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A-type cranberry proanthocyanidins and uropathogenic bacterial anti-adhesion activity

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Abstract

Clinical, epidemiological and mechanistic studies support the role of cranberry (*Vaccinium macrocarpon* Ait.) in maintaining urinary tract health. Cranberry proanthocyanidins contain A-type linkages and have been associated with preventing adhesion of P-fimbriated uropathogenic *Escherichia coli* to uroepithelial cells. It is not known if the presence of the A-type linkage is a prerequisite for anti-adhesion activity. Other commercial sources of proanthocyanidins with all B-type linkages have not previously been screened for this activity. The goals of this study were to compare the in vitro anti-adhesion activity of A-linked proanthocyanidins from cranberry juice cocktail with the anti-adhesion activities of B-linked proanthocyanidins from commercial grape and apple juices, green tea and dark chocolate, and determine if anti-adhesion activity is detectable in human urine following consumption of single servings of each commercial food product. Structural heterogeneity and presence of the A-type linkage in cranberry proanthocyanidins was confirmed utilizing MALDI-TOF/MS and DI/ESI MS, as was the presence of all B-type linkages in the proanthocyanidins from the other commercial products. The isolated A-type proanthocyanidins from cranberry juice cocktail elicited in vitro anti-adhesion activity at 60 μg/ml, the B-type proanthocyanidins from grape exhibited minor activity at 1200 μg/ml, while other B-type proanthocyanidins were not active. Anti-adhesion activity in human urine was detected following cranberry juice cocktail consumption, but not after consumption of the non-cranberry food products. Results suggest that presence of the A-type linkage in cranberry proanthocyanidins may enhance both in vitro and urinary bacterial anti-adhesion activities and aid in maintaining urinary tract health.

Keywords: *Vaccinium macrocarpon* Ait; Ericaceae; Cranberry; Bacterial adhesion; Urinary tract infection; Proanthocyanidin; Condensed tannin; A-type linkage

1. Introduction

Clinical evidence supports the role of cranberry in the prevention of urinary tract infections (UTIs) (Avorn et al., 1994; Walker et al., 1997; Kontiokari et al., 2001; Stothers, 2002). Adhesion of bacteria to the uroepithelium is the initial step in development of mammalian UTI. Cranberry may act by inhibiting the adhesion of P-fimbriated uropathogenic strains of *Escherichia coli* to uroepithelial cells (Sobota, 1984; Zafriri et al., 1989; Ofek et al., 1991). *E. coli* strains that express P-fimbriae are associated with both cystitis and pyelonephritis

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(Roberts et al., 1989). The majority of P-fimbriated *E. coli* that cause UTIs bind glycosphingolipid receptor sites (i.e., α-Gal(1 → 4)β-Gal disaccharide) on the uroepithelium that are similar in structure to the P blood group antigens on the surface of A_1, Rh+ human red blood cells (HRBCs) (Kallenius et al., 1980; Leffler and Svanborg-Eden, 1980).

Howell et al. (1998) determined that proanthocyanidins isolated from cranberry fruit inhibited P-fimbrial adhesion in vitro, and thus may be the compounds responsible for the beneficial effect on UTI prevention. Mice fed isolated cranberry proanthocyanidins in their drinking water exhibited bacterial anti-adherence activity in their urine (Howell et al., 2001). Howell and Foxman (2002) found that the urine of humans that consumed cranberry juice cocktail exhibited anti-adhesion activity. This suggests that a certain level of absorption occurred and that bioactive proanthocyanidins and/or their metabolites may have been excreted in the urine to inhibit adhesion. Larger proanthocyanidin oligomers may undergo some degradation by colonic microflora and further biotransformation forming sulphate ester or glucoronide-conjugated metabolites (Harmand and Blanquet, 1978).

Cranberry proanthocyanidins have unusual A-type linkages (Foo et al., 2000a,b) compared to the more common B-type linkages found in proanthocyanidins from other tannin-rich foods. No data exist on the anti-adhesion activity of B-linked proanthocyanidins. It is important to determine if molecular linkage type is associated with anti-adhesion activity. If the A-type linkage was found to be prerequisite for anti-adhesion activity, especially in urine, it could help to explain why cranberry consumption prevents UTIs and other foods containing all B-linked proanthocyanidin do not. The purpose of this study was to compare the in vitro anti-adhesion activity of cranberry juice cocktail with those of several non-cranberry food products containing proanthocyanidins with B-type linkages and attempt to detect anti-adhesion activity in human urine following consumption of these products. Confirmation of proanthocyanidin linkage type and structural complexities in commerical food products requires sophisticated analytical techniques. Mass spectrometry that uses "soft" ionization techniques such as matrix assisted laser desorption ionization (MALDI-TOF MS) or electrospray ionization (DI/ESI MS) is capable of elucidating presence of A- or B-type linkages (Foo et al., 2000a; Porter et al., 2001; Krueger et al., 2000b, 2003, 2004). To associate linkage type with anti-adhesion activity, extracts enriched for proanthocyanidins were isolated from cranberry juice cocktail and other commercial food products and general structural heterogeneities and linkage types determined utilizing MALDI-TOF MS and DI/ESI MS.

2. Results and discussion

2.1. Urinary bacterial anti-adhesion activity following product consumption

Bacterial anti-adhesion activity was detectable only in the urines of those volunteers that consumed a single serving (240 ml) of the cranberry juice cocktail (Fig. 1). This 240-ml dose contained the equivalent of 83 mg of proanthocyanidin with at least one A-type linkage. Detection of the activity continuously increased in a regular progression, peaking at 4–6 h post-consumption and persisting in the urine for at least 8 h, suggesting potential protection against bacterial attachment in the uroepithelium during this period. No activity was detected at any time period in the urines of volunteers that consumed the commercial apple juice (0.27 mg PAC/240 ml serving), purple grape juice (39.1 mg PAC/240 ml serving), green tea (4.4 mg/2g serving) or dark chocolate (106 mg/40 g serving) products, suggesting that the B-linked proanthocyanidins and/or their metabolites either do not have bacterial anti-adhesion activity or the compounds are not bioavailable. No anti-adhesion activity was detected in urines prior to consumption of products. The bacterial anti-adhesion activity elicited by the cranberry juice cocktail was not due to the acidity of the urine, as pH of all urine samples was 6.5 (pH 5.9 has been shown to be bacteriostatic (Kraemer, 1964)).

