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Comparison of Isolated Cranberry (*Vaccinium macrocarpon* Ait.) Proanthocyanidins to Catechin and Procyanidins A2 and B2 for Use as Standards in the 4-(Dimethylamino)cinnamaldehyde Assay

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ABSTRACT: The 4-(dimethylamino)cinnamaldehyde (DMAC) assay is currently used to quantify proanthocyanidin (PAC) content in cranberry products. However, this method suffers from issues of accuracy and precision in the analysis and comparison of PAC levels across a broad range of cranberry products. Current use of procyanidin A2 as a standard leads to an underestimation of PACs content in certain cranberry products, especially those containing higher molecular weight PACs. To begin to address the issue of accuracy, a method for the production of a cranberry PAC standard, derived from an extraction of cranberry (c-PAC) press cake, was developed and evaluated. Use of the c-PAC standard to quantify PAC content in cranberry samples resulted in values that were 2.2 times higher than those determined by procyanidin A2. Increased accuracy is critical for estimating PAC content in relationship to research on authenticity, efficacy, and bioactivity, especially in designing clinical trials for determination of putative health benefits.

KEYWORDS: *cranberry (Vaccinium macrocarpon Ait), 4-(dimethylamino)cinnamaldehyde, procyanidin A2, procyanidin B2, proanthocyanidins, PAC, elemental analysis, MALDI-FT-ICR MS, MALDI-TOF MS*

INTRODUCTION

Recent research suggests that cranberry proanthocyanidins (PAC) inhibit tumor cell growth[1] and decrease the risk of cardiovascular diseases,[2] periodontal diseases,[3] gastrointestinal diseases caused by *Helicobacter pylori*,[4] and noroviruses.[5] In addition, A-type PAC are the putative bioactive component in the use of cranberries for prevention of urinary tract infections because A-type PAC prevent adherence of uropathogenic *Escherichia coli* to uroepithelial cells,[6] including multidrug-resistant *E. coli*.[7]

4-(Dimethylamino)cinnamaldehyde (DMAC) reacts with flavan-3-ols and proanthocyanidins to form a green chromophore that has a maximum absorbance at approximately 640 nm.[8] This wavelength effectively excludes the spectra of anthocyanins, which are a source of interference in other quantification assays for PAC,[9] such as the vanillin and butanol−HCl assays. Postcolumn derivatizations with DMAC have been used to detect PAC by HPLC[8,10,11] and Sephadex G-25 chromatography[12] and to detect PAC accumulation in plant seeds.[13] Cell-specific localization of PAC is possible with DMAC staining in plant tissues.[14]

In strongly acidic solutions, the DMAC reagent is highly reactive via the formation of a strongly reactive electrophilic carbocation.[15] Due to the delocalization of the positive charge on the DMAC molecule and consequently reduced electrophilicity, the reaction is specific for phenolic compounds with meta-oriented di- or trihydroxy phenols, as found in PAC.[16,17] DMAC does not react with hydroxycinnamic acids, hydroxybenzoic acids, flavones, and flavonols[8,12] and is more accurate and sensitive for PAC than the acid−butanol and vanillin assays.[15]

The reaction appears to be limited to the C_8 position of the A-ring of PAC terminal units; therefore, as degree of polymerization (DP) increases, molar absorptivity decreases. Thus, the use of monomers and procyanidin dimers as DMAC standards to estimate the content of PAC oligomers of higher DP may be inaccurate and may greatly underestimate PAC content.[15,18,19]

Recently, a multilaboratory validation study was conducted to evaluate the use of procyanidin A2 standards in the DMAC method for the determination of PAC content in cranberry powders. The results of this study showed intralaboratory variation of 16% and interlaboratory variations of 32%. The variations were most evident in cranberry powders containing higher PAC oligomers. No linear relationship between PAC content estimated gravimetrically and by the DMAC method was found.[20]

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Increasing the accuracy of the DMAC assay through development of a more robust standard will improve the marketing and regulation of cranberry products. Ideally, standards should express the complex nature of the specific food component being assayed. Commercially available flavan-3-ols and procyanidin dimers are not representative of the structural heterogeneity of PAC found in cranberries. As PAC with higher DP are not commercially available, isolation of PAC from the food being studied is recommended to obtain accurate results.[15] For this specific application, a standard for the DMAC assay would be most accurate when based on purification of PAC oligomers from cranberries.[21]

A similar problem existed in which the use of gallic acid as a standard in the Folin−Ciocalteu assay resulted in the total polyphenol content of pomegranate powders being underestimated by up to 30%. We addressed this issue by developing and validating a novel pomegranate standard.[15] The pomegranate standard was reflective of the complex nature of oligomeric ellagitannins found in pomegranate source material, and it was an improvement over the gallic acid standard for accurately quantifying polyphenols in pomegranate powders.

