

Review

MALDI-TOF mass spectrometry of oligomeric food polyphenols

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Abstract

The 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. Spectrometric 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- and B-types) 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 that correspond to previously described structures were detected, such as punicalagin, but others that correspond to oligomeric ellagitannins in which two to five core glucose units are cross-linked by dehydrodigalloyl and/or valoneoyl units were also observed. Results demonstrate that large heterogeneity occurs in degree of polymerization, intermolecular bonds, pattern of hydroxylation, and substitution with monosaccharides and gallic acid.

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Keywords: Proanthocyanidins; Ellagitannins; Cranberry; Sorghum; Grape seed extract; Pomegranate; Tannins

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1. 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 and Williamson, 2000). However, tannins are diverse compounds with great 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 matrix assisted laser desorption time of flight mass spectrometry (MALDI-TOF MS) for analysis of oligomeric polyphenols in foods have appeared within the last 7 years (Ohnishi-Kameyama et al., 1997; Krueger et al., 2000; 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 degree of polymerization (DP) and structure of cranberry PA oligomers that are associated with the in vitro inhibition of adhesion of *Escherichia 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 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 demon-

strated 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 an HP 1100 single quadrupole 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; Hayasaka and Asenstorfer, 2002; Mateus et al., 2002; Remy-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 (Montaudou et al., 2002). Alternatively, MALDI-TOF MS is ideally suited for characterizing polydispersed oligomers (Hanton, 2001) and is considered the mass spectrometric 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 (Montaudou et al., 2002).

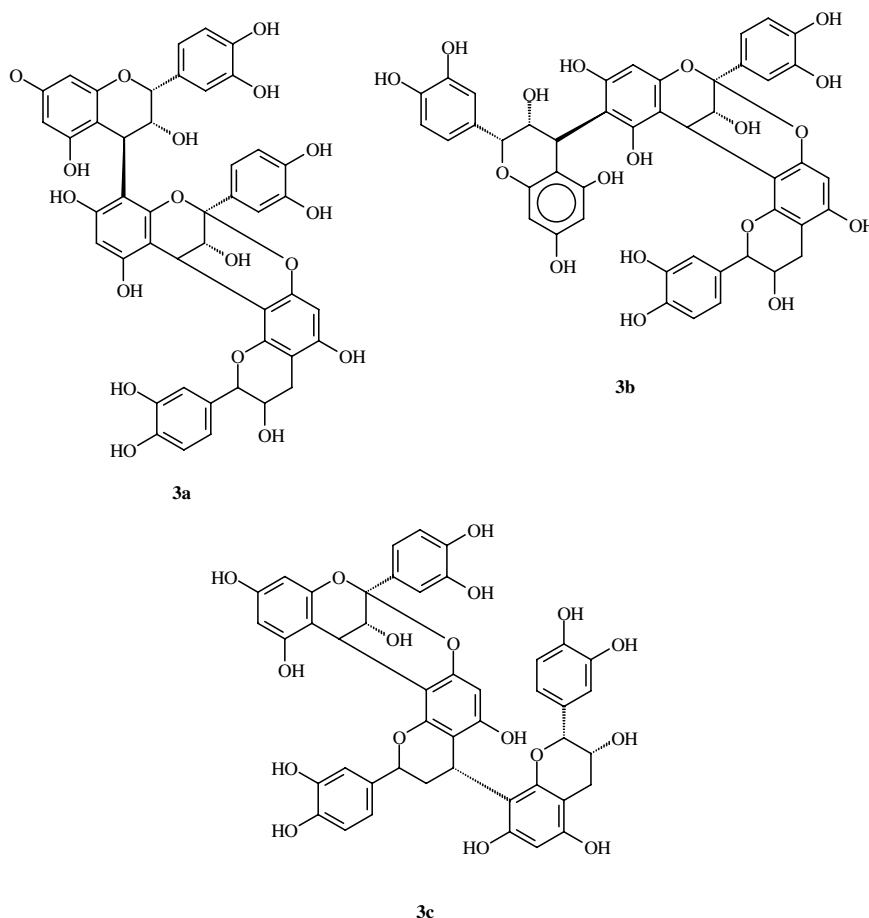
In this paper we report our research on the characterization of complex mixtures of oligomeric polyphenolic compounds in fruits using a combination of liquid chromatography and MALDI-TOF MS. The advantages of MALDI-TOF MS are; only one molecular ion is formed from each parent molecule, high sensitivity 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 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. Of course, MS alone does not distinguish among isomers and gives no information regarding regiochemistry of C–C and C–O bonds. These limitations present a problem to the application of MS to the analysis of oligomeric polyphenols because, as the DP increases, the number of possible chiral carbon atoms also increase. In addition, the position of monomer additions may differ among oligomers of the same DP, as in the case of 4 β -8 or 4 β -6 addition of epicatechin to cranberry procyanidin trimers, **3a** and **3b**, respectively (Foo et al., 2000b). Isolation of individual isomers and

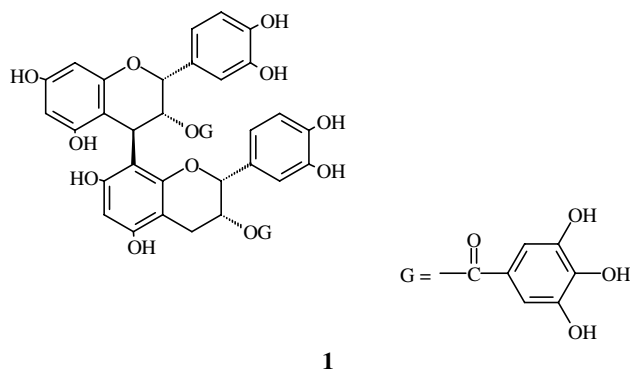
characterization by a combination of chemical and physical techniques are required to determine absolute structure. However, the large number and structural heterogeneity of oligomeric polyphenols in foods makes chromatographic isolations difficult. On the other hand, interest in the nutritional and health related effects have created the need for methods to better characterize structural features of mixtures of oligomeric polyphenols that may be related to bioactivity. Time of flight analysis of molecular ions coupled with a knowledge of dimer and trimer structure provides a powerful tool for characterization of the higher oligomers. This review presents a summary of our previously published research on the use of MALDI-TOF MS to characterize tannins from grapes, sorghum, cranberries and pomegranates.

