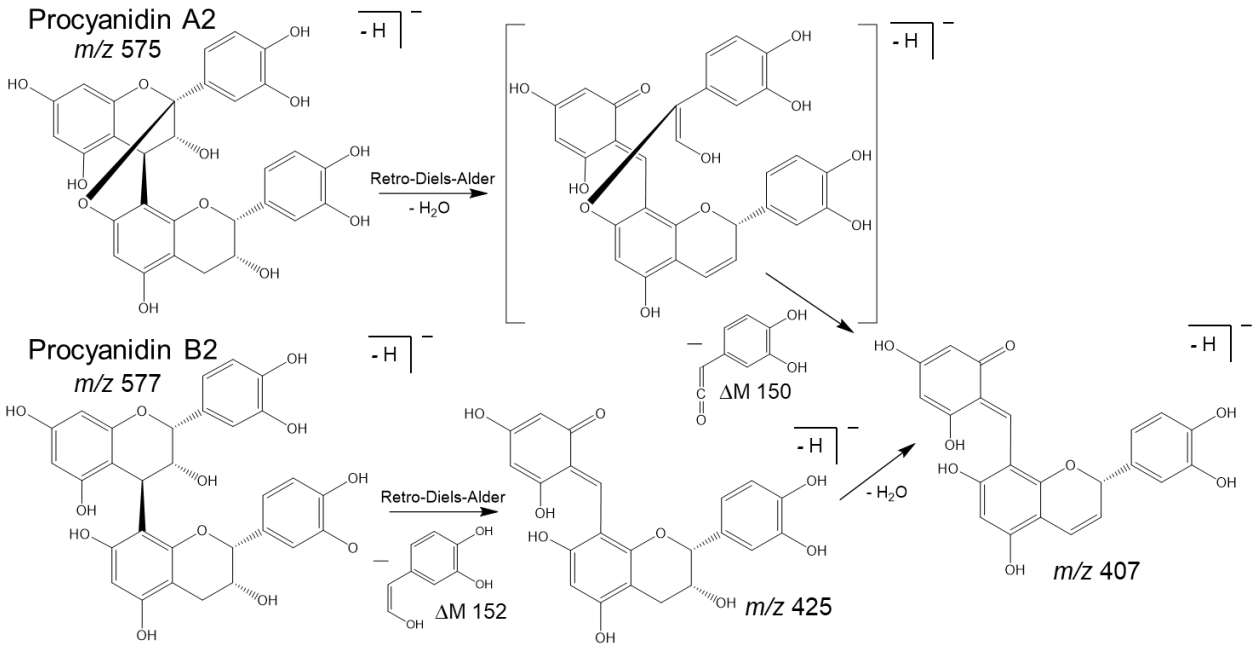

Figure 3.4 The retro-Diels-Alder fragmentation pathway for A) A-type procyanidins; and B) B-type procyanidins. B-type procyanidins undergoing RDA fragmentation will yield two ions, one losing hydroxyvinyl benzenediol ($C_8H_8O_3$), $[M-H-152]^-$, and another losing both hydroxyvinyl benzendiol and water $[M-H-150-18]^-$. A-type procyanidins yield only one ion corresponding to the combined loss of $C_8H_6O_3$ and water, $[M-H-168]^-$.

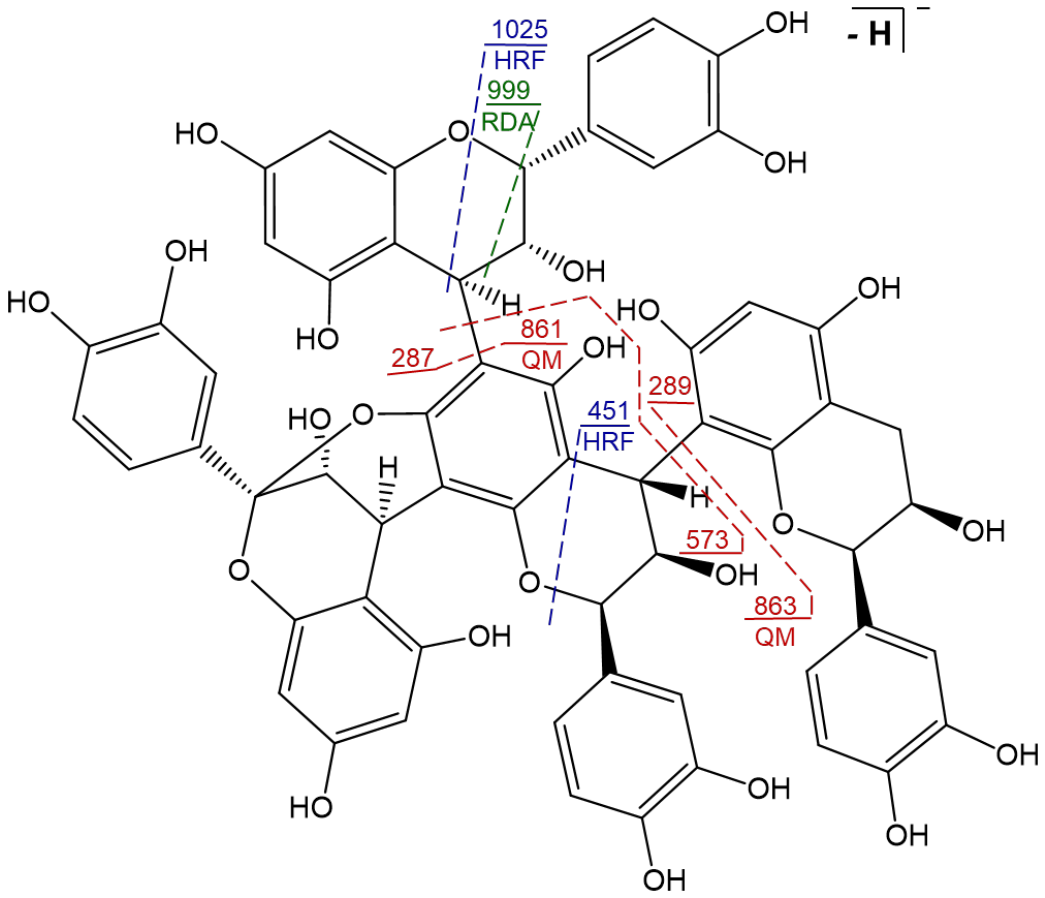

Figure 3.5 The connectivity of catechin/epicatechin subunits within the mixed, branched procyanidin tetramer parameritannin A1 can be determined based on the deprotonated molecule of *m/z* 1151 and the combination of HRF, QM, and RDA fragmentation during high-energy negative ion MALDI ToF/ToF mass spectrometry.

