International Food Research Journal 20(5): 2023-2034 (2013)
Journal homepage: *http://www.ifrj.upm.edu.my*

Review

MALDI-TOF mass spectrometry of oligomeric food polyphenols

[1*]Madrigal-Carballo, S., [1]Rodriguez, G., [1]Vega-Baudrit, J.R.,
[2]Krueger, C.G. and [2]Reed, J.D.

[1]*School of Chemistry, National University, Heredia, Costa Rica*
[2]*Department of Animal Science, University of Wisconsin-Madison, Madison, WI, USA*

Article history

Received: 9 November 2012
Received in revised form: 22 May 2013
Accepted: 29May 2013

Keywords

Proanthocyanidins
Ellagitannins
Cranberry
Sorghum
Grape seed extract
Pomegranate
Tannins

Abstract

Structural heterogeneity of polyphenols from cranberries, grape seed extracts, sorghum and pomegranate was characterized by MALDI-TOF MS. Polyphenolics were isolated by liquid chromatography and subjected to MALDI-TOF MS using trans-3-indoleacrylic acid as the matrix. Spectral analysis gave information on degree of polymerization, monomeric substitution, and the nature of intermolecular bonds. Cranberry polyflavan-3-ols had variation in interflavan bonds (A-type and B-type) and contained polyflavan-3-ols linked to anthocyanins through a CH3-CH bridge. Polygalloyl-polyflavan-3-ols in grape seed extract had large variation in the degree of galloyl substitution. Sorghum polyflavans had structural heterogeneity in glycosylation and hydroxylation. Pomegranate hydrolyzable tannins were detected that correspond to previously described structures, such as punicalagin, but others that correspond to oligomeric ellgitannins in which two to five core glucose units are cross linked by dehydrodigalloyl and or valoneoyl units. Results demonstrate that large heterogeneity occurs in degree of polymerization, intermolecular bonds, pattern of hydroxylation, and substitution with monosaccharides and gallic acid.

© All Rights Reserved

Introduction

Oligomeric plant polyphenols (tannins) are present in many foods, botanicals and nutritional supplements. A growing body of research indicates that increased dietary consumption of tannins is associated with a decreased risk of diseases such as coronary artery disease, cancer, urinary tract infections, and ulcers (Scalbert *et al.*, 2000). However, tannins are diverse compounds with high variation in structure and concentration within and among plant species. Therefore, biomedical research on the health benefits and risks of increased tannin consumption is severely limited by lack of methods for rapid characterization and standardization.

Reports on the application of MALDI-TOF MS for analysis of oligomeric polyphenols in foods have appeared in the literature within the last 7 years (Ohnishi-Kameyama *et al*., 1997; Krueger *et al.*, 2000b; Foo *et al*., 2000a; Yang and Chien, 2000). The first publication demonstrated the power of MALDI-TOF MS to determine range in degree of polymerization of proanthocyanidin (PA) oligomers in apples (Ohnishi-Kameyama *et al*., 1997). These researchers detected oligomers from the dimer to the pentadecamer, whereas previous research with fast atom bombardment (FAB) MS had only detected up to the pentamer. Our laboratory applied MALDI-TOF MS to characterize the structural heterogeneity of galloyl procyanidins from grape seed extract (GSE) (Krueger *et al*., 2000). The method was able to detect a series of PA oligomers up to the nonamer in the positive-ion reflectron mode and up to the undecamer in the positive-ion linear mode. Additional masses corresponding to a series of galloyl procyanidins were seen; the highest degree of galloylation observed was six. Yang and Chien (2000) subsequently published similar results and indicated that MALDI-TOF MS had potential as a sensitive and quantitative method. However, the authors cautioned that the difficulty in preparing standards to develop response factors for each group of oligomers is a major problem to overcome.

Both electrospray and MALDI-TOF were used to characterize the DP and structure of PA oligomers in cranberries that are associated with the *in vitro* inhibition of adhesion of *E. coli* to uroepithelial cells (Foo *et al.*, 2000a). The methods detected oligomers up to a DP of 7 in the PA fraction that had the greatest anti-adhesion activity. Foo *et al.* (2000a) concluded from the interpretation of the MALDI-TOF MS spectra that observed peak distribution reflected the quantity of various PA oligomers present in the cranberry fraction. Therefore, the tetramer, which

*Corresponding author.
Email: *sergio.madrigal.carballo@una.cr*

was the first mass detected and had the greatest signal height, represented the most abundant oligomer in the sample. The application of MALDI-TOF MS clearly demonstrated the presence of A-type linkages in the tetramer through hexamer. Our lab used MALDI-TOF MS to characterize the PA oligomers that are associated with decreased susceptibility of human LDL to in vitro Cu^{2+} induced oxidation (Porter *et al.*, 2001).

The application of atmospheric pressure ionization electrospray and LC/MS techniques to characterize the PA oligomers in foods is also recent (Guyot *et al.*, 1997; Lazarus *et al.*, 1999; Hammerstone *et al*., 1999; Hammerstone *et al.*, 2000). Hammerstone *et al.* (1999) and Lazarus *et al.* (1999) used normal phase HPLC interfaced with a HP 1100 single quadrapole mass spectrometer (MS) with an atmospheric pressure ionization (API) source. These authors used positive and negative ion detection and the negative mode showed better responses for PA oligomers than positive mode. Detection was in both scan mode and selected ion mode (SIM). Yang and Chien (2000) used this method and the same instrumentation with an atmospheric pressure chemical ionization (APCI) source to characterize PA oligomers in grape. Negative ion detection gave good responses for PA oligomers. Recent advances in mass spectrometry have also allowed for the characterization of complex mixtures of anthocyanin-polyflavan-3-ol pigments in wine (Asenstorfer *et al.*, 2001; Hayaska *et al.*, 2002; Mateus *et al*., 2002; Reme-Tanneau *et al*., 2003). Both ESI and MALDI-TOF have been applied to the analysis of tannins in foods and beverages. While ESI and MALDI-TOF MS are both capable of detecting intact molecular ions with high molar mass (>100,000 Da), ESI is best suited for analysis of monodispersed biopolymers because of complications arising from the formation of multiply charged ions (Montaudo *et al.*, 2002). Alternatively, MALDI-TOF MS is ideally suited for characterizing polydispersed oligomers (Hanton, 2002) and is considered the mass spectral method of choice for analysis of tannins which exhibit large structural heterogeneity. MALDI-TOF MS produces only a singly charged molecular ion for each parent molecule and allows detection of high mass with precision (Montaudo *et al*., 2002).

In this paper we report our research on the characterization of complex mixtures of oligomeric polyphenolic compounds in fruits using a combination of liquid chromatography and MALDI-TOF MS. The advantages of MALDI-TOF MS are; only one molecular ion is formed from each parent molecule, high sensitivity across a broad range of masses allows detection of oligomeric series of compounds, ability to detect compounds of high molecular weight, and interpretation of isotope patterns allows the detection of oligomers with small differences in mass. Several factors must be optimized in order to develop MALDI-TOF MS techniques. These factors include the selection of an appropriate matrix, optimal mixing and drying of matrix and sample, optimal selection of alkali metal complex, adjustment of laser strength, selection of calibration standards, and correct interpretation of the spectra. The application of MALDI-TOF MS to characterize the heterogeneity of tannins is possible by using published research on structures of isolated dimers and trimers. These structures were determined on isolated compounds using a NMR, MS, chemical degradation and MS fragmentation studies. However, our ability to determine structures of tannins with higher degrees of polymerization is severely limited by the difficulty in separation and isolation of individual isomers from complex oligomeric mixtures. In addition, the complexity of chemical shifts in NMR of higher oligomers makes the spectra difficult to interpret. However, TOF analysis of molecular ions coupled with a knowledge of dimer and trimer structure provides a powerful tool for characterization of the higher oligmers. We have applied this approach to tannins from grapes, sorghum, cranberries and pomegranate.