2. 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 B₂ 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. Based on the galloylated structures described by Ricardo-Da-Silva et al. (1992), an equation was developed to predict the mass distribution of poly-galloyl-poly-flavans 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 weight of sodium.



The MALDI-TOF spectrum of the grape seed extract in *trans*-indole acrylic acid detected masses that correspond to an oligomeric series of catechin/epicatechin units up to the nonamer in the positive-ion reflectron mode (Fig. 1) and up to the undecamer in the

positive-ion linear mode (Fig. 2) (Krueger et al., 2000). Additionally masses corresponding to a series of poly-galloyl poly-flavans were 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. The 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 (Fig. 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; $290 + 288c + 152g + 23$ (Fig. 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

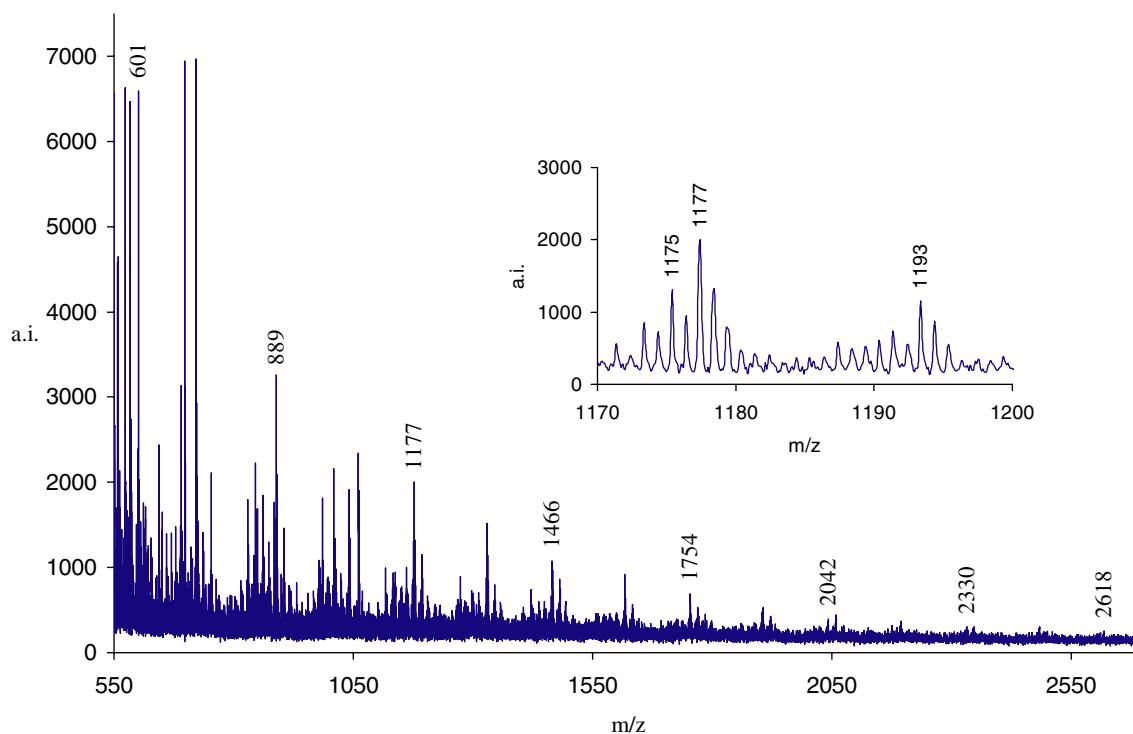


Fig. 1. MALDI-TOF mass spectrum in positive reflectron mode, showing a procyanidin series $[M + Na^+]$ from the dimer (601 m/z) to the nonamer (2618 m/z). Inset is enlarged spectrum of the tetramer (1177 m/z) and the digalloyl-trimer (1193 m/z) representing isotope distribution patterns and an uncharacterized mass series (1175 m/z) two molecular weight units lower than predicted values.