Table 3.1 Negative ion MALDI high-energy collision-induced dissociation ToF/ToF diagnostic fragment ions of procyanidins.

Procyanidin	$[M-H]^-$	Quinone Methide (QM)	Retro Diels-Alder (RDA) ($RDA–H_2O$)	Heterocyclic Ring Fragmentation (HRF)	QM +RDA +HRF	Type
Procyanidin B1	577.160	287, 289	425 (407)	451		1B
Procyanidin B2	577.152	287, 289	425 (407)	451		1B
Procyanidin B5	577.153	287, 289	425 (407)	451		1B
Procyanidin C1	865.191	287, 289, 575, 577	713 (695), 425 (407)	739, 451, 413		2B
Cocoa tetramer D	1153.245	287, 289, 575, 577, 863, 865	1001 (983), 713 (695), 425 (407)	1027, 739, 451		3B
Cocoa pentamer E	1441.305	287, 289, 575, 577, 863, 865, 1151, 1153	1289 (1271), 1001 (983), 713 (695), 425 (407)	1315, 1027, 739		4B
Procyanidin A1	575.127	285, 289	407	449		1A
Procyanidin A2	575.130	285, 289	407	449		1A
Cinnamtannin D1	863.170	285, 289, 573, 577	711 (693)	737, 451, 411	299	1A1B
Cinnamtannin B1	863.151	285, 289, 573, 577	711 (693)	737, 451, 411	299	1A1B
Lindetannin	863.145	285, 289, 573, 577	711 (693)	737, 451, 411	299	1A1B
Aesculitannin B	863.140	285, 289, 573, 577	711 (693)	737, 451, 411	299	1A1B
Parameritannin A1	1151.235	287, 289, 573, 861, 863	999 (981), 711 (693)	1025, 735, 451, 411	299	1A2B
Cassiatannin A	1151.235	287, 289, 573, 861, 863	999 (981), 711 (693)	1025, 735, 451, 411	299	1A2B
Peanut trimer B[24]	861.163	285, 289, 571, 575	693	735, 449, 411		2A
Peanut trimer D[24]	863.184	287, 289, 573, 575	711 (693)	737, 449		1A1B
Peanut trimer A[24]	863.189	287, 289, 573, 577	711 (693)	737, 449, 411	299	1A1B
Peanut trimer C[24]	863.188	285, 289, 573, 577	711 (693)	737, 451, 411	299	1A1B
Peanut tetramer E[24]	1149.217	285, 289, 573, 575, 859, 863	997 (979), 711 (693)	1023, 411		2A1B
Peanut tetramer F[24]	1149.218	285, 289, 573, 575, 859, 863	997 (979), 711 (693)	1023, 411		2A1B

CHAPTER 4

AUTHENTICATION OF CRANBERRY BOTANICAL DIETARY SUPPLEMENTS UTILIZING LIQUID CHROMATOGRAPHY MASS SPECTROMETRY WITH NEUTRAL LOSS SCANNING

4.1 Abstract

The potential health benefits of the American cranberry (*Vaccinium macrocarpon* Aiton) have led to the popularity of cranberry botanical dietary supplements in America. A-type procyanidins found in cranberries can inhibit the adhesion of *E. coli* to uroepithelial cells thus preventing urinary tract infections. To enhance profits, some manufacturers of cranberry dietary supplements may be adulterating their products with other botanicals lacking A-type procyanidins. Procyanidins are found in commonly consumed food making them readily available, but few foods contain high quantity of A-type procyandins like cranberries, therefore adulteration leads to supplements with reduced health benefits. Validated analytical methods are needed for the authentication of cranberry botanical dietary supplements. In this study, liquid chromatography tandem mass spectrometry (LC-MS/MS) was investigated as an approach to authenticate cranberry botanical dietary supplements by analyzing procyanidin content. Neutral loss scanning (NL) and multiple reaction monitoring (MRM) were examined to develop methods that could distinguish A-type from B-type procyanidins as an alternative to commonly used methods which look specifically for a known adulterant. MRM was able to quantitate A-type dimeric procyanidins in eight dietary supplements, whereas NL provided a method that is capable of profiling procyanidins found in cranberry botanical dietary supplements.

4.2 Introduction

Urinary infections are one of the most common bacterial infections in humans occurring in approximately 40-50% of women and 5% of men causing 150 million cases per year globally.[1,2] Urinary tract infections can be caused by a variety of pathogens; however, the primary cause is *Escherichia coli.* The American cranberry, *Vaccinium macrocarpon* Aiton, is a natural medicinal fruit which is indigenous to northern North America and has been used to prevent and treat urinary tract infections.[3,4] The cranberry contains substantial amounts of proanthocyanidins, organic acids (malic, quinic and citric) and resveratrol which give the berry an astringent taste making it unappealing for direct consumption. Therefore, cranberry food products are typically sweetened for consumption. Alternatively, cranberry botanical dietary supplements are a convenient source of these phytochemicals.[5,6] The increased demand and rising cost of cranberry raw material has driven economically motivated adulteration with proanthocyanidin rich extracts and materials from less expensive botanical sources. The inclusion of proanthocyanidins from other sources are difficult to detect and require advanced instrumentation or a combination of analytical techniques.[7-9]

Cranberry secondary metabolites are dominated by polyphenolics, mainly procyanidins.[6] Procyanidins are polymeric flavan-3-ols with two linkage types. The A-type linkage consists of single carbon-carbon bonds in addition to a carbon-oxygen bond between subunits while B-type consists of a single carbon-carbon

bond.[10,11] The A-type procyanidins have been reported to be essential for preventing adhesion of uropathogenic *E. coli* in the urinary tract, while B-type procyanidins has shown no anti-adhesion activity.[12,13] Common adulterants in cranberry products are grape seed extract, maritime and Masson pine bark extract, apple, and green tea, which contain predominantly B-type procyanidins, as well as peanut skin and almond skin extracts containing both A and B-type procyanidins.[9]

