REVIEW

Methods to determine effects of cranberry proanthocyanidins on extraintestinal infections: Relevance for urinary tract health

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Urinary tract infections (UTI) are one of the most frequent extraintestinal infections caused by *Escherichia coli* (ExPEC). Cranberry juice has been used for decades to alleviate symptoms and prevent recurrent UTI. The putative compounds in cranberries are proanthocyanidins (PAC), specifically PAC with "A-type" bonds. Since PAC are not absorbed, their health benefits in UTI may occur through interactions at the mucosal surface in the gastrointestinal tract. Recent research showed that higher agglutination of ExPEC and reduced bacterial invasion are correlated with higher number of "A-type" bonds and higher degree of polymerization of PAC. An understanding of PAC structure–activity relationship is becoming feasible due to advancements, not only in obtaining purified PAC fractions that allow accurate estimation, but also in high-resolution MS methodologies, specifically, MALDI-TOF MS. A recent MALDI-TOF MS deconvolution method allows quantification of the ratios of "A-type" to "B-type" bonds enabling characteristic fingerprints. Moreover, the generation of fluorescently labeled PAC allows visualization of the interaction between ExPEC and PAC with microscopy. These tools can be used to establish structure–activity relationships between PAC and UTI and give insight on the mechanism of action of these compounds in the gut without being absorbed.

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1 Overview

The cranberry (*Vaccinium macrocarpon* Aiton) is a native North American fruit [1–3] whose production is largely limited to Northern US and Canada (~99.5%) [4]. Cranberries are a rich source of a class of plant secondary metabolites called phenolic compounds. Among the most consumed fruits in the US, cranberries have the highest phenolic content, apart from wild blueberries [5]. Flavonoids constitute the major subgroup of phenolic compounds with over 9000 structurally distinct molecules identified [6]. Amidst flavonoids, proanthocyanidins (PAC) found in cranberries assume special relevance for their antimicrobial properties in regards to uropathogenic *Escherichia coli* and urinary tract infections (UTI).

2 Proanthocyanidins

2.1 Chemistry and structural heterogeneity

Flavan-3-ols such as (−)-epicatechin and (+)-catechin are the monomeric units used by the plant to assemble oligomeric

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Mol. Nutr. Food Res. 2015, *59*, 1292–1306

1293
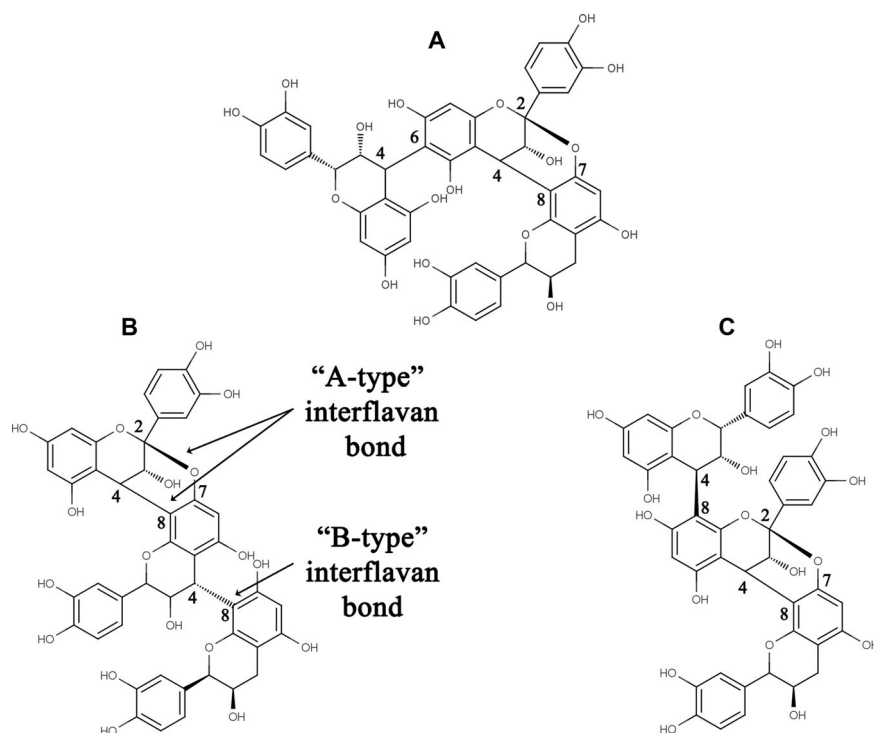
Figure 1. Structures of PAC trimers isolated from cranberries. (A) epicatechin-(4-6)-epicatechin-(4-8,2-*O*-7)-epicatechin, (B) epicatechin-(4-8,2-*O*-7)-epicatechin-(4-8)-epicatechin, (C) epicatechin-(4-8)-epicatechin-(4-8, 2-*O*-7)-epicatechin. Adapted from Foo et al. [11]

structures known as PAC. The mechanism by which this reaction happens is unknown and there is still debate concerning if the condensation reactions occur in the presence of an enzyme or not, although no enzyme has been isolated so far [7–10]. In most fruits and vegetables, PAC oligomerization occurs through an interflavan bond between each flavan-3-ol unit between C4–C6 or C4–C8 and these PAC may be described as having "B-type" interflavan bonds. In addition to this type of bond, some fruits, such as avocados, plums and cranberries, [11–16] contain an additional ether bond (C2-*O*-C7) leading to PAC with "A-type" interflavan bonds (Fig. 1). The molecular weight (MW) difference between a "B-type" and an "A-type" interflavan bond is due to the loss of two hydrogen atoms in formation the ether bond [15, 17].