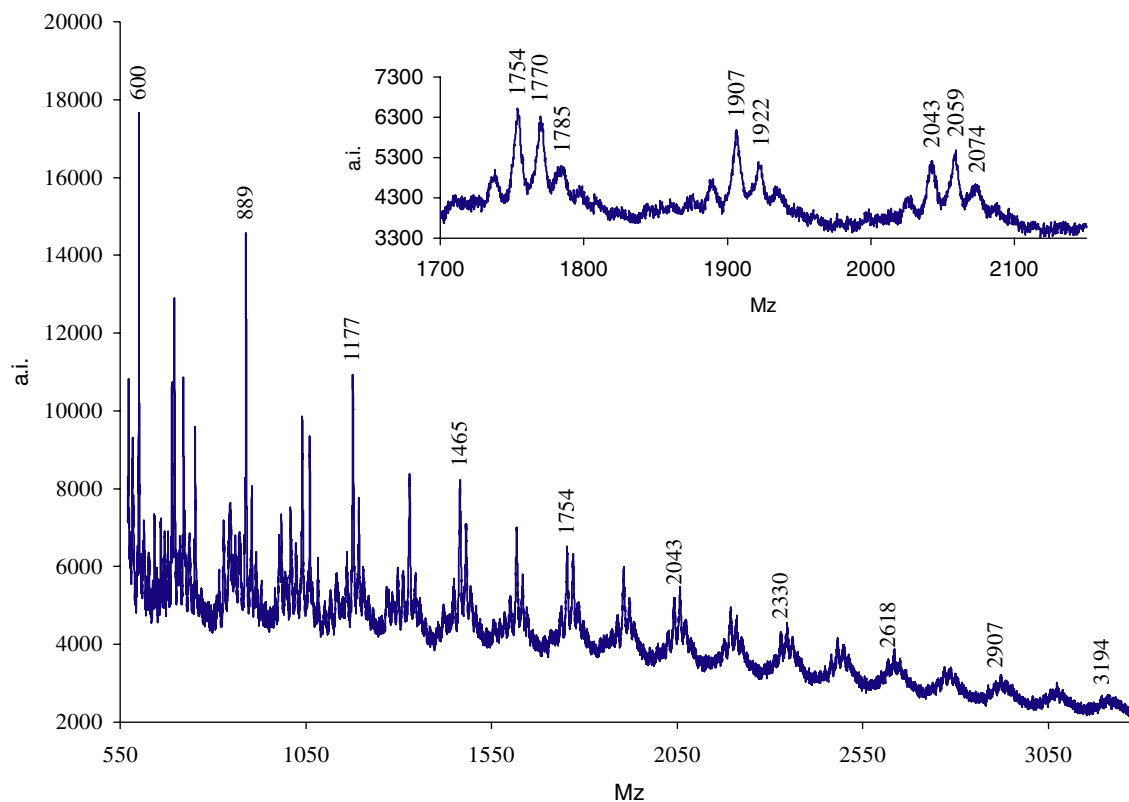


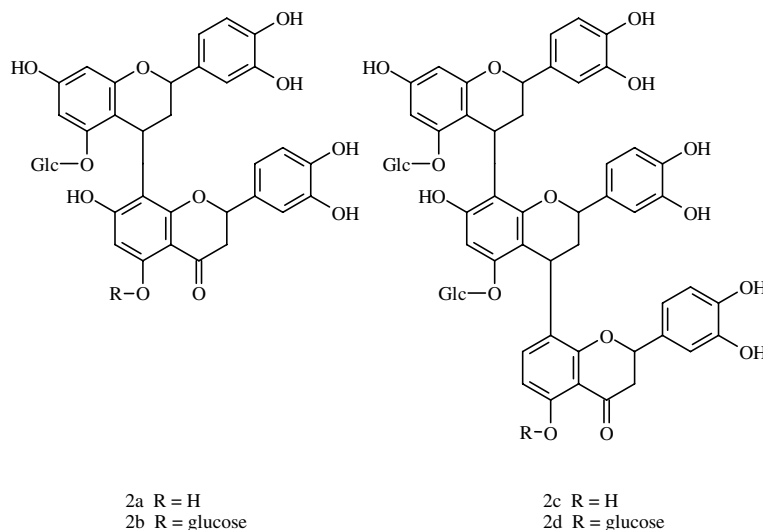
Fig. 2. MALDI-TOF mass spectrum in positive linear mode, showing a procyanidin series $[M + Na^+]$ from the dimer ($600\text{ }m/z$) to the undecamer ($3194\text{ }m/z$). Inset is an enlarged spectrum of masses representing a procyanidin series with varying degree of galloylation.

improve our understanding of the structural complexity of grape seed tannins.

3. 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 glycosylated

at 5-OH with a flavanone, eriodictyol or eriodictyol-5-*O*- β -glucoside, terminal unit (**2**). We also detected heterogeneity in the 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 polyvinylpyrrolidone column and subjecting the anthocyanins to MALDI-TOF MS. The anthocyanins produced were luteolinidin (m/z 271) and apigeninidin (m/z 255).



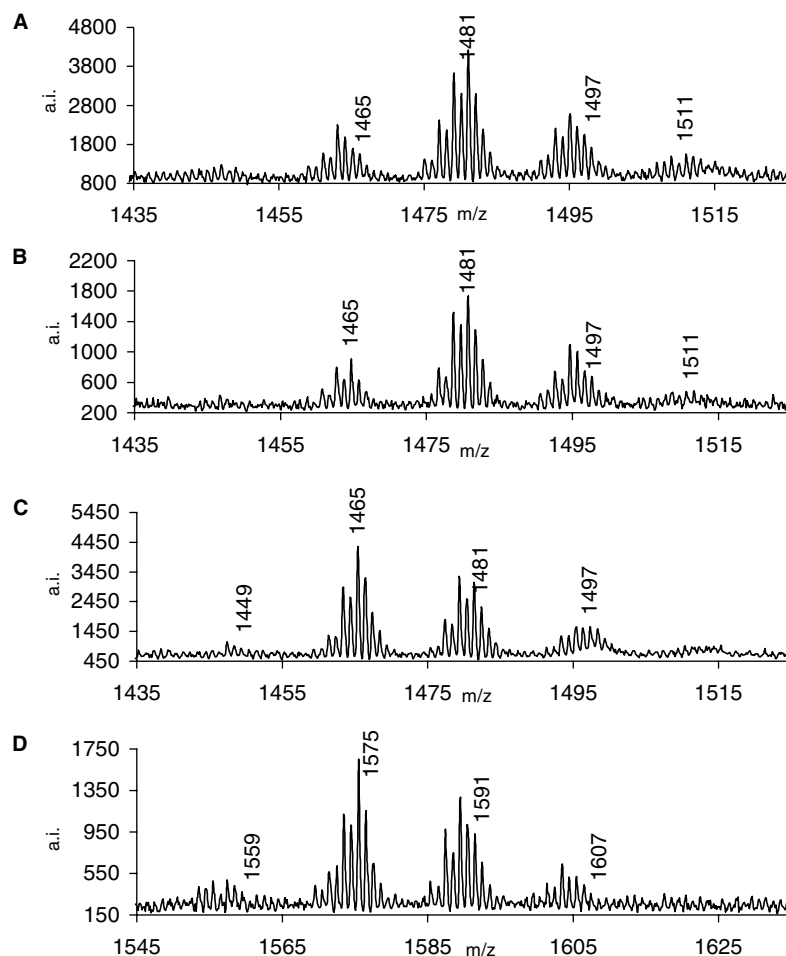


Fig. 3. MALDI-TOF positive reflectron mode mass spectra of the heteropolyflavan-3-ol pentamers in the Sephadex LH20-methanol eluate of Ruby Red sorghum (*Sorghum bicolor* (L.) monech). (A) No deionization or addition of cation to the matrix:analyte prior to deposition on the target. (B) Deionization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

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) (Fig. 3). The second series, separated by a glucose substitution ($\Delta 162$ amu), represents glucosylated heteropolyflavans containing the flavanone, eriodictyol 5-*O*- β -glucoside as the terminal unit and extend from the trimer (m/z 1451) to the heptamer (m/z 3188).