2.2. In vitro bacterial anti-adhesion activity of isolated proanthocyanidins

Isolated cranberry juice cocktail proanthocyanidins with A-type linkages exhibited in vitro bacterial anti-adhesion activity in the HRBC hemagglutination assay, with a bioactivity detection threshold of 60 μg proanthocyanidin/ml PBS. This activity threshold is similar to

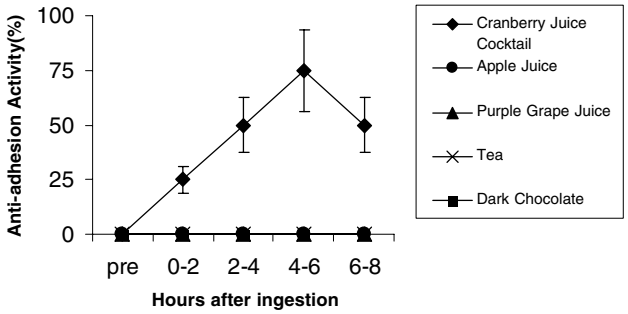

Fig. 1. Detection of bacterial anti-adhesion activity of human urine pre- and post-consumption of single servings of proanthocyanidin-containing juice and food products. Average percent inhibition of anti-adhesion activity was recorded over an 8-h period for each set of urine samples tested. Urine collected during each 2-h time period was pooled for each participant prior to testing for anti-adhesion activity.

previously reported research on anti-adhesion of cranberry proanthocyanidins, representing the dilution at which 50% agglutination is recorded (Howell et al., 1998). The proanthocyanidins isolated from the purple grape juice had a bioactivity detection threshold of 1200 μg/ml, indicating that the cranberry proanthocyanidins were significantly more active. The proanthocyanidins isolated from the apple juice, green tea and dark chocolate products had no anti-adhesion activity at any of the dilutions tested.

2.3. Structural heterogeneity and confirmation of linkage type in isolated proanthocyanidins

The mass spectrometry indicated that there were large differences in the proanthocyanidin structures among the commercial food products assayed. In general, there was agreement between the masses detected by both MALDI-TOF MS and DI/ESI MS. Both methods gave spectra that could be interpreted based on knowledge of the structures of lower molecular weight oligomers, familiarity with the chemistry of the proanthocyanidins present in each food and an understanding of mass spectral ionization events. However, the MALDI-TOF MS method provided superior resolution than the DI/ESI MS, allowing for a more detailed understanding of the structural variation occurring in proanthocyanidins from the different food products.

Proanthocyanidins from cranberry juice cocktail were characterized by a series of polyflavan-3-ol oligomers based on a repeating unit structure of (epi)catechin with one or more A-type interflavanyl linkages present in the oligomer. MALDI-TOF MS and DI/ESI MS of the cranberry proanthocyanidins detected oligomers with a

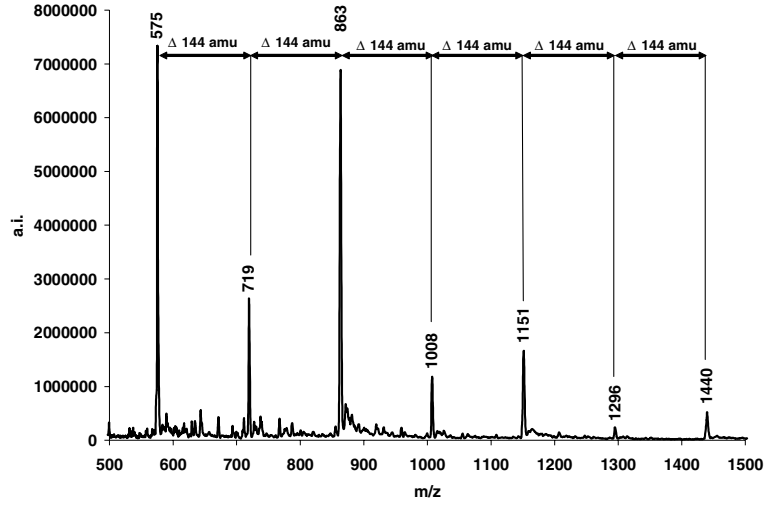

Fig. 2. Negative mode DI/ESI spectra of cranberry juice cocktail proanthocyanidins. Masses are assigned as doubly deprotonated molecular ions $[M - 2H]^{2-}$ and represent variation in degree of polymerization (Δ144 amu) of the polyflavan-3-ol oligomers.

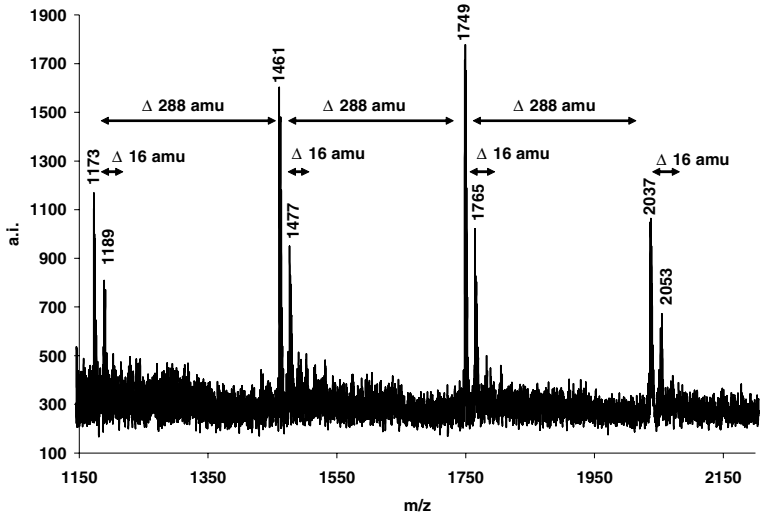

Fig. 3. Positive reflectron mode MALDI-TOF spectra of the cranberry juice cocktail proanthocyanidins. Masses represent variation in degree of polymerization (Δ288 amu) and degree of hydroxylation (Δ16 amu) of the polyflavan-3-ol oligomers $[M + Na]^{+}$.

degree of polymerization (DP) of 4–10 (Figs. 2 and 3, Table 1). Cranberry proanthocyanidins also contained masses that corresponded to oligomers with one (epi)gallocatechin unit.