Various analytical methods have been developed to authenticate cranberry products. For example, the quantitation of cranberry products relies on a non-specific spectrophotometric 4-demethylaminocinnamaldehyde method,[14-17] which cannot detect adulteration with B-type procyanidins. Other approaches to detect adulteration of cranberry products have included measuring the ratio of procyanidin A2/ epicatechin using high-performance thin layer chromatography,[18] DNA analysis,[19] and mass spectrometry. By facilitating the detection, identification, and quantification of multiple procyanidins simultaneously, mass spectrometry-based methods have the potential to detect adulterants while establishing the A-type procyanidin levels in cranberry extracts.[20] Different mass spectrometry approaches have been developed to detect adulteration of cranberry products including measuring the ratio of A-type/ B-type procyanidins,[21] anthocyanidin fingerprinting,[22-23] proanthocyanidin determination,[24] and phenolic profiling.[6,25-27]

In this study, a triple quadrupole mass spectrometer was utilized by employing multi-stage mass spectrometry experiments. Triple quadrupole mass

spectrometers are capable of various tandem mass spectrometric measurements in which the first and third quadrupole are linked in different ways while collision-induced dissociation is used to produce fragment ions in the middle quadrupole (q2) (Figure 4.1).[28] Precursor ion tandem mass spectrometry on this type of instrument involves scanning the first quadrupole while the third quadrupole is fixed to transmit ions of a preselected *m/z* value. This results in ions that are detected selectively that produce product ions of this particular *m/z* value. Product ion scanning tandem mass spectrometry uses the first quadrupole to select a particular precursor ion while the third quadrupole is scanned. This results in mass selected precursor ions that are induced to dissociate into structurally related product ions.

During Multiple Reaction Monitoring (MRM), the first and last quadrupoles are set to transmit precursor ions and product ions of particular *m/z* values while collision-induced dissociation is used in the middle quadrupole. This triple quadrupole configuration is extremely sensitive and selective for chromatographic detection, because no ions are discarded during scanning, and only ions of particular precursor and product ion *m/z* value combinations are measured. Neutral loss scanning involves scanning both the first and third quadrupoles at the same rate while linking them such that the precursor ion and product ion are offset by a fixed mass corresponding to the loss of a neutral molecule. Although not as sensitive as MRM, neutral loss scanning is ideal for experiments where a group of structurally related compounds, such as procyanidins, fragment to eliminate

identical neutral molecules. By utilizing neutral loss scanning, procyanidins of varying degrees of polymerization can be analyzed simultaneously without the need to program specific precursor or product ion *m/z* values in advance, as would be necessary when using MRM, product ion scanning, or precursor ion scanning.

4.3 Experimental

4.3.1 Materials

Procyanidin standards were obtained from Planta Analytica (New Milford, CT), and botanical dietary supplements were purchased from various online vendors. Cranberry supplements were labeled to contain 100% *Viccinium macrocarpon* Aiton (American cranberry), and mixed fruit supplements were labeled to contain cranberry in addition to other fruits. Hershey's unsweetened cocoa powder was purchased from a local retail store. HPLC-grade methanol, hexane, and formic acid were purchased from Sigma-Aldrich (St. Louis, MO). Purified water was prepared using a Milli-Q water purification system (Millipore, MA, USA).

4.3.2 Preparation of Samples

Cranberry dietary supplements

Six cranberry dietary supplements labeled to contain 100% *vaccinium macrocarpon* Aiton and two mixed fruit dietary supplements labeled to contain cranberry were randomly selected from online vendors and given a code S1-S8. For each supplement the contents of ten capsules were combined; for S4 ten tablets were crushed using a mortar and pestle. For each supplement, the contents were combined, and 50 mg of each supplement was used for extraction. The samples were vortexed for 1 min with 10 mL methanol: water (50:50). Samples were sonicated for 30 min at 45 ºC then cooled to room temperature. Samples were vortexed for 10 min then centrifuged at 1,252 *g* for 10 min.

Defatting cocoa powder

Cocoa powder (50 mg) was placed in a 50 mL centrifuge tube, 45 mL *n*-hexane was added, and the mixture was vortexed for 1 min, sonicated for 5 min, and then vortex mixed again for 1 min. The mixture was centrifuged for 10 min at 1,252 *g* and the hexane layer was decanted. This extraction process was repeated more 2 times, and remaining hexane was allowed to evaporate from the defatted cocoa powder. Procyanidins were extracted from the defatted cocoa powder by

adding 10 mL methanol followed by sonication, vortex mixing, and centrifugation as described above for the fruit botanical dietary supplements.

4.3.3 LC-MS/MS

All cranberry products and cocoa were analyzed in duplicate immediately following extraction using a Shimadzu LCMS-8060 triple quadruple mass spectrometer equipped with a Shimadzu Nexera UHPLC system. HPLC separation was carried out with a YMC (Wilmington, NC) ODS-AQ (150 x 2 mm, 5 µm) column utilizing a binary gradient elution system consisting of (A) 0.1% formic acid in water and (B) 0.1% formic acid in methanol. The elution gradient was as follows: 12% B at 0-1 min, 12-25% B at 1-1.5 min, 25-30% B at 1.5-3 min, 30-50% B at 3-6 min, 50-12% B at 6-7 min, 12% B at 7-9 min with a flow rate of 0.8 mL/min. The column was heated to 35 ^{o}C, and the sample injection volume was 1 µL for MRM and 2 µL for neutral loss analyses.

Negative ion electrospray was used for procyanidin ionization. Nitrogen was used for nebulization (2 L/min), heating gas (10 L/min) and drying gas (10 L/min). The temperatures of the interface, the desolvation line and the heat block were 300, 250, and 400 ^{o}C, respectively. Argon was used for collision-induced dissociation at 230 kPa. The following parameters were used for MRM: *m/z* 575 >285, A-type dimer; *m/z* 577>287, B-type dimer; and *m/z* 863> 289, A-type trimer. For neutral loss scanning, the following neutral losses were used 126 u to

monitor for HRF; 290 u to monitor for QM fragmentation; and 152 u to detect procyanidins fragmenting via RDA.