Polygalloyl-polyflavan-3-ols in grape seed extract

Ricardo-Da-Silva *et al.* (1992) elucidated the structure of compounds such as epicatechin 3-*O*-gallate, procyanidin B_2 3,3'di-*O*-gallate (1), and procyanidin trimer isolated from grape seeds. Galloylation always appears to occur on an epicatechin unit, to date no compound with catechin-*O*-gallate has been described (Santos Buelga, 1995). Based on the galloylated structures described by Ricardo-Da-Silva *et al.* (1992) an equation was developed to predict the mass distribution of PGPF in grape seed extracts. The equation is; $290 + 288c + 152g + 23$, where 290 represents the molecular weight of the terminal catechin/epicatechin unit, c is the degree of polymerization of catechin/epicatechin units, g is the number of galloyl esters, and 23 is the molecular weight of sodium.

The MALDI-TOF spectrum of the GSE in *t*-IAA detected masses that correspond to an oligomeric series of catechin/epicatechin units up to the nonamer in the positive-ion reflectron mode (Figure 1) and up to the undecamer in the positive-ion linear mode (Figure 2) (Krueger *et al*., 2000). Additionally masses corresponding to a series of poly-galloyl proanthocyanidins (PGPA) were

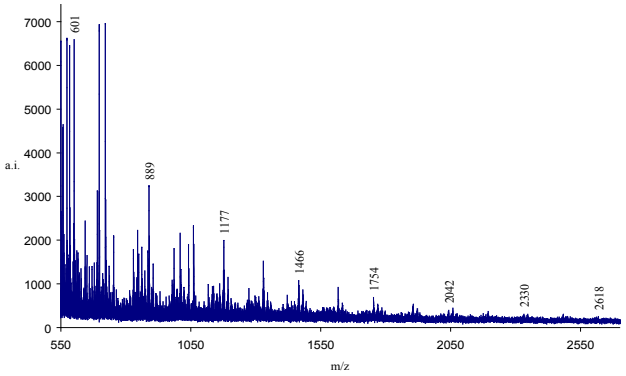

Figure 1. Matrix assisted laser desorption ionization time-of-fight mass spectrum in positive reflectron mode, showing a procyanidin series [M + Na^+] from the dimer (601 *m/z*) to the nonamer (2618 *m/z*). Insert is enlarged specturm of the tetramer (1177 *m/z*) and the digalloyl-trimer (1193 *m/z*) representing isotope distribution patterns and an uncharacterized mass series (1175 *m/z*) two molecular weight units lower than predicted values.

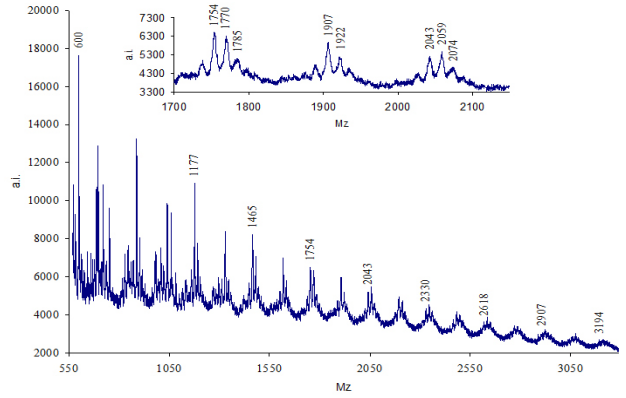

Figure 2. Matrix assisted laser desorption ionization time-of-fight mass spectrum in positive linear mode, showing a procyanidin series [M + Na^+] from the dimer (600 *m/z*) to the undecamer (3194 *m/z*). Insert is an enlarged spectrum of masses representing a procyanidin series with varing degree of galloylation.

also detected. Sodium adduct ions [M + Na^+] were detected in positive-ion linear and reflectron modes. The highest degree of galloylation observed was six, regardless of the degree of polymerization. Degree of galloylation decreases as the extent of polymerization increases past the heptamer. We speculated that the lower molecular weight compounds are saturating the detector, preventing the observation of higher polymers and galloylation patterns. Improved separations of narrower mass ranges by liquid chromatography prior to MALDI-TOF MS may allow for detection of additional galloylation patterns.

Positive-ion reflectron mode resolved masses that correspond to an isotope pattern representative of carbon, hydrogen and oxygen (Figure 1). Positive-ion linear mode gave less resolution, but allowed detection of masses corresponding to higher degree of polymerization. Mass spectra also provide evidence for a series of compounds that are two mass units lower than those described by the equation; 290 + 288*c* + 152*g* + 23 (Figure 1). This series of compounds may represent A-type linkages or oxidation reaction products, although neither of these structures have previously been described in grape seed extracts. Additional characterization by liquid chromatography coupled to mass spectrometry is necessary to improve our understanding of the structural complexity of grape seed tannins.

Glycosylated polyflavans in sorghum

Gujer *et al.* (1986) elucidated the dimeric and trimeric structures of a unique class of heteropolymeric 3-deoxyproanthocyanidins in sorghum. This class of PA consisted of repeating monomeric flavan units glucosylated at carbon-5 with a flavanone, eriodictyol or eriodictyol-5-*O*-ß-glucoside, terminal unit (2 a-d). We also detected heterogeneity in pattern of hydroxylation in the B ring by heating the LH20-ethanol:methanol eluate with acid (Krueger *et al*., 2003), separating reaction products on a polyvinylpolypyrrolidone column and subjecting the anthocyanins to MALDI-TOF MS. The anthocyanins produced were luteolinidin (*m/z* 271) and apigeninidin (*m/z* 255).

Positive-ion reflectron mode MALDI-TOF spectrum of the Sephadex-ethanol:methanol eluate contained masses corresponding to two distinct oligomeric series of glucosylated heteropolyflavan units. The first series represents glucosylated heteropolyflavans containing the flavanone; eriodictyol as the terminal unit. The mass distribution extends from the trimer (*m/z* 1289) to the heptamer (*m/z* 3025) (Figure 3). The second series, separated by a glucose substitution (Δ 162 amu), represents glucosylated heteropolyflavans containing the flavanone; eriodictyol 5-*O*-ß-glucoside as the terminal unit and extended from the trimer (*m/z* 1451) to the heptamer (*m/z* 3188).

Each DP within glucosylated heteropolyflavan mass series had a subset of masses (Δ 16 amu) lower than the proluteolinidin oligomer in which all glucosylated flavan units are hydroxylated at both the 3′ and 4′ positions of the B-ring. This pattern of hydroxylation gives predicted 4 tetramer masses that were also observed; *m/z* 1837 (3 proluteolinidin + 0 proapigeninidin), *m/z* 1853 (2 proluteolinidin + 1 proapigeninidin), *m/z* 1869 (1 proluteolinidin + proapigeninidin) and *m/z* 1885 (0 proluteolinidin + 3 proapigeninidin) each with eriodictyol 5-*O*-ß-glucoside as the terminal unit. The mass differences are explained by the hydroxyl substitutions (Δ 16 amu) at either the 3′ or 3′ and 4′ positions of the B-ring of the

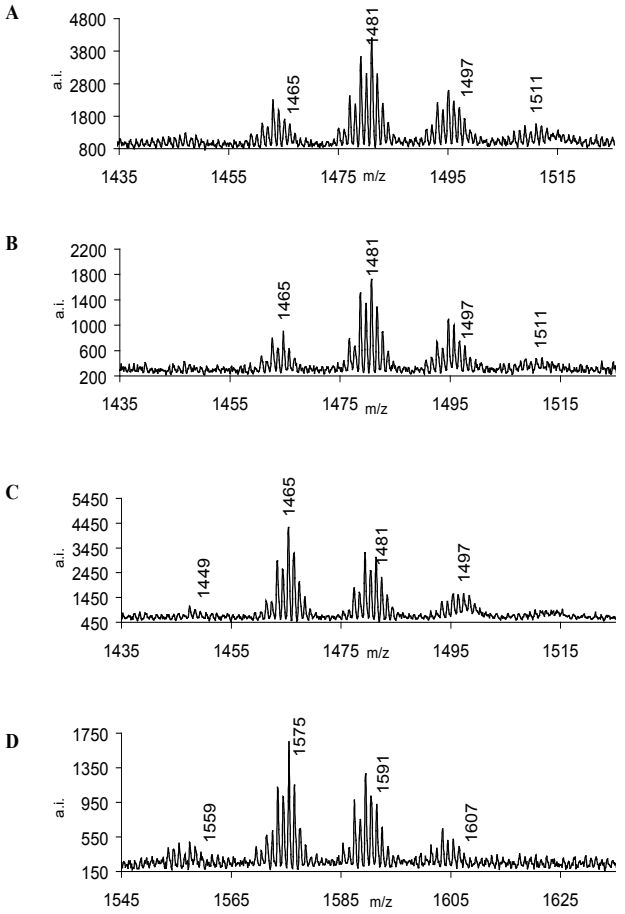

Figure 3. MALDI-TOF positive reflectron mode mass spectra of the heteropolyflavan-3-ol pentamers in the Sephadex LH20-methanol eluate of Ruby Red sorghum (*Sorghum bicolor* (L.) monech). (A) No deionization or addition of cation to the matrix:analyte prior to deposition on the target. (B) Deionization and addition of K^+. (C) Deionization and addition of Na^+. (D) Deionization and addition of Cs^+ provides evidence the mass distribution (Δ 16 amu) is due to heterogeneity of the repeating flavan-3-ol units.

repeating flavan unit. In addition, a mass that was 16 amu lower than the proapigeninidin homopolyflavan was also detected at each DP. Although no predictive equation was formulated to account for these masses, it is speculated that the masses represent a compound with one fewer hydroxyl (Δ 16 amu) substitution.