In this work, we investigated the suitability of PAC that were isolated from cranberry (c-PAC) press cake for use as a standard in the DMAC assay to more accurately quantify PAC in cranberry powders and juices. Mass spectrometry analysis corroborated PAC composition and confirmed cranberry-specific structures, that is, A-type proanthocyanidins. This method can be easily applied to the generation of PAC from other food sources (chocolate, grapes, etc.) and further applied to other food industries.

MATERIALS AND METHODS

Chemicals and Reagents. Water, methanol, acetone (HPLC grade), hydrochloric acid (37.4% v/v), sodium carbonate, and formaldehyde solution (37% w/w) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Ethanol (200 proof) was obtained from Decon Laboratories Inc. (King of Prussia, PA, USA). The reagent 4-(dimethylamino)cinnamaldehyde was purchased from Acros Organics (Geel, Belgium). The standards procyanidins A2 and B2 were purchased from Indofine Chemical Co. Inc. (Hillsborough, NJ, USA). (+)-Catechin hydrate, bradykinin, 2,5-dihydroxybenzoic acid (DHB), gallic acid, glucagon, angiotensin II, and the Folin−Ciocalteu reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sephadex LH-20 was purchased from GE Healthcare (Uppsala, Sweden).

Samples. Cranberry press cake was obtained from Ocean Spray Cranberries, Inc. (Wisconsin Rapids, WI, USA) from a nondepectinized production line of North American cranberries (*Vaccinium macrocarpon* Ait.). The press cake was ground to a fine powder with liquid nitrogen and frozen at −80 °C until further use. Cranberry powders (powders A, B, and C) were commercially sourced. One hundred percent cranberry juice (juice B) and three different brands of cranberry juice blends (juices A, C, and D) were purchased from a local supermarket.

Preparation of c-PAC. Frozen cranberry press cake powder (100 g) was extracted with 70% aqueous acetone (v/v; 400 mL) in an ultrasonic bath for 15 min. The extract was centrifuged at 400*g* at 15 °C for 10 min and the supernatant collected. The extraction was repeated twice, and the supernatants were combined. After filtration with cellulose paper, acetone was removed by rotary evaporation under vacuum at 35 °C, and the remaining suspension was solubilized in ethanol. The extract was then centrifuged at 13416*g* at 0 °C for 10 min to eliminate insoluble material. The supernatant was used to isolate PAC by chromatography on Sephadex LH-20.

The ethanolic cranberry press cake extract was loaded on a glass column (2.5 cm i.d. × 60 cm length, Kontes, Chromaflex) packed with Sephadex LH-20 that was previously swollen and washed in water and equilibrated with ethanol for 45 min at a flow rate of 4 mL/min. Preliminary work had shown that a combination of three solvents was sufficient to achieve a PAC fraction that was devoid of other classes of polyphenols. The resin bed was sequentially eluted with ethanol, ethanol/methanol (1:1), and 80% aqueous acetone (v/v). The 80% aqueous acetone fraction that contained PAC was evaporated as described above and solubilized in absolute methanol and abbreviated c-PAC. c-PAC (82.6 g dry matter L^{-1}) was diluted into six aliquots (C1−C6, 16.3, 32.6, 48.9, 65.2, 81.5, and 97.8 mg dry matter L^{-1}, respectively) for the DMAC assay.

Stability and Characterization of c-PAC. The Folin−Ciocalteu method was used to evaluate c-PAC stability over 12 consecutive months. Results are expressed as gallic acid equivalents (GAE). c-PAC characterization was accomplished with high-performance liquid chromatography with diode array detection (HPLC-DAD), formaldehyde−HCl precipitation, elemental analysis, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), and matrix-assisted laser desorption/ionization−Fourier transform ion cyclotron resonance mass spectrometry (MALDI-FT-ICR MS). Folin−Ciocalteu[22] and formaldehyde−HCl[23] methods were based on previous works without modifications.

Prior to characterization by HPLC-DAD, c-PAC was diluted 10-fold to reduce the amount of methanol to 10%. One hundred microliters was injected onto a Waters Spherisorb 10 μm ODS2 RP-18 column (4.6 × 250 cm). The solvents for elution were 0.1% trifluoroacetic acid/water (solvent A) and methanol (solvent B). The HPLC elution program was as follows: the first 10 min isocratic at 10% B; 10−25 min, B increased linearly from 10 to 28%; 25−45 min, B increased linearly from 28 to 55%; 45−50 min, B increased linearly from 55 to 99%; and isocratic at 99% B for 5 min, followed by reconditioning of the column. The HPLC system consisted of a Waters automated gradient controller, two Waters 501 HPLC pumps, and a Rheodyne 7125 manual injector. The flow rate was maintained at 2 mL/min, and the elution was monitored by a Waters 996 diode array detector using Waters Empower software for collecting and analyzing three-dimensional chromatograms.

