

# Comparison of A-type Proanthocyanidins in Cranberry and Peanut Skin Extracts Using Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry

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## Abstract

Cranberry products have long been used to treat urinary tract infections. It is believed that the A-type proanthocyanidins in cranberries contribute to this function. Peanut is one of the other, few food sources that primarily contain A-type proanthocyanidins. The skin on the outside of the peanut kernels (testa), which is treated as an agriculture waste product, contains high levels of A-type proanthocyanidins. In this study, an HPLC diol column separation method and MALDI-TOF MS were used to characterize and compare the proanthocyanidin compositions of peanut skins and cranberries. MALDI-TOF MS in linear mode was able to detect a group of proanthocyanidins with DP (degree of polymerization) 10 in peanut skin extract, but was only able to detect DP 8 in cranberry extract. The reflectron mode showed clusters of clear narrow peaks at DP 7 in peanut skin extract, while the highest DP resolved for cranberry extract was only 3 in reflectron mode. This might be due to the low response intensity of the cranberry samples with the current cleanup method and the matrix. Based on the resolved peaks in reflectron mode, pPeanut skins and cranberries have similar proanthocyanidins composition; they contain both A-type and B-type proanthocyanidins, with the A-type being predominant. This result may inspire future studies on the comparison of biological functions between peanut skins and cranberries and further comparison of their polymeric proanthocyanidin composition.

**Keywords:** Peanut skin; Cranberry; A-type proanthocyanidins; MALDI-TOF

## Introduction

Cranberry proanthocyanidins are well known for their anti-UTI (urinary tract infection) properties. UTIs, primarily caused by uropathogenic *Escherichia coli* in the kidney, prostate, urinary bladder, or genitourinary system [1], affect 8 million women annually in the USA and exhibit a high recurrence rate: >25% recur within 6 months [2]. Worldwide, UTIs affect 150 million people and account for 6 billion US dollars (equivalent) in direct costs per year [3]. The development of bacterial resistance to traditional antibiotics makes the treatment of UTI recurrences very challenging, and demands the exploration of alternative remedies such as herbs [4]. The American red cranberry (*Vaccinium macrocarpon*) has been recognized for its benefits to the urinary tract [3]. Consumption of cranberry juice helps prevent UTIs [5]. A few theories regarding the mechanism have been proposed. At one time, the acidity of cranberry was believed to provide antibacterial effects. However, later research showed that cranberries do not alter the pH of urine, and that the prevention effect is not due to change in the physical properties of urine, but to specific compounds that inhibit the adherence of *E. coli* to uroepithelial cells [6]. Proanthocyanidins were identified and considered to be the main active ingredient for inhibiting P-fimbriated *E. coli* adherence to uroepithelial cells [7]. In later studies, proanthocyanidins with "unique" molecular structures, the A-type interflavan bonds, were isolated from cranberry fruit that exhibited potent bacterial anti-adhesion activity. These proanthocyanidins consisted predominantly of epicatechin units with mainly a DP of 4 and 5 and at least one A-type linkage [8,9].

Peanut is one of the few common foods that have been reported to contain primarily A-type proanthocyanidins besides cranberry products [10]. The reddish-brown skins, a byproduct of the peanut industry, represent 2.0-3.5% by weight of peanut kernels [11] and contain 17% by weight of proanthocyanidins [12]. It was reported that A-type proanthocyanidin oligomers were predominant in a 20% (v/v)

methanol extract of peanut skins and represented 95% (w/w) of the extract on a dry weight basis [13]. Although other nut skins contain proanthocyanidins as well, the B-type proanthocyanidins are often predominant [14]. Compared to cranberry proanthocyanidins, the bioactivities of peanut skin proanthocyanidins are far less studied, possibly because peanut skins are less commonly consumed than cranberry products in a western diet.

Peanut skins have been used for centuries in many Chinese medicine formulas; the recommended usage can be found in ancient medical books. Interestingly, one of peanut skin functions is that it could help with hematuria, to which UTI could be one of the contributing factors. However, like many other ingredients used in Chinese medicines, its function and mechanism hasn't been thoroughly studied in contemporary lab setting or clinical trials. In this study, we compared the proanthocyanidin profiles of peanut skins and cranberry, with the hypothesis that there might be some structural and compositional similarities between the A-type proanthocyanidins.

## Materials and Methods

### Cranberry extracts from fresh cranberry and sample cleanup

Fresh cranberries (Decas Cranberry Products, Inc., Carver,

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MA) were purchased from a local grocery store. For each replicate extraction, 300 g cranberries were ground using a Waring blender and extracted twice with acidified aqueous acetone (acetone: water: acetic acid = 70: 29.5: 0.5, v/v/v) for 15 minutes in a Fisher (Pittsburgh, PA) FS20 ultrasonic water bath. A total of 750 mL solvent was used for each batch. Liquid extracts were collected, filtered, and evaporated at 40°C for 2 hours using a rotary evaporator. The volume of the liquid extract was brought to 50 mL with Milli-Q water. Extracts were frozen at -20°C until use.