Another possible explanation for detection of (Δ 16 amu) differences in the mass spectrum must be addressed. MALDI-TOF MS of polyflavans tend to favor an association with sodium $[M + Na]^+$ and potassium $[M + K]^+$ ions over the formation of a protonated molecular ion $[M + H]^+$ (Porter *et al.*, 2001, Takahata *et al.*, 2001). If Na^+ and K^+ are present at the time of the desorption/ionization event the signal of the polyflavan will be split and detected as both $[M + Na]^+$ and $[M + K]^+$ adduct ions. The atomic mass difference between the monoisotope of Na^+ (22.9900 amu) and monoisotope of K^+ (39.0980 amu) is Δ 15.9739 amu. The molecular weight difference of two polyflavans, differing by one hydroxyl group substitution, is equal to the atomic mass of oxygen (15.9949 amu).

To solve the problem of distinguishing between the formation of both $[M + Na]^+$ and $[M + K]^+$ adduct ions from one species, and the presence of two species differing in the number of hydroxyl groups, the analyte was first mixed with matrix and applied directly to the target with no deionization or addition of cations. The predicted mass value for a homopolyflavan-3-ol with a DP of 5 and all B-type interflavan bonds is (*m/z* 1481; $[M + K]^+$) assuming that naturally abundant K^+ are the most abundant ions . The predicted value was observed as the most abundant mass (*m/z* 1481; $[M + K]^+$). However, masses 16 amu lighter, 16 amu and 32 amu heavier (m/z 1465, m/z 1497, and m/z 1511) than the predicted homopolymer were also observed. To determine if these masses were due to heterogeneity in hydroxyl substitution (Δ 16 amu) or multiple cation adducts ($[M + Na]^+$ and $[M + K]^+$) the analyte was deionized with cation exchange resin and K^+ was added. The mass spectra was found to give a similar mass distribution as the original spectra, with the predicted value (*m/z* 1481; $[M + K]^+$) remaining as the most abundant mass (Figure 3).

Ambiguity remained as to whether the mass at (*m/z* 1465) was due to a compound containing one fewer hydroxyl substitutionss (Δ 16 amu) than the predicted homopolymer or the inability of the cation exchange resin to remove all Na^+ (*m/z* 1465; $[M + Na]^+$). The Sephadex-methanol analyte was again deionized and this time Na^+ was added. The predicted mass value for a homopolyflavan-3-ol with a DP of 5 and all B-type interflavan bonds is now (*m/z* 1465; $[M + Na]^+$) assuming that Na^+ is the only ion present. The entire mass distribution shifted Δ 16 amu lower than the original spectra (Figure 3). The predicted value was again observed as the most abundant mass (*m/z* 1465; $[M + Na]^+$). Again, masses 16 amu lighter, 16 amu and 32 amu heavier (*m/z* 1449, *m/z* 1481, and *m/z* 1497) than the predicted homopolymer were also observed (Figure 3). Ambiguity remained as to whether the mass at (*m/z* 1481) was due to a compound containing one greater hydroxyl substitutions (Δ 16 amu) than the predicted homopolymer or the inability of the cation exchange resin to remove all K^+ (*m/z* 1481; $[M + K]^+$). In order to overcome this problem a cation must be added to the analyte such that the mass distribution is shifted sufficiently from the range in which $[M + K]^+$ and $[M + Na]^+$ confound interpretation.

Onishi-Kameyama *et al.* (1997) and Takahata *et al.* (2001) employed silver trifluoroacetate to

suppress cationization with Na^+ and K^+ [M^+ alkali metal]$^+$. However, addition of Ag^+ created two new problems. Silver has two naturally occurring isotopes; 107Ag (51.839% abundance) and 109 Ag (48.161% abundance). The addition of Ag^+ will effectively split the signal of the analyte, leading to the detection of $[M + Ag]^+$ ions differing by 2 amu. This is particularly confusing for analysis of polyflavans, which contain both B-type and A-type interflavan linkages (Porter *et al*., 2001, Takahata *et al*., 2001). A second disadvantage of Ag^+ is the possible production of silver cluster ions in the presence of acidic matrices such as *t*-IAA. Silver clusters may form preferentially, as opposed to the formation of silver-adducted oligomer ions (Macha *et al*., 2001).

In order to eliminate the problem of cationization with Na^+ and K^+ we first deionized the polyflavan solutions by addition of a cation exchange resin and second, added cesium trifluoroacetate to the analyte: matrix solution prior to deposition on the target. Cesium has the attribute of having a single isotope (133 Cs; 100% abundance). This approach resulted in the detection of exclusively $[M + Cs]^+$ molecular ions (Figure 3), eliminating the possibility that the Δ 16 amu was due to the formation of both $[M + Na]^+$ and $[M + K]^+$ adduct ions from one species. The mass distribution provides evidence for a class of heteropolyflavan-3-ols that have one fewer hydroxyl substitution (Δ 16 amu) than the predicted equations that were developed based on the structures previously described by Gupta and Haslam (1978), and later by Brandon *et al.* (1982) and also provides evidence for the presence of the prodelphinidin gallocatechin/epigallocatechin hydroxylation patterns.

The combination of liquid chromatographic separation and MALDI-TOF MS to characterize sorghum proanthocyanidins indicates that the structural heterogeneity is much greater than previously described. Structural heterogeneity occurs in the nature of repeating monomeric units (flavan, flavan-3-ol and flavanone), pattern of hydroxylation, type of interflavan bonds (A-type and B-type) and substitutions with moieties such as glucose. In light of this structural diversity a more descriptive nomenclature must be employed when describing heteropolyflavans.

A-type interflavan bonds in cranberry polyflavan-3-ols

Foo *et al*. (1986) elucidated the trimeric structures of "A-type" proanthocyanidins (3) from cranberries that are associated with the inhibition of adhesion of p-fimbriated Escherichia coli to uroepithelial cells. We fractionated cranberry flavonoids on sephadex LH-20

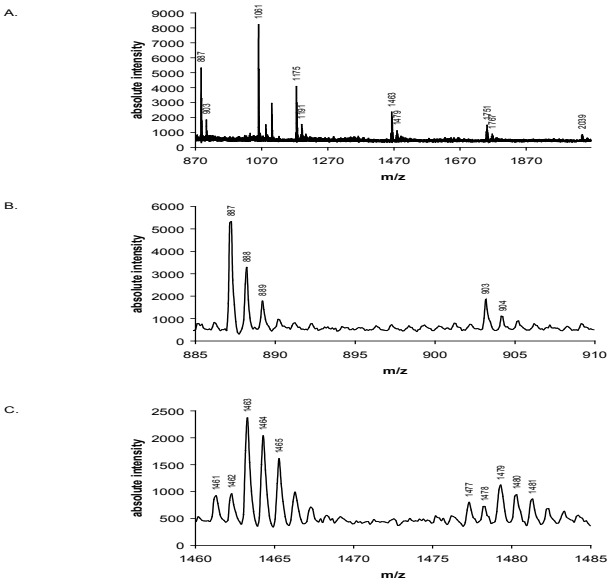

Figure 4. Matrix assisted laser desorption-time of flight mass spectra of proanthocyanidins from cranberries. The proanthocyanidins were obtained by elution from a Sephadex LH-20™ column with methanol (fraction 5). A) Spectra of the trimers through heptamers. The signal at *m/z* 1061 is bradykinin, used for internal calibration. B) Spectra of the trimers showing the typical isotope pattern for m/z of less than 2000. The mono-isotopic signal is the first and highest. C) Spectra of the pentamer that show a more complex isotope pattern and signals that suggest structures that differ by 2 mass units. This difference may result from proanthocynidins with 2 A-type interflavan bonds.

to determine which compounds would associate with low-density lipoprotein in serum and subsequently inhibit Cu^{2+} induced oxidation after isolation of the LDL by ultracentrifugation (Porter *et al*., 2001). Only the two fractions that contained proanthocyanidins significantly increased lag time of oxidation. The MALDI-TOF MS spectra of these fractions showed that both fractions contained a series of oligomeric PA. The fraction that eluted in methanol contained trimers through heptamers with at least one-A-type interflavan bond. This series of masses closely resembled the epicatechin structures reported by Foo *et al.* (2000a) to be present in cranberries.