Elemental analysis of c-PAC was performed on a Perkin-Elmer 2400 II elemental analyzer (carbon, hydrogen, and nitrogen) and a LECO CHNS 932/VTF 900 (oxygen) (Atlantic Microlab, Norcross, GA, USA). Positive ion linear mode mass spectra were collected on a Bruker Reflex II MALDI-TOF mass spectrometer (Billerica, MA, USA) equipped with delayed extraction and a N_2 laser (337 nm). Mass spectra were calibrated with bradykinin and glucagon as external standards. The analyte (0.5 μL) was first deposited onto a stainless steel target followed by deposition of 1.0 μL of DHB matrix (50 mg/mL). Three hundred shots were acquired, and $[M + Na]^+$ and $[M + K]^+$ ion adducts were detected. Only the sodium adducts were used for assigning peak m/z to each PAC DP. Positive mode MALDI-FT-ICR spectra were collected on an IonSpec ProMALDI FT mass spectrometer equipped with a MALDI source with hexapole ion accumulation. Mass spectra were calibrated with angiotensin II as an internal standard. The analyte (0.3 μL) was mixed with 0.3 μL of DHB (100 mg/mL) and deposited on a stainless steel target. Fifty shots were collected, and $[M + Na]^+$ and $[M + K]^+$ ion adducts were detected.

DMAC Assay. *Standard Preparation.* Stock solutions of catechin (510 mg L^{-1}), procyanidin A2 (480 mg L^{-1}), and procyanidin B2 (360 mg L^{-1}) were prepared by dissolving the standard in methanol. Stock solutions were subsequently diluted with methanol to produce six levels of concentrations (C1−C6): 2.6, 5.1, 7.7, 10.2, 12.8, and 15.3 mg L^{-1} for catechin; 4.8, 9.6, 14.4, 19.2, 24.0, and 28.8 mg L^{-1} for procyanidin A2; and 5.0, 10.1, 15.1, 20.2, 25.2, and 30.2 mg L^{-1} for procyanidin B2.

Preparation of Samples. Approximately 100 mg of each cranberry powder was solubilized in 20 mL of a PAC extraction solution (75% acetone/24.5% H_2O/0.5% acetic acid (v/v)), placed in an ultrasonic bath for 30 min at 22 °C, placed on a shaker for 1 h, and finally centrifuged at 1840*g* for 10 min at 20 °C.[20] These extracts were diluted 4-, 10-, or 50-fold, and juices were submitted to 2-, 10-, or 50-fold for DMAC analysis.

Protocol. The procedure used in this experimental work was based on the previously published DMAC protocol.[20] An acidified ethanol solution was prepared by adding 12.5 mL of hydrochloric acid (37%) to 12.5 mL of deionized water and 75 mL of ethanol. A 0.1% (w/v) DMAC reagent solution was prepared immediately prior to use.

Polystyrene 96-well plates were utilized in conjunction with a Thermo Scientific Varioskan Flash plate reader (Hudson, NH, USA) to measure absorbance. Seventy microliters of blanks (80% ethanol), calibration standards, and samples was dispensed into individual wells. A multichannel pipet was used to dispense 210 μL of DMAC reagent solution into each well, excluding blanks. Immediately after the addition of DMAC reagent, the plate was placed into a plate reader and shaken for 10 s at 600 shakes min^{-1}. Absorbance readings at 640 nm were taken every 30 s for 1 h. The corresponding blank absorbance readings at each time point were subtracted from those from the standard and sample wells. For powders and juices, sample blanks (diluted samples only) were also analyzed to correct for interference from sample color.

Data and Statistical Analysis. All data were reported as the mean ± standard deviation of at least three replicates. mMass version 3.9.0[24] was used for mass spectra analysis.

RESULTS

The stability of c-PAC was evaluated monthly over the course of a year (70.9 ± 5.2 g GAE/L), and the results showed a coefficient of variation of <7.5%. Over that same period, MALDI-TOF MS spectra did not show any new peaks arising from degradation reactions (data not shown).

Results from HPLC-DAD showed two unresolved humps, characteristic of PAC. Absorbance at wavelengths higher than 280 nm that would be typical of other cranberry polyphenols such as hydroxycinnamic acids (320 nm), flavonols (360 nm), and anthocyanins (520 nm) was low compared to absorbance at 280 nm (Figure 1). The purity of c-PAC was estimated to be 99.0 ± 1.3% by the formaldehyde−HCl precipitation test, and elemental analysis showed 58.2% C, 4.7% H, and 37.3% O.