Each DP within the glucosylated heteropolyflavan mass series had a subset of masses ($\Delta 16$ amu) lower than the proluteolinidin oligomer in which all of the glucosylated flavan units are hydroxylated at both the 3' and 4' positions of the B-ring. This pattern of hydroxylation gives four predicted tetramer masses that were also observed; m/z 1837 (three proluteolinidin + 0 proapigeninidin), m/z 1853 (two proluteolinidin + one proapigeninidin), m/z 1869 (one proluteolinidin + pro-

apigeninidin) and m/z 1885 (0 proluteolinidin + three proapigeninidin), each with eriodictyol 5-*O*- β -glucoside as the terminal unit. The mass differences are explained by the hydroxyl substitutions ($\Delta 16$ amu) at either the 3' or 3' and 4' positions of the B-ring of the 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 less hydroxyl ($\Delta 16$ amu) substitution.

Another possible explanation for detection of ($\Delta 16$ 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 $\Delta 15.9739$ 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 $[\text{M} + \text{Na}]^+$ and $[\text{M} + \text{K}]^+$ 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; $[\text{M} + \text{K}]^+$) assuming that naturally abundant K^+ are the most abundant ions. The predicted value was observed as the most abundant mass (m/z 1481; $[\text{M} + \text{K}]^+$). 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 ($\Delta 16$ amu) or multiple cation adducts ($[\text{M} + \text{Na}]^+$ and $[\text{M} + \text{K}]^+$) 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; $[\text{M} + \text{K}]^+$) remaining as the most abundant mass (Fig. 3).

Ambiguity remained as to whether the mass at (m/z 1465) was due to either a compound containing one fewer hydroxyl substitutions ($\Delta 16$ amu) than the predicted homopolymer or to the inability of the cation exchange resin to remove all Na^+ (m/z 1465; $[\text{M} + \text{Na}]^+$). 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; $[\text{M} + \text{Na}]^+$) assuming that Na^+ is the only ion present. The entire mass distribution shifted $\Delta 16$ amu lower than the original spectra (Fig. 3). The predicted value was again observed as the most abundant mass (m/z 1465; $[\text{M} + \text{Na}]^+$). Again, masses 16 amu lighter, 16 and 32 amu heavier (m/z 1449, m/z 1481, and m/z 1497) than the predicted homopolymer were also observed (Fig. 3). Ambiguity remained as to whether the mass at (m/z 1481) was due to a compound containing one greater hydroxyl substitution ($\Delta 16$ amu) than the predicted homopolymer or the inability of the cation exchange resin to remove all K^+ (m/z 1481; $[\text{M} + \text{K}]^+$). 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 $[\text{M} + \text{K}]^+$ and $[\text{M} + \text{Na}]^+$ confound interpretation.

Ohnishi-Kameyama et al. (1997) and Takahata et al. (2001) employed silver trifluoroacetate to suppress cationization with Na^+ and K^+ $[\text{M} + \text{alkali metal}]^+$. However, addition of Ag^+ created two new problems. Silver has two naturally occurring isotopes; ^{107}Ag (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 $[\text{M} + \text{Ag}]^+$ ions differing by 2 amu. This is particularly confusing for analysis of polyflavans which contain both B- and A-types 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 $[\text{M} + \text{Cs}]^+$ molecular ions (Fig. 3), eliminating the possibility that the $\Delta 16$ amu was due to the formation of both $[\text{M} + \text{Na}]^+$ and $[\text{M} + \text{K}]^+$ adduct ions from one species. The mass distribution provides evidence for a class of heteropolyflavan-3-ols that have one fewer hydroxyl substitution ($\Delta 16$ 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 gallo catechin/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- and B-types) and substitutions with moieties such as glucose. In light of this structural diversity a more descriptive nomenclature must be employed when describing heteropolyflavans.

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

Foo et al. (2000a,b) elucidated the trimeric structures of cranberry "A-type" proanthocyanidins **3** that are associated with the inhibition of adhesion of p-fimbriated *E. coli* to uroepithelial cells. We fractionated cranberry flavonoids on Sephadex LH-20 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 the lag time of oxidation. The MALDI-TOF MS spectra 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 four (Fig. 4). The pentamer and hexamer had more peaks than could be explained by a predicted isotope pattern for the simple oligomers with one A-type interflavan bond (Fig. 4B and C). These peaks were interpreted to represent PA with two A-type ether linkages present in the oligomer because the pattern corresponded to a two 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 two or three A-type interflavan bonds in each oligomer. These results indicated that the DP of PA in the acetone fraction was greater than the DP of PA in the methanol fraction and that the acetone fraction contained several oligomers with more than one A-type interflavan bond.