The degree of polymerization (DP) of PAC depends on the fruit source [16] and processing [18]. PAC with DP = 23 [15] and 26 [19] were detected in cranberries by MS. Since each monomeric unit can be either (+)-catechin or (−)-epicatechin and the oligomerization can occur via C4–C6 or C4–C8, Cheynier et al. developed a formula to calculate the number of possible combinations of PAC per DP [20]. For instance, for PAC with DP = 23 with only "B-type" interflavan bonds, the number of theoretical structures surpasses 1×10^{13} (see Supporting Information). Cranberries have both "A-type" and "B-type" interflavan bonds so the number of possible combinations is likely even higher. PAC trimers assume a helical structure in water and the complexity of the three dimensional structure increases with DP [21, 22]. Since isolation and characterization of cranberry PAC with DP above

3 has not been reported, the only way to predict 3D structure for higher MW PAC is to use in silico tools as PRODRG, as recently done for a PAC tetramer with "A-type" linkages [23] and a PAC hexamer with "B-type" linkages [24].

In addition, molecules corresponding to anthocyanins bound directly to PAC or via a vinyl linkage have been detected in cranberry fruit and juice (Fig. 2) [25, 26], increasing molecular complexity.

PAC are responsible for astringency felt in the oral cavity after consumption of food products rich in PAC, due to their complexation with proline-rich proteins found in saliva [27, 28]. It was suggested that different binding properties, leading to different astringency sensations, could arise from the preferential conformation adopted by PAC with different DP [21]. This protein–PAC interaction can also cause haze in beverages [29]. Since ancient times, this ability to precipitate proteins (e.g., collagen [30]) has been used to transform animal skins into leather by adding powdered vegetable sources rich in PAC (e.g., oak bark) [31, 32], in a process known as tanning, and hence PAC are also referred to as "tannins" [33, 34]. The term "PAC" arises from the fact that when these compounds are treated with concentrated acid they form red-colored anthocyanidins, which is the basis of the butanol-HCl assay to detect PAC [35]. Since PAC are not hydrolyzed with dilute acids, some authors refer to PAC as "condensed tannins." However, the term "tannin" should be used with caution to avoid confusion with "hydrolyzable tannins," which are structurally different from PAC as they contain multiple esters of gallic acid with glucose and products of their oxidative reactions [32].
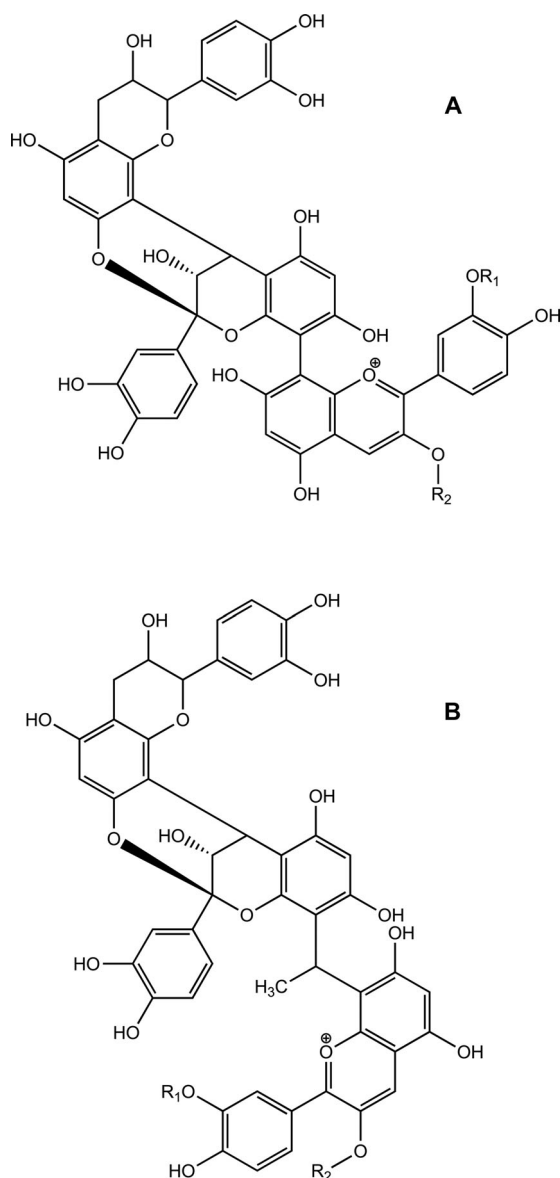
Figure 2. Structure of PAC directly bound to anthocyanins (A) or via an ethyl bridge (B). Each of these structures correspond to six molecules depending on the combination of the substitution in R_1 and the sugar moiety present in R_2: (I) $R_1 = H$, $R_2 = $ glucose, (II) $R_1 = H$, $R_2 = $ galactose, (III) $R_1 = H$, $R_2 = $ arabinose, (IV) $R_1 = CH_3$, $R_2 = $ glucose, (V) $R_1 = CH_3$, $R_2 = $ galactose, and (VI) $R_1 = CH_3$, $R_2 = $ arabinose. Adapted from Reed et al. [15].

2.2 Benefits for human health

Recent research suggests that cranberry PAC decrease the risk of cardiovascular diseases [14, 36], dental caries [37], periodontal diseases [37–39], gastrointestinal diseases caused by *Helicobacter pylori* [40] and noroviruses [41], inhibit enzymes, such as pancreatic lipase [42], α-amylase, and glucoamylase [43], reduce inflammation at the gut level [44], and improve the gut mucus layer morphology and homeostasis [45, 46]. In

vitro, cranberry PAC have demonstrated anticancer activities in esophagus [47], colon [13], prostate [48], ovaries [49], and lung cancer cell lines [13, 50]. Depending on the type of cancer cell line, the beneficial role of cranberry PAC seems to be related to induced apoptosis and inhibition/suppression of cell proliferation. The best-known and most extensively documented cranberry PAC health benefits concern the prevention of UTI, which will be discussed in Section 3.