Crude extract from fresh cranberries was cleaned up using Waters (Milford, MA) Sep-Pak C18 cartridges (5 g, 20 cc) using a 24-position vacuum manifold. Columns were conditioned with 2 column volumes (about 20 mL) of methanol and 2 column volumes (about 20 mL) of Milli-Q water before use. Each column was loaded with 2 mL crude extract and eluted with Milli-Q water to remove sugar and small phenolics. A refractometer was used to measure the sugar content in each eluent. Elution with Milli-Q water continued until the Brix value reached zero. Then each column was washed with two column volumes (20 mL) of aqueous acetone (acetone: water: acetic acid = 70: 28: 2, v/v/v) to collect proanthocyanidins. Aqueous acetone fractions from multiple columns were combined, evaporated at 40°C, and freeze-dried. This dry extract was stored at -20°C before use.

### Cranberry extracts from cranberry juice and sample cleanup

Ocean Spray Cranberry Juice Cocktail was purchased from a local grocery store. Waters Sep-Pak C18 SPE cartridges (5 g, 20 cc) were used to concentrate proanthocyanidins and remove the sugar in the juice cocktail. Columns were conditioned with 2 column volumes (about 20 mL) of methanol and 2 column volumes (about 20 mL) of Milli-Q water before use. Each column was loaded with 10 mL cranberry juice cocktail and immediately flushed with vacuum; this was repeated 4 times for each column. So, essentially, 40 mL of cranberry juice cocktail was loaded onto each column. During this process, most proanthocyanidins were concentrated on the column while most sugar was eluted out of the column.

To clean up the extract, Milli-Q water was used to rinse off the sugar and other small molecules. A refractometer was used to measure the sugar content of the eluents. Elution continued till Brix value reached zero. Then each column was washed with two column volumes (20 mL) of aqueous acetone (acetone: water: acetic acid = 70: 28: 2) to collect proanthocyanidins. Aqueous acetone fractions from multiple columns were combined, evaporated at 40°C, and freeze-dried. This powdered extract was stored at -20°C before use.

### Peanut skins extract and sample cleanup

One hundred grams of peanut skins was defatted with 250 mL n-hexane 3 times and air dried in a fume hood overnight. The defatted peanut skin was then extracted with 250 mL acetone/water/acetic acid (70:28:2, v/v/v) 3 times. The skins were extracted in an ultrasonic bath for 5 min and in a stomacher for 2 min. Liquid extract was combined and centrifuged. The clear liquid was collected, evaporated, and freeze-dried. The extract was stored at -20°C until use.

One gram of the crude extract from peanut skins was dissolved in 10 mL Milli-Q water and cleaned up using Waters (Milford, MA) Sep-Pak C18 cartridges (5 g, 20 cc). Columns were conditioned with 2 column volumes (about 20 mL) of methanol and 2 column volumes (about 20 mL) of Milli-Q water before use. Each column was loaded with 2 mL extract and eluted with 30 mL Milli-Q water. Then each column was washed with two column volumes (20 mL) of aqueous acetone (acetone:

water: acetic acid = 70: 28: 2) to collect proanthocyanidins. Aqueous acetone fractions from multiple columns were combined, evaporated at 40°C, and freeze-dried. This dry extract was stored at -20°C before use.

### HPLC analysis

A normal-phase HPLC-fluorescence detection method developed by Gu [15] was used to compare the extracts before and after cleanup to see if any major proanthocyanidins of interest were lost. Analysis was performed using an Agilent 1260 Infinity HPLC equipped with a solvent degasser, a quaternary pump, an autosampler with temperature control, column oven, diode array detector, and a fluorescence detector. A Develosil Diol column (100 Å, 250 × 4.6 mm, 5 µm particle size) and a Luna HILIC guard column (4 × 3.0 mm ID SecurityGuard cartridge and cartridge holder) were purchased from Phenomenex (Torrance, CA, USA) and installed in this HPLC system. The columns were held at 35°C throughout runs. A binary mobile phase consisting of solvent A (acetonitrile: acetic acid = 98:2, v/v) and solvent B (methanol: water: acetic acid = 95:3:2, v/v/v) was run at 1 mL/min. Solvent B was held at 7% for 3 min, increased to 37.6% over the next 57 min, increased again to 100% in the next 3 min, held at 100% for 7 min, and then returned to 7% then held for 6 min. DAD detection, FLD excitation, and FLD emission wavelength was set to 280 nm, 230 nm, and 321 nm, respectively. Standards, consisting of flavanols (DP 1) and B-type procyanidins (DP 2-10), were purchased from Planta Analytica (Danbury, CT) and used as references. All samples and standards were held at 5°C in the autosampler before injection. Injection volume was set to 5 µL.