Fig. 5 shows previously unpublished MALDI-TOF MS spectra of cranberry PA that were extracted from press cake and fractionated on Sephadex LH-20 by the methods described by Porter et al. (2001). Proanthocyanidin oligomers with a DP of four to 13 (1191–3788 m/z) were detected in the fraction that eluted with methanol (Fig. 5A). Proanthocyanidin oligomers with a DP of five to 23 (1476–6662 m/z) were detected in the fraction that eluted with aqueous acetone (Fig. 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 (Fig. 5C). The broad peaks in these spectra 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 four to 13 in the methanol fraction were three 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 subsequently will be tested in an *in vitro* bacterial anti-

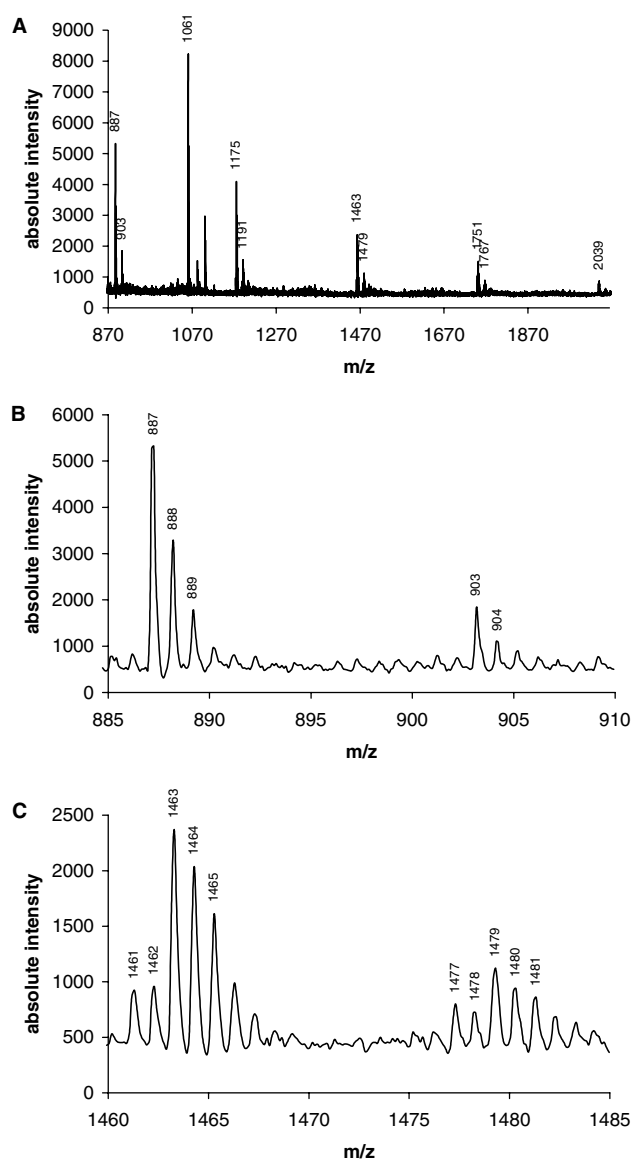


Fig. 4. Matrix assisted laser desorption-time of flight mass spectra of proanthocyanidins from cranberries. The proanthocyanidins were obtained by elution from a Sephadex LH-20TM column with methanol (fraction 5). (A) Spectra of the trimers through heptamers. The signal at m/z 1061 is bradykinin, used 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 mass units. This difference may result from proanthocyanidins with 2 A-type interflavan bonds.

adherence assay to determine the relationship between PA structure and potential beneficial effects of cranberries on urinary tract infections.

5. Anthocyanin-polyflavan-3-ols in cranberries

The pigmentation of fruits such as cranberries and grapes is attributed to anthocyanins. However, our

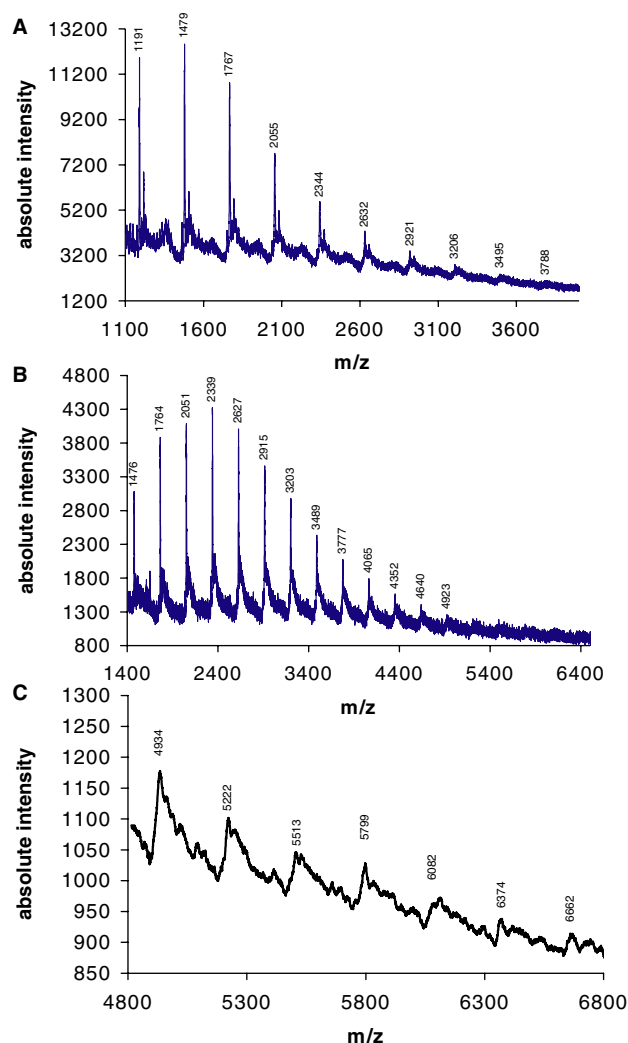


Fig. 5. MALDI-TOF mass spectra of proanthocyanidins from the aqueous acetone extraction of cranberry press cake. The proanthocyanidin fractions were obtained by elution from a Sephadex LH-20TM column with methanol (A) or aqueous acetone (B). The spectrum of the aqueous acetone fraction indicates that 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 4800–6800 enlarged. A smoothing function was applied to the spectrum in order to assist in visualizing the peaks for m/z greater 4900.