2.3 Bioavailability

To exert these health benefits, PAC have to be bioavailable in the target tissue and most research conducted thus far has not taken into consideration this crucial aspect. The research regarding PAC bioavailability is scarce because it is difficult to obtain sufficient quality and quantity of these molecules due to their complex chemical nature [30, 51, 52]. The concept of bioavailability is intimately related with the concentration of the molecule at the target cell or tissue after absorption, metabolism and excretion. After absorption, molecules are sulfated, glucuronidated, or methylated [53, 54] and transferred to either the bloodstream or back to the colon via the bile. This enterohepatic circulation is a common route to excrete xenobiotics but does not seem to play a relevant role in PAC with high MW because they have limited absorption [51, 55–57]. This PAC feature is paramount to the development of new research hypotheses.

While in vitro research shows that PAC are degraded in gastric juice, [58] studies performed with human volunteers show that PAC remain stable until they reach the small intestine, [59, 60] probably because acid secretion in the stomach is buffered by the food bolus, exposing PAC to less acidic conditions than in vitro.

In vitro research has been conducted using epithelial intestinal cell lines (e.g., Caco-2) to assess the absorption of PAC with low DP. (+)-Catechin, (−)-epicatechin and PAC dimers with one "B-type" interflavan bond are transported across Caco-2 cells, with a rate of 5–10% [61, 62]. A ten-fold decrease in permeability for PAC with DP = 6 was reported when compared to PAC with low MW [61]. These results were confirmed by Zumdick et al. who detected an eight-fold decrease in permeability when comparing PAC with DP = 6 and DP = 2 [63].

PAC with DP = 3 and 6 were found adsorbed to Caco-2 cells in concentrations significantly higher than (+)-catechin and PAC with DP = 2 [61], suggesting that PAC with higher DP can affect cell biochemistry more pronouncedly than oligomers with lower MW.

PAC dimers, trimers, and tetramers with "A-type" interflavan bonds have transport rates in Caco-2 cells of 0.6, 0.4, and 0.2%, respectively [64], indicating that "A-type" PAC have lower absorption rates than "B-type" PAC. However, perfusion experiments using rat small intestine showed that PAC dimers with "A-type" interflavan bonds are better absorbed

Mol. Nutr. Food Res. 2015, 59, 1292–1306

1295

than dimers with "B-type" interflavan bonds, but only 5–10% of the rate found for (−)-epicatechin [55].

In vivo, (+)-catechin, (−)-epicatechin, and PAC with DP = 2 and 3 with "B-type" interflavan bonds were detected intact in plasma at concentrations between 0.9 and 9.0 µM in rats fed with grape seed extract [65]. Lower values, in the order of nanomolar, were found in plasma after interventions with other PAC with "B-type" interflavan bonds such as in rats fed with apple [66] and in humans after cocoa ingestion [67]. Recently, procyanidin A2 was found in human urine in a concentration of 24.4 ± 13.8 ng/mg creatinine after consumption of cranberry juice [68]. However, the concentration of procyanidin A2 in the cranberry beverage was not reported making it impossible to estimate the bioavailability of this compound. These results show the lack of consensus about to what extent, if any, PAC are absorbed in the gut. Nonetheless, it is commonly accepted that for PAC with DP > 2 and 3, the absorption from the small intestine is very low.

In vivo and in vitro experiments show a large discrepancy in absorption rates of PAC with different types of interflavan bonds. Both in vitro and in vivo research in this field suffers from methodological biases because PAC quantification and characterization of PAC interflavan bond types are not adequate.

Perez-Maldonado et al. showed that when plants were grown with $^{14}CO_2$, it was possible to generate PAC fractions enriched in [^{14}C] to study the fate of PAC in the gut. After feeding sheep and goats with radioactive PAC fractions, high MW PAC were found intact in the cecum [69]. These results were corroborated by Gonthier et al. who demonstrated that intact PAC reach the colon after feeding rats with PAC with different DP [56]. In humans, 90% of apple PAC are recovered in the ileostomy effluent and therefore would reach the colon under physiologic circumstances [70]. Along with the poor absorption in the small intestine, these results suggest that PAC are found mainly intact in the large intestine and can serve as substrates for gut microbiota activity [51, 54, 71–73]. The influence of the intestinal microbiota on bioavailability of phenolic compounds was recently reviewed [74, 75].

Dimers with "A-type" and "B-type" interflavan bonds were degraded between 30–50% and 80%, respectively, using a pig cecum model, yielding hydroxylated low MW phenolic acids as hydroxyphenlyacetic, hydroxyphenlypropionic, and hydroxybenzoic acids [76, 77]. Similarly, 40% percent of a PAC trimer with "B-type" interflavan bonds was degraded by intestinal microbiota in a pig cecum model generating hydroxylated low MW phenolic acids [77]. The degradation rate decreases with the increase in PAC DP [56, 78] and metabolites of microbial origin as phenylvaleric, phenylpropionic, phenylacetic, and benzoic acid derivatives were identified in mice urine [56]. Since PAC exhibit strong complexation with proteins, it is extremely difficult to quantify free PAC in biological fluids. It is known that flavonoids strongly bind to albumin and other plasma proteins [79]. Up to 92% of apple PAC can circulate in the bloodstream bound to plasma proteins but unlike low MW flavonoids such as rutin, apple PAC

tend to bind to HDL instead of albumin [80]. For PAC with "A-type" linkages little research has been conducted but PAC from persimmon [81] are able to strongly bind to albumin [82]. In order to release PAC bound to protein in biological fluids, the addition of phosphoric acid is general performed in the sample preparation step [83]. Therefore, it is likely that PAC degradation reported in the previous studies might correspond to the limitations of analytical methods. The low MW phenolic degradation products may be excreted in the feces or absorbed from the large intestine, enter the bloodstream, and then excreted in the urine. Thus, peripheral blood, tissue and urinary concentrations of PAC are lower than concentrations that have in silico antimicrobial effects, indicating that PAC may not exert a biological effect outside of the gut, unless low concentration of PAC affect other aspects of a postabsorptive response in tissue such as gene expression.