### MALDI-TOF analysis

An Applied Biosystems 4800 MALDI TOF/TOF Analyzer was used for the analysis of proanthocyanidins. The preschedule was modified from a method developed by Stringano et al. [16]. Lithium chloride (0.2 M in Milli-Q water) was used as the cationization agent and was spotted (about 1 µL) on the plate first. The s-DHB reagent (9:1 mixture of 2,5-dihydroxybenzoic acid and 2-hydroxy-5-methoxybenzoic acid) was purchased from Sigma-Aldrich (St. Louis, MO). It was prepared into a 20 mg/mL solution in 70% aqueous acetonitrile (v/v) with 0.1% TFA and used as the matrix. About 1 µL s-DHB was spotted on each dried target with lithium chloride. Each sample was prepared into 4 mg/mL, in triplicates, in acetonitrile/methanol (1:2, v/v), and was spotted on the dried target that had cationization agent and matrix agent on it. Both linear and reflectron modes were applied on each target during the analysis.

The predicted molecular weight of proanthocyanidins was calculated according to the following formula:

$$m/z = 288.0634 \times DP - 2.0157 \times A + 7.0160 + 2.0157$$

where 288.0634 corresponds to the molecular weight of one catechin or epicatechin extension unit (C<sub>15</sub>H<sub>12</sub>O<sub>6</sub>), DP is the degree of polymerization, 2.0157 accounts for the 2 lost H for each additional A-type interflavan bond, A represents the number of A-type bonds, 7.0160 denotes the molecular weight of lithium, the end 2.0157 accounts for that additional H on the two end units of the proanthocyanidin molecule. Molecular weights used in the calculation are based on the monoisotopic mass. The calculated molecular weights of proanthocyanidins were rounded to 2 decimal points.

## Results and Discussion

### Sample cleanup

Cranberries and cranberry juice have high sugar contents. If sugar

was not removed, the sample after lyophilization would be very sticky and hard to separate by chromatography. Also, based on literature, cleanup improves resolution and appearance of MALDI-TOF MS spectra [17]. Commercial SPE cartridges and LH-20 columns have been used in combination with various solvent systems to purify proanthocyanidin-rich plant extracts. In our study, we compared C18 and tC18, and selected C18 for sample cleanup; the separation results were similar, but C18 was much faster. After loading on the C18 columns, samples were eluted with Milli-Q water until the eluent Brix reached 0 ° as the wash step. It usually took 3 column volumes of water to bring down the Brix's value to 0 °. Then, one column volume of aqueous acetone was used elute the proanthocyanidins. Aqueous methanol and aqueous acetone were compared and it was decided to use acetone. While the solvents were equally effective in concentrating the proanthocyanidins, acetone evaporated much faster during rotary evaporation. Two more column volumes of aqueous acetone solution were used to rinse the columns. All eluents from the columns were analyzed using HPLC to see if proanthocyanidins were lost during cleanup. The collected proanthocyanidins fractions had very similar proanthocyanidin profiles as the crude extracts based on the HPLC chromatograms. Eluents before and after the collected fraction contained little or no proanthocyanidins.

Although peanut skin extract does not contain as much sugar as cranberries, it was cleaned following the same procedure used for cranberries because it was thought that it might help with MALDI-TOF MS resolution and lead to a more fair comparison with the cleaned cranberry extracts. However, both HPLC and MALDI-TOF results showed that cleanup method was not feasible for the peanut skin extract: proanthocyanidins started eluting off the column with water before switching to aqueous acetone; and some proanthocyanidins were still left on the column after eluted with one column volume of aqueous acetone. Also, the resolution of peanut skin MALDI-TOF spectra was not improved after this cleanup step. Instead, the cleanup seemed to lead to the loss of a few peaks in the MALDI-TOF spectra.

### Proanthocyanidins in fresh cranberry extract vs. commercial cranberry juice

The HPLC Diol phase method that we used in this study has been used to separate proanthocyanidins based on the degree of polymerization [15]. Robbins et al. [18] compared the separation efficiency of different brands of columns with similar packing material and found the Develosil diol phase column yielded the best chromatograms. Thus,

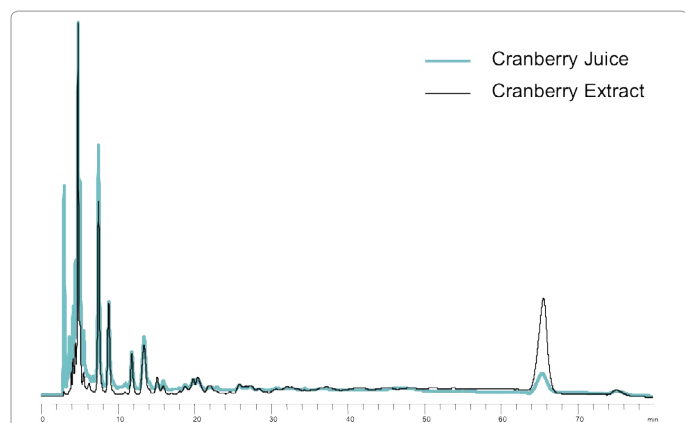


Figure 1: Overlay of HPLC chromatograms of cranberry juice and fresh cranberry extract.