4.3.4 Calibration curve

A-type dimeric procyanidins in the cranberry botanicals were quantified utilizing MRM. Procyanidin A2 was used as an external standard for quantification. Serial dilutions were made from a 1 mg/mL solution in methanol and injected twice. The calibration curve was generated from the corresponding peak areas of individual standards. The coefficient of determination (R^2) was greater than 0.99. The limit of detection (LOD) and limit of quantification (LOQ) were 0.01 µg/mL. The concentrations of procyanidins in extracts of cranberry botanical dietary supplements were determined using this standard curve after accounting for dilution during sample preparation.

4.4 Results and Discussion

Multiple reaction monitoring

Multiple reaction monitoring (MRM) was used with procyanidin standards to develop and optimize the LC-MS/MS parameters. Procyanidins have three characteristic fragmentation pathways consisting of quinone methide (QM)

formation, heterocyclic ring fission (HRF), and retro-Diels-Alder (RDA). Quinone methide formation produces the most abundant fragment ions and therefore was used in this study when selecting precursor and product ions to monitor. For A-type dimers, the MRM transition of *m/z* 575> 285 was used, the transition of *m/z* 577> 287 was used for B-type dimers, and for A-type trimers the transition of *m/z* 863> 289 was used.

Figure 4.2A demonstrates this LC-MS/MS MRM method for the analysis of a procyanidin A2 standard. Note that a single peak was detected at 4.7 min representing an A-type dimeric procyanidin. Applying this MRM method to the analysis of a cranberry supplement (S1; Figure 4.2B) demonstrates how an A-type trimeric procyanidin (retention time 2.8 min) and an A-type dimeric procyanidin (retention time 4.7 min) may be detected selectively during the same analysis when the MRM transitions for each procyanidin are programmed in advance. For comparison, this same LC-MS/MS MRM method was used to analyze a mixed fruit supplement (S7), which contained both A- and B-type procyanidins. Note the greater complexity of the mixed fruit chromatogram compared with the cranberry sample (Figure 4.2C). The chromatogram in Figure 4.2C is enhanced to show the multiple isomeric B-type dimeric procyanidins, the isomeric A-type trimeric and dimeric procyanidins present in this sample. A major challenge in the analysis of procyanidins is distinguishing between isomeric compounds with different linkage type. Unfortunately, without the availability of corresponding procyanidin standards or a method to differentiate between the C4>C8 and C6>C8 interflavan bonds,

these isomeric compounds cannot be identified using this LC-MS/MS MRM approach.

Multiple HPLC columns were tested when developing this method. The complexity of procyanidin chemical structures encompass varying degrees of polymerization that result in different polarities. Reversed phase HPLC columns efficiently resolve the lower procyanidins (monomers through tetramers), while under the same conditions, the higher order oligomeric procyanidins tend to co-elute (9). As an alternative to reversed phase chromatography, hydrophilic interaction liquid chromatography (HILIC) was evaluated for procyanidin separation.[29] However, procyanidin retention was excessively long using HILIC and was not investigated further. Reverse phase ultrahigh pressure liquid chromatography (UHPLC) was examined, but without a long column containing more theoretical plates, which would have produced excessive back pressure, the procyanidins were not retained well enough for adequate separation. Ultimately, a reversed phase HPLC column was identified that provided sufficient theoretical plates for baseline resolution, which was used to generate the chromatograms shown in Figure 4.2. Acetonitrile and methanol are commonly used solvents in procyanidin analysis with the addition of an acid; in this application, methanol was used.

Accurate labeling of the quantities of procyanidins in dietary supplements is essential for consumers to compare products. For the eight cranberry dietary supplements evaluated in this study, the stated procyanidin content varied from 40

– 1,200 mg total procyanidins per daily serving, and these levels are summarized in Table 4.1. Using our LC-MS/MS assay, the concentration of A-type dimers like procyanidin A2 in each of these products was determined to range from 2.0 – 75.2 mg/daily serving (Table 4.1). Note that standardization to A-type procyanidins would be preferable to total proanthocyanidin content to minimize the impact of economic adulteration on human health and studies of efficacy.

For example, an ex vivo study of the efficacy of human urine from women who had consumed cranberry dietary supplements found that 72 mg of total cranberry procyanidins was effective in preventing *E. coli* adhesion to human T34 epithelial cells daily.[30] In this ex vivo study, the 4-demethylaminocinnamaldehyde colorimetric method (14–17) was used to standardize the cranberry product with respect total procyanidin content. However, this type of standardization does not selectively measure the A-type procyanidin content that is believed to be effective in treating or preventing urinary tract infections. The main A-type procyanidin in cranberries is procyanidin A2,[31] however, neither the investigators in this ex vivo study nor manufacturers determined procyanidin A2 or total A-type procyanidin content of their cranberry supplements. Instead, products are usually labeled according to total proanthocyanidins which includes both A-type and B- type of all degrees polymerization.[32]

Using this LC-MS/MS MRM assay to determine the concentration of A-type dimeric procyanidins exceeds the ability of current methods by providing more specific data that can be related to botanical efficacy. A-type procyanidins are

responsible for the anti-adhesion activity that is desired in cranberry dietary supplements, and therefore, a method which quantifies dimeric A-type procyanidins would be better for the standardization of cranberry supplements. Currently, cranberry botanicals are standardized using a total proanthocyanidin content, however, the most important compound, dimeric A-type procyanidins, should be the focus when developing a supplement aimed to prevent and treat urinary tract infections. Although this method is better for standardization, more reliable methods need to be developed to detect adulteration.

Currently, there are two general approaches to determine adulteration. One approach includes methods that are used to detect known adulterants, the other approach examines botanicals with a focus on cranberry procyanidin composition. A method that would provide the ability to not only distinguish A- and B-type procyanidins as well as degree of polymerization but also determine the presence of A-type adulterants with varying degrees of polymerization would be monumental in this area.