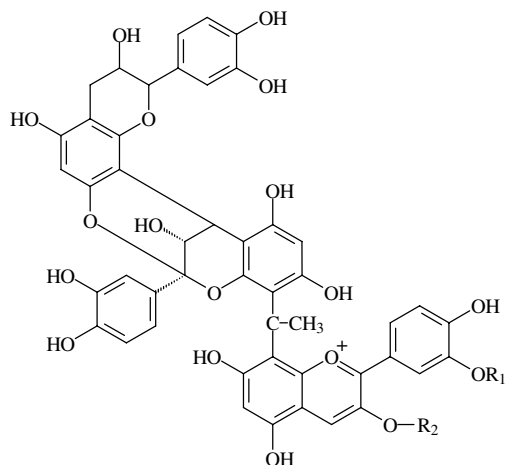
results indicate that cranberries contain oligomeric pigments that are similar to structures found in wine (Krueger et al., 2004). Kennedy et al. (2002) reported that anthocyanins are incorporated into proanthocyanidins during fruit ripening. While there are few reports of anthocyanin-polyflavan-3-ol oligomers occurring in fruits and unfermented beverages, there are well documented accounts of complex pigments forming in alcoholic beverages such as red wine (Wildenrad and Singleton, 1974; Timberlake and Bridle, 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 (Wildenrad and Singleton, 1974; Timberlake and Bridle, 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 (Wildenrad and Singleton, 1974). Using a rose cider model, Shoji et al. (2002) elucidated the structure of such oligomeric pigments by high resolution FAB-MS, ^1H and ^{13}C NMR analysis. The dimeric pigments consisted of an anthocyanin linked by a $\text{CH}_3\text{--CH}$ 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 spectra 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-arabinoside, peonidin-3-galactoside, peonidin-3-glucoside and peonidin-3-arabinoside (Fig. 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- and A-type interflavan bonds ($\Delta 2$ 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- and B-types; 2Δ 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 $\text{CH}_3\text{--CH}$ 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 DP when condensation products involving a $\text{CH}_3\text{--CH}$ 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 **4a–d**. Whereas MALDI-TOF MS has the power to distinguish molecular weight differences due to extent of hydroxylation ($\Delta 16$ amu), nature of interflavan bonds (A- or B-types; $\Delta 2$ amu), degree of polyflavan-3-ol polymerization ($\Delta 288$ amu) and substitutions by hexosides ($\Delta 162$ amu) or arabinose ($\Delta 132$ 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.



- 4a $R_1 = H$, $R_2 = \text{glucose}$ ($m/z = 1051$)
 4b $R_1 = H$, $R_2 = \text{galactose}$ ($m/z = 1051$)
 4c $R_1 = H$, $R_2 = \text{arabinose}$ ($m/z = 1021$)
 4d $R_1 = CH_3$, $R_2 = \text{glucose}$ ($m/z = 1065$)
 4e $R_1 = CH_3$, $R_2 = \text{galactose}$ ($m/z = 1065$)
 4f $R_1 = CH_3$, $R_2 = \text{arabinose}$ ($m/z = 1035$)

An equation was formulated to predict the series of anthocyanins linked to polyflavan-3-ols via a CH_3-CH bridge. The equation is: $Anthocyanin + 28 + 288a - 2b$, where *anthocyanin* represents the molecular weight of the terminal anthocyanin [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 and Sporns, 1999) and are detected as $[M]^+$ ions by MALDI-TOF mass spectral analysis (Wang and Sporns, 1999; Wang et al., 2000). However, anthocyanins, in the same manner as polyflavan-3-ols (Krueger et al., 2003; Takahata et al., 2001; Ohnishi-Kameyama et al., 1997), may also associate with naturally abundant sodium $[M + Na]^+$ and potassium $[M + K]^+$ to form 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), cyani-

din-hexoside (m/z 449.3) and peonidin-hexoside (m/z 463.3) (Fig. 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 of 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 ($\Delta 288$ amu) unit via a CH_3-CH ($\Delta 28$ amu) bridge were also observed; 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) (Fig. 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 two degrees of polymerization (DP2) with A- and B-types interflavan bonds ($\Delta 2$ 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) (Fig. 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 (4 β -8) and an ether linkage (2 β -O-7) between adjacent flavan-3-ol subunits (Fig. 6B). The mass difference between a B-type interflavan bond and an A-type interflavan bond is due to the loss of two hydrogen atoms ($\Delta 2$ 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 two 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.