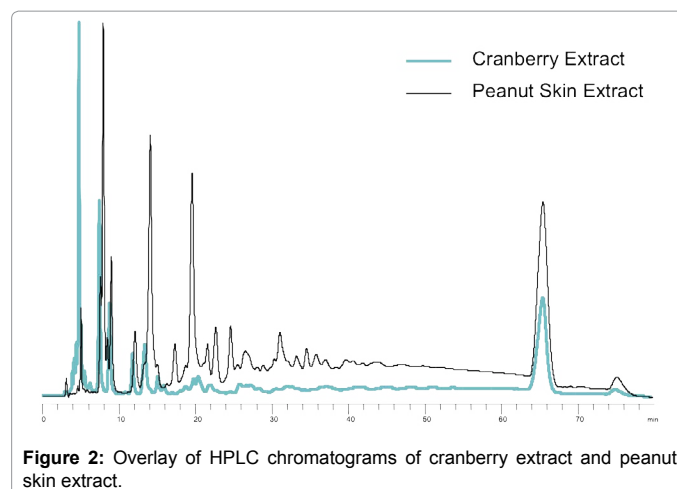


Figure 2: Overlay of HPLC chromatograms of cranberry extract and peanut skin extract.

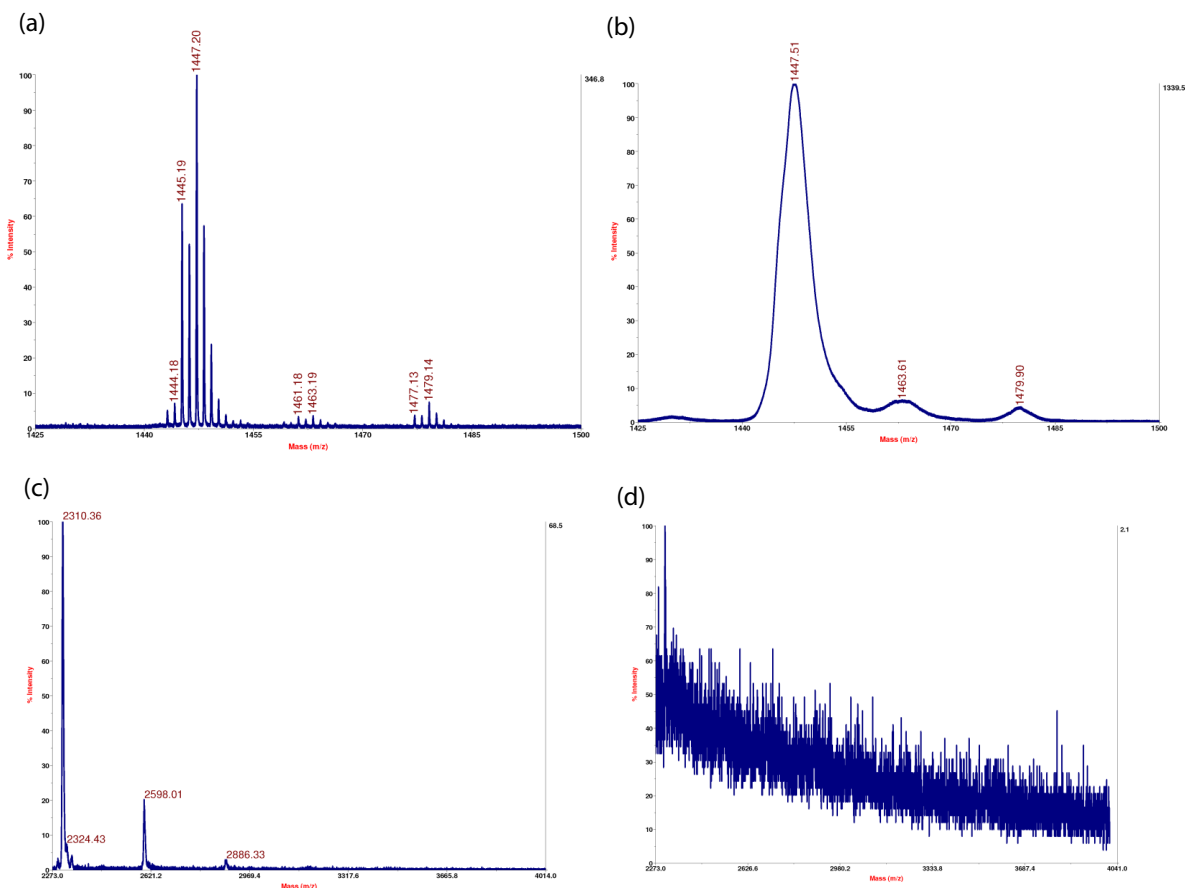
the Develosil column was used for our study. The eluting order of the procyanidin standards followed the degree of polymerization. Monomers eluted at the beginning of the run with nice sharp peaks, while oligomers eluted later with wider peaks that overlapped with each other. Polymers eluted after oligomers. It is worth noticing that the response intensity gets lower as the degree of polymerization gets higher. So the relative peak height does not indicate relative abundance. As the peak resolution became too low to be identified in the later part of the runs, the polymers were eluted together by drastically changing the mobile phase ratios. Although it has been argued that, due to the structure heterogeneity, proanthocyanidins derived from different plants sources with the same DP may have very different elution times, the general elution pattern (from low DP to high DP) has been verified in quite a few studies [15].