The persistence of PAC in the gut would allow these compounds to exert biological effects on the epithelium and absorption would not be required for a potential health benefit associated with cranberry PAC [22]. As almost unabsorbable molecules, PAC can induce local antioxidant, antimicrobial, antiviral and antimutagenic effects in the gut [34]. Some authors have suggested the potential role of PAC against inflammation-mediated diseases, such as colorectal cancer [60].

2.4 Methods of extraction and isolation

High MW PAC tend to bind to cell wall material and are difficult to extract by conventional methods [84, 85]. Approximately 40% of total PAC in cranberries are retained in the press cake [18], after juicing, leaving a substantial amount of insoluble (nonextractable) PAC unquantified. Alkaline hydrolysis and acid depolymerization have been proposed to quantify insoluble PAC [84, 86], but they can alter structural information. Analytic methods, such as butanol-HCl [35], can be used to quantify insoluble PAC if appropriate standard reference materials are used [87–89]. Low MW PAC are water-soluble, whereas high MW PAC are partially insoluble in water. Because water-soluble PAC are easily extracted during juicing, the press cake is relatively enriched in high MW PAC. This complex solubility of PAC poses significant challenges in terms of extracting these compounds from different commodities.

Lipophilic compounds are removed from the plant material with dichloromethane or hexane and PAC are extracted with a mixture of water and organic solvents such as methanol or acetone [90]. Partitioning of phenolic compounds is performed using resin beds, such as Sephadex LH-20 or Toyopearl HW-40 [14, 19, 91, 92], which allow for PAC isolation. PAC structural intricacy makes the isolation of single compounds with DP > 2 very difficult. Foo et al. isolated three trimers (DP = 3) with "A-type" interflavan bonds from cranberries using Sephadex LH-20 (Fig. 1) [11]. More recently, a trimer with two "A-type" interflavan bonds was isolated from

the geranium *Ixora coccinea* [93] and dimers, trimers and tetramers with "B-type" interflavan bonds were isolated from black soybean seed coats using Sephadex LH-20 [94]. Eleven tetramers with "B-type" interflavan bonds were isolated from apples *Malus pumila* cv. Fuji by SPE [95].

2.5 Methods of analysis

2.5.1 Spectrophotometry

A review on PAC methods of analysis was published by Schofield et al. [96]. Apart from lignin, PAC represent the most abundant class of phenolic compounds found in plants [97] and they can be analyzed by the Folin–Ciocalteu method [98]. However, this method does not distinguish between PAC and other cranberry phenolics because most phenolic hydroxyl groups reduce the phosphomolybdate reagent used in this assay, as well as other redox active compounds, such as sugars and vitamin C [99]. More specific spectrophotometric assays such as butanol-HCl [35], vanillin [100] and 4-(dimethylamino)cinnamaldeyde (DMAC) [19, 101, 102] methods are used for PAC quantification. The DMAC method is currently the most used assay [103] because it does not react with hydroxycinnamic acids, hydroxybenzoic acids, flavones, and flavonols [101, 102] and is more accurate and sensitive for PAC than other colorimetric assays [97]. However, due to the structural complexity associated with PAC found in cranberry products, the simple PAC dimer standard commonly used (e.g., procyanidin A2) gives inaccurate results for PAC content. Commercial cranberry products found in the market include supplements completely devoid of flavan-3-ols to highly purified ones [104] and PAC dosage on European cranberry supplements labels has been found to be unreliable [105]. It is also important to note that none of these colorimetric methods are capable of authenticating cranberry PAC from other sources of PAC.

To address the lack of appropriate standards for cranberry PAC analysis, a PAC fraction was isolated from cranberry press cake, characterized, and quantified by different analytical methods [19]. When used as a standard for the DMAC method, this PAC fraction was more accurate in estimating PAC content in different cranberry juices and powders than commercially available standards. The nature of the interflavan bond ("A-type" and "B-type") did not influence DMAC regression curve slopes, which indicates that PAC DP has the greatest influence on accurate estimates of PAC content. This same PAC standard finds application in the butanol-HCL assay, allowing for quantification of insoluble PAC. These results illustrate the importance of utilizing a PAC standard that is isolated from the sample to avoid underestimation of PAC content when using colorimetric analyses. Using a previous published method [19], we isolated PAC from different cranberry sources and apples and verified that the slope of DMAC calibration curves decreased when compared to procyanidin A2 (Table 1). Unlike in cranberries, PAC in apples are mainly

Table 1. Slopes of regression curves obtained in the DMAC method for procyanidin A2 and PAC isolated from different fruit sources

Standard	Calibration curve slope	SD
Apple	0.1327	0.0066
Cranberry (fruit)	0.1111	0.0080
Cranberry (press cake)	0.1612	0.0132
Procyanidin A2	0.4308	0.0419

At least four independent curves were evaluated and results are expressed in micrograms of dry matter.

composed of "B-type" interflavan bonds [106]. This suggests that interflavan bond type does not affect the reactivity toward DMAC since cranberry and apple PAC purified fractions displayed similar regression curve slopes. This observation is in agreement with a previous work that shows that the reactivity of procyanidin B2 and A2 standards is similar in terms of DMAC reactivity [19]. Hence, PAC DP has greater influence than the type of interflavan bond in estimating total PAC content using the DMAC method.