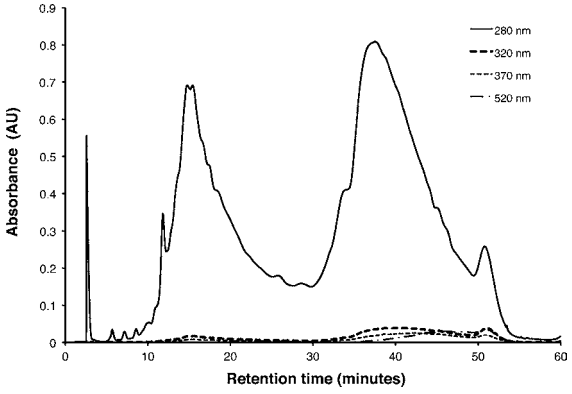

Figure 1. HPLC chromatogram recorded at 280, 320, 370, and 520 nm of c-PAC.

The MALDI-TOF MS spectrum in linear mode showed a wide range of PAC with DP between 3 and 26 (Figure 2). Peaks consistent with anthocyanidin moieties bound to PAC (antho-PAC) were also detected.[25] However, the MALDI-TOF MS spectra did not have high enough resolution to assign accurate masses to these peaks, so MALDI-FT-ICR MS was used to characterize these compounds. The major peaks seen between each PAC series correspond to anthocyanin−polyflavan-3-ol oligomers that have previously been described in cranberries (Figure 3).[25−27] High-resolution mass spectrometry techniques have been used in proteomics, but as far as we know this is the first report of the application of MALDI-FT-ICR MS to the study of PAC from cranberries.

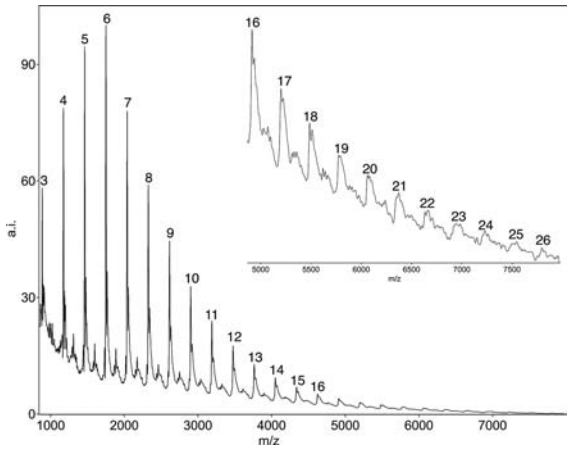

Figure 2. MALDI-TOF MS spectrum in positive linear mode, showing PAC oligomers with degree of polymerization between 3 and 26. (Inset) Enlarged spectrum after a Savitzky−Golay smoothing function was applied to assist with visualizing the peaks for m/z >4900.

Standard curves were produced for catechin, procyanidin A2, procyanidin B2, and c-PAC. Intercept coefficients were very small compared to the slope values, and therefore their impact on estimating PAC content was negligible. The slope of the c-PAC standard curve was 2.5 times lower than those of procyanidins A2 and B2 and 7.1 times lower than that of catechin (Figure 4), indicating that c-PAC content would be underestimated by 7.1- and 2.5-fold if these standards were used for the DMAC assay.

The theoretical detection (LOD) and quantification (LOQ) limits of the method were calculated as described previously.[28] As an example, for procyanidin A2, LOD was 1.94 mg/L and LOQ was 6.47 mg/L. To determine if these values were detected above the equipment noise level, a lower linear range between 1.0 and 6.0 mg/L of procyanidin A2 was prepared. The results showed a very similar regression curve fit to the linear range shown in Figure 4 (data not shown), suggesting that the DMAC method is sensitive enough to detect c-PAC concentrations of 1−6 mg/L.

A 96-well plate reader increased efficiency and output and allowed for monitoring reaction kinetics (Figure 5). Our results show that procyanidin B2 reaches its maximum absorbance more quickly than the other standards and 6.5 times more quickly than procyanidin A2 (Figure 6). These times required to reach maximum absorbance (t_{max}) were consistent across different days of analysis (data not shown). When using the c-PAC standard, cranberry powders and juices had on average 2.2 more PAC than if procyanidin A2 standard was used (Table 1).

DISCUSSION

In this work, we isolated PAC from cranberry press cake for use as a standard in the DMAC assay for quantifying PAC content in cranberry products. This strategy may also be applied to whole fruit and juice. The isolation of standards for use in colorimetric assays has already been pursued in other food sources such as carob pods[29] and pomegranates.[22]

Characterization of c-PAC. The HPLC chromatogram of c-PAC at 280 nm showed two unresolved humps and minor absorbance at 520 nm due to the presence of antho-PAC. The poorly resolved chromatogram at 280 nm reflects the large