In contrast to cranberries, proanthocyanidins in purple grape juice were characterized as a series of heteropolyflavan-3-ols (procyanidin/prodelphinidin oligomers substituted with gallic acid) (Figs. 4 and 5, Table 2). MALDI-TOF MS detected a series of oligomers with a DP of 5–7 while DI/ESI MS detected oligomers from DP 4–8. At each DP, oligomers that contain galloyl residues were detected at 152 amu greater mass relative to molecular ions without galloyl substitutions for MALDI-TOF MS and 76 amu greater mass for the DI/ESI MS because doubly charged molecular ions were detected. Gallocatechin constituent unit in an oligomer was detected as a series of 16 amu greater masses than the homopolymer of catechin at each DP in the

Table 1
Calculated and observed masses for proanthocyanidins from cranberry juice cocktail

DP	EC	A-type	EGC	MALDI TOF MS		DIESI	
				Calculated	Observed	Calculated	Observed
4	4	0	0	1177	NO	576	NO
	4	1	0	1175	1175	575	575
	4	2	0	1173	1173	574	NO
	3	0	1	1193	NO	584	NO
	3	1	1	1191	NO	583	NO
	3	2	1	1189	1189	582	NO
5	5	0	0	1465	NO	720	NO
	5	1	0	1463	1463	719	719
	5	2	0	1461	1461	718	NO
	4	0	1	1481	NO	728	NO
	4	1	1	1479	NO	727	NO
	4	2	1	1477	1477	726	NO
6	6	0	0	1755	NO	865	NO
	6	1	0	1753	NO	864	NO
	6	2	0	1751	1751	863	863
	6	3	0	1748	1749	862	NO
	5	0	1	1771	NO	873	NO
	5	1	1	1769	NO	872	NO
	5	2	1	1767	NO	871	871
	5	3	1	1764	1764	870	NO
7	7	0	0	2043	NO	1009	NO
	7	1	0	2041	NO	1008	1008
	7	2	0	2039	2038	1007	NO
	7	3	0	2037	2036	1006	NO
	6	0	1	2059	NO	1017	NO
	6	1	1	2057	NO	1016	NO
	6	2	1	2055	2055	1015	NO
8	8	0	0	2331	NO	1153	NO
	8	1	0	2329	NO	1152	NO
	8	2	0	2327	2327	1151	1151
	8	3	0	2325	2325	1150	NO
	7	0	1	2347	NO	1161	NO
	7	1	1	2345	NO	1160	NO
	7	2	1	2343	2343	1159	NO
9	9	0	0	2619	NO	1297	NO
	9	1	0	2617	NO	1296	1296
	9	2	0	2615	NO	1295	NO
	9	3	0	2613	2613	1294	NO
10	10	0	0	2908	NO	1441	NO
	10	1	0	2906	NO	1440	1440
	10	2	0	2904	NO	1439	NO
	10	3	0	2902	2901	1438	NO

The observed masses were determined by matrix assisted laser desorption-time of flight mass spectrometry (MALDI-TOF MS) and direct infusion electrospray mass spectrometry (DI/ESI/MS). The calculated masses were based on predicted values for proanthocyanidins with 1, 2, or 3 A-type interflavan bonds (4β-2, 2β-O-7) and homopolymers of all epicatechin units (EC) or heteropolymers with one epigallocatechin unit (EGC). NO indicates that the mass was not observed.

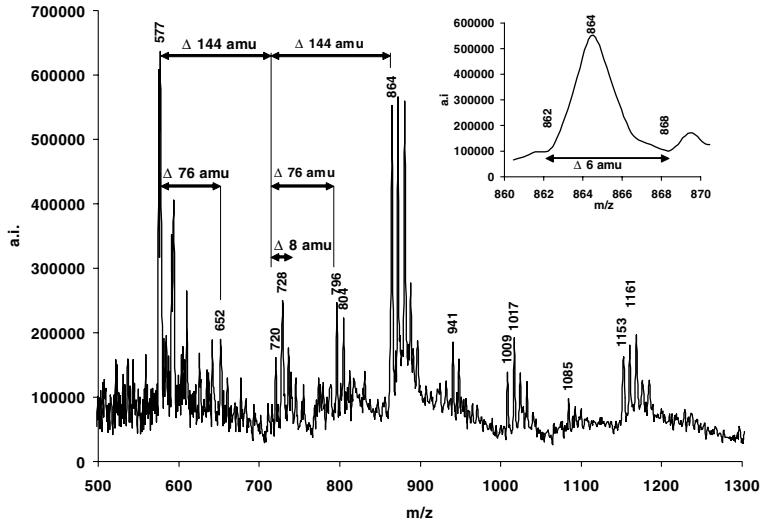

Fig. 4. Negative mode DI/ESI spectra of grape juice proanthocyanidins. Masses are assigned as doubly deprotonated molecular ions $[M - 2H]^{2-}$. A mass difference (Δ144 amu) represents variation in degree of polymerization of procyanidin (epicatechin) homopolymers. A mass difference of (Δ76 amu) represents gallic acid substitution. A mass difference of (Δ8 amu) represents heteropolymer oligomers of procyanidin (epicatechin) and prodelphinidin (epigallocatechin), which differ by one or more hydroxyl substitutions. Inset (*m/z* 864) shows lack of unit mass resolution due to overlapping isotope distributions of multiply charged molecular ions.