2.5.2 Chromatography

Separation and quantification of phenolics is done by HPLC using RP columns. However, due to the high structural complexity of PAC, separation of individual peaks it is not possible and PAC elute as a single hump [107, 108]. Normal-phase columns allow the separation of PAC with DP = 7 (heptamers) [109], 8 (octamers) [110], and even 10 (decamers) [111], but not only the peak resolution decreases as DP increases, but also for oligomers DP > 10 a broad peak elutes, showing an unresolved hump [110, 112–116]. Hydrophilic interaction chromatography is used to detect PAC from cocoa and apples with DP = 10 (decamers) [117]. PAC detection is frequently done by DAD, fluorescence (FD), or MS methods [19, 96, 99]. The structural diversity of PAC is much greater than previously appreciated [15, 118] and unlike DAD or FD, MS can provide structural information about PAC.

2.5.3 Mass spectrometry

ESI is a technique used in MS that has been used for PAC analysis [119] but it generates multiple charged species [120–123] making spectra analysis more cumbersome [124]. For instances, singly charged PAC with DP = 6 have the same *m/z* as doubly charged PAC with DP = 12 (*m/z* = 1729.3882) (Fig. 3).

Since two cranberry products (e.g., dietary supplements) with the same PAC content can have different ratios of "A-type" and "B-type" interflavan bonds, MS tools, such as MALDI-TOF MS, which fingerprint PAC composition are necessary. MALDI-TOF MS was first used to characterize PAC from apples in 1997 [125]. It is ideally suited for
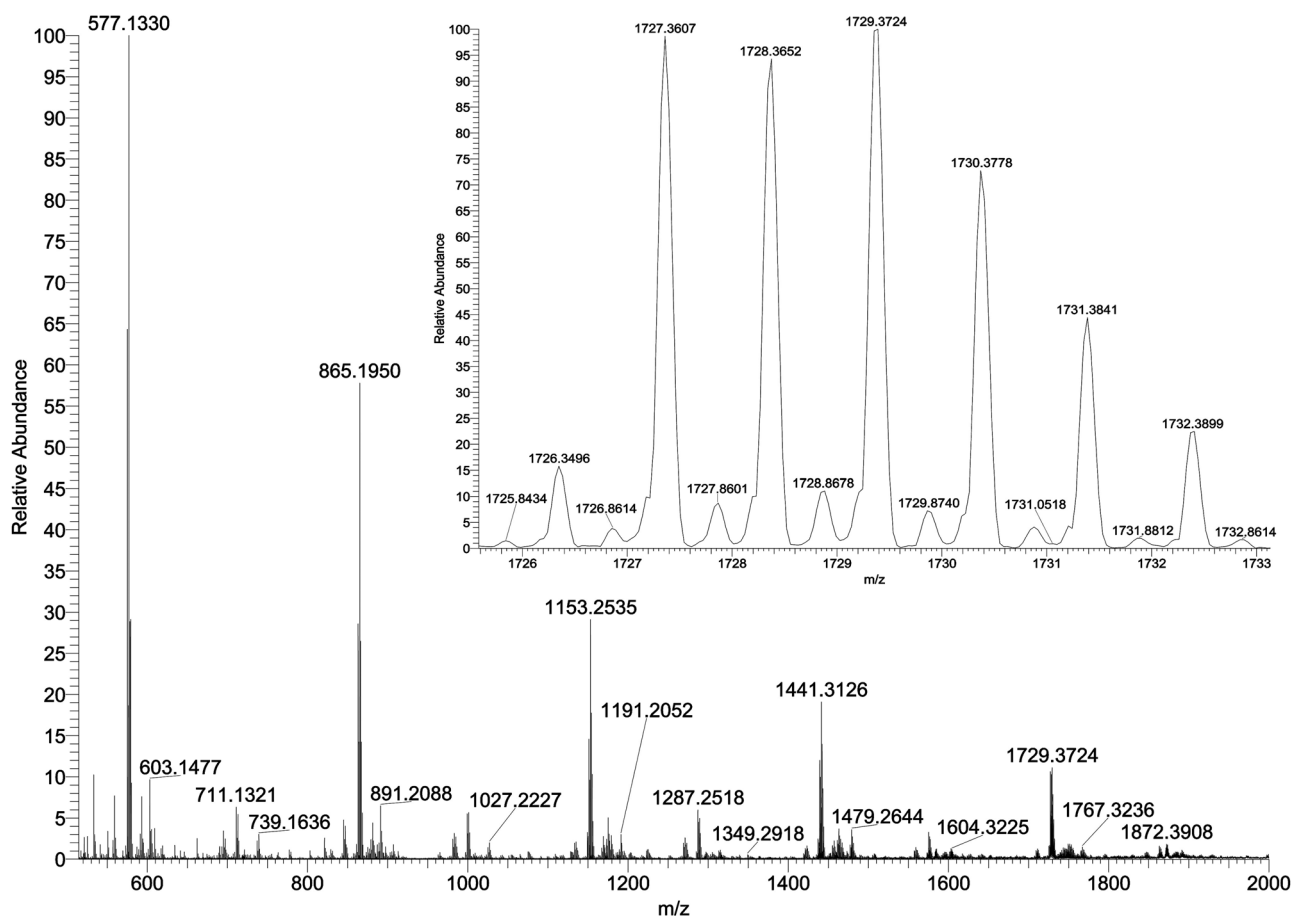
Figure 3. Linear ion trap Fourier transformed ion cyclotron resonance (FT-ICR) mass spectra of a cranberry PAC fraction displaying oligomers with between DP = 2 (*m/z* = 577.1330) and DP = 6 (*m/z* = 1729.3724). The differences between expected and observed *m/z* was <0.02 Da. The enlargement is a set of peaks that corresponds to both singly charged PAC with DP = 6 and doubly charged PAC with DP = 12 due to the intermediary minor peaks which differ 0.5 Da from the more abundant peaks.

characterizing polydispersed oligomers [126] and is considered the mass spectral method of choice for analysis of PAC with large structural heterogeneity [15]. In addition, TOF analyzers detect masses up to 500 000 Da [127] and unlike thiolysis [122], they are not destructive. MALDI-TOF MS produces only a singly charged molecular ion for each parent molecule and allows detection of high mass with precision [128]. MALDI-TOF MS application to cranberry PAC has been extensively described [12–14, 25, 116, 120, 129] and it provides identical mass spectra for different cranberry cultivars [129]. MALDI-TOF MS is an analytic tool that can be used for the authentication of cranberry PAC and compliments spectrophotometric methods that quantitate PAC.