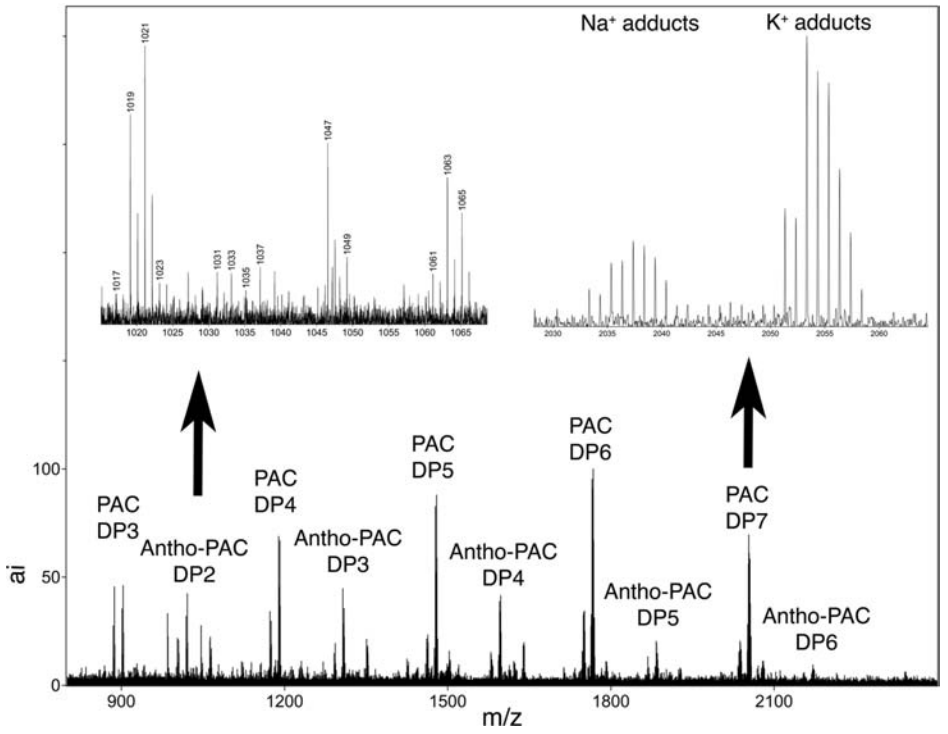

Figure 3. MALDI-FT-ICR MS spectrum, showing PAC and antho-PAC: *m/z* 1017, pyranocyanidin-arabinoside-A2; *m/z* 1019, pyranoanthocyanidin-arabinoside-DP2B; *m/z* 1021, A2-ethyl-cyanidin-arabinoside; *m/z* 1023, DP2B-ethyl-cyanidin-arabinoside; *m/z* 1031, pyranopeonidin-arabinoside-A2; *m/z* 1033, pyranopeonidin-arabinoside-DP2B; *m/z* 1035, A2-ethyl-peonidin-arabinoside; *m/z* 1037, DP2B-ethyl-peonidin-arabinoside; *m/z* 1047, pyranocyanidin-hexoside-A2; *m/z* 1049, pyranocyanidin-hexoside-DP2B; *m/z* 1061, pyranopeonidin-hexoside-A2; *m/z* 1063, pyranopeonidin-hexoside-DP2B; *m/z* 1065, A2-ethyl-peonidin-hexoside.

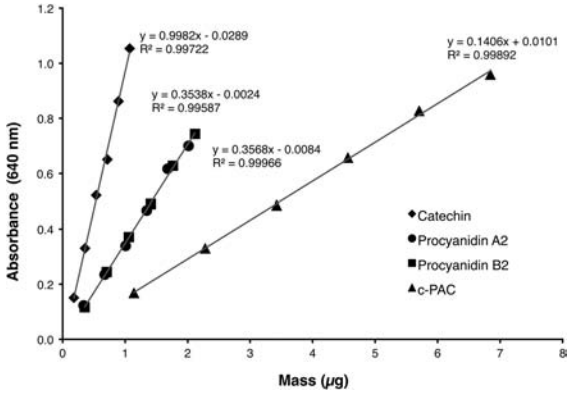

Figure 4. Regression curves for catechin, procyanidin A2, procyanidin B2, and c-PAC after reaction with 4-(dimethylamino)cinnamaldehyde.

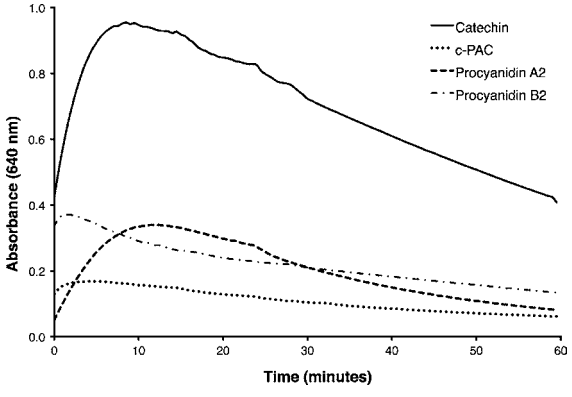

Figure 5. Absorbance at 640 nm over 60 min after reaction of 4-(dimethylamino)cinnamaldehyde with four different standards: catechin (1.07 μg); c-PAC (1.14 μg); procyanidin A2 (1.09 μg); procyanidin B2 (1.06 μg).

structural heterogeneity of c-PAC.[25] Reverse-phase HPLC is unable to separate PAC higher than trimers due to the large number of possible isomers that increase exponentially with PAC chain length; more than a trillion PAC molecules can exist for DP above 21.[30] No peaks with an absorbance maximum that is typical of the other classes of cranberry polyphenolic compounds (anthocyanins, hydroxycinnamic acids, and flavonols) were observed, and the UV spectrum during the entire HPLC elution was identical to that of PAC. These initial chromatographic data suggest that c-PAC did not contain other classes of flavonoids.

