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Determination of proanthocyanidin A2 content in phenolic polymer isolates by reversed-phase high-performance liquid chromatography

Jayma L. Koerner[a], Victor L. Hsu[b], Jungmin Lee[c], James A. Kennedy[a,*,1]

[a] Department of Food Science and Technology, Oregon State University, Corvallis, OR, 97331, USA
[b] Department of Biochemistry and Biophysics, Oregon State University, OR, 97331, USA
[c] United States Department of Agriculture, Agricultural Research Service, Horticultural Crops Research Unit Worksite, 29603 U of I Lane, Parma, ID, 83660, USA

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ABSTRACT

This article summarizes the development of an analytical method for the determination of proanthocyanidin (PAC) A2 in phenolic polymer isolates following acid-catalyzed degradation in the presence of excess phloroglucinol. Isolates from concentrated cranberry juice (CCJ) were extensively characterized and molar extinction coefficients were determined for the terminal A2 and phloroglucinol adduct of the extension A2 unit. Peanuts were also found to contain both extension and terminal A-type PACs and therefore a total peanut system (TPS) was chosen to test the effectiveness of the HPLC method that was developed with the CCJ system. Kinetic studies were conducted and reaction conditions were optimized for the A2 units in both CCJ and TPS. The optimized method provides quantitative and reproducible information on the A2 content of proanthocyanidin isolates.

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1. Introduction

Proanthocyanidins (PACs), also known as condensed tannins, are polymeric flavan-3-ols. PACs are the second most abundant group of natural phenolics [1] and are found in a wide variety of plants and plant-derived products, including fruits, vegetables, legume seeds, cereal grains and some beverages including tea, wine, cocoa, beer, and cider [2]. PACs have the ability to interact with and precipitate proteins and it is believed that this property gives rise to the astringent mouth-feel of PAC-rich consumables [2]. PACs are also effective antioxidants [3] and it is believed that PACs provide several health benefits, including the prevention of urinary tract infections (UTI) [4], cancer, and cardiovascular disease, as well as the inhibition of LDL oxidation and platelet aggregation [2,5,6].

In the most common PAC polymerization linkage, known as B-type, monomers are linked via a single bond from the C-4 on the upper unit to either C-6 or C-8 of the lower unit. Another less common linkage, know as A-type (Fig. 1), has the same B-type linkage, with an additional ether bridge from the C-2 on the upper unit to the C-7 hydroxyl of the lower unit [1]. Studies suggest that A-type PACs may be beneficial in the prevention of UTIs [7,8]. Tools for studying these polymers are vital for improving our understanding of potential health benefits.

Characterizing and quantifying PACs has historically been difficult, largely due to inadequate analytical methods and lack of commercial standards. Characterization difficulties are compounded by the fact that PACs are reactive, being susceptible to acid-catalyzed and oxidation reactions [1], and additionally due to the complex mixtures found in food extracts. Several methods have been developed to analyze PACs, but each method has limitations and not one of them is considered fully satisfactory. Historically, HPLC combined with mass spectrometry has been used to detect the presence of A-type PACs [9–11] and quantification has been based upon non A-type flavan-3-ol extinction coefficient information [10,11]. However, an HPLC method for the qualitative and quantitative analysis of A2-containing PACs (A-type dimers of (−)-epicatechin) has up-to-now not been presented.

Cranberries contain both extension and terminal A2 PACs (Fig. 1) [7,8,10], which makes concentrated cranberry juice (CCJ) ideal for the development of an HPLC method for the analysis of either or both A2 PAC units. Peanuts have also been reported to contain both terminal and extension A-type PACs [11] therefore a total peanut system (TPS) was used to test the effectiveness of the method presented here. Reported here is the development of a method that maximizes the cleavage of A2-containing PACs into A2 subunits by acid-catalyzed depolymerization in the presence of excess

* Corresponding author. Tel.: +61 8 8303 6600; fax: +61 8 8303 6601.
E-mail address: james.kennedy@awri.com.au (J.A. Kennedy).
[1] Current address: The Australian Wine Research Institute, P.O. Box 197, Glen Osmond (Adelaide), SA 5064, Australia.

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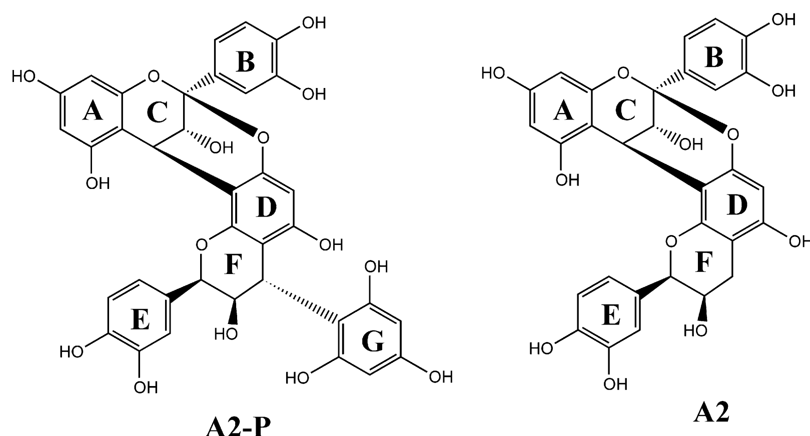

Fig. 1. Structure of A2-P and A2.

phloroglucinol (phloroglucinolysis), and quantifies the terminal and extension A2 subunits products using measured extinction coefficients of purified and characterized standard material.

