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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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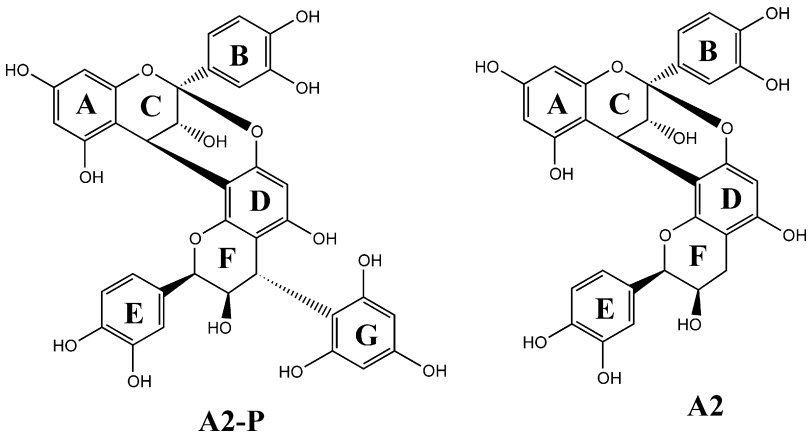

Fig. 1. Structure of A2-P and A2.

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

water was added to solubilize the products. The remainder of the ethyl acetate was then evaporated. The solubility of the depolymerized CCJ products in water was approximately 29 mg/mL. The saturated aqueous solution was then injected directly onto the semi-preparative column as discussed below.

2.3.3. Semi-preparative HPLC

Peaks of interest were isolated using a LiChrospher 100 RP-18 (10 μm) semi-preparative column (250 mm × 10 mm) with a guard column of the same material. The mobile phases were 0.1% (v/v) aqueous TFA (A) and acetonitrile containing 0.1% (v/v) TFA (B) with a flow rate of 5 mL/min. The stepwise gradient was as follows: time in min (%B), 0–3 (3%), 3–11 (10%), 11–19 (12%), 19–24 (15%), 24–35 (16.5), and 35–42 (80%). DAD detection was at 280 nm. The peaks of interest were collected and the fractions were lyophilized. Isolates were then subjected to this method two additional times to obtain >99% purity (by HPLC) products. Purified products were lyophilized to white powders and stored at −80 °C (isolated approximately 15 mg of each product). Based upon the optimized method presented here, CCJ consisted of approximately 3.35 g/L A2 extension units, and 2.17 g/L A2 terminal units.

2.4. Compound characterization

Preliminary HPLC/DAD/ESI-MS/MS identification of CCJ PAC reaction products was performed using previously described HPLC [14] and MS [15] conditions. The mobile phases were 1.0% (v/v) aqueous acetic acid (A) and acetonitrile containing 1.0% (v/v) acetic acid (B). The column temperature was 30 °C; flow rate, 3 mL/min; and with a linear gradient as follows: time in min (%B), 0 (3%), 4 (3%), 14 (18%), 16 (80%). The following modifications were made to the previously described conditions [14,15]: two serially connected Oynx Monolithic columns (100.0 mm × 4.6 mm), fitted with a guard column (5.0 mm × 4.6 mm) from Phenomenex (Torrance, CA, USA) were used; scan range was m/z 50–2200; DAD detection at 280, 320, 370, and 520 nm; and a graduated micro-splitter valve (P-470, Upchurch Scientific, Oak Harbor, WA, USA) reduced the flow from the column to the ion trap MS.

Final HPLC/DAD/ESI-MS/MS conditions for the identification of purified A2 and A2-P differed from the preliminary identification as follows: The mobile phases were 0.1% (v/v) aqueous formic acid (A) and acetonitrile (B) with a flow rate of 0.1 mL/min. The linear gradient was as follows: time in min (%B), 0 (10%), 10 (50%), 12 (20%). The column was a single Zorbax SB-C18 (1 mm × 15 mm, 5 μm) column from Agilent (Santa Clara, CA, USA). Scan range was m/z 200–1200 for A2-P and 200–1000 for A2.

2.5. Analytical HPLC

The previously published method for the analytical preparation of PACs [12] was optimized for PAC A2 quantification in both CCJ and TPS. For CCJ, 10 mg crude cranberry PAC was dissolved in 2.0 mL of a 0.1N methanolic hydrochloric acid solution containing 100 mg/mL phloroglucinol and 10 mg/mL ascorbic acid. The reaction mixture was vortexed to solubilize (~3 s) and then allowed to react for 135 min at 50 °C. The reaction was quenched with 10.0 mL of 40 mM aqueous sodium acetate. The quenched reaction mixture was centrifuged (RCF = 16,128 g) to remove gross particulates. The reaction mixture was then subjected to the analytical HPLC method discussed below. The same conditions were used for TPS, except a concentration of 50 mg/mL phloroglucinol was found to be optimal.

The previously published analytical HPLC method [14] was modified by extending the end time to assure removal of all late eluting material. Additionally, a 3 min post-run was added to equilibrate the columns prior to the next injection. Briefly, two 100 mm × 4.6 mm Chromolith RP-18e columns, purchased from EM Science (Gibbstown, NJ, USA), were connected in series with a guard column of the same material. The mobile phases were 1.0% (v/v) aqueous acetic acid (A) and acetonitrile containing 1.0% (v/v) acetic acid (B). The column temperature was 30 °C, flow rate was 3 mL/min, and with a linear gradient as follows: elution time in min (%B), 0 (3%), 4 (3%), 14 (18%), 14.01 (80%), 20 (80%). Eluting peaks were monitored at 280, 320 and 520 nm to identify peaks of interests in the CCJ PAC system. The final method has an approximate runtime of 25 min, from injection to conclusion of the post-run. This same method was applied unmodified to the TPS.