Despite the broad range of applications for MALDI-TOF MS, TOF analyzers have inherently lower sensitivity at the higher MW region, which is due to lower impact velocity of high MW ions [130], compromising the detection of higher MW PAC. Furthermore, the matrix necessary for ionization of the analytes generates high intensity signals below 500 Da, resulting in detector saturation [127] and decreased PAC

signals. Recent MALDI-TOF MS equipment has the capacity of deflecting masses below a certain *m/z*, therefore increasing the overall analyte response. By deflecting *m/z* below 3000 Da, and using a cleanup method (Amberlite FPX66) prior to Sephadex LH-20 PAC isolation, we were able to detect in linear mode PAC with DP = 38, the highest DP ever reported for cranberry PAC using MALDI-TOF MS (Fig. 4).

Semiquantitative approaches were attempted by using the signal height as a measure of relative abundance. The contributions of various PAC in the MALDI-TOF MS spectrum of cranberries were determined [120], but it is noteworthy that peak heights for equimolar loadings of different analytes may vary significantly and are not representative of their abundance in solution [131].

An understanding of the natural abundance of different isotopes of C, O, and H within a specific PAC DP permitted the development of a mathematical model to calculate the ratios of "A-type" to "B-type" interflavan bonds utilizing MALDI-TOF MS [132]. This novel MS deconvolution method was validated by using known amounts of procyanidin A2 and
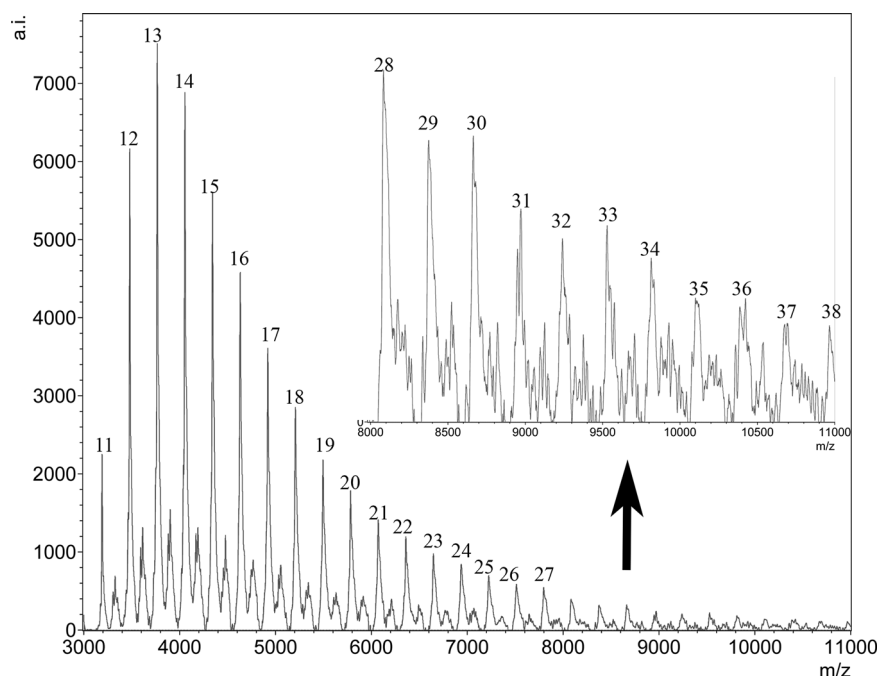
Figure 4. MALDI-TOF-MS of PAC isolated from cranberries after cleanup with Amberlite FPX66 and fractionation on Sephadex LH-20. Data were collected in positive linear mode and deflection set at 3000 Da and each number corresponds to a specific PAC DP. The region of the spectrum between 8000 and 11 000 Da is enlarged to assist in visualization. "a.i" stands for absolute intensity.

procyanidin B2 and applied to cranberry PAC. This simple, repeatable, robust, precise, and accurate methodology allows determination of structural diversity of PAC in food products other than cranberries. We recently applied this deconvolution method to other food sources to assess PAC fingerprinting and demonstrate the broad field of application of this tool (Table 2).

Nonetheless, MALDI-TOF MS lacks resolution to resolve compounds above the baseline, which have lower absolute

intensities [15]. When dealing with complex mixtures, such as PAC, ultrahigh resolution allows signals of two ions with similar *m/z* to be detected as distinct ions. Orbitrap [124] and triple quadrupole [133] mass spectrometers have been used to characterize cranberry PAC. Fourier transformed ion cyclotron resonance provides ultrahigh resolution and high mass accuracy [134] and was recently applied to study PAC in lychees [135], grape seeds [136], and cranberries [19]. However, like Orbitrap and triple quadrupoles, when using

Table 2. Percentage of PAC with at least 1 "A-type" and 2 "A-type" interflavan bonds after applying the MALDI-TOF MS deconvolution method [132] between DP 3 and 8 for cinnamon, cranberry and blueberry PAC fractions extracted with 70% acetone and isolated with Sephadex LH-20 as described previously [19]