The HPLC chromatograms of the commercial cranberry juice and our fresh cranberry extract showed some general differences (Figure 1). Based on an overlay of the chromatograms from the fluorescence detector, cranberry juice contains relatively more monomers and small oligomers and less polymers compared with the fresh cranberry extract.

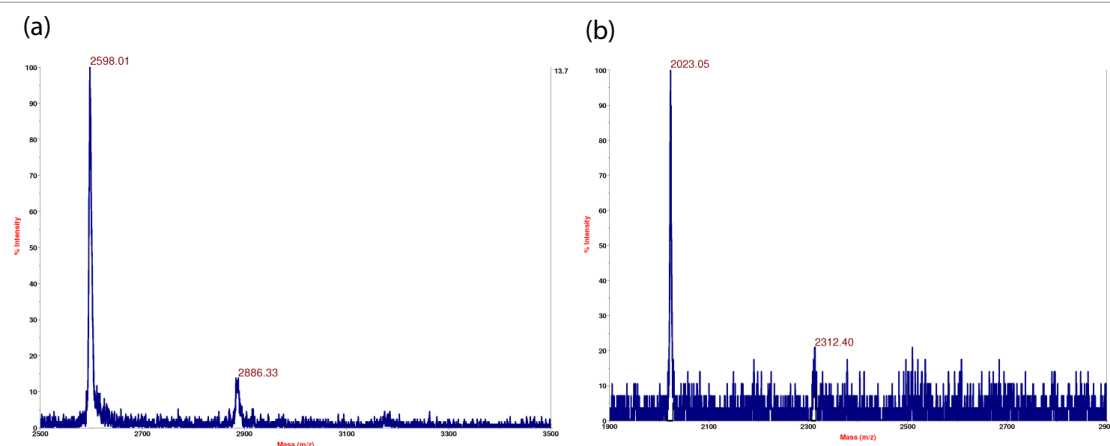
### Proanthocyanidins in cranberries vs. peanut skin

HPLC chromatograms showed that the proanthocyanidin composition of peanut skins differs markedly from cranberry proanthocyanidins (Figure 2). Although, due to structure heterogeneity, it may be unfair to compare proanthocyanidins from different sources using this HPLC method, it was used to tentatively estimate the composition difference and the relative distribution of monomers, oligomers, and polymers in these two extracts. Compared to cranberry extract, peanut skin extract seems to contain relatively more oligomers and polymers. To further elucidate the proanthocyanidin compositions of these two, MALDI-TOF MS was applied.

Each spotted sample on the MALDI-TOF plate was analyzed using both reflectron mode and linear mode, which were complementary to each other, as the reflectron mode could resolve peaks with one atomic mass unit apart, whereas linear mode could detect proanthocyanidins with higher degrees of polymerization. For example, in the spectrum of peanut skin extract, proanthocyanidins with a degree of polymerization of 5 were detected in both reflectron mode (Figure 3a) and linear mode (Figure 3b). The reflectron mode provided very narrow peaks with good resolution, so that we could easily see proanthocyanidin ions at 1447.20, 1445.19, 1461.18, 1463.19, 1477.13, and 1479.14 m/z. The linear mode was able to detect proanthocyanidins with higher degree of