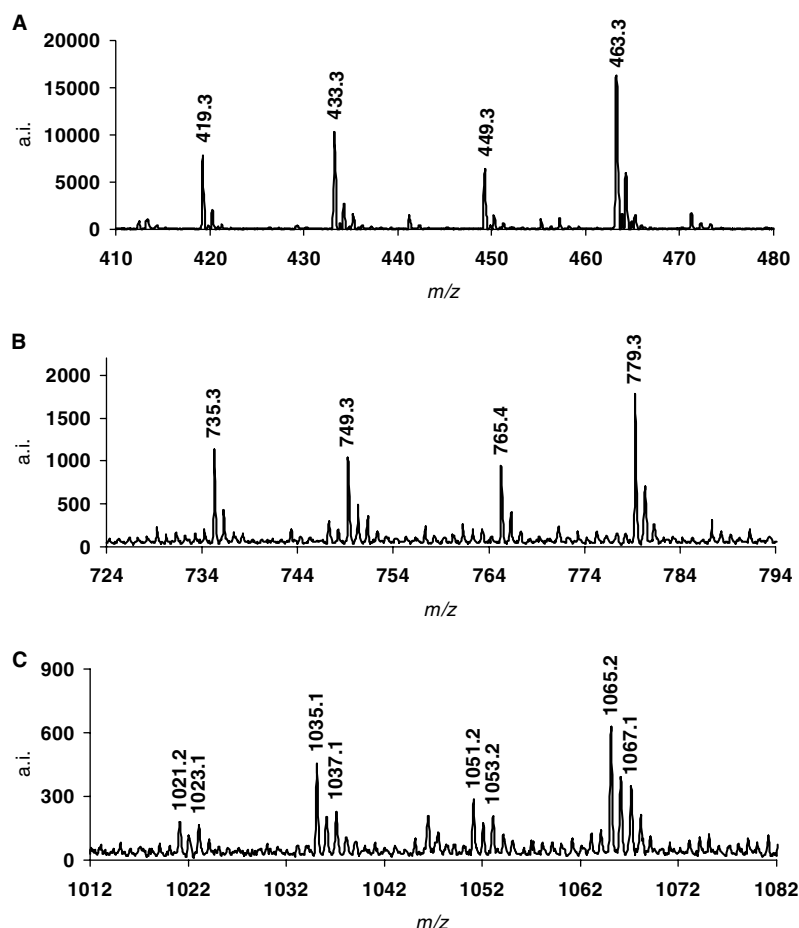


Fig. 6. MALDI-TOF positive reflectron mode mass spectra of the anthocyanin-polyflavan-3-ol oligomers of Hyred cranberry fruit and spray dried juice. (A) Anthocyanins $[M]^+$. (B) Anthocyanin linked to a single flavan-3-ol through a CH_3-CH bridge $[M]^+$. (C) Anthocyanin linked to a polyflavan-3-ol of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

The MALDI-TOF mass spectra of an ethanol eluate contained a series of masses corresponding to anthocyanins linked to polyflavan-3-ols of DP1–DP4, whereas the ethanol:methanol (1:1) eluate contained a series masses corresponding to anthocyanins linked to polyflavan-3-ols of DP3–DP5. As the degree of polymerization of the polyflavan-3-ol units increased, the predicted iterations of A- and B-types interflavan bonds ($\Delta 2$ 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 two B-type interflavan bonds (m/z 1311.2), one A-type:one B-type interflavan bond (m/z 1309.3) and two 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 that were previously described (Porter et al., 2001) as well as 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 MS 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- and B-types interflavan bonds. However, MALDI-TOF MS only allows for tentative structural assignments, as this analytic technique suffers from the inability to assign specific conformation 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

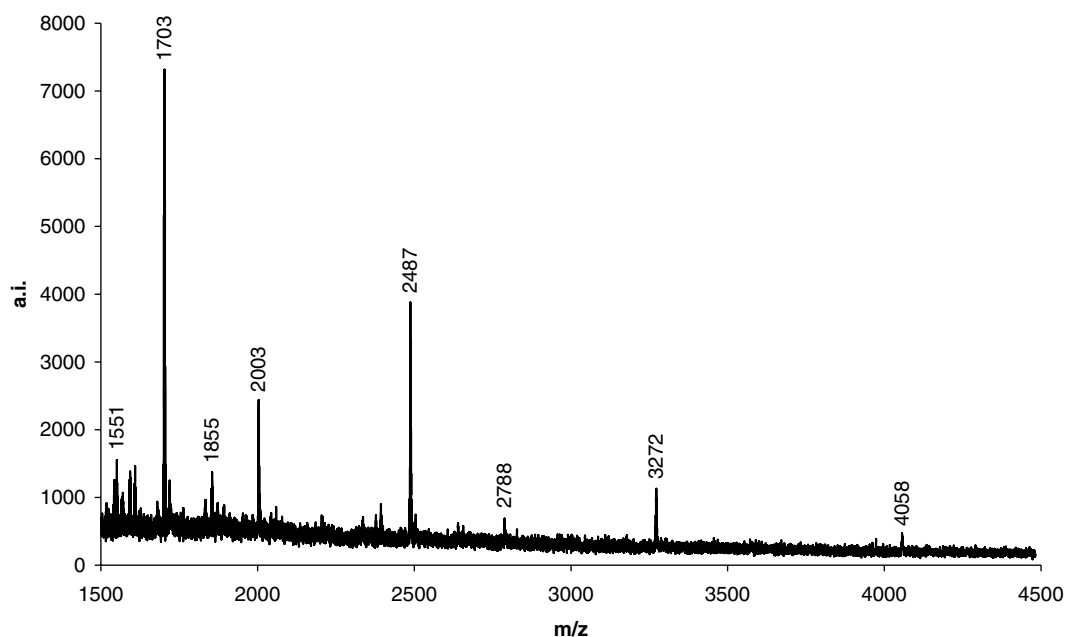


Fig. 7. Matrix assisted laser desorption time of flight mass spectrum of oligomeric ellagitannins in pomegranate juice. The spectrum is the sum of 100–500 shots for positive reflectron mode, calibrated with bradykinin (1060.6 MW) and glucagon (3483.8 MW). Labeled masses are the molecular ions minus 1 proton plus Cs^+ . Masses between 1551 and 2003 correspond 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).

Table 1

Calculated and observed masses for oligomeric ellagitannins in pomegranate juice and possible monomeric composition

Mass + Cs	Observed mass	Monomeric composition				
		Glucosyl	Gallagic acid	Ellagic acid	Gallic acid	Dehydrodigallic acid
<i>Dimers</i>						
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
<i>Trimers</i>						
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
<i>Tetramers</i>						
3270	3270	4	0	5	0	3
3272	3272	4	0	4	2	3
<i>Pentamers</i>						
4055	4055	5	0	6	0	4
4057	4056	5	0	5	2	4

anthocyanins and flavan-3-ols via acetaldehyde condensation reactions has been well described in fermented beverages such as red wine. The presence of