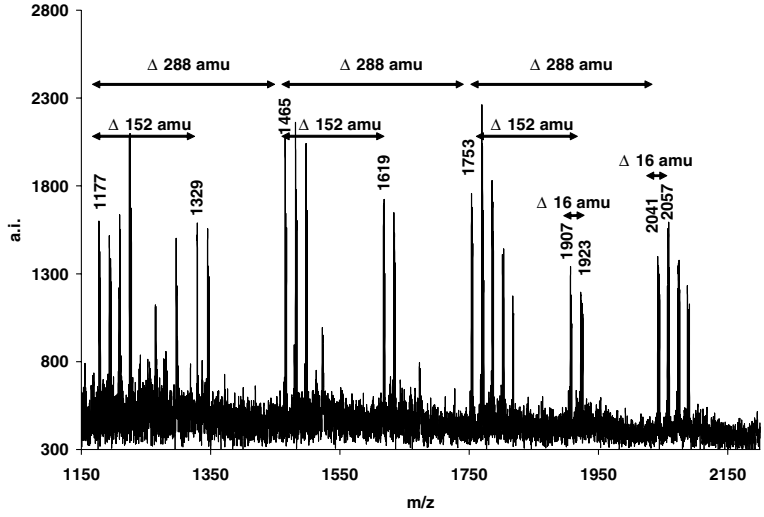

Fig. 5. Positive reflectron mode MALDI-TOF spectra of the grape juice proanthocyanidins. Masses represent variation in degree of polymerization (Δ288 amu), galloyl residues (Δ152 amu) and degree of hydroxylation (Δ16 amu) of the polyflavan-3-ol oligomers $[M + Na]^{+}$.

MALDI-TOF MS and a series of 8 amu greater masses for the DI/ESI MS.

The dark chocolate product contained the least complex series of proanthocyanidin oligomers. MALDI-TOF MS detected a simple series of polyflavan-3-ol oligomers of DP 4 to 10 with no A-type linkages. DI/ESI MS detected the same series of oligomers up to DP11.

The MS of green tea proanthocyanidins showed a very complex series of oligomers from trimers to hexamers with (epi)catechin/(epi)gallocatechin monomeric units and galloyl residues. No A-type linkages were detected.

The MS of apple juice proanthocyanidins gave a series of procyanidin masses from the tetramer to octamer. A single (epi)gallocatechin substitution was also observed within each DP for both the MALDI-TOF MS and the DI/ESI MS. However, all of the observed masses were 4–6 amu less than the calculated masses. Unlike the cranberry proanthocyanidins, these masses did not have a typical isotope pattern that would indicate multiple A-type linkages. Therefore, at present, there is no explanation for the discrepancy between the calculated masses and the observed masses for the apple juice proanthocyanidins.

Table 2
Calculated and observed masses for proanthocyanidins from grape juice

DP	EC	EGC	G	MALDI TOF MS		DIESI	
				Calculated	Observed	Calculated	Observed
4	4	0	0	1177	1178	576	577
	3	1	0	1193	1193	584	584
	2	2	0	1209	1209	592	593
	1	3	0	1225	1225	600	
	4	0	1	1329	1330	652	652
	3	1	1	1345	1345	660	660
5	5	0	0	1465	1465	720	720
	4	1	0	1481	1481	728	728
	3	2	0	1497	1497	736	736
	2	3	0	1513	1514	744	
	1	4	0	1529	1523	752	
	5	0	1	1618	1619	796	796
	4	1	1	1634	1634	804	804
6	6	0	0	1755	1754	864	864
	5	1	0	1771	1770	872	872
	4	2	0	1787	1786	880	880
	3	3	0	1803	1802	888	888
	2	4	0	1819	1818	896	896
	6	0	1	1907	1907	941	941
	5	1	1	1923	1923	949	949
7	7	0	0	2043	2042	1009	1009
	6	1	0	2059	2059	1017	1017
	5	2	0	2075	2075	1025	1025
	4	3	0	2091	2091	1033	1033
	7	0	1			1085	1085
	6	1	1			1093	1093
8	8	0	0			1153	1153
	7	1	0			1161	1161
	6	2	0			1169	1169
	5	3	0			1177	1177
	4	4	0			1185	1185

The observed masses were determined by matrix assisted laser desorption-time of flight mass spectrometry (MALDI-TOF MS) and direct infusion electrospray mass spectrometry (DI/ESI/MS). The calculated masses were based on predicted values for procyanidin homopolymers of all epicatechin units (EC), procyanidin and prodelphinidin heteropolymers with EC units and epigallocatechin units (EGC) substituted with gallic acid (G) and with all B-type linkages. NO indicates that the mass was not observed.

2.4. Interpretation of DI/ESI mass spectral data

While DI/ESI MS and MALDI-TOF MS are both capable of detecting intact molecular ions with high mass (>100,000 Da), ESI is best suited for analysis of monodispersed biopolymers because of the complications arising from the formation of multiply charged ions (Montaudo et al., 2002). The DI/ESI MS of proanthocyanidins run in negative ion mode experiments appears to have produced primarily doubly deprotonated molecular ions $[M - 2H]^{2-}$ (Figs. 2 and 4, Tables 1 and 2). Predicted structural variation; number of catechin/epicatechin monomers (Δ144 amu), galloyl residues (Δ76 amu), hydroxyl substitutions (Δ8 amu) and difference in A-type vs. B-type interflavan bonds (Δ1 amu) were based on the assumption that compounds were detected as doubly deprotonated molecular ions. However, a closer investigation of individual peaks revealed poor mass resolution and peak broadening, most likely attributed to the formation of multiply charged ions. Observation of the peak at *m/z* 864 (Fig. 4 inset) showed the peak beginning at *m/z* 862 and continuing to *m/z* 868 (6 amu). This broadening could be explained by overlapping isotope clusters of multiply charged molecular ions; $[DP3 - H]^{1-} = m/z$ 865, $[DP6 - 2H]^{2-} = m/z$ 864, $[DP9 - 3H]^{3-} = m/z$ 863 and $[DP12 - 4H]^{4-} = m/z$ 862. The lack of unit mass resolution results in the observation of an average molecular mass calculated across the entire width of the peak base. Lack of resolution prevented the ability to detect differences in A-type vs. B-type interflavan bonds (Δ1 amu) as unit mass isotope distributions were not resolved.