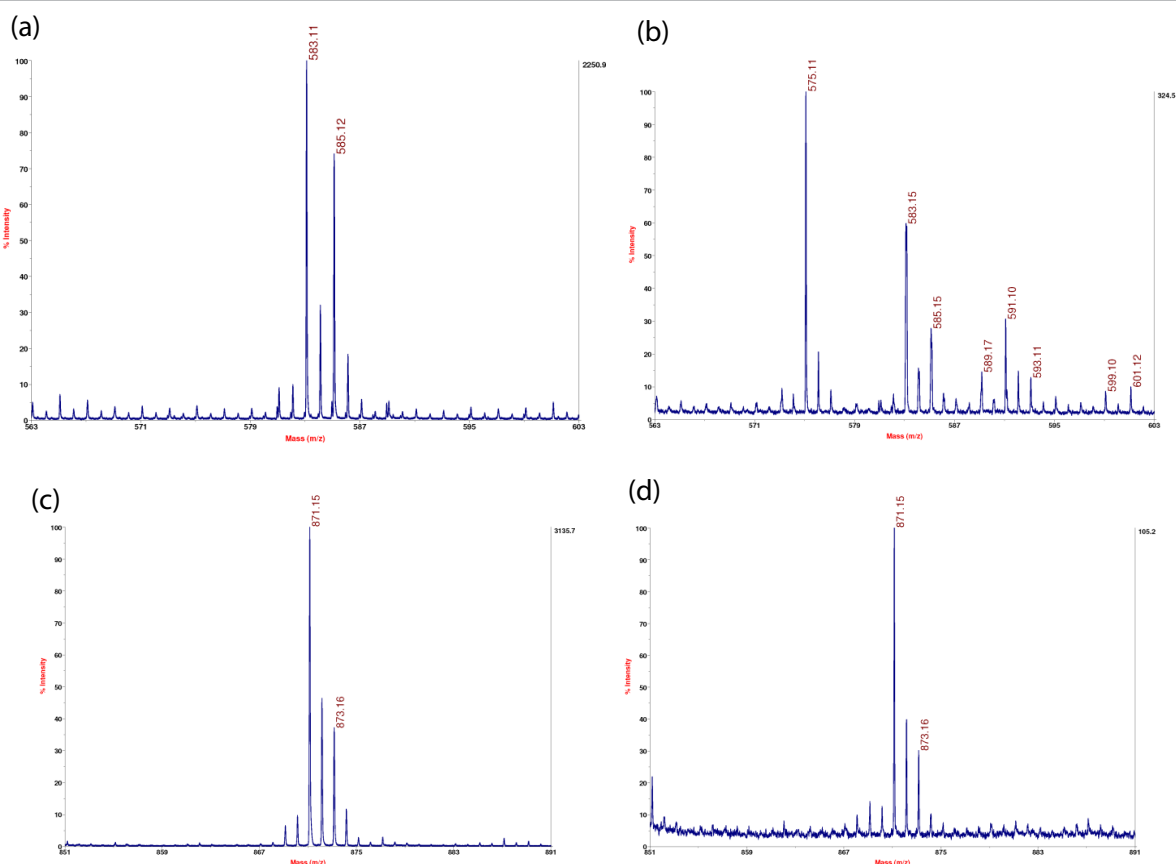
**Figure 3:** Selected regions in MALDI spectra of peanut skin extract (a) DP 5 in reflectron mode, (b) DP 5 in linear mode, (c) DP 10 in reflectron mode, (d) DP 10 in linear mode.



**Figure 4:** MALDI spectra showing the highest DP detected in the linear mode in (a) peanut skin extract, (b) cranberry extract.

polymerization than the reflectron mode. For example, the linear mode identified proanthocyanidins ions with DP up to 10 (average MW 2886.33) in the peanut skin extract and even showed small peaks with DP higher than 10 (Figure 3c), which were not automatically identified, whereas reflectron mode was not able to detect any peaks in the same range (Figure 3d). However, for structure elucidation, the wide humps in the linear spectra were less informative than the clusters of narrow peaks in the reflectron mode spectra.

More oligomers were identified in the peanut skin extract than the cranberry extract. The linear mode was able to detect a group of proanthocyanidins with DP 10 in peanut skin extract (Figure 4a) but was only able to detect DP 8 in cranberry (Figure 4b). In the reflectron mode spectra, peanut skin proanthocyanidins with DP up to 7 showed clusters of clear narrow peaks, while the highest DP identified for cranberry extract was only 3 in the same mode. This may indicate the relatively higher content of oligomers and polymers in the peanut skin



**Figure 5:** MALDI spectra in the reflectron mode for (a) proanthocyanidins with DP2 in peanut skin extract, (b) proanthocyanidins with DP 2 in cranberry extract, (c) proanthocyanidins with DP3 in peanut skin extract, (d) proanthocyanidins with DP3 in cranberry extract.

		B-type	A-type
DP 2	Predicted	585.08	583.06
	Peanut Skin	585.12	583.11*
	Cranberry	585.15	583.15*
DP 3	Predicted	873.14	871.12
	Peanut Skin	873.16	871.15*
	Cranberry	873.16	871.15*

\*the highest peak in the peak cluster.