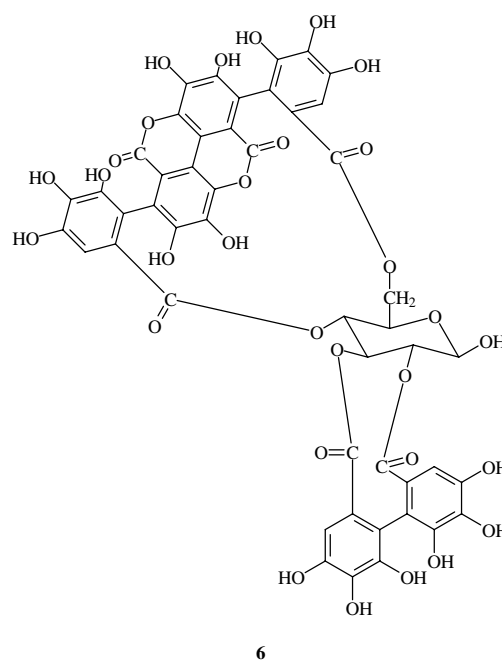
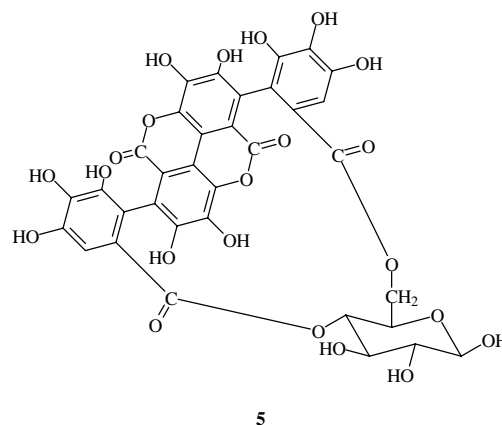
acetaldehyde in alcoholic solutions is attributed to either oxidative products of ethanol or microbial byproducts. In the case of cranberry fruit and spray

dried juice neither ethanol nor microbial fermentation were present. It therefore becomes a concern that the observed anthocyanin-pigments may be an artifact of harvest, storage, juice processing or analytic techniques.

Ethanol was also used in the elution of phenolics from the Sephadex LH20 column. Whereas acetaldehyde is generated from oxidation of ethanol in the presence of phenolic compounds (Wildenradt and Singleton, 1974), the model reactions used to investigate this chemistry are typically carried out under conditions of high temperature (>50 °C) and for long periods of time (>30 days). Our analysis of anthocyanin-polyflavan-3-ols from cranberry fruit was completed in less than 12 h, from the time of extraction to the time of MS analysis. We propose it is more likely that the anthocyanin-polyflavan-3-ol oligomers arise as a result of fruit ripening and not as an artifact of 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).

6. Ellagitannins in pomegranate

Consumption of pomegranate juice by healthy male volunteers, and also by atherosclerotic apolipoprotein E deficient (E⁰) mice, decreased LDL atherogenic modifications, including its oxidation, aggregation and retention (Aviram et al., 2000; Kaplan et al., 2001). Tanaka et al. (1985, 1986a,b) 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 characterized the structural diversity of ellagitannins in pomegranate juice (Afaq et al., 2005). A crude preparation of the polyphenolic fraction of pomegranate juice was obtained by absorption on to Sephadex LH20. An oligomeric series of at least 22 hydrolyzable tannins were detected with masses between 500 and 4058. Masses less than 1500 corresponded to known pomegranate ellagitannin and gallotannin structures, such as punicalagin as described by Tanaka et al. (1985, 1986a,b). The higher masses correspond to structures of oligomeric ellagitannins in which two or more core glucose units are cross-linked by dehydrodigalloyl or valoneoyl units (Fig. 7 and Table 1). This study 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.



7. Conclusion

The excellent research carried out on the absolute structures of dimers and trimers of oligomeric polyphenols indicates that there is great 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 stereoisomers and structural isomers of dimers and trimers have been described. Therefore, the number of possible structures greatly increases by isomerization at each of the masses detected by MALDI-TOF MS. For instance, in cranberries Foo et al.

(2000a,b) determined the structures of three 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.

We have developed several techniques that have allowed us to apply MALDI-TOF MS to characterize oligomeric polyphenols. The ability to obtain baseline resolution of one atomic mass unit in the reflectron mode allowed us to interpret isotope patterns. Therefore, we can use a knowledge of predicted isotope patterns based on isotope abundance to deconvolute overlapping isotope patterns of oligomers that differ by a few mass units. In addition, linear mode allowed us to detect oligomeric polyflavans with masses greater than 6000 (DP > 20). Control of cationization by deionizing the matrix/analyte mixture allowed easy detection of anthocyanin-polyflavan oligomers. Cationization by addition of Cs⁺ allowed us to eliminate the interference of the $\Delta 16$ mass differences between Na⁺ and K⁺ with $\Delta 16$ mass differences that results from pattern of hydroxylation. As a result of these techniques, we have observed larger structural heterogeneity of oligomers than is generally appreciated in the literature on food tannins. For instance, we have detected oligomeric anthocyanin-polyflavans in non-fermented fruits and beverages. We detect structural heterogeneity in the nature of the interflavan bonds in proanthocyanidins. Our results suggest that there is much variation in the pattern of hydroxylation of flavan monomers within the same oligomer, especially in the sorghum 3-deoxyproanthocyanidins. We also observe large variation in substitution with non-flavan moieties in polyflavans, such as gallic acid in grape seed polyflavan-3-ols and glycosylation in sorghum polyflavans. Recently, we also observed oligomeric ellagitannins with DP up to five.

The results from the application of MALDI-TOF MS clearly demonstrate its power as a tool to characterize the nature of tannins. 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 (Wang and Sporns, 1999; Foo et al., 2000a; Ohnishi-Kameyama et al., 1997). On the other hand, a pattern of decreasing signal intensity with increasing DP is characteristic of MALDI-TOF MS spectra of polydisperse oligomers (Montaudou 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 yield 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 separations technology advances along with improved instrumentation for NMR and MS, our ability to determine structure/function 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.

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