Additional evidence for the formation of multiply charged molecular ions arose from the absolute peak intensities observed (Fig. 4). The observed mass (*m/z*

864) produced a more intense signal than (*m/z* 1009) and mass (*m/z* 577) was greater than (*m/z* 720) due to the summation of overlapping multiply charged molecular ions. The signal at *m/z* 864 was stronger than the signal at *m/z* 719 because more iterations of multiply charged molecular ions may have occurred; $[DP3 - H]^{1} + [DP6 - 2H]^{2-} + [DP9 - 3H]^{3} + [DP12 - 4H]^{4-} =$ *m/z* 864 vs. $[DP7 - 2H]^{2-} + [DP14 - 4H]^{4-} =$ *m/z* 1009.

2.5. Interpretation of MALDI-TOF mass spectral data

MALDI-TOF MS is ideally suited for characterizing polydispersed oligomers (Hanton, 2001), and is considered the mass spectral method of choice for analysis of proanthocyanidins which exhibit greater structural heterogeneity (Krueger et al., 2000a,b, 2003, 2004). MALDI-TOF MS produces only a singly charged molecular ion for each parent molecule and allows detection of high masses with precision (Montaudo et al., 2002). MALDI-TOF MS of the proanthocyanidins in this experiment were detected in the positive ion mode as singly charged ions produced through cationization with naturally abundant sodium ion adduct. Structural variation of proanthocyanidins, number of catechin/epicatechin monomers (Δ288 amu), galloyl residues (Δ156 amu), hydroxyl substitutions (Δ16 amu) and difference in A-type vs. B-type interflavan bonds (Δ2 amu) could be predicted (Tables 1 and 2). MALDI-TOF MS equipped with delayed extraction provided the unit mass resolution necessary for the visualization of isotopic distributions, allowing for characterization of mass differences due to variation of A-type vs. B-type interflavan bonds in cranberry juice cocktail proanthocyanidin (Table 1), a level of sensitivity that DI/ESI was not able to accomplish.

2.6. Unusual A-type linkages in cranberry juice cocktail proanthocyanidins

Because of the superior resolution, MALDI-TOF MS data was referenced for comparison of the structural feature of cranberry proanthocyanidins believed to be responsible for both in vitro and urinary anti-adhesion activity, namely the A-type interflavan linkage. The following discussion on isotope distribution and characterization of A-type vs. B-type interflavan linkages may be applied to the interpretation of any mass spectral data set.

The observed MALDI masses (*m/z*) corresponded to the monoisotope of the predicted compound. For example; the predicted and observed monoisotope of grape juice proanthocyanidins of 5 DP containing four B-type interflavan bonds was (*m/z* 1465), representing the contribution of ^{12}C, ^{1}H and ^{16}O to the compound (Fig. 6). The mass at (*m/z* 1466) represented the contribution of one ^{13}C, or one ^{2}H, or one ^{17}O. The mass at (*m/z* 1467) represented two ^{13}C, or one ^{13}C and one ^{2}H, or one ^{18}O or two ^{2}H. Mass calculating programs such as IsoPro 3.0 [Shareware at:_http://members.aol.com/msmsoft] can be used to predict such isotopic distribution of compounds and allow for comparison between predicted and observed isotopic distributions (Krueger et al. (2004)).

The enlarged MALDI-TOF spectrum of the isotope cluster of the purple grape juice proanthocyanidin (*m/z* 1465; Fig. 6) represents a compound of DP5 with four B-type interflavan bonds and was in agreement with the predicted isotopic distribution generated with IsoPro 3.0.

The enlarged MALDI-TOF spectrum of the isotope cluster of the cranberry juice cocktail proanthocyanidin

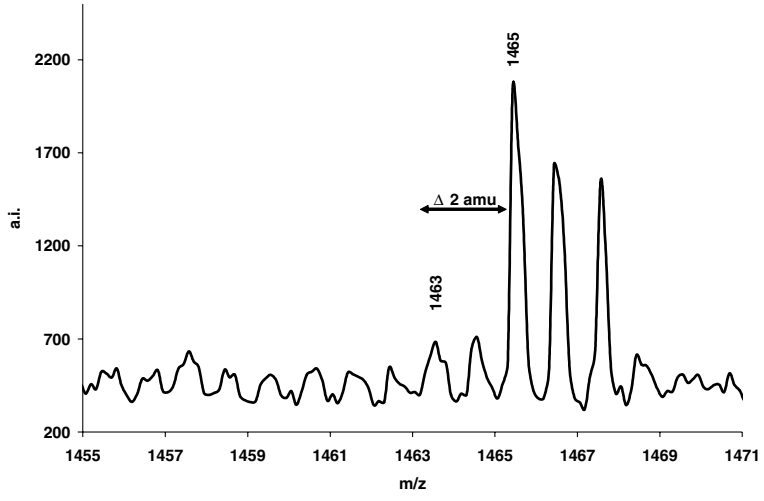

Fig. 6. Enlarged positive mode reflectron MALDI-TOF spectrum showing the isotope cluster of the grape juice proanthocyanidins of DP5. Masses represent variation in the nature of interflavan bonds (Δ2 amu) $[M + Na]^{+}$. Grape juice proanthocyanidins are composed primarily of B-type (*m/z* 1465) bonds.