However, our spectra also had masses that were 16 mass units greater than the masses reported by Foo *et al.* (2000a). These masses may correspond to oligomers with one epigallocatechin unit. Although Foo *et al.* (2000a) reported the presence of epigallocatechin subunits in their cranberry juice fraction, they did not detect heteropolymers with both epicatechin and epigallocatechin units by mass spectrometry.

Our MS results also showed the presence of more complex heteropolymers for PA with a DP greater than 4 (Figure 4). The pentamer and hexamer had

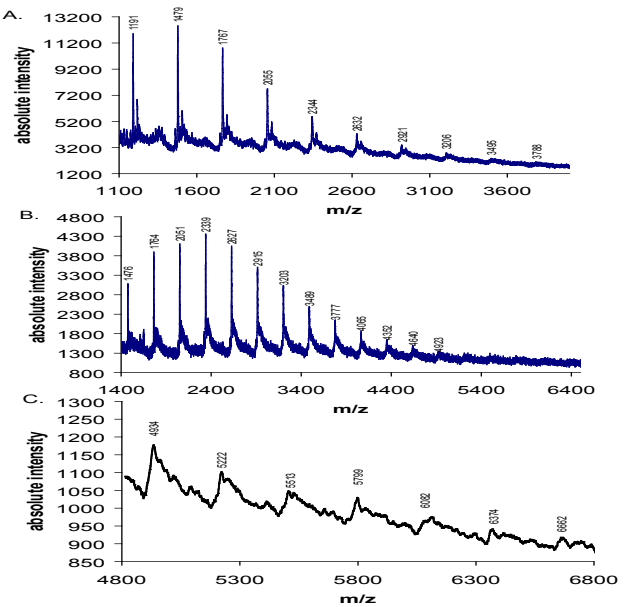

Figure 5. Matrix assisted laser desorption-time of flight mass spectra of proanthocyanidins from the aqueous acetone extraction of cranberry press cake. The proanthocyanidin fractions were obtained by elution from a Sephadex LH-20™ column with methanol (A) or aqueous acetone (B). The spectrum of the aqueous acetone fraction indicates that the press cake contained oligomers with a degree of polymerization of up to 23. The spectrum that is shown in (C) is the area from spectrum (B) for *m/z* of 4800 to 6400 enlarged. A smoothing function was applied to the spectrum in order to assist in visualizing the peaks for *m/z* greater 4900.

more spectral peaks than could be explained by a predicted isotope pattern for the simple oligomers with one A-type interflavan bond (Figure 4B and 4C). These peaks were interpreted to represent PA with 2 A-type ether linkages present in the oligomer because the pattern corresponded to a 2 mass units lower molecular weight. The fraction that eluted in aqueous acetone contained pentamers through nonamers. Masses with at least one epigallocatechin substitution were detected for the hexamer, heptamer and octamer. The masses were closer to calculated masses for PA with either 2 or 3 A-type interflavan bonds in each oligomer. These results indicated that the DP of PA in the acetone fraction was greater than PA in the methanol fraction and the acetone fraction contained several oligomers with more than one A-type interflavan bond.

Figure 5 shows the MALDI-TOF MS spectra of cranberry PA that were extracted from press cake and fractionated by chromatography on Sephadex LH-20. Proanthocyanidin oligomers with a DP of 4 to 13 (1191 to 3788 *m/z*) were detected in the fraction that eluted with methanol (Figure 5A). Whereas, PA oligomers with a DP of 5 to 23 (1476 to 6662 *m/z*) were detected in the fraction that eluted with aqueous acetone (Figure 5B). These spectra indicate that MALDI-TOF MS is capable of detecting PA oligomers of much greater mass than a DP of 10 and as high as DP of 23 (Figure 5C). The broad peaks in these sprectra also indicate that there is large structural heterogeneity within each DP. The results also show that chromatography of PA on Sephadex LH-20 separates fractions that differ in range of DP and other structural characteristics. For instance, oligomers with a DP of 4 to 13 in the methanol fraction were 3 to 11 mass units heavier than the corresponding oligomers in the aqueous acetone fraction. These results suggest that the PA oligomers that elute with aqueous acetone contain more A-type interflavan bonds. We are currently using reverse phase and normal phase HPLC of Sephadex LH-20 fractions to further separate the cranberry PA by the nature of the interflavan bond and pattern of hydroxylation. These PA fractions are subsequently tested in an in vitro bacterial anti-adherence assay to determine the relationship between PA structure and potential beneficial effects of cranberries on urinary tract infections.

Anthocyanin-polyflavan-3-ols in cranberries

The pigmentation of fruits such as cranberries and grapes is attributed to anthocyanins. However, our results indicate that cranberries contain oligomeric pigments that are similar to structures found in wine (Krueger *et al*., 2004). Kennedy *et al.* (2002) reported that anthocyanins are incorporated in to proanthocyanidins during fruit ripening. While there are few reports of anthocyanin-polyflavan-3-ol oligomers occurring in fruits and unfermented beverages, there are well documented accounts of complex pigments forming in alcoholic beverages such as red wine (Wildenradt et al., 1974; Timberlake *et al*., 1976; Remy *et al.*, 2000) and rose cider (Shoji *et al.*, 1999). During the aging and storage of red wines, anthocyanins are converted to new pigments through reactions with other phenolics such as polyflavan-3-ols. Condensation of an anthocyanin and a polyflavan-3-ol via an ethyl bridge, arising from acetaldehyde, is one mechanism by which anthocyanin-polyflavan-3-ol oligomers may occur (Wildenradt *et al*., 1974; Timberlake *et al.*, 1976; Rivas-Gonzalo *et al*., 1995; Dallas *et al*., 1996; Saucier *et al.*, 1997; Es-Safi *et al.*, 1999). Acetaldehyde is found naturally in wine as either a by-product of yeast metabolism or as an oxidation product of ethanol (Wildenradt *et al*., 1974). Using a model rose cider, Shoji *et al.* (2002) elucidated the structure of such oligomeric pigments by high resolution FAB-MS, ^{1}H and ^{13}C NMR analysis. The dimeric pigments consisted of an anthocyanin linked by a CH_3-CH bridge to

a flavan-3-ol. In addition direct linkages between anthocyanins and PA in wines have been described (Remy *et al*., 2000). Anthocyanin-vinyl-PA linkages were also discovered and may occur as a result of the acetaldehyde condensation reaction (Mateus *et al*., 2002).

Tentative structures were assigned to cranberry anthocyanin-polyflavan-3-ol oligomers by comparing MALDI-TOF mass spectral distributions to predictive equations. In the case of cranberries, Hong and Wrolstad (1990) described the anthocyanins which occur in cranberry fruit; cyanidin-3-galactoside, cyanidin-3-glucoside, cyanidin-3-arabnoside, peonidin-3-galactoside, peonidin-3-glucoside and peonidin-3-arabinoside (Figure 6A). Foo *et al.* (2000b) elucidated the structure of trimeric polyflavan-3-ols from cranberries (3), showing that cranberry poly-flavan-3-ols contained both B-type and A-type interflavan bonds (Δ2 amu). MALDI-TOF MS showed spray dried cranberry juice contained polyflavan-3-ol heteropolymers with structural variation in the degree of hydroxylation of the B-ring (epicatechin or epigallocatechin) and nature of the interflavan bond (A-type and B-type; Δ 2 amu) (Porter *et al.*, 2001). Shoji *et al.* (2002) identified pigments in model rose cider consisting of anthocyanins linked to a flavan-3-ol by a CH_3-CH bridge.