**Table 1:** Predicted and observed molecular weight of proanthocyanidins with DP 2 and DP 3 in peanut skin extract and cranberry extract.

extract, which would agree with the HPLC chromatograms. However, there are other potential causes. For example, the selected matrix and salt may facilitate the ionization of peanut skin proanthocyanidins more efficiently than cranberry proanthocyanidins. For another example, there might be more non-proanthocyanidin impurities in cranberry extract that saturated the MALDI-TOF detector so that less proanthocyanidins was detected.

To analyze the proanthocyanidin compositions of peanut skin extract and cranberry extract, we zoomed in individual major clusters of peaks in the reflectron mode spectra. The spectrum region below m/z 500 was not considered as it is often saturated with signals from matrix-derived ions [17]. As the highest DP detected in cranberry extract in the reflectron mode was 3, we were only able to compare the proanthocyanidin compositions with DP 2 and 3. Peanut skins and cranberries contain both A-type and B-type proanthocyanidins, with the A-type being dominant, at both DP 2 and DP 3 (Figure 5). The observed

MW was very close to the predicted MW of proanthocyanidins (Table 1). It worth noticing that the response intensity of cranberry samples was much lower than that of peanut skin, and that more unknown compounds were detected in the cranberry spectra. More thorough cleanup might be necessary for further elucidation of cranberry proanthocyanidins using this MALDI-TOF MS method.

More peanut skin proanthocyanidins with higher DP were identified in the spectra (Figures 6 and 7), with A-type being dominant (Table 2). It seems that with the increase of DP, the A-type linkage in peanut skin proanthocyanidins increases. For example, the dominant peaks at both DP 4 and DP 5 were proanthocyanidins with one A-type linkage, while the dominant peaks at DP 6 and DP 7 were proanthocyanidins with two A-type linkages.

Although we were not able to compare the proanthocyanidin compositions of peanut skins and cranberries at higher DP, the spectra at lower DP clearly showed the similarity between these two. As it has been believed that the A-type proanthocyanidins in cranberry juice contribute to its anti-UTI effect, it would be interesting to investigate if the peanut skin extract could have a similar function towards UTIs.

## Conclusion

Although we were not able to compare the proanthocyanidin compositions of peanut skins and cranberries at higher DP, the spectra at lower DP clearly showed the similarity between these two. As it has been believed that the A-type proanthocyanidins in cranberry juice contribute to its anti-UTI effect, it would be interesting to investigate if

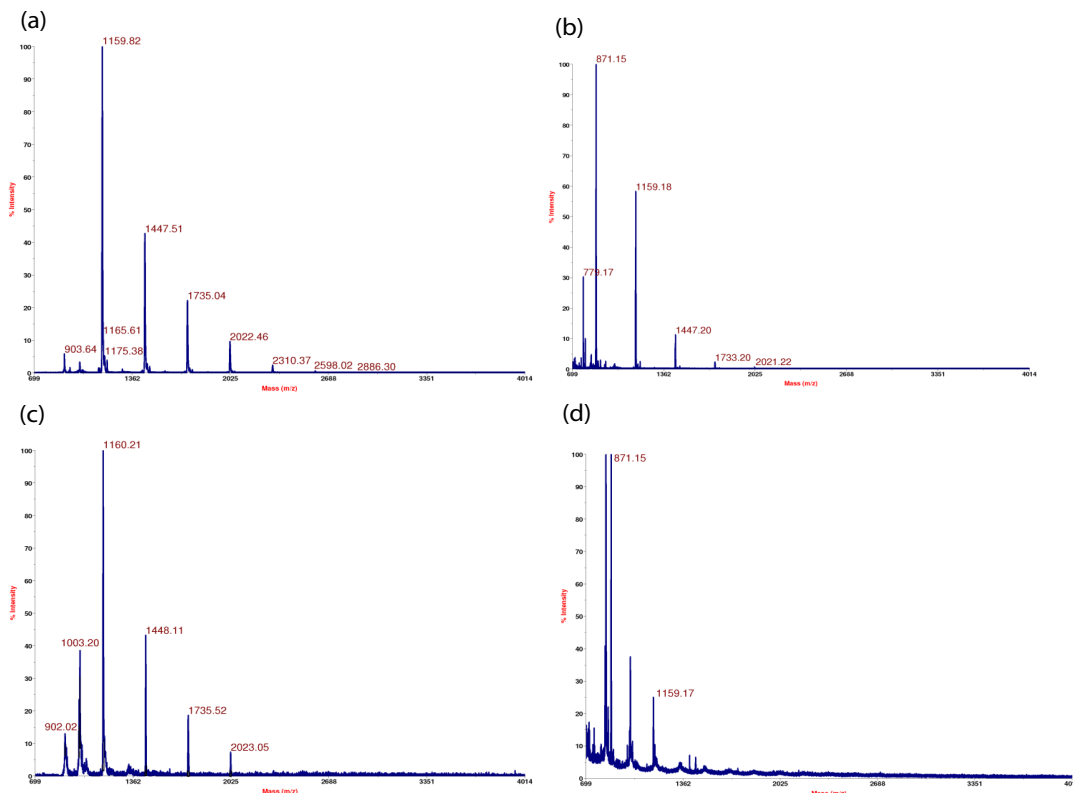


Figure 6: Full spectra of (a) peanut skin extract in the linear mode, (b) peanut skin extract in the reflectron mode, (c) cranberry extract in the linear mode, (d) cranberry extract in the reflectron mode.