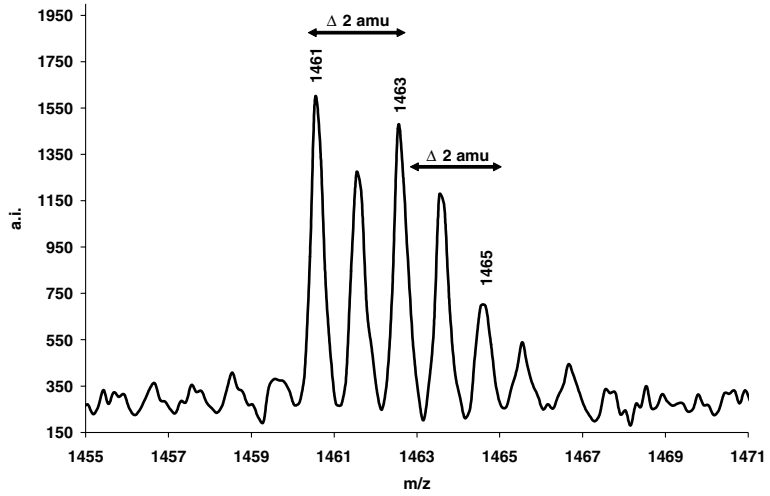

Fig. 7. Enlarged positive mode reflectron MALDI-TOF spectrum showing the isotope cluster of the cranberry juice cocktail proanthocyanidins of DP5. Masses represent variation in the nature of interflavan bonds (Δ2 amu) $[M + Na]^+$. Cranberry juice cocktail proanthocyanidins contain at minimum one (*m*/*z* 1463) but often two (*m*/*z* 1461) or more A-type bonds at each degree of polymerization.

of the same degree of polymerization (DP5) was much more complex than the isotope cluster of the grape juice proanthocyanidin (Fig. 7). The increased complexity arises from overlapping isotopic distributions of proanthocyanidin with structural heterogeneity expressed in the nature of cranberry interflavan bonds (Δ2 amu). In the case of cranberry proanthocyanidin, the assignment of compounds to the monoisotopic peaks at *m*/*z* 1461 and *m*/*z* 1463 represent proanthocyanidin of DP5 which contain (2 A-type:2 B-type and 1 A-type:3B-type linkages) respectively.

The isotopic distribution of a proanthocyanidin containing two A-type interflavan linkages will overlap with the isotopic distribution of a proanthocyanidin of the same DP, containing one A-type interflavan linkage. The overlapping isotope distributions result in a summation of the observed intensity. For example the mass observed at (*m*/*z* 1463) is the summation of the monoisotope (^{12}C, ^{1}H, and ^{16}O) of a cranberry proanthocyanidin of DP5 with one A-type interflavan bond and the contribution of the isotope distribution (two ^{13}C, or one ^{13}C and one ^{2}H, or one ^{18}O or ^{2}H) attributed to the cranberry proanthocyanidin of DP5 with two A-type interflavan linkages (Krueger et al. (2004)). The summation of the predicted isotopic distributions generated by IsoPro 3.0 was in agreement with the observed overlapping isotopic clusters.

3. Conclusions

It was not our intent to perform structural elucidation of proanthocyanidins in this study, but rather attempt to associate the A-type linkage in cranberry proanthocyanidins with greater bacterial anti-adhesion activity than that expressed by proanthocyanidins with B-type linkages. The spectrometry techniques were used to confirm the presence of the A- or B-type linkages and ascertain any unusual structural features of the molecules that could help explain the differences in biological activity. If it had been our intent to do structural elucidation of proanthocyanidin oligomers, we would probably not have utilized processed products, nor would we have employed MALDI, which is somewhat limited in its ability to yield complete structural information. Moreover, current elucidation techniques would make it very difficult (if not impossible) to determine putative structures of individual proanthocyanidin oligomers beyond DP3. In 2000, Foo et al. isolated and identified structures of several A-linked trimers from cranberry, but were not able to continue the laborious and expensive work needed to identify the structure of a tetramer.

The current study found that cranberry juice cocktail was the only food product tested that prevented bacterial anti-adhesion in human urine following consumption. Further studies are needed on a broad range of participants before conclusions can be made regarding dose–response or pharmacokinetics of the cranberry juice cocktail proanthocyanidins. Consumption of purple grape juice, apple juice, green tea or dark chocolate products did not result in urinary bacterial anti-adhesion activity, nor did the isolated proanthocyanidins from these products demonstrate significant in vitro activity. These data suggest that other proanthocyanidin-rich foods may not possess beneficial effects on maintenance of urinary tract health, possible due to the absence of the A-type linkage in the proanthocyanidins in these products. The spectrometry data suggest that the structures of the cranberry juice cocktail proanthocyanidins are heterogeneous compared to the

structures of the proanthocyanidins isolated from the other food products. The MALDI-TOF analyses indicate that cranberry juice cocktail proanthocyanidins are composed primarily of oligomers containing at minimum one A-type interflavan bond, but often multiple A-type interflavan linkages at each degree of polymerization within the proanthocyanidin oligomeric series. In previous research, A-linked dimers from cranberry were shown to be more effective at inhibiting in vitro bacterial adherence than the B-linked dimers (Foo et al., 2000a), suggesting that the conformational rigidity that the A-linkage affords (Foo et al., 2000b) to the cranberry proanthocyanidin molecules may play a role in bioactive urine metabolite formation. More research is required to determine if other, recently identified food sources of A-type proanthocyanidins have anti-adhesion activity and whether number and location of A-type linkages in the oligomers or other structural features such as 4β-8 vs. 4β-6 B-type interflavan bonds and DP have any influence on anti-adhesion activity.

4. Experimental

4.1. Proanthocyanidin-containing foods

Four different juice products and one chocolate product were purchased from the local supermarket. A portion of each product sample was used for human consumption to detect anti-adhesion activity in urine and the remainder was used to extract the proanthocyanidins for structural feature studies. The same manufacturing code (lot) of each sample was used for both the proanthocyanidin fractions and the urine activity studies. Products tested included: (1) Ocean Spray Cranberry Juice Cocktail (27% cranberry juice), (2) Welch's Purple 100% Grape Juice (made with Concord grapes), (3) Mott's 100% Apple Juice, (4) Lipton's Green Tea (100% Natural), (5) Dove Dark Chocolate Promises.