Predictive equations were formulated to describe the heteropolymeric nature of cranberry anthocyanin-polyflavan-3-ol oligomers based on the assumption that the structural diversity seen in polyflavan-3-ols (Porter *et al.*, 2001) and anthocyanins (Hong and Wrolstad, 1990) can be extrapolated to higher degrees of polymerization (DP) when condensation products involving a CH_3-CH bridge (Shoji *et al*., 2002) are applied. Condensation between the A2 dimer described by Foo *et al.* (2000b) and known cranberry anthocyanins through an ethyl bridge would yield (4 a-f).

Whereas MALDI-TOF MS has the power to distinguish molecular weight differences due to extent of hydroxylation (Δ 16 amu), nature of interflavan bonds (A-type or B-type; Δ 2 amu), degree of polyflavan-3-ol polymerization (Δ 288 amu) and substitutions by hexosides (Δ 162 amu) or arabinose (Δ 132 amu), it lacks the ability to assign specific stereochemistry to the molecule. Thus, glucosides cannot be differentiated from galactosides and are simply referred to as hexosides.

An equation was formulated to predict the series of anthocyanins linked to polyflavan-3-ols via a CH_3-CH bridge. The equation is: Anthocyanin + 28 + 288*a* - 2*b*, where anthocyanin represents the molecular weight of the terminal anthocyanin

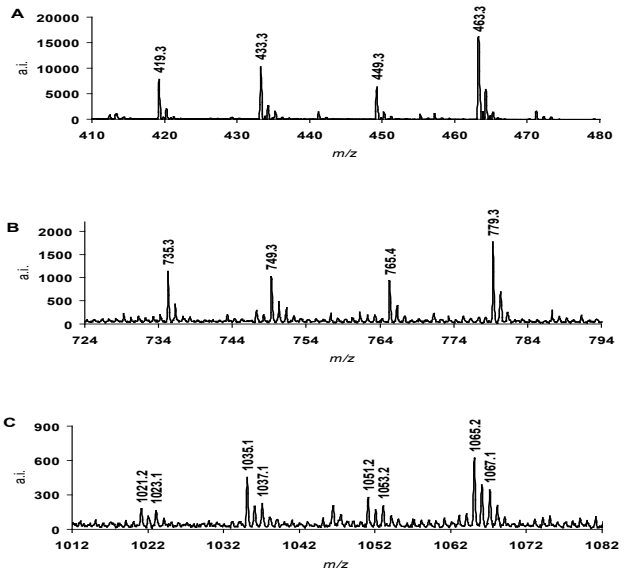

Figure 6. MALDI-TOF positive reflectron mode mass spectra of the anthocyanin-polyflavan-3-ol oligomers of Hyred cranberry fruit and spray dried juice. (A) Anthocyanins $[M]^+$. (B) Anthocyanin linked to a single flavan-3-ol through a CH_3-CH bridge $[M]^+$. (C) Anthocyanin linked to a polyflavan-3-ol of 2 degrees of polymerization through a CH_3-CH bridge, containing either an A-type or a B-type interflavan bond $[M]^+$.

[cyanidin-3-galactoside (449.4 amu), cyanidin-3-glucoside (449.4 amu), cyanidin-3-arabinoside (419.4 amu), peonidin-3-galactoside (463.4 amu), peonidin-3-glucoside (463.4 amu) and peonidin-3-arabinoside (433.4 amu)], 28 is the molecular weight of the CH_3-CH bridge, a is the DP contributed by the repeating flavan-3-ol unit and b is the number of A-type interflavan bonds.

Anthocyanins in the presence of acidic matrixes such as *t*-IAA are predominately in the aromatic oxonium ion form (Wang *et al*., 1999) and are detected as $[M]^+$ ions by MALDI-TOF mass spectral analysis (Wang *et al*., 1999; Wang *et al*., 2000). However, anthocyanins (*23*), in the same manner as polyflavan-3-ols (Ohnishi-Kameyama *et al.*, 1997; Takahata *et al*., 2001; Krueger *et al*., 2003), may also associate with naturally abundant sodium $[M + Na]^+$ and potassium $[M + K]^+$ forming alkali metal adducts. To suppress the formation of alkali metal adducts, we deionized the anthocyanin-polyflavan-3-ol/matrix solution prior to deposition on the target. This approach resulted in the detection of anthocyanin-polyflavan-3-ols in the oxonium ion form $[M]^+$. The MALDI-TOF mass spectra of the water/ethanol (1:1) eluate of both the spray dried cranberry juice and cranberry fruit showed a series of anthocyanins; cyanidin-pentoside (*m/z* 419.3), peonidin-pentoside (*m/z* 433.3), cyanidin-hexoside (*m/z* 449.3) and peonidin-hexoside (*m/z* 463.3) (Figure 6A). MALDI-TOF MS equipped with delayed extraction provide

unit mass resolution, allowing for the visualization of isotopic distribution. The reported observed masses (*m/z*) correspond to the monoisotope of the predicted compound. For example; the predicted and observed monoisotope of cyanidin-pentoside is (*m/z* 419.3), the mass at (*m/z* 420.3) represents the contribution a one heavier isotope to the compound and the mass at (*m/z* 421.3) represents the contribution of two heavier isotopes. Mass calculating programs such as IsoPro 3.0 can be used to predict the isotopic distribution of compounds and allow for comparison between predicted and observed isotopic distributions.

Masses that correspond to a series of anthocyanins linked to a single flavan-3-ol (Δ 288 amu) unit via a CH_3-CH (Δ 28 amu) bridge were also seen; cyanidin-pentoside-flavan-3-ol (*m/z* 735.3), peonidin-pentoside-flavan-3-ol (*m/z* 749.3), cyanidin-hexoside-flavan-3-ol (*m/z* 765.4) and peonidin-hexoside-flavan-3-ol (*m/z* 779.3) (Figure 6B). Again, the observed isotopic distribution is in agreement with the predicted distribution. Masses that correspond to a series of anthocyanins linked to polyflavan-3-ols of 2 degrees of polymerization (DP2) with either A-type and B-type interflavan bonds (Δ 2 amu) were observed; cyanidin-pentoside-DP2 (A-type = *m/z* 1021.2, B-type = *m/z* 1023.1), peonidin-pentoside-DP2 (A-type = *m/z* 1035.1, B-type = *m/z* 1037.1), cyanidin-hexoside-DP2 (A-type = *m/z* 1051.2, B-type = *m/z* 1053.2) and peonidin-hexoside-DP2 (A-type = *m/z* 1065.2, B-type = *m/z* 1067.1) (Figure 6C). The B-type interflavan bonds consist of flavan-3-ol units linked by a carbon-carbon bond at positions C4-C6 or C4-C8, while A-type interflavan bonds contain both a carbon-carbon bond (4β-8) and an ether linkage (2β-*O*-7) between adjacent flavan-3-ol subunits (Figure 6B). The mass difference between a B-type interflavan bond and an A-type interflavan bond is due to the loss of 2 hydrogen atoms (Δ 2 amu) during the formation of the ether linkage.

The isotopic distribution of the anthocyanin-polyflavan-3-ol containing an A-type interflavan linkage will overlap with the isotopic distribution of the anthocyanin-polyflavan-3-ol containing a B-type interflavan linkage. The overlapping isotope distributions results in a summation of the observed intensity. For example, the mass observed at (*m/z* 1023.1) is the contribution of the monoisotope of a cyanidin-pentoside-DP2 with a B-type interflavan bond and the contribution of the 2 amu heavier isotope of the cyanidin-pentoside-DP2 with an A-type interflavan bond. The summation of predicted isotopic distributions generated by IsoPro 3.0 was in agreement with the observed overlapping isotopic clusters.