It should be noted that sodium acetate (pH 4.00) and phosphate (pH 2.00) buffered mobile phases were tested in an attempt to reduce peak drifting due to environmental fluctuations, however both buffers resulted in a co-elution of A2 with an as yet unidentified flavonol.

The determination of the molar extinction coefficients allowed for the quantification of A2 and A2-P using the readily available (−)-epicatechin to calibrate the HPLC's DAD. A 100 ppm (−)-epicatechin standard underwent the described HPLC analysis in 20 μL increments from 20 to 100 μL. A calibration curve was generated by plotting the mass of injected (−)-epicatechin (in ng) versus the integral of the absorbance over time (in pAU). The slope of the regression line was then multiplied by the integral of the A2 or A2-P absorbance over time and the respective corrected relative mass response (Table 1) to give the mass (in ng) found in the injected volume. For mole quantification, the determination was conducted in a similar fashion using moles of (−)-epicatechin and the relative molar response.

Table 1
Retention properties, molar absorptivities, and response factors for the major CCJ and grape proanthocyanidin cleavage products.

Compound	Retention factor (k)	Extinction coefficient (ε280)[a,b]	Relative molar response[a,c]	Corrected relative mass response[a,c,d]
EGC-P	1.0	1,344	0.34	0.32
C-P	2.6	4,218	1.06	1.06
EC-P	2.9	4,218	1.06	1.06
C	5.8	3,988	1.00	1.00
A2-P	7.5	8,003	2.01	1.01
ECG-P	7.8	14,766	3.70	2.44
EC	8.9	3,988	1.00	1.00
ECG	11.8	12,611	3.16	2.07
A2	12.6	5,857	1.47	0.74

[a] Non-A2 values previously published in [12].
[b] In methanol.
[c] Relative to EC.
[d] Not including the phloroglucinol (G-ring) moiety.

3. Results and discussion

3.1. Characterization of cleavage products

The analytical HPLC method [14] was directly applied to the depolymerized CCJ PAC mixture. The CCJ PAC mixture was also co-injected with grape seed and skin standards produced in this lab. Comparison of the chromatograms with previously published data [7,8,14] resulted in a probable identification of peak 1 as epigallocatechin-phloroglucinol adduct (EGC-P), peak 2 as catechin-phloroglucinol adduct (C-P), peak 3 as epicatechin-phloroglucinol adduct (EC-P), peak 5 as catechin (C), and peak 7 as epicatechin (EC) (Fig. 2). Identification of these peaks was confirmed by LC/MS. Four new peaks (4, 6, 8 and 9) were targeted as peaks of potential interest. Preliminary LC/MS (M–H) data gave a major ion peak of 987.6 for peak 4, 699.3 for peak 6, 863.6 for peak 8 and 575.4 for peak 9, which were tentatively identified

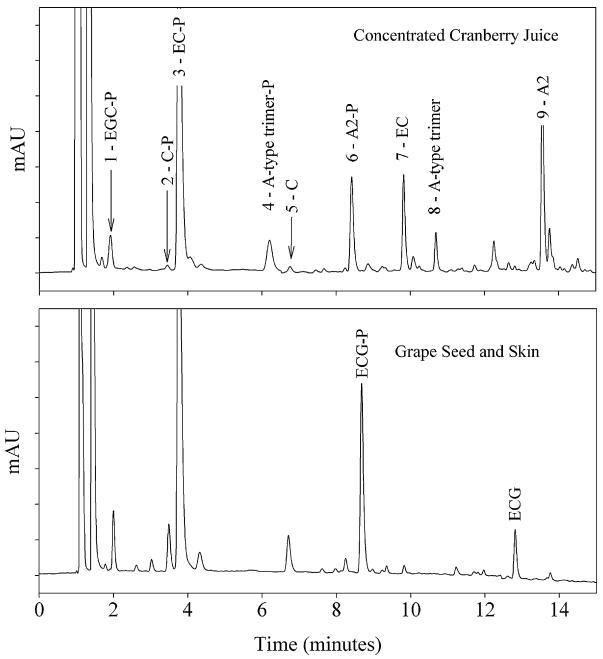

Fig. 2. Analytical HPLC chromatograms (280 nm) for CCJ and grape seed and skin at 20 min reaction time. P = phloroglucinol adduct, EGC = epigallocatechin, C = catechin, E = epicatechin, and ECG = epicatechin gallate. Peaks 4 and 8 are tentatively identified as A-type trimers.

as an A-type trimer-phloroglucinol adduct (A-type trimer-P), A2-phloroglucinol adduct (A2-P), an A-type trimer (A-type trimer) and A2, respectively. Kinetic studies showed the disappearance of the two potential trimer peaks as the A2 and A2-P peaks increased in area (Fig. 3), which improves the preliminary identification of the A-type trimer-P and A-type trimer. However, further studies are needed to confirm the trimers identity. The tentatively identified A2-P and A2 peaks were chosen to undergo further characterization.