4.2. Urinary bacterial anti-adhesion activity following product consumption

This study protocol was reviewed by IRB and received an exemption. Urine was self-collected from six healthy volunteers (4 women and 2 men) between the ages of 25 and 45 following consumption of the various food products. Each urine sample obtained from the volunteers served as an experimental unit to detect the presence or absence of bacterial anti-adhesion activity. Volunteers refrained from consuming flavonoid-rich foods for a 3-day wash-out period prior to consuming the proanthocyanidin-containing products and throughout the study period. On urine collection days, additional fluid consumption was standardized among volunteers to avoid dilution of urine samples and allow for detection of anti-adhesion activity, if present. Single serving-sizes of each product were designated as follows: 240 ml of cranberry juice cocktail, purple grape and apple juices, the aqueous extract from 2 g of brewed tea (1 bag), and 40 g of dark chocolate (5 pieces). A single serving of each product was individually administered in the morning to the volunteers with a 3-day wash-out period between each product. Urine (50 ml) was collected (clean-catch) before product consumption and every 2 h, for a total of 8 h following consumption. Urine collected during each 2-h time-period was pooled for each participant. Urine was immediately frozen at −20 °C for subsequent anti-adhesion testing. At the conclusion of the feeding study, the urines were removed from the freezer and allowed to come to room temperature (21 °C). Uropathogenic P-fimbriated *E. coli* bacteria isolated from women with clinical UTIs (isolate cryogenically stored at Marucci Center for Blueberry Cranberry Research at Rutgers University, Chatsworth, NJ) were cultured according to Foo et al. (2000b) and incubated in 1-ml aliquots of each urine at a concentration of 10^5/ml (to mimic bacterial concentration of clinical UTI) for 20 min. Bacteria were harvested from the urines by centrifugation (2500 rpm), the urine supernatants removed and the bacteria suspended in phosphate buffered saline solution (PBS) at pH 7.0 at a concentration of 5×10^8 bacteria/ml. The bacterial suspensions were tested for presence of anti-adhesion activity utilizing a mannose-resistant human red blood cell (HRBC) hemagglutination assay specific for P-fimbriated bacteria, according to Foo et al. (2000b). Bacterial anti-adhesion activity was scored microscopically and recorded as the percentage of anti-adhesion activity detected for each undiluted sample. Assays were repeated 4 times on triplicate urine samples and the standard error calculated. Urinary activity measured at each time collection period was plotted to show persistence of bacterial anti-adhesion activity over time for each product consumed (Fig. 1).

4.3. Proanthocyanidin extractions from food products

A total proanthocyanidin extract was isolated each food product to provide a source of material for the in vitro anti-adhesion assay and the mass spectral analyses of linkage type and molecular heterogeneity. Prior to proanthocyanidin extraction, the chocolate sample was de-fatted and acetone extracted (Me_2CO:dH_2O:HOAc (70:29:5:0.5) (v/v/v) according to Hammerstone et al. (2000) and turbovapped at 60 °C under nitrogen. The green tea sample was brewed with distilled water at 100 °C for 2 min with agitation and filtered through 5 layers of cheesecloth to remove tea leaves. The juice samples were subjected to the chromatography steps without pre-treatment. Prepared products were applied to 10-g C18 SepPak® cartridges (Waters Corp.,

Milford, MA) that were equilibrated in 20 ml MeOH followed by 40 ml dH_2O. The cartridges were washed with 40 ml dH_2O then 40 ml dH_2O:MeOH (85:15) (v/v) and the elutants discarded. The polyphenolic fraction was eluted from each cartridge with 40 ml 1% HOAc in MeOH (v/v) and reduced to 10 ml by turbovap to remove solvent. Fractions were applied to 35-cc cartridges of Sephadex™ LH-20 (Sigma Chemical Co., St. Louis, MO) (5 g dry Sephadex per column) that was equilibrated overnight in EtOH:dH_2O (50:50) (v/v). Columns were washed with 20 ml EtOH:dH_2O (50:50) (v/v) and the proanthocyanidin fractions eluted with two column volumes Me_2CO:dH_2O (80:20) (v/v). Aqueous acetone fractions were turbovapped to 10 ml to remove the acetone, and freeze-dried for the subsequent analyses.

4.4. In vitro bacterial anti-adhesion testing of proanthocyanidin extracts

Proanthocyanidins isolated from the food products were tested for in vitro bacterial anti-adhesion activity on a per weight basis. Samples were suspended (1 mg/ml) in PBS (2-fold dilution series), and tested for anti-adhesion activity utilizing an HRBC hemagglutination assay specific for uropathogenic P-fimbriated *E. coli* according to Foo et al. (2000b). The final proanthocyanidin dilution at which hemagglutination activity was suppressed was an indicator of the strength of the bacterial anti-adhesion activity (0% hemagglutination of HRBCs = 100% bacterial anti-adhesion activity). Dilutions (2-fold) of each proanthocyanidin extract (starting concentration of 1 mg/ml) were scored for anti-adhesion activity and the dilution endpoints recorded when 50% bacterial anti-adhesion activity was detected. Anti-adhesion assays were repeated four times on duplicate proanthocyanidin samples and the results averaged.

4.5. MALDI-TOF mass spectrometry

MALDI-TOF mass spectra were collected on a Bruker Reflex II-TOF mass spectrometer (Billerica, MA) equipped with delayed extractions and an N_2 laser set at 337 nm. In the positive reflectron mode, an accelerating voltage of 25.0 kV and a reflectron voltage of 26.5 kV were used. Spectra were the sum of 300 laser shots. Spectra were calibrated with bradykinin (1060.6 MW) and glucagon (3483.8 MW) as external standards. In accordance with previously published results (Krueger et al. (2000a,b)), *trans*-3-indoleacrylic acid (*t*-IAA; 5 mg/100 μl 80% aq. acetone) was used as a matrix. Ten microliters of the proanthocyanidin fractions (1 mg/40 μl 80% aq. acetone) from cranberry juice cocktail, purple grape juice, apple juice, green tea and dark chocolate products were mixed with the matrix solution at volumetric ratios of 1:2. The proanthocyanidin:matrix mixture was applied directly (0.2 μl) to a stainless steel target and dried at room temperature. *trans*-3-Indoleacrylic acid (Aldrich Chemical Co., Milwaukee, WI), bradykinin and glucagon (Sigma Chemical Co., St. Louis, MO) were used as received.