Neutral loss scanning

As a novel approach to measure all A-type procyanidins in cranberry products, LC-MS/MS with neutral loss scanning was investigated. Although neutral loss (NL) scanning is inherently less sensitive than non-scanning approaches such as MRM, neutral loss tandem mass spectrometry has the benefit of detecting all A-

type and/or B-type procyanidins simultaneously, regardless of their degrees of polymerization. This is especially important when the identities of specific procyanidins are unknown. For comparison, MRM analysis requires that the *m/z* values of each precursor ion and corresponding product ion be programmed into the tandem mass spectrometer data system prior to analysis. Furthermore, the sensitivity of MRM decreases with each MRM ion pair transition that is measured. In contrast, NL scanning facilitates the detection of all analytes exhibiting a preselected structural feature that results in the facile elimination of a neutral molecule during collision-induced dissociation. In the case of procyanidins, NL scanning may be used to detect and measure any procyanidin with varying degrees of polymerization.

The fragmentation pathways of procyanidins, QM, HRF and RDA, are characteristic of procyanidin structure and are not unique to any particular degree of polymerization. A-type and B-type procyanidins undergo all three types of fragmentation and eliminate neutral, even electron molecules, through each pathway. However, there are small differences which might enable the selective detection of A-type versus B-type procyanidins using neutral loss tandem mass spectrometry.

Heterocyclic ring fission results in the elimination of 1,3,5-trihydroxybenzene (weighing 126 u) from both A-type and B-type procyanidins. Quinone methide fragmentation cleaves inter-subunit bonds resulting in a loss of 290 u for both A-type and B-type procyanidins, but B-type procyanidins uniquely

eliminate 288 u while the A-type eliminate neutrals weighing 286 u. A-type and B-type procyanidins fragment via two different retro-Diels-Alder (RDA) pathways. A-type procyanidins undergo an initial loss of water followed by RDA loss of a neutral molecule weighing 150 u. In the case of procyanidin A2, RDA fragmentation produces a fragment ion of *m/z* 407 (Figure 3.4). However, B-type procyanidins undergo RDA fragmentation to eliminate hydroxyvinylbenzenediol (152 u) followed by a subsequent loss of water loss. In the case of procyanidin B2, fragment ions of both *m/z* 425 and 407 are observed (Figure 3.4).

In this study, a neutral loss LC-MS/MS method was developed utilizing three neutral losses. For QM, NL scanning of 290 u was used because it is the most abundant type of procyanidin fragmentation. For HRF, neutral losses of 126 u was selected because 1,3,5-trihydroxybenzene is lost from both A-type and B-type procyanidins. For the RDA pathway, NL scanning of 152 u was used for the selective detection of B-type procyanidins from cranberries. The mass range *m/z* 250 - 900 was selected to include the most abundant procyanidins found in cranberries, which are dimers and trimers. Extending the scan range would enable the detection of a wider range of oligomeric procyanidins but would result in lower sensitivity.

The neutral loss LC-MS/MS chromatograms of a procyanidin A2 standard are shown in Figure 4.3. This analysis establishes that this tandem mass spectrometry approach can detect A-type procyanidins according to HPLC retention time while measuring their elimination of neutral molecules formed via

QM, HRF, or RDA fragmentation pathways. Utilizing the RDA neutral losses of 150 u and 152 u, a neutral loss method was created to determine if the ratio between A- and B-type procyanidins can be used to identify adulterants. Note that due to the relatively low resolving power of triple quadrupoles in scan mode, abundant RDA signals were detected corresponding to both loss of 150 u, which was expected for procyanidin A2, but also for loss of152, which is not an abundant fragmentation pathway for A-type procyanidins (Figure 4.3B). Therefore, neutral loss scanning using LC-MS/MS on a triple quadrupole mass spectrometer will not selectively detect A-type over B-type procyanidins. Nevertheless, this approach may still be used for the selective detection of all types of procyanidins in cranberry extracts, as shown in Figure 4.4.

This negative ion electrospray LC-MS/MS NL method demonstrates the ability to detect the procyanidin class of molecules. In Figure 4.4 this method (using three programmed neutral losses) is demonstrated using a procyanidin A2 standard. Eluting as a single peak at 4.5 min, procyanidin A2 was detected using neutral losses of 126 u (HRF), 290 u (QM), and 152 u (RDA), . Analysis of cranberry supplements S2 and S6 using this LC-MS/MS NL method (Figure 4.4B and 4.4C) showed a peak for an A-type trimer at 2.8 min and an A-type dimeric procyanidin peak at 4.5 min. The mixed fruit supplement S7 showed peaks for A- and B-type dimeric and trimeric procyanidins with corresponding isomeric pairs (Figure 4.4D). For comparison, cocoa powder (which contains only B-type procyanidins), showed trimeric procyanidins at 2.05 and 2.7 min and dimeric

procyanidin at 2.25 min. Although only procyanidins are detected, this LC-MS/MS NL method does not provide the ability to differentiate between A- and B-type or identify specific isomeric procyanidins, except based on their retention times. However, the ability to differentiate between cranberry and adulterated supplements based on the UHPLC profile using this method may be valuable.

4.5 Conclusion

A targeted analysis was performed utilizing LC-MS/MS with NL scanning on a triple quadrupole mass spectrometer to determine the feasibility of selectively measuring A-type procyanidins as a means of authenticating cranberry dietary supplements. Characteristic fragmentation pathways of procyanidins including QM, HRF, and RDA were used to establish three corresponding NL scans. The intention was first, to measure all procyanidins in a complex sample without interference from other classes of compounds, and second, to differentiate between A-type and B-type procyanidins. The ability to measure multiple A-type procyanidins independently of B-type procyanidins could be a novel approach to confirm the quality and integrity of cranberry dietary supplements.

Retro Diels-Alder fragmentation of procyanidins results in two distinct neutral loss compounds of 150 u for A-type and 152 u for B-type. Unfortunately, the resolving power of triple quadrupole mass spectrometers during neutral loss scanning was insufficient to discriminate between the elimination of these two

neutral molecules. Therefore, this approach would not be suitable for distinguishing procyanidin type.