2. Experimental

2.1. Chemicals

Acetone, acetonitrile, diethyl ether, ethyl acetate, glacial acetic acid, high purity hydrochloric acid, trifluoroacetic acid (TFA), and methanol were all HPLC grade and 0.1% aqueous formic acid was LC/MS grade and all were purchased from VWR (Tualatin, OR, USA). Reagent grade phloroglucinol and (−)-epicatechin were purchased from Sigma–Aldrich (St. Louis, MO, USA). L-(+)-ascorbic acid (99.8%) was purchased from J.T. Baker (Phillipsburg, NJ, USA). Ammonium acetate (HPLC grade) was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Anhydrous sodium acetate (ACS grade) was purchased from Mallinckrodt (Paris, KY, USA). Water was purified using a Millipore Milli-Q system (Bedford, MA, USA). Toyopearl HW 40F chromatography resin was purchased from Supelco (Bellefonte, PA, USA).

2.2. Instrumentation

Analytical HPLC with diode array detection (DAD) was performed on an Agilent 1100 series HPLC (Palo Alto, CA, USA) composed of a degasser (G1322A), quaternary pump (QuatPump G1311A), autosampler (G1313A ALS), and column heater (G1316A ColCom) controlled by Agilent's ChemStation for LC Rev. A.08.04 software. Semi-preparative HPLC was performed on the same Agilent system with the addition of a 1400 μL seat capillary and the replacement of the column heater by an Eppendorf CH-430 column heater with TC-50 temperature control unit (Westbury, NY, USA).

Preliminary LC/MS of the CCJ PAC reaction products was performed using an Agilent HP1100 system equipped with a DAD and XCT ion trap (Palo Alto, CA, USA). Final LC/MS of the purified products was performed using Analyst 1.2.1 software to control an Applied Biosystems 4000 Q-Trap (Foster City, CA, USA) with Shimadzu SIL-HTC autosampler (Columbia, MD, USA), Shimadzu LC-20AD pump, and Shimadzu DGU-20A5 prominence degasser. Accurate mass was determined on a Waters Micromass LCT Classic (Milford, MA, USA) using a polypropylene glycol standard.

^{13}C NMR data were acquired on a Bruker Avance 600 MHz spectrometer. ^{1}H NMR, heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond correlation (HMBC) data were acquired on a Bruker Avance 600 MHz spectrometer. All experiments were internally referenced to acetone, using acetone-d_6 as the solvent purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA).

Molar extinction coefficient determinations in methanol were performed using 282 QS 1.000 cuvettes on a Shimadzu UV-3101 PC UV–VIS–NIR Scanning Spectrophotometer (Columbia, MD, USA).

2.3. Sample preparation

2.3.1. PAC isolation and purification

CCJ received from Ocean Spray Cranberries, Inc. (Boston, MA, USA) was diluted to 25% (v/v) concentration with water and applied to a 5 cm × 30 cm Toyopearl HW-40F column pre-equilibrated with 0.05% (v/v) aqueous TFA. As previously described [12], the loaded column was rinsed with 1.0 L 0.05% (v/v) aqueous TFA to remove sugars and organic acids, followed by 1.0 L of 1:1 methanol:water containing 0.05% (v/v) TFA to remove low molecular weight phenolics. Finally 300 mL of 2:1 acetone:water containing 0.05% (v/v) TFA was used to elute the PAC fraction [13]. The PAC fraction was then lyophilized to a magenta colored powder and stored at −80 °C. The yield was approximately 16 g/L CCJ. The solubility of the crude PAC powder was approximately 8 mg/mL in water.

For the TPS, shelled, whole peanuts with skins were purchased from a local market (Corvallis, OR, USA). The peanuts were broken apart and extracted in 2:1 acetone:water under argon and in reduced light. The liquid extract was vacuum filtered and the acetone was removed under reduced pressure at 40 °C. The aqueous solution was then applied to the above column. The fraction elution and additional purification steps were the same as for CCJ (see above) [12,13]. The PAC fraction was lyophilized to a pale yellow powder and stored at −80 °C.

2.3.2. Phloroglucinolysis of PACs for isolation of A2 and A2-P

Phloroglucinolysis for analytical samples has been previously described [12]. Large-scale phloroglucinolysis of the CCJ PAC powder was performed for the semi-preparative HPLC purification of compounds of interest. 8 g CCJ PAC was dissolved in 1.6 L of 0.1N methanolic HCl. 80 g of phloroglucinol was added and the reaction flask was placed in a 50 °C water bath and allowed to react for 130 min. Ascorbic acid was not used in order to simplify the purification process, therefore special care was taken to reduce oxidation in the depolymerized PACs by minimizing post-phloroglucinolysis light and heat exposure. The reaction was quenched with 4.93 g ammonium acetate. The methanol was removed under reduced pressure and the products were taken up in Milli-Q water and washed with diethyl ether. The aqueous layer was then extracted with ethyl acetate. The ethyl acetate portions were combined and concentrated under reduced pressure. A minimal amount of

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