4.6. Direct infusion electrospray mass spectrometry

For DI/ES MS analysis of the proanthocyanidin extracts, solutions were prepared by either rinsing the freeze dry vessel with methanol or by dissolving an amount of proanthocyanidin extract in methanol. Proanthocyanidin solution (300 μl) was mixed with 100 μl of 10 mM ammonium acetate in a 1-ml Hamilton syringe. The syringe was placed in a syringe pump and connected to the TurboIonSpray (TIS) atmospheric pressure ionization (API) electrospray source of an Applied Biosystems API2000 Triple Quadrupole mass spectrometer. The sample was pumped into the source at 5 μl/min. The mass spectral data was collected using a 250 amu *m/z* to 1800 amu *m/z* Q1 scan in negative ion mode. One hundred scans were collected at 3 s/scan and summed together in MCA mode to provide the mass spectra.

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References

Avorn, J., Monane, M., Glynn, R.J., Lipsitz, L.A., Gurwitz, J.H., Choodnovskiy, I., 1994. Reduction of bacteriuria and pyuria after ingestion of cranberry juice. JAMA 271, 751–754.

Foo, L.Y., Lu, Y., Howell, A.B., Vorsa, N., 2000a. A-type proanthocyanidin trimers from cranberry that inhibit adherence of uropathogenic P-fimbriated *Escherichia coli*. J. Nat. Prod. 63, 1225–1228.

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

Hammerstone, J.F., Lazarus, S.A., Schmitz, H.H., 2000. Procyanidin content and variation in some commonly consumed foods. J. Nutr. 130, 2086S–2092S.

Hanton, S.D., 2001. Mass spectrometry of polymers and polymer surfaces. Chem. Rev. 101, 527–569.

Harmand, M.F., Blanquet, P., 1978. The fate of total flavanolic oligomers (OFT) extracted from '*Vitis Vinifera*' in the rat. Eur. J. Drug. Metab. Pharmacokin. 1, 15–30.

Howell, A.B., Foxman, B., 2002. Cranberry juice and adhesion of antibiotic-resistant uropathogens. JAMA 287, 3082.

Howell, A.B., Vorsa, N., DerMarderosian, A., Foo, L.Y., 1998. Inhibition of adherence of P-fimbriated *Escherichia coli* to uroepithelial-cell surfaces by proanthocyanidin extracts from cranberries. New England J. Med. 339, 1085–1086.

Howell, A.B., Leahy, M., Kurowska, E., Guthrie, N., 2001. *In vivo* evidence that cranberry proanthocyanidins inhibit adherence of

p-fimbriated *E. coli* bacteria to uroepithelial cells. Fed. Am. Soc. Exp. Biol. J. 15, A284.

Kallenius, G., Mollby, R., Svenson, S.B., Winberg, J., Lundblad, A., Svenson, S., Cedergren, B., 1980. The P antigen as receptor for the haemagglutinin of pyelonephritic *Escherichia coli*. Microbiol. Lett. 7, 297–302.

Kontiokari, T., Sundqvist, K., Nuutinen, M., Pokka, T., Koskela, M., Uhari, M., 2001. Randomised trial of cranberry-lingonberry juice and *Lactobacillus* GG drink for the prevention of urinary tract infections in women. Brit. Med. J. 322, 1571–1573.

Kraemer, R.J., 1964. Cranberry juice and the reduction of ammoniacal odor of urine. S.W. Med. 45, 211–212.

Krueger, C.G., Porter, M.L., Weibe, D.A., Cunningham, D.G., Reed, J.D., 2000a. Potential of cranberry flavonoids in the prevention of copper-induced LDL oxidation. Polyphen. Commun. 2, 447–448.

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

Krueger, C.G., Vestling, M.M., 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]. J. Agric. Food Chem. 51, 538–543.

Krueger, C.G., Vestling, M.M., Reed, J.D., 2004. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of anthocyanin-polyflavan-3-ol polymers in cranberry fruit (*Vaccinium macrocarpo*n, Ait] and spray dried cranberry juice. In: Waterhouse, A.L., Kennedy, J.A. (Eds.), Red Wine Color, Revealing the Mysteries, ACS Symposium Series 886.

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

Leffler, H., Svanborg-Eden, C., 1980. Chemical identification of a glycosphingolipid receptor for *Eschericia coli* attaching to human urinary tract epithelial cells and agglutinating human erythrocytes. FEMS Microbiol. Lett. 8, 127–134.

Ofek, I., Goldhar, J., Zafriri, D., Lis, H., Adar, R., Sharon, N., 1991. Anti-*Escherichia coli* adhesin activity of cranberry and blueberry juices. New England J. Med. 324, 1599.

Porter, M.L., Krueger, C.G., Wiebe, D.A., Cunningham, D.G., Reed, J.D., 2001. Cranberry proanthocyanidins associate with low-density lipoprotein and inhibit *in vitro* Cu^{2+}-induced oxidation. J. Sci. Food Agric. 81, 1306–1313.

Roberts, J.A., Kaack, M.B., Fussell, E.N., 1989. Bacterial adherence in urinary tract infections: Preliminary studies in a primate model. J. Clin. Study Treat. Infect. 17, 401–403.

Sobota, A.E., 1984. Inhibition of bacterial adherence by cranberry juice: potential use for the treatment of urinary tract infections. J. Urol. 131, 1013–1016.

Stothers, L., 2002. A randomized trial to evaluate effectiveness and cost effectiveness of naturopathic cranberry products as prophylaxis against urinary tract infection in women. Can. J. Urol. 9, 1158–1162.

Walker, E.B., Barney, D.P., Mickelsen, J.N., Walton, R.J., Mickelsen, R.A., 1997. Cranberry concentrate: UTI prophylaxis. J. Fam. Prac. 45, 167–168.

Zafriri, D., Ofek, I., Adar, R., Pocino, M., Sharon, N., 1989. Inhibitory activity of cranberry juice on adherence of type 1 and type P fimbriated *Escherichia coli* to eucaryotic cells. Antimicrob. Agents Chemother. 1989, 92–98.