The MALDI-TOF mass spectra of ethanol eluate contained a series of masses corresponding to anthocyanins linked to polyflavan-3-ols of DP1 to DP4 and the ethanol:methanol (1:1) eluate contained a series masses corresponding to anthocyanins linked to polyflavan-3-ols of DP3 to DP5. As the degree of polymerization of the extending polyflavan-3-ol units increased, the predicted iterations of A-type and B-type interflavan bonds (Δ 2 amu) at each degree of polymerization also increased. As an example; cyanidin-pentoside attached to a polyflavan-3-ol of DP3 is predicted to have 2 B-type interflavan bonds (*m/z* 1311.2), 1 A-type: 1 B-type interflavan bond (*m/z* 1309.3) and 2 A-type interflavan bonds (*m/z* 1307.2), all three predicted masses were observed.

The methanol eluate and 80% aqueous acetone (v/v) eluate contained a series of heteropolyflavan-3-ols which were previously described (Porter *et al*., 2001) and indications of anthocyanin-polyflavan-3-ol oligomers of DP6 and greater. However, due to the large number of individual compounds within the heteropolyflavan-3-ol series and anthocyanin-polyflavan-3-ol series it was difficult to obtain high mass spectral resolution of any individual anthocyanin-polyflavan-3-ol oligomers of higher degrees of polymerization.

The combination of liquid chromatographic separation and MALDI-TOF MS indicates that the structural heterogeneity of cranberry pigments is much greater than previously described. Mass spectral data indicates that cranberry contains a series of anthocyanin-polyflavan-3-ol oligomers, linked through an ethyl bridge, with both A-type and B-type interflavan bonds. However, MALDI-TOF MS only allows for tenative structural assingments, as this analytic technique suffers from the inability to assign specific stereochemistry to the observed compounds. Confirmational NMR analysis of isolated anthocyanin-polyflavan-3-ols of DP2 or DP3, used in concert with MALDI-TOF MS would provide the most solid foundation for the interpretation of novel compounds such as the proposed anthocyanin-polyflavan-3-ols in cranberry fruit.

The incorporation of ethyl linkages between anthocyanins and flavan-3-ols via acetaldehyde condensation reactions has been well described in fermented beverages such as red wine. The presence of acetaldehyde in alcoholic solutions is attributed to either oxidatative products of ethanol or microbial byproducts. In the case of cranberry fruit and spray-dried juice neither ethanol nor microbial fermentation were present. It becomes a concern then that the observed anthocyanin-pigments may be an artifact of harvest, storage, juice processing or analytic

Table 1. Calculated and observed masses for oligomeric elagitannins in pomegranate juice and possible monomeric composition

		Monomeric Composition				
Mass + Cs	Observed Mass	Glucosyl	Gallagic acid	Ellagic acid	Gallic acid	Dehydrodigallic acid
Dimers						
1551	1551	2	0	2	1	1
1701	1701	2	0	3	0	1
1703	1703	2	0	2	2	1
1853	1853	2	0	3	1	1
1855	1855	2	0	2	3	1
2003	2003	2	0	4	0	1
Trimers						
1881	1881	3	0	2	0	2
2335	2336	3	0	3	1	2
2486	2485	3	0	4	0	2
2488	2487	3	0	3	2	2
2640	2639	3	0	3	3	2
2788	2787	3	1	2	2	2
Tetramers						
3270	3270	4	0	5	0	3
3272	3272	4	0	4	2	3
Pentamers						
4055	4055	5	0	6	0	4
4057	4056	5	0	5	2	4

techniques.

Ethanol was also used in the elution of phenolics from the Sephadex LH20 column. While acetaledhyde is generated from oxidation of ethanol in the presence of phenolic compounds (Wildenradt *et al.*, 1974), the model solutions used to investigate this reaction are typically carried out under conditions of high temperature (> 50ºC) and for long periods of time (> 30 days). Our analysis of anthocyanin-polyflavan-3-ols from cranberry fruit was completed in less than 12 hours, from the time of extraction to the time of mass spectral analysis. We propose it is more likely that the anthocyanin-polyflavan-3-ol oligomers arise as a result of fruit ripening than as an artifact of analytic analysis. Biologically available acetaldehyde in the ripening berry may be used in the condensation reaction between anthocyanins and polyflavan-3-ols. Cranberries produce acetaldehyde during ripening and harvest (Krueger *et al.*, 2004).

Ellagitannins in pomegranate

Consumption of pomegranate juice by healthy male volunteers and in the atherosclerotic apolipoprotein E deficient (E^o) mice decreased LDL atherogenic modifications, including its oxidation, aggregation and retention (Aviram *et al*., 2000; Kaplan *et al*., 2001). Tanaka *et al*. (1985, 1986a, and 1986b) determined the structures of several ellagitannins isolated from pomegranate fruits leaves and stems. These compounds include punicalin (5) and punicalagin (6), which are esters of gallagic acid.

We recently characterize the structural diversity of ellagitannins in pomegranate juice. A crude preparation of the polyphenolic fraction of PJ was obtained by absorption on to Sephadex LH20. An oligomeric series of at least 22 hydrolyzable tannins

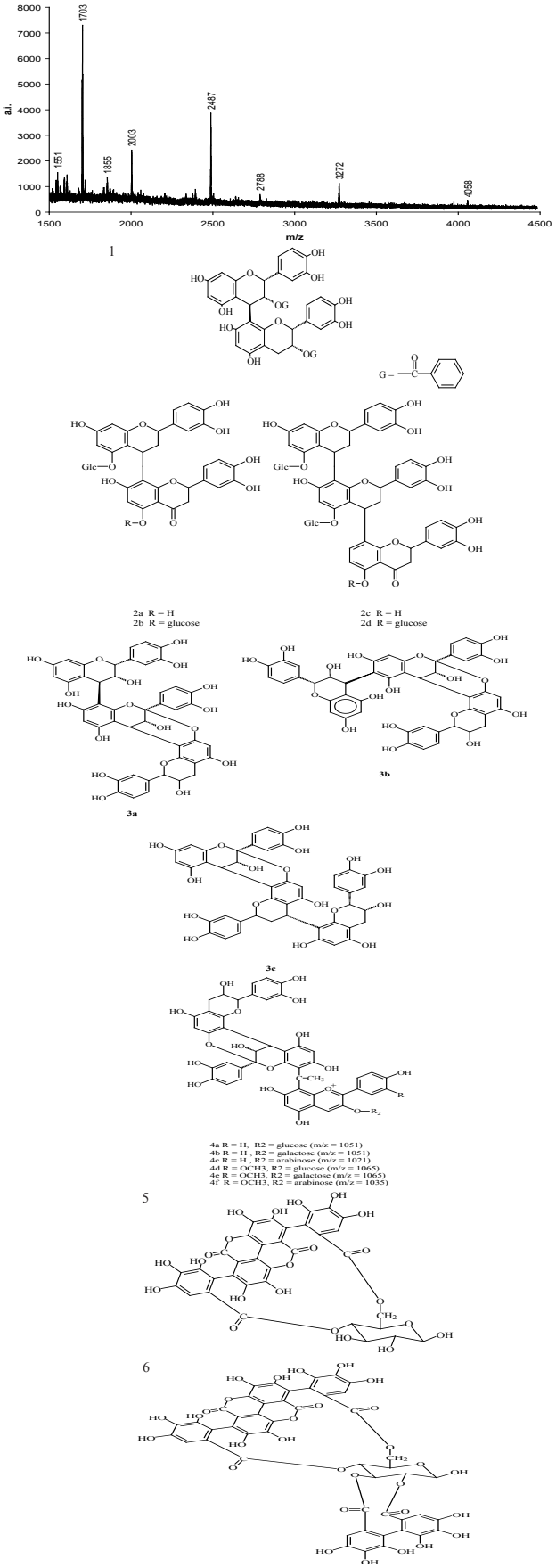

Figure 7. Matrix assisted laser desorption time of flight mass spectrum of olligomeric ellagitannins in pomegranate juice. The spectrum is the sum of 100 to 500 shots for positive reflectron mode, calibrated with bradykinin (1060.6 MW) and glucagon (3483.8 MW). Labeled masses are the molecular ions minus 1 proton plus Cs^+. Masses between 1551 and 2003 correpond to dimers, masses between 1881 and 2788 correspond to trimers, the mass at 3272 corresponds to a tetramer and the mass at 4058 corresponds to a pentamer (see table 1 for more explanation).

were detected with masses between 500 and 4058. Masses less than 1500 corresponded to known pomegranate ellagitannins and gallotannin structures, such as punicalagin as described by Tanaka *et al.* (1985, 1986a, 1986b).