Additionally, an MRM LC-MS/MS assay was developed successfully that is suitable for the quantitative analysis of A-type procyanidin dimers in cranberry extracts and dietary supplements. This quantitative assay was applied to the standardization of eight commercial dietary supplements containing cranberry procyanidins. The A-type procyanidin content of these cranberry dietary supplements varied greatly between products and relatively low compared with the total procyanidin levels. This highlights the importance of not only botanically authenticating cranberry supplements but chemically standardizing them to specific procyanidins that are important for health such as anti-adhesion activity necessary to prevent urinary tract infections.

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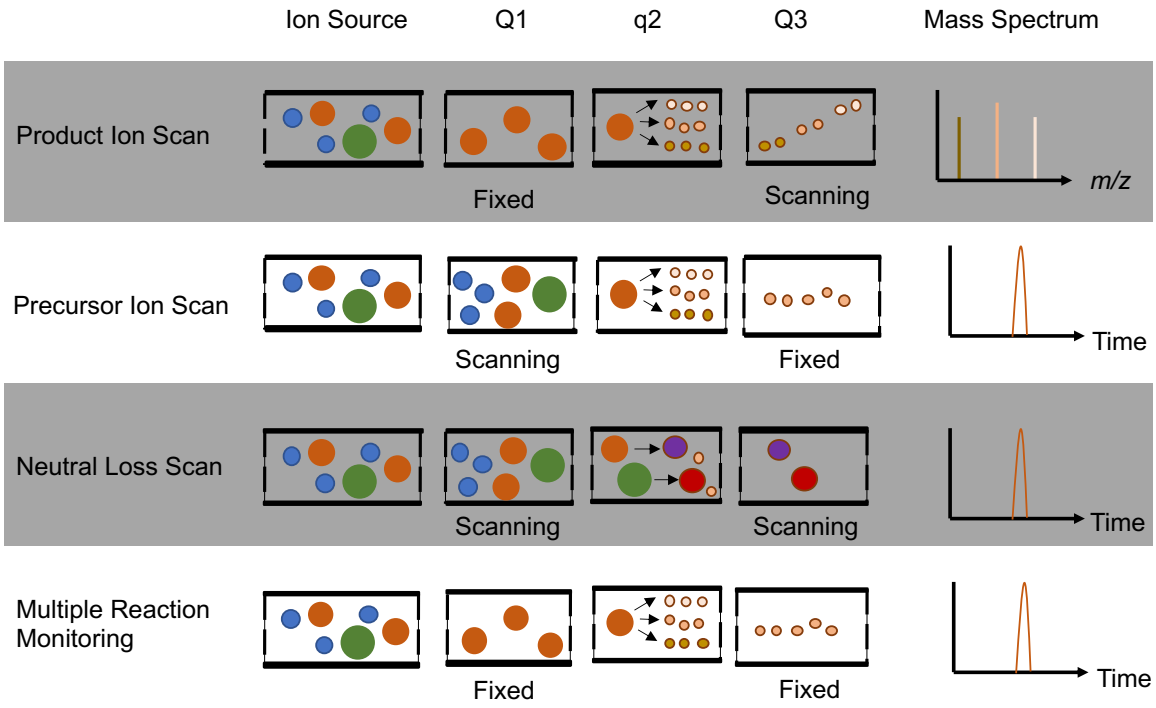

Figure 4.1 Four tandem mass spectrometry modes of triple quadrupole mass spectrometers. A) Product ion scan: select precursor ion and scan Q3; B) Precursor ion scan: scan Q1 and select product ion in Q3; C) Neutral loss scan: link scans of Q1 and Q3 such that they are offset by the mass of a neutral molecule loss; D) Multiple reaction monitoring: select precursor ion in Q1 and select product ion in Q3. Collision induced dissociation is carried out in q2 for all modes.

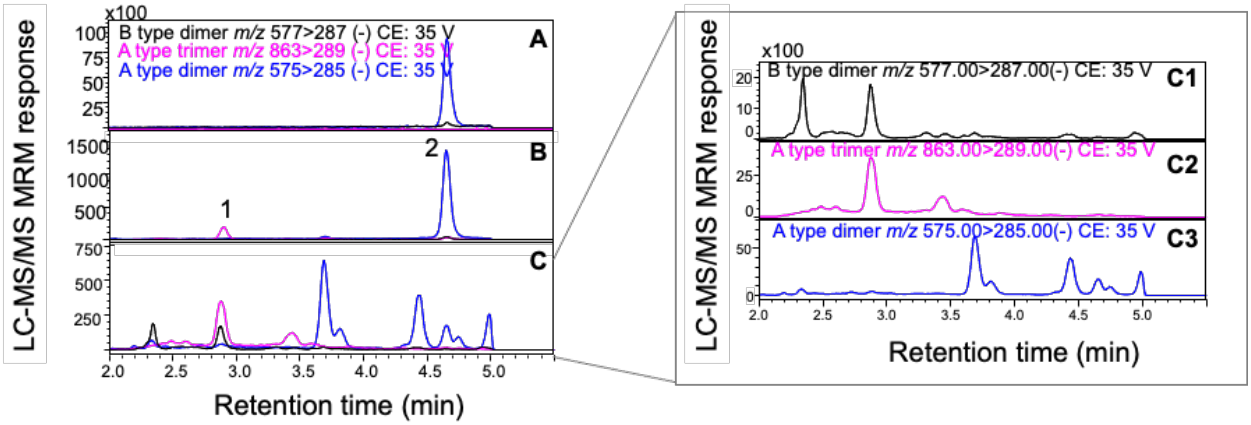

Figure 4.2 Negative ion electrospray LC-MS/MS MRM chromatograms of A) Procyanidin A2 standard (0.01 μg/mL), B) S1 (cranberry) containing peak 1 an A-type trimeric procyanidin and peak 2 an A-type dimeric procyanidin and C) S7 (mixed fruit) containing: C1) Isomeric B-type dimeric procyanidins at 2.3 and 2.9 min C2) Isomeric A type trimeric procyanidins at 2.9 and 3.4 min and C3) Isomeric A-type dimeric procyanidins.