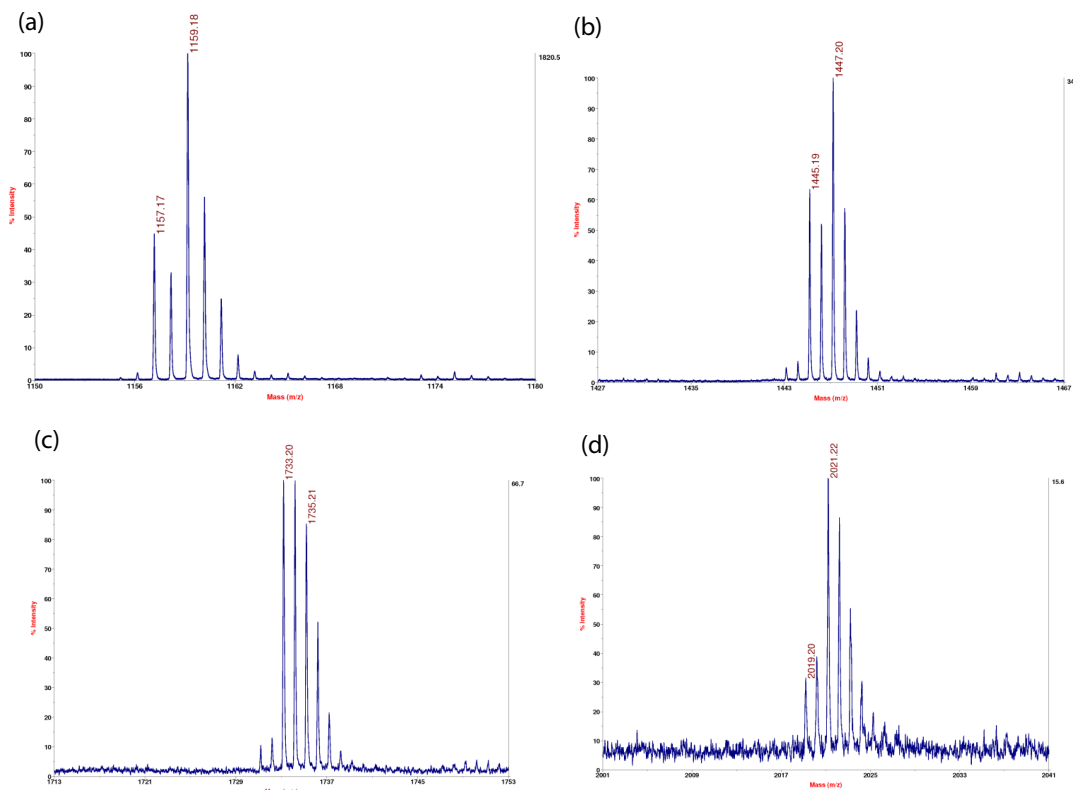


Figure 7: Selected ranges in reflectron mode MALDI spectrum that shows the proanthocyanidins with (a) DP 4, (b) DP 5, (c) DP 6, (d) DP 7.

		B-type	A-type		
			1 A-link	2 A-link	3 A-link
DP 2	Predicted	585.08	583.06		
	Observed	585.12	583.11*		
DP 3	Predicted	873.14	871.12		
	Observed	873.16	871.15*		
DP 4	Predicted	1161.20	1159.18	1157.16	
	Observed	--	1159.18*	1157.17	
DP 5	Predicted	1449.26	1447.24	1445.22	1443.20
	Observed	--	1447.20*	1445.19	--
DP 6	Predicted	1737.32	1735.30	1733.28	1731.26
	Observed	--	1735.21	1733.20*	--
DP 7	Predicted	2025.38	2023.36	2021.34	2019.32
	Observed	--	--	2021.22*	2019.2

\*the highest peak in the peak cluster.

**Table 2:** Predicted and observed molecular weight of proanthocyanidins in peanut skin extract.

the peanut skin extract could have a similar function towards UTIs. It would also be interesting to investigate the sample cleanup method and matrix selection for MALDI-TOF MS analysis of proanthocyanidins with higher DP, so that a more complete comparison of their proanthocyanidin composition could be made.

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