The higher masses correspond to structures of oligomeric ellgitannins in which two or more core glucose units are cross-linked by dehydrodigalloyl or valoneoyl units (Figure 7, Table 1). This is the first time that these higher oligomers have been detected in pomegranates, although they are known to occur in other plants (Quideau and Feldman, 1996), and more research is required to elucidate their structure.

Conclusions

The excellent research carried out on the absolute structures of dimers and trimers of oligomeric polyphenols indicates that there is large structural heterogeneity within and among plant species. Our research using MALDI-TOF MS indicates that this structural heterogeneity increases with degree of polymerization. Mass spectrometry does not distinguish between isomers, yet several stereo and structural isomers of dimers and trimers have been described. Therefore, the number of possible structures greatly increases by isomeriazation at each of the masses detected by MALDI-TOF MS. For instance, in cranberries Foo *et al*. (2000a, 2000b) determined the structures of 3 trimeric proanthocyanidins 3 with the same mass. If this structural diversity continues with each degree of polymerization then the number of possible compounds becomes very large indeed.

There also appears to be much structural heterogeneity of oligomeric polyphenols among plants species (Ferreira and Bekker, 1996). This fact suggests that methods for quantification and characterization need to be plant specific. General methods that attempt to quantify and compare effects of oligomeric plant polyphenols across plant species may be misleading. For instance, methods that use a combination of LC-MS with homopolymeric procyanidins standards (Hammerstone *et al*., 2000) are not applicable to common foods and nutritional supplements such as grapes, cranberries, and sorghum, because these foods do not contain homopolymers and simple liquid chromatographic methods will not be capable of adequate separations in these plant species.

The results on the application of MALDI-TOF MS clearly demonstrate its power as tool to characterize the nature of PA oligomers. However, the problem of its usefulness as a quantitative tool remains a critical aspect of its application. Several of the above cited authors have claimed that the pattern of observed masses in MALDI-TOF MS spectra reflect the composition of the sample (Ohnishi-Kameyama *et al.*, 1997; Wang *et al*., 1999; Foo *et al.*, 2000a). On the other hand, a pattern of decreasing signal intensity with increasing DP is characteristic of MALDI-TOF MS spectra of polydisperse oligomers (Montaudo *et al*., 1995; Chan and Chan, 2000). This characteristic pattern results from detector response because the detector has a finite capacity and the lower masses reach the detector first. This phenomenon results in a spectrum in which the lower masses have a greater peak response than higher masses even when the oligomers are present in the sample at similar concentrations. Our results from applications of MALDI-TOF MS to characterize PA in sorghum, GSE, cranberries and several forage legumes typically yields spectra in which intensity declines in a regular pattern as DP of PA oligomers increase. Therefore, the peak response may not give a true indication of relative concentration of oligomers in the sample. However, the repeatability of peak response allows rapid characterization of the range of DP within PA fractions. This information can be used along with knowledge of dimer and trimer structure to obtain a more precise description of the higher oligomers. As separation technology advances along with improved instrumentation for NMR and MS our ability to determine structure functions relationships of oligomeric polyphenols in foods will improve. Although advances in this research will be slow, it is greatly needed to determine the effects of polyphenols in foods on nutrition and health. MALDI TOF MS is a valuable tool for this research.

Acknowledgements

We thank Dr. Martha Vestling, Department of Chemistry, University of Wisconsin-Madison, for her collaboration on MALDI-TOF MS. Dr. Amy Howell, Marucci Center for Blueberry Cranberry Research, Rutgers University NJ and Dr. David Cunningham, Ocean Spray Cranberries, Inc., MA, for their insights and collaboration on proanthocyanidins in cranberries. Our research on cranberries was funded by Ocean Spray Cranberries, Inc., USDA award number 2002-35503-12297, and the University of Wisconsin-Madison Hatch grant WISO4525. The authors thank Dr. John Folts for collaboration on the studies of grape seed extracts. We wish to thank Paul Stittt of Natural Ovens for stimulating our interest in s=high tannin sorghums. Our sorghum research was funded by Natural Ovens Inc. Manitowoc, WI, The National Grain Sorghum Producers, TX, and

the University of Wisconsin I&EDR grant 0310. We thank Prof. Michael Aviram for supplying pomegranate samples and for his collaboration on our pomegranate research.

References

Asenstorfer, R. E., Hayasaka Y. and Jones, G. P. 2001. Isolation and structures of oligomeric wine pigmetns by bisulfite-mediated ion-exchange chromatography. Journal of Agricultural and Food Chemistry 59: 5957-5963.

Aviram M., Dorenfeld, L., Rosenblat, M., Volkova, N., Kaplan, M., Hayek, T., Presser, D. and Fuhrman, B. 2000. Pomegranate juice consumption reduces oxidative stress, atherogenic modifications to LDL, and platelet aggregation: studies in humans and in the atherosclerotic apolipoprotein E deficient mice. American Journal of Clinical Nutrition 71: 1062-1076.

Brandon, M.J., Foo, L.Y., Porter, L.J. and Meredith, P. 1982. Proanthocyanidins of barley and sorghum composition as a function of maturity of barley ears. Phytochemistry 21: 2953-2957.

Chan, P. K. and Chan, T. W. D. 2000. Effect of sample preparation methods on the analysis of dispersed polysaccharides by matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry. Rapid Communications in Mass Spectrometry 14: 1841-1847.

Dallas, C., Ricardo-da-Silva, J. M. and Laureano, O. 1996. Products formed in model wine solutions involving anthocyanins, procyanidin B2, and acetaldehyde. Journal of Agricultural and Food Chemistry 44: 2402-2407.

Es-Safi, N., Fulcrand, H., Chenynier, V. and Moutounet, M. 1999. Studies on the acetaldehyde-induced condensation of (-)epicatechin and malvidin3-O-glucoside in a model solution system. Journal Agricultaul and Food Chemistry 47: 2096-2102.

Ferreira, D. and Bekker, R. 1996. Oligomeric proanthocyanidins: naturally occuring o-heterocycles. Natural Product Reports 13: 411-433.

Foo, L.Y., Lu Y., Howell, A.B. and Vorsa, N. 2000a. The structure of cranberry proanthocyanidins which inhibit adherence of uropathogenic p-fimbriated *Escherichia coli in vitro.* Phytochemistry 54: 173-181.

Foo, L.Y., Lu, Y., Howell, A.B. and Vorsa, N. 2000b. A-Type Proanthocyanidin Trimers from Cranberry that Inhibit Adherence of Uropathogenic P-Fimbriated *Escherichia coli.* Journal of Natural Products 63: 1225-1228.

Gujer, R., Magnalato, D. and Self, R. 1986. Glucosylated flavonoids and other phenolic compounds from sorghum. Phytochemistry 25: 1431-1436.

Gupta, R.K. and Haslam, E. 1978. Plant proanthocyanidins 5. sorghum polyphenols. Journal of the Chemical Society- Perkin Transactions 1 8: 892-896.

Guyot, S., Doco, T., Souquet, J., Moltounet, M., Drilleau, J. 1997. Characterization of highly polymerized procyanidins in cider apple (*Malus Sylvestris* Var. Kermerrien) skin and pulp. Phytochemistry 44, 351-357.

Hammerstone, J.F., Lazarus, S.A. and Schmitz, H.H. 2000. Procyanidin content and variation in some commonly consumed foods. Journal of Nutrition 130: 2086S-2092S.

Hammerstone, J.F., Lazarus, S.A., Mitchell, A.E., Rucker, R. and Schmitz, H. H. 1999. Identification of procyanidin in cocoa (*Theobroma cacao*) and chocolate using high-perfrormance liquid chromatography/ mass spectrometry. Journal of Agricultual and Food Chemistry 47: 490-496.

Hanton, S.D. 2001. Mass spectrometry of polymers and polymer surfaces. Chemical Reviews 101, 527-569.

Hayasaka, Y. V. and Asenstorfer, R.E. 2002. Screening for potential pigments derived from anthocyanins in red wine using nanoelectrospray tandem mass spectrometry. Journal of Agricultural Food Chemistry 50: 756-761.