An absolute measurement of PAC was done gravimetrically by using the formaldehyde−HCl precipitation test. Under acidic conditions, formaldehyde reacts with flavonoids, leading to polymerization and precipitation.[31] PAC purity is estimated by weighing the lyophilized supernatant that contains non-flavonoid components.[23] Applying this methodology, the present work suggested that c-PAC had a purity of 99.0%, reinforcing the chromatographic data that indicated the absence of other flavonoids. This value is higher than in previous works in which the purity of grape seed PAC obtained by RP-18 chromatography was 93%.[23]

Elemental analysis was utilized to determine the purity of PAC similar to the approach taken for PAC isolated from grapes and highbush blueberries.[32,33] The percentages of C, H, and O are not altered with an increase in DP, as the addition of one repeating flavan-3-ol unit does not change the elemental analysis. Calculated elemental analysis for cranberry PAC

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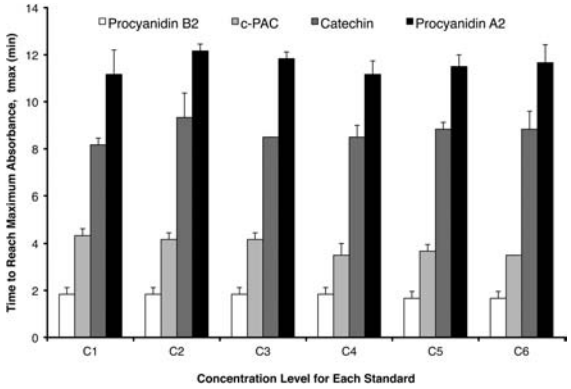

Figure 6. Variation of time required to reach maximum absorbance (t_{max}) with increasing levels of concentration (C1−C6, see Materials and Methods) for catechin, procyanidin A2, procyanidin B2, and c-PAC.

Table 1. PAC Content Estimated with the 4-(Dimethylamino)cinnamaldehyde Method in Cranberry Powders and Cranberry Juices Using Procyanidin A2 and c-PAC as Standards[a]

	procyanidin A2	c-PAC
powder A	198.8 ± 3.5	421.8 ± 51.2
powder B	123.0 ± 13.6	271.7 ± 37.0
powder C	146.2 ± 9.1	316.9 ± 22.1
juice A	174.3 ± 16.5	381.1 ± 11.3
juice B	777.2 ± 79.9	1716.2 ± 107.6
juice C	95.5 ± 9.1	207.4 ± 3.1
juice D	43.9 ± 4.6	94.3 ± 0.6

[a]Results are expressed as the average of three independent replicates and standard deviation in mg/g for powders and mg/L for juices.

dimers showed 62.4% C, 4.3% H, and 33.3% O. For dimeric antho-PAC, 60.9% C, 4.4% H, and 34.7% O were calculated. In antho-PAC the amount of C is slightly lower and the percentage of O is slightly higher than in PAC due to the increase in O/C ratio with the introduction of anthocyanin moieties. c-PAC showed the following elemental composition: C, 58.2%; H, 4.7%; O, 37.3%. Nitrogen detection was 0.1%, leading to the conclusion that few, if any, nitrogenous compounds were present in c-PAC. In addition to the lower amount of C and slightly higher amounts of H and O obtained experimentally, a weight loss of 11.7% was observed when c-PAC was evaporated under nitrogen and dried overnight at 100 °C, suggesting that c-PAC contained water. Previous works with purified polymeric PAC fractions from different sources have shown a 13−15% weight loss upon drying and that anhydrous PAC polymers are highly hygroscopic.[33] Assuming a molecule of water per each flavan unit, PAC would have 58.9% C, 4.6% H, and 36.6% O and antho-PAC would have 59.2% C, 4.6% H, and 36.2% O. The discrepancy between these values and the experimental values is ≤1.1%. Other authors have reported PAC fractions with 2.5−3 mol of water per monomeric unit.[33] The deviation of our elemental analysis of c-PAC from theoretical values is lower than published results for purified grape seed PAC, in which the error for C content was 7%.[23]