DP	Cinnamon				Cranberries		Blueberries	
	Branches	Leaves	Wood	Bark	Press cake	Fruit	Wild	Domestic
PAC with at least 1 "A-type" interflavan bond								
3	97.8	96.2	93.8	90.1	94.8	93.5	0.0	0.0
4	86.6	80.0	86.1	90.4	96.0	88.0	34.4	32.0
5	87.6	77.9	89.8	98.1	96.3	90.1	34.3	26.2
6	89.3	74.2	95.5	97.4	96.7	95.4	35.6	33.8
7	88.5	71.0	96.2	94.4	99.2	99.6	31.3	0.0
8	85.1	69.0	100.0	98.3	97.1	97.5	0.0	0.0
Median	88.0	76.0	94.7	95.9	96.5	94.5	32.8	13.1
PAC with at least 2 "A-type" interflavan bonds								
3	0.9	6.3	2.4	28.7	12.5	1.7	0.0	0.0
4	5.5	12.5	6.6	29.1	34.6	5.4	7.8	8.5
5	10.2	19.1	15.0	41.4	42.5	13.1	6.5	7.2
6	31.2	24.3	47.9	68.0	61.8	39.0	0.0	0.0
7	36.3	30.1	63.1	78.0	65.6	51.7	0.0	0.0
8	41.1	41.1	3.4	72.3	44.7	60.1	0.0	0.0
Median	20.7	21.7	10.8	54.7	43.6	26.0	0.0	0.0

Mol. Nutr. Food Res. 2015, *59*, 1292–1306

1299

Fourier transformed ion cyclotron resonance the upper mass range is limited to 2000 Da and singly charged PAC with DP > 6 are not detected using this technique. Accurate quantification and characterization of PAC are extremely important due to the putative health benefits associated with the consumption of cranberry PAC.

3 ExPEC and UTI

Extraintestinal pathogenic *E. coli* (ExPEC) are part of the gastrointestinal microbiota. Although ExPEC are not associated with acute disease in the gut, they have the capacity to colonize niches outside of the gut, including the urinary tract [137,138]. UTI is the most common disease caused by ExPEC [139–141] and in 2010 UTI had an estimated annual direct and indirect cost of approximately $2.3 billion [142]. These pathogens are believed to originate within the gut [143] and there is evidence for an intestinal habitat for uropathogenic bacteria [144]. Furthermore, isolates from individuals with UTI often match rectal isolates from the same person [145].

Bacterial adherence to the host tissue is one of the most important prerequisites for bacteria colonization and subsequent infection. Inability of the pathogen to bind to the cell surface causes microbial clearance. Investigations into the mechanism of adherence to host tissue showed that the primary adherence factors are supramolecular filamentous adhesive organelles known as fimbriae. Fimbriae can specifically bind to sugars present on the mucosal or intestinal cell surfaces of the host tissue [146]. This type of binding occurs via specific types of fimbriae-mediated adhesion: type 1 fimbriae (mannose sensitive) and type P fimbriae, which is mannose resistant [147,148]. In UTI, P fimbriae are associated with adhesion to both vaginal and bladder epithelium cells [149].

3.1 Effect of PAC on ExPEC

PAC may act at the initial step, by inhibiting strains of *E. coli* from adhering to uroepithelial cells after consumption of cranberry products [12,149–151], in a linear dose-dependent manner [149], but the exact mechanism remains unknown [152–154]. PAC bind to *E. coli* and render bacteria nonadherent, possibly by attaching to fimbrial tips [11,152] and compressing them, thus diminishing the adhesive forces [155], affecting the first step in the infection process [156]. In addition to P fimbriae compression, PAC can also reduce the number of P fimbriae in *E. coli* as recently demonstrated by electron microscopy [157]. These mechanisms do not kill bacteria, so there is less chance of selection for resistant bacterial strains. PAC from cranberries have shown inhibition of adherence of multi-drug resistant *E. coli* strains on uroepithelial cells by 70% [158]. "A-type" PAC are able to affect ExPEC agglutination probably by binding to P fimbriae and

subsequently reducing the availability of these virulence factors to adhere to epithelial cells in the gut [106]. The prevention of transient gut colonization decreases the likelihood that ExPEC will be present on the perineum and gain access to the genitourinary tract, thus eliciting a prophylactic role in UTI. It is also possible that cranberry PAC can affect ExPEC beyond a physical–chemical mechanism by changing the genetic expression of virulence factors at the gut level, although this hypothesis has not been tested. In vitro research showed that cranberry PAC negatively affect the swimming and swarming motility of ExPEC by downregulating the *fliC* gene, which is responsible for the production of flagellin subunits which compose the long curved flagellum found in some ExPEC [159]. Other studies with tea polyphenols have reported inhibition of specific genes associated with the growth of cariogenic bacteria [160]. Cranberry PAC decrease virulence of ExPEC responsible for oropharyngeal colonization and subsequent prevention of pneumonia in a mouse model [161] but no changes in virulence factors were reported. Cranberry PAC altered virulence factors and decreased biofilm formation by ExPEC, but the genetic expression of virulence factors was not studied [162].

Evidence from a meta-analysis of clinical studies indicated that cranberry juice and cranberry dietary supplements decrease the number of symptomatic UTI over a 12-month period in women with recurrent UTI [163]. However, recent evidence shows that cranberry products are unlikely to prevent UTI, except for specific patient sub-populations, according to an updated systematic review published in The *Cochrane Library* [164]. Not only did the studies included in this meta-analysis utilize different administration vehicles (e.g., tablets, capsules, juices) but also the dosage and concentration of the cranberry products were never accurately and properly determined. Due to the substantial variability across trials in the quantitative and qualitative chemical composition of the cranberry products, it is difficult to draw valid conclusions about the effectiveness of cranberry products, as was also pointed out by the authors of the *Cochrane* study. Although promising results concerning the interaction between PAC and UTI have been published in the past two decades, this research field still lacks qualitative and quantitative methods to allow PAC standardization and comparison between different formulations used in clinical trials.