Hong, V. and Wrolstad, R. 1990. Use of HPLC separation/ photodiode array detection for characterization of anthocyanins. Journal of Agricultural and Food Chemistry 38: 708-715.

Kaplan, M., Hayek, T., Raz, A., Coleman, R., Dornfeld, L., Vaya, J. and Aviram, M. 2001. Pomegranate juice supplementation to atherosclerotic mice reduces macrophage lipid peroxidation, cellular cholesterol accumulation and development of atherosclerosis. Journal of Nutrition 131: 2082-2089.

Kennedy, J.A., Matthews, M.A. and Waterhouse, A.L. 2002. Effect of maturity and vine water status on grape skin and wine flavonoids. American Journal Enolology and Viticulture 53: 268-274.

Krueger, C.G., Dopke, N.C., Treichel, P.M., Folts, J. and Reed, J.D. 2000. Matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry of polygalloyl polyflavan-3-ols in grape seed extract. Journal of Agricultural and Food Chemistry 48: 1663-1667.

Krueger, C.G., Vestling, M.M. and Reed, J.D. 2003. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of heteropolyflavan-3-ols and glucosylated heteropolyflavans in sorghum [*Sorghum bicolor* (L.) Moench)]. Jouranl of Agricultural and Food Chemistry 53: 538-543.

Krueger, C.G., Vestling, M.M. and Reed, J.D. 2004. Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry of Anthocyanin-Polyflavan-3-ol Oligomers in Cranberry Fruit [*Vaccinium macrocarpon*, Ait.] and Spray Dried Cranberry Juice. In: Red Wine Color: Revealing the Mysteries. ACS Symposium Series 886. A. L. Waterhouse and J. A Kenedy, eds. American Chemical Society, Washington , DC. pp. 232-246.

Lazarus, S.A., Adamson, G.E., Hammerstone, J.F. and Schmitz, H. H. 1999. High-performance liquid chromatography/mass spectrometry analysis of proanthocyanidins in foods and beverages. Journal of

Agricultural Food Chemistry 47: 3693-3701.

Macha, S.F., Limbach, P.A., Hanton, S.D. and Owens, K.G. 2001. Silver cluster interferences in matrix-assisted laser desorption/ionization (MALDI) mass spectrometry of nonpolar polymers. Journal of the American Society for Mass Spectrometry 12: 732-743.

Mateus, N., Silva, A.M.S.C., Santos-Buelga, Rivas-Gonzalo, J.C. and De Freitas, V.. 2002. Identification of anthocyanin-flavanol pigments in red wines by NMR and mass spectrometry. Journal of Agricultural and Food Chemistry 50: 2110-2116.

Montaudo, G., Garozzo, D., Montaudo, M.S., Puglisi, C. and Samperi, F. 1995. Molecular and structural characterization of polydisperse polymers and copolymers by combining MALDI-TOF mass spectrometry with GPC fractionation. Macromolecules 28: 7983-7989.

Montaudo, G., Montaudo, M.S. and Samperi, F. 2002. Martix-assisted laser desorption/ionization mass spectrometry of polymers (MALDI-MS). In Mass Spectrometry of Polymers; Montaudo, G., Lattimer, R. P., Eds.; CRC Press: Boca Raton, FL, pp 419-521.

Ohnishi-Kameyama, M., Yanagida, A., Kanda, T. and Nagata, T. 1997. Identification of catechin oligomers from apple (*Malus pumila* cv. Fuji) in matrix-assisted laser desroption/ionization time-of-flight mass spectrometry and fast-atom bombardment mass spectrometry. Rapid Communications in Mass. Spectrometry 11: 31-36.

Porter, M.L., Krueger, C.G., Wiebe, D.A., Cunningham, D.G. and Reed, J. D. 2001. Cranberry Proanthocyanidins Associate with Low-Density Lipoprotein and Inhibit In Vitro Cu^{2+}-Induced Oxidation. Journal of the Science of Food and Agriculture 81: 1306-1313.

Quideau, S. and Feldman, K. 1996. Ellagitannin chemistry. Chemical Reviews 96: 475-503.

Remy S., Fulcrand, H., Labarbe, B., Cheynier, V. and Moutounet, M. 2000. First confirmation in red wine of products resulting from direct anthocyanin-tannin reactions. Journal of the Science of Food and Agriculture 80: 745-751.

Remy-Tanneau, S., Le Guerneve, C., Meudec, E. and Cheynier, V. 2003. Characterization of a colorless anthocyanin-flavan-3-ol dimer containing both carbon-carbon and ether interflavanoid linkages by NMR and mass spectrometry. Journal of Agricultural and Food Chemistry 51: 3592-3597.

Ricardo-Da-Silva, J.M., Riguad, J., Cheynier, V., Chemiant, A. and Moutounet, M. 1992. Procyanidin dimers and trimers from grape seeds. Phytochemistry 30: 1259-1264.

Rivas-Gonzalo, J.C., Bravo-Haro, S. and Santos-Buelga, C. 1995. Detection of compounds formed through the reaction of malvidin 3-monoglucoside and catechin in the presence of acetaldehyde. Journal of Agricultural and Food Chemistry 43: 1444-1449.

Santos-Buelga, C. and Scalbert, A. 2000. Proanthocyanidins and tannin-like compounds-nature, occurrence, dietary intake and effects on nutrition and health. Journal of the Science of Food and Agriculture 80: 1094-1117.

Saucier, C., Guerra, C., Pianet, I., Laguerre, M. and Glories, Y. 1997. (+)-catechin-acetaldehyde condensation products in relation to wine-aging. Phytochemistry 46: 229-234.

Scalbert, A. and Williamson, G. 2000. Dietary intake and bioavailability of polyphenols, Journal of Nutrition 130: 2073S-2085S.

Shoji, T., Yanagida, A. and Kanda, T. 1999. Gel permeation chromatography of anthocyanin pigments from rose cider and red wine. Journal of Agricricultural and Food Chemistry 47: 2885-2890.

Shoji, T., Goda, Y., Toyota, M., Yanagida, A. and Kanda, T. 2002. Characterization and structures of anthocyanin pigmetns generated in rose cider during vinification. Phytochemistry 59: 183-189.

Takahata,Y. Ohnishi-Kameyama, M., Furuta, S., Takahashi, M. and Suda, I. 2001. Highly polymerized procyanidins in brown soybean seed coat with a high radical-scavenging activity. Journal of Agricultural and Food Chemistry 49: 5843-5847.

Tanaka, T., Nonaka, G. and Nishioka, I. 1985. Punicafolin, an ellagitannin from the leaves of *Punica granatum*. Phytochemistry 24: 2075-2078.

Tanaka, T., Nonaka, G. and Nishioka, I. 1986a. Tannins and related compounds. XL. Revision of the structures of punicalin and punicalagin, and isolation and characterization of 2-O-galloylpunicalin from the bark on *Punica granatum* L. Chemical and Pharmaceutical Bulletin 34: 650-655.

Tanaka, T., Nonaka, G. and Nishioka, I. 1986b. Tannins and related compounds. XLI. Isolation and characterization of novel ellagitannins, puicacorteins A, B, C and D, and punigluconin from the bark of *Punicum granatum* L. Chemical and Pharmaceutical Bulletin 34: 656-663.

Timberlake, C.F. and Bridle, P. 1976. Interactions between anthocyanins, phenolic compounds, and acetaldehyde and their significance in red wines. American Journal Enology and Viticulture 27: 97-105.

Wang, J. and Sporns, P. 1999. Analysis of anthocyanins in red wine and fruit juices using MALDI-TOF MS. Journal of Agricultural and Food Chemistry 47: 2009-2015.

Wang, J., Kalt, W. and Sporns, P. 2000. Comparison between HPLC and MALDI-TOF MS analysis of anthocyanins in highbush blueberries. Journal of Agricultural and Food Chemistry 48: 3330-3335.

Wildenradt, H.L. and Singleton, V. L. 1974. The production of aldehydes as a result of oxidation of polyphenolic compounds and its relation to wine aging. American Journal Enology and Viticulture 25: 119-126.

Yang, Y. and Chien, M. 2000. Characterization of grape procyanidins using high-performance liquid chromatography/mass spectrometry and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Journal of Agricultural and Food Chemistry 48: 3990-3996.