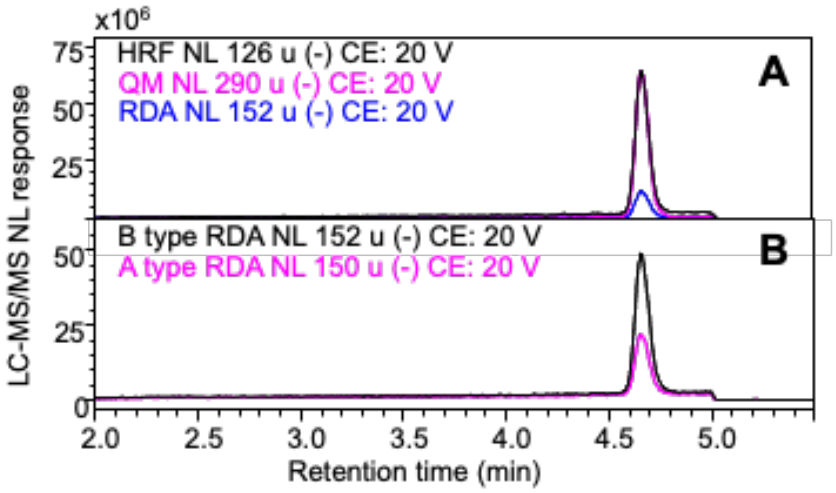

Figure 4.3 Negative ion electrospray LC-MS/MS NL chromatograms for a procyanidin A2 standard (1 μg/mL). A) Neutral losses of 126 u during HRF, 290 u during QM, and 152 u for RDA fragmentation. B) RDA neutral losses of 150 u for A-type and 152 u for B-type procyanidins.

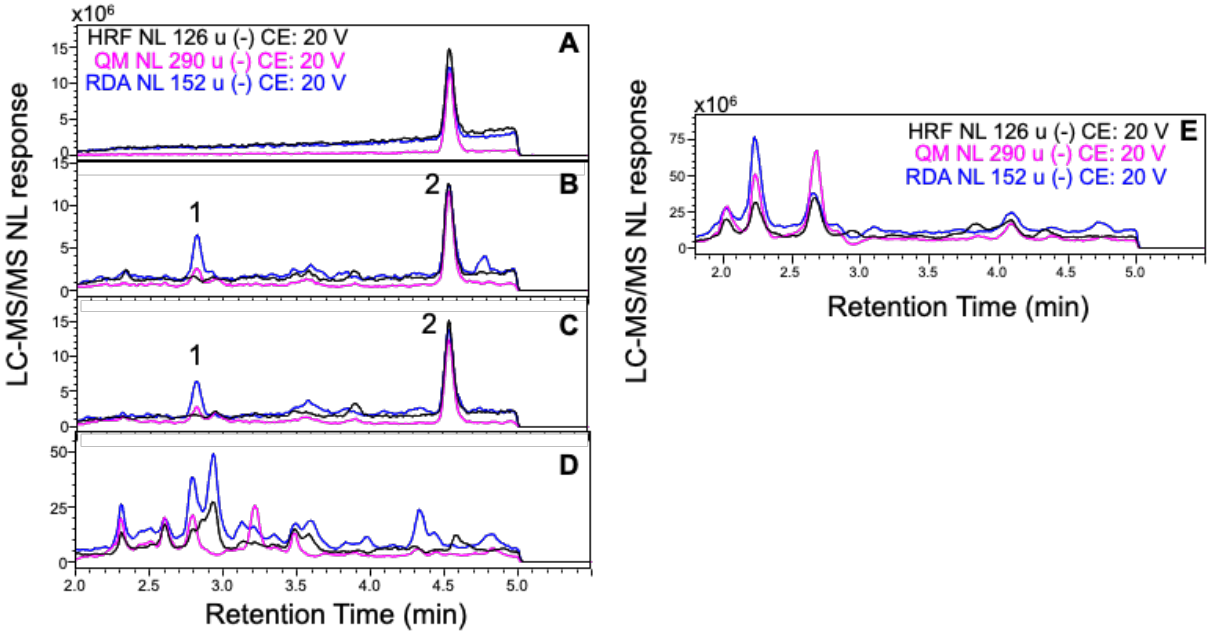

Figure 4.4 Negative ion electrospray LC-MS/MS NL chromatograms of A) Procyanidin A2 standard (0.01 μg/mL), B) S2 (cranberry) containing peak 1 an A-type trimeric procyanidin and peak 2 an A-type dimeric compound, C) S6 (cranberry) containing peak 1 an A-type trimeric procyanidin and peak 2 an A-type dimeric compound, D) S7 (mixed fruit) containing unidentified isomeric A- and B-type procyanidins and E) Cocoa powder containing B-type trimeric procyanidins at 2.05 and 2.7 min and B-type dimeric procyanidin at 2.25 min.

Sample	Form	Cranberry ingredient	Daily serving	Dry weight of single serving	Concentration of A-type dimers (per daily serving)
S1	Capsule	Cranberry concentrate	700 mg	448 mg	30.3 mg
S2	Capsule	Cranberry concentrate (50:1)	600 mg	1033 mg	50.7 mg
S3	Capsule	Cranberry juice extract	425 mg	377 mg	2.4 mg
S4	Tablet	Cranberry extract	1200 mg	1602 mg	75.2 mg
S5	Capsule	Cranberry concentrate (37:1)	400 mg	401 mg	20.6 mg
S6	Capsule	Cranberry concentrate (50:1)	500 mg	504 mg	38.8 mg
S7	Capsule	Cranberry extract	40 mg	473 mg	2.0 mg
S8	Capsule	N.D.	N.D.	498 mg	2.1 mg

*N.D.- not defined

Table 4.1 Product information for the six cranberry supplements (S1- S6) and two mixed fruit supplements (S7 & S8) as well as the concentrations of A-type dimers that were determined during this study.

CHAPTER 5

CONCLUSIONS

5.1 Summary

Here I have reviewed literature to recognize the need for further investigation of complex procyanidins and developed mass spectrometry-based methods of analysis. Procyanidins constitute a unique class of polymeric plant metabolite commonly found in a wide variety of foods we consume and are composed of monomeric catechin and epicatechin with varying interflavan bonds. The oligomeric and polymeric nature of these compounds have driven challenges in structure determination. Given the complexity of procyanidins there is a gap in knowledge where more accurate methods of analysis and validated standardization are imperative.