In this research, we used both linear and reflectron MALDI MS modes because they provide complementary information; high sensitivity was obtained in the linear mode, particularly at high molecular weights, whereas high resolution was achieved in the reflectron mode. MALDI-TOF MS linear mode spectra had m/z peaks that corresponded to PAC with a range of 3−26 DP. Our previous works have shown PAC from cranberry press cake with DP up to 23,[25] so the present work shows the highest DP reported in the literature using MS techniques. Spectra in linear mode also displayed m/z peaks that correspond to antho-PAC molecules. Accurate mass assignment of these peaks was possible only in reflectron mode and with the use of FT-ICR MS, which provides increased mass resolution and accuracy.[34] Antho-PAC are thought to be derived from ripening and postharvest processing and storage steps[26] and can account for up to 10% of sample weight.[35] Antho-PAC, in which an anthocyanin moiety occupies the C_8 position of the terminal catechin molecule, cannot react with DMAC because the C_8 position is not avaiable for the reaction. The presence of antho-PAC also contributes to an underestimation of PAC when using standards such as catechin and procyanidins A2 and B2 in the DMAC assay.

Influence of Different Standards on the DMAC Reaction Kinetics. Catechin was used in this work because it has historically been the standard of choice[9,36−38] for the DMAC reaction. Procyanidin A2 was investigated, as it has recently gained acceptance as an improvement over the catechin standard for the quantification of cranberry PAC.[20] Procyanidin B2 was investigated to determine if the nature of the interflavan bond influences reaction kinetics. Finally, c-PAC was investigated to determine the reaction kinetics of a standard that represents the structural heterogeneity of PAC found in cranberry.

Catechin had a much greater slope ($y = 0.9982x - 0.0289$) than the other standards, which agrees with recently published research.[16] The slopes of procyanidins A2 ($y = 0.3538x - 0.0024$) and B2 ($y = 0.3568x - 0.0084$) regression curves were very similar and about 2.8 times lower than that of catechin. A previous publication indicated slightly different slopes for procyanidins A2 and B2. However, absorbance was read after 30 min instead of at t_{max} in that study;[11] as regression equations were not provided in that publication, no quantitative comparisons to our results can be made.

The slope of the c-PAC regression curve ($y = 0.1406x + 0.0101$) was 7.1 times lower when compared with catechin and 2.5 times lower when compared with procyanidin A2 and B2 standards (Figure 4). Prior et al.[20] previously speculated that large polymeric PAC compounds may have a lower response per unit weight than monomers or dimeric procyanidins. The use of PAC oligomers as standards was suggested as a better approach for estimating chocolate content and confectionary products rich in PAC with high molecular weights.[16] The reduced response of c-PAC is likely due to the proportion of C_8 reactive sites on the PAC that are available to participate in the DMAC reaction. If, as previously reported, the C_8 terminal unit is the only position available for DMAC reaction, then as the PAC grows linearly in polymer length, each additional DP adds weight but no additional DMAC reactivity. Thus, it is not unexpected that when c-PAC, which represents the structural heterogeneity and oligomeric dispersion (DP 3−26) of cranberry PAC, is used as a standard in the DMAC reaction, the response will be lower than that of the currently accepted procyanidin A2 standard. This is precisely the justification for investigating the development of robust standards, consisting of the natural structural heterogeneity of PAC found in the source material. It is imperative that the standard of choice has similar

reaction kinetics to the product for which accurate quantification of PAC content is desired.

The end point of the DMAC reaction that was previously used varied from 5,[37,39] to 20[40] and to 35 min.[11] However, the chromophore generated in the DMAC reaction is not stable[15] and reaches its maximum absorbance at different time points, depending on the flavan-3-ols used in the reaction.[16,20] Our kinetics for procyanidins A2 and B2 have similar shapes as in a previous work; t_{max} was not described in that research.[11] For the same concentration of procyanidins A2 and B2, similar maximum absorbance values were attained, but procyanidin B2 reached maximum absorbance more quickly than procyanidin A2 (Figure 5). PAC with A-type bonds have less freedom to rotate around their bond and therefore may have more steric hindrance when interacting with the DMAC molecule. Differences in spatial configuration between A- and B-type bonds may only affect the rate of the reaction, making the reaction faster for PAC with B-type bonds. Our results also show differences between the four standards used in this work in terms of t_{max}. However, t_{max} does not change with increasing concentrations of each standard (Figure 6).