Furthermore, the research conducted thus far has systemically ignored the low bioavailability of PAC and in vitro models using kidney [165], vaginal [149] and bladder cells [158] have been used. To overcome this limitation, it has been suggested that PAC could bind to ExPEC rendering them antiadherent before introduction in the urinary tract or PAC could modify bacterial selection pressure in the colon to favor nonadherent ExPEC strains [152]. Recently, it was shown that cranberry PAC supplementation can positively restore mucosal integrity in immunocompromised interventions [45] and improve gut-associated lymphoid tissue functionality [46], indicating that PAC can elicit health benefits in the gut level without being absorbed.

3.1.1 Effects of PAC structure on ExPEC

The chemistry of cranberry PAC is essential to understand the bioactivity of these compounds but most research has failed in connecting PAC chemistry and biology. A new paradigm is needed to advance the structure–function understanding of how PAC affect UTI. This knowledge is critical for the implementation of clinically effective and reproducible therapies that utilize standardized cranberry dietary supplements. PAC effects on mechanisms involved in intestinal colonization by ExPEC offer a new physiologically relevant paradigm for understanding of how PAC affect UTI. To test the hypothesis that "A-type" PAC effectively associate with specific ExPEC surface virulence factors to reduce adherence to and invasion of enterocytes, novel methods that simulate the gastrointestinal tract were recently developed [106]. In this context, agglutination and invasion assays using ExPEC were developed to simulate what may occur in the gut after PAC consumption [106]. Under these in vitro conditions, PAC with "A-type" interflavan bonds are able to interact with ExPEC virulence factors. On one hand, ExPEC agglutination was significantly higher for cranberry PAC then for apple PAC and on the other hand, ExPEC invasion in Caco-2 cells was significantly lower for cranberry PAC than for apple PAC [106]. Although structure–activity relationships of cranberry PAC have not been studied in detail yet, it seems that the "A-type" interflavan bond is responsible for the outcomes seen in in vitro and in vivo interventions. Some authors suggested that the similarity of "A-type" PAC with the galabiose lectin [39], to which P fimbriae attach in epithelial cells, could represent a competitive mechanism.

Recently, it was demonstrated that not only the nature of the interflavan bond ("A-" or "B-type") in PAC is crucial, but also other PAC structural features, like the number of "A-type" interflavan bonds and DP. Higher ExPEC agglutination and lower bacterial invasion were reported for cranberry fractions that contained higher number of "A-type" interflavan bonds and higher DP [166]. These two novel in vitro methods represent a change in the scientific paradigm underlying the relationship between cranberry and UTI because most available literature neglects that PAC have low or no absorption from the gut. Through induction of bacterial agglutination, PAC can limit ExPEC ability to invade enterocytes and colonize niches outside of the gut. The prevention of ExPEC gut colonization by PAC might also be related with changes in immune function, namely regulation of intestinal secretory immunoglobulin A and interleukins 4 and 13 production [45,46]. These studies illustrate that supplementation of cranberry PAC to mice attenuates immunosuppression induced by enteral nutrition and likely normalizes mucosal integrity to levels comparable to a solid standard diet, highlighting the concept that PAC affect gut barrier function and may have health benefits in the gut level without being absorbed [167].

The cranberry industry and consumers require information on the minimum PAC DP and number of "A-type" inter-flavan bonds in a product that positively influence urinary tract health in order to more effectively market and promote cranberries. However, due to difficulties in isolating PAC with DP > 3 this task might be compromised until advances in separation and characterization technology are achieved.

Methods that allow visualization of PAC interactions with ExPEC are also required. Fluorescently labeled PAC were synthetized via an aromatic nucleophilic substitution in alkaline media and characterized by MALDI-TOF MS [168]. This fluorescent PAC fraction was able to agglutinate ExPEC and bacterial-PAC aggregates were successfully detected by fluorescence microscopy [168]. This novel tool can be used along with normal-phase chromatography to isolate fluorescent labeled PAC with DP <10. The effect of PAC DP and number of "A-type" interflavan bonds on ExPEC agglutination and cell invasion can be investigated by using this technique. An alternative approach would be to radiolabel or fluorescently label ExPEC [169], but no information about structure–activity relationship would be obtained.

Although it has been known since the pioneer work of Howell et al. [12] that PAC are likely responsible for beneficial effects of cranberries on the urinary tract health, one should not discard the fact that, in intervention trials that use whole juice, other phytochemicals such as anthocyanins, phenolic acids, terpenes, and flavonols [170] may also have an important role. Terpenes (e.g., ursolic acid and esters) from cranberries failed to affect ExPEC viability but a significant reduction in inflammatory markers was reported [171].

In conclusion, the potential for synergistic effects of cranberry PAC and other cranberry phytochemicals should be studied. For instances, cranberry PAC and licochalcone A from licorice reduce the virulence of periodontitis-inducing bacteria [172]. In parallel, future work should also focus on other sources of "A-type" PAC such as cinnamon (Table 2). To our best understanding, the nature of the interflavan bond is crucial but most importantly the number of "A-type" bond combined with the larger DP found in cranberries make this source unique in terms of relevance for the urinary tract health.

The elucidation of mechanisms by which PAC affect ExPEC transient gut colonization and persistence in the gut serves as a conceptual model to study, in a larger context, how PAC may affect other ExPEC strains and extraintestinal infections and can explain the potential of cranberry PAC in urinary tract health despite their poor bioavailability. The interactions of PAC with ExPEC are relevant to study other intestinal and extraintestinal pathogenic bacteria. Future studies should also focus on the effects of cranberry PAC on gene expression of bacterial virulence factors and host response in the gut epithelium.

Mol. Nutr. Food Res. 2015, *59*, 1292–1306

1301

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1303

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