The second chapter of this dissertation investigates ion mobility as a chromatographic method for the rapid separation of complex procyanidins. Ion mobility is capable of detecting multi-charged compounds, varying degrees of polymerization and linkage types. Cranberry fractions exhibit the capability of ion mobility to be applied to natural products. Here we utilized traveling wave ion mobility with a short drift tube which demonstrated remarkable results for complex procyanidins. A longer tube could improve the separation of more complex polymeric procyanidins without the use of sample purification which will expedite the analysis of these time-consuming high molecular weight compounds.

The third chapter investigated MALDI to simplify the chromatographs identifying single-charged species which highlighted the characteristic fragmentation pathways of procyanidins. Given their complex structures MALDI was an easy approach to determine how many A- and B-type bonds were in each compound while molecular weight expressed the degree of polymerization. The downside of MALDI is the inability at this time to quantify the samples however, MALDI demonstrate its importance in its ability to rapidly identify procyanidins.

The fourth chapter addressed adulteration, a major issue with botanical dietary supplements. Without proper regulation and standardization manufacturers have added lower cost procyanidins in cranberry botanical supplements. Over the counter supplements from a local store were analyzed with tandem mass spectrometry. The neutral loss method is capable of identifying procyanidin standards with varying degrees of polymerization. Multiple reaction monitoring was capable of identifying procyanidins based on specific degree of polymerization which provided more sensitivity and selectivity to aid in the quantitation of procyanidins.

Together these results highlight the efficiency of mass spectrometry as a powerful tool at the identification of procyanidins. This research addresses the gap in knowledge by providing faster approaches to previous methods of analysis. The development of rapid methods is immensely beneficial to enable faster screening and limit lengthy sample preparation.

5.2 Future Direction

Our long-term goal is to improve the methods of analysis for complex procyanidins. The objective of this research was to test the feasibility of improved methods of analysis that aid in the detection of adulterants in cranberry botanicals. This research focused on mass spectrometry techniques to advance procyanidin identification. Our data proves that rapid methods of analysis are possible and demonstrates that further research needs to be performed on complex procyanidins.

Within the complexity of procyanidins, isomers have become the most challenging to distinguish. More specifically, the inter monomeric linkages C4-C6 and C4-C8 (Figure 5.1). These bonds have been challenging for nuclear magnetic resonance as well as mass spectrometry. Our group looked to differentiate these two bonds by high resolution mass spectrometry utilizing sodium and potassium adducts to distinguish two fragmentation patterns, however, more will need to be investigated. An instrument with higher energy such as MALDI will provide more fragments leading to unique fragments where electrospray produced the same fragments. The ability to distinguish these bonds will give rise to an undervalued area of research in which the complete structure and absolute configuration of procyanidins can be properly identified where currently, procyanidins are being identified based on degree of polymerization and bond type. Furthermore, scientists will be able to properly identify specific sources of procyanidins in plant products, monitor procyanidin content throughout growth to determine the best

time to harvest, determine the best region to grow the highest procyanidin content food, as well as determine specifically which procyanidin is linked to an individual therapeutic effect to create targeted botanical supplements.

Procyanidins are important flavonoids that play critical roles in the plant kingdom and human life. The numerous therapeutic effects make them attractive to scientists in pharmaceutical science and homeopathic medicine as well as health-conscious individuals looking for alternatives to traditional medicine and supplements to increase their overall wellbeing. The complexity of procyanidins offers a lot to be discovered. This research highlights the potential of procyanidin research specifically focused on cranberry botanicals however these methods are not limited to cranberries. With the wide distribution of procyanidins in various plant-based items these methods are applicable to all procyanidin based research making them profoundly versatile.

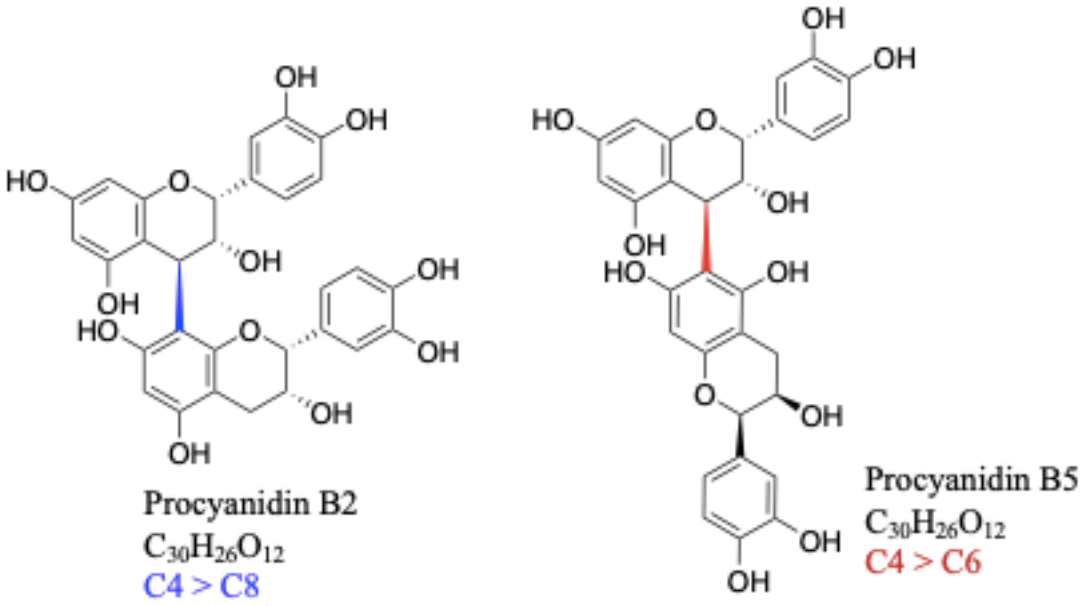

Figure 5.1 Isomeric B-type dimeric procyanidins with varying linkage types: procyanidin B2 (C4>C8) and procyanidin B5 (C4>C6).