Whereas some authors have reported that concentrations of DMAC reagent ≥2.0% should be used to prevent underestimation of PAC content,[9] other authors have used concentrations as low as 0.1%.[20] Our results showed that a DMAC concentration of 2.0% greatly decreased t_{max}. In fact, all of the standards analyzed with DMAC concentration of 2.0% reached t_{max} between 0.5 and 0.7 min, suggesting that the colorimetric reaction occurred quickly, contradicting the suggestion by Wallace et al. that absorbance should be read between 15 and 35 min for monomeric PAC and between 20 and 35 min for oligomeric and polymeric PAC to achieve maximum color development and the lowest standard deviation.[9] Applying this rationale to our data would bias the estimation of PAC content as the absorbance at 35 min is 30, 48, 47, and 44% of the maximum absorbance registered at t_{max} for catechin, procyanidin A2, procyanidin B2, and c-PAC, respectively (Figure 5). Furthermore, our data show that between 15 and 35 min after the onset of the DMAC reaction, the coefficient of variation is actually higher than at the t_{max}, undermining the lowest standard deviation criterion chosen by Wallace et al.[9]

Implications of the Use of c-PAC as a Standard. Most clinical studies with cranberries have used juice, with intake based on volume consumed[41−43] and with no proper qualitative or quantitative characterization of the PAC consumed. On the basis of efficacy in clinical trials, a daily dosage of 36 mg of PAC measured by DMAC has been proposed as prophylaxis against urinary tract infections.[20] The accuracy with which the PAC content of cranberry products is determined is critical for comparing the efficacy of the products in laboratory and clinical research.

The accurate determination of PAC content is dependent on the standard utilized for the DMAC assay. A previous DMAC method (DMAC/PAC003) using a commercially unavailable proprietary standard yielded the same PAC content as the DMAC method using procyanidin A2 as a standard.[20,44] However, PAC contents in cranberry powders and juices were 2.2 times higher in our method using c-PAC. Although the biological efficacy, including antiadhesion activity of a dose of cranberry powder or juice (with 36 mg PAC as measured by DMAC with the A2 standard) will not change, only the reported PAC content will be higher when using a PAC standard that was isolated from a similar product matrix.

The development of new standards for the measurement of polyphenols in pomegranate samples has shown that using a commercially available standard in the Folin−Ciocalteu can underestimate the polyphenolic content up to 30%.[22] The use of c-PAC in the DMAC assay showed a more pronounced effect on PAC estimation because cranberry powders and juices were underestimated up to 47% compared to procyanidin A2 results. The daily dosage of 36 mg would correspond to almost 80 mg by using the c-PAC standard. However, within a clinical study the actual amount of cranberry product that was used to obtain an effect would not be different. However, the accuracy problem becomes more important when cranberry products are compared across clinical studies and is a serious problem in meta analysis of the efficacy of cranberries in clinical studies on urinary tract infections.[45] Accurate estimation of PAC content is important for standardizing products for use in clinical research and in compositional analysis of products for GRAS approval. Underestimation of PAC content by 40% would lead to a large amount of unidentified components in a cranberry powder supplement.

We developed a c-PAC standard from cranberry press cake as a first step toward better understanding the influence PAC oligomer length and PAC structural complexity have on the kinetics of the DMAC reaction. A potential problem may exist in the choice of press cake as the source material for the isolation of cranberry PAC for use as a standard, as PAC composition may differ between fruits, juices, and press cake. Low molecular weight PAC are water-soluble, whereas higher molecular weight PAC are partially insoluble in water. Because water-soluble PACs are easily extracted during juicing, the press cake is relatively enriched in higher molecular weight PAC. The differences in the reaction kinetics of PAC standards derived from whole fruit, juice, and press cake are currently being researched in our laboratory. It may be necessary to develop several PAC standards derived from different cranberry products to accurately measure PAC content in products with variable PAC structural profiles, along with mass spectrometry techniques. Preliminary MALDI-TOF MS data indicate that the range of DP of standards prepared on different days is very similar.

Although c-PAC is a complex mixture of closely related oligomers, analysis by several analytic techniques showed that the standard may be reliably reproduced and represents the structural heterogeneity of cranberry PAC from press cake including degree of polymerization, A-type interflavan linkages, and antho-PAC. The DMAC method as it stands is extremely rapid as all samples (juices and powders) and standards reach their t_{max} before 15 min and no temperature control is necessary. The use of c-PAC as a standard improves the accuracy of PAC quantification when compared to the use of the procyanidin A2 standard. A reference standard for the DMAC assay is most accurate when based on the partial purification of a PAC from the food source, which could constitute a hurdle for the implementation of this methodology as routine. However, due to the long-term stability of c-PAC standard, it seems that the work involved in preparing the standard is warranted and will result in an invaluable tool for estimating PAC in complex samples such as cranberries.

The intent of this paper is not to advocate the use of the c-PAC standard that was developed in this research as the reference standard to be used for the DMAC assay, but rather

to present the necessary approach and tools to investigate the kinetics of a complex standard and compare the reaction kinetics to single compounds such as procyanidins A2 and B2 and catechin, which are currently used by industry for assessing PAC content in food and dietary supplements. More research is required to determine the variability in DMAC reaction kinetics for PAC isolated from fruit, juice, press cake, and powders to determine the most appropriate standard.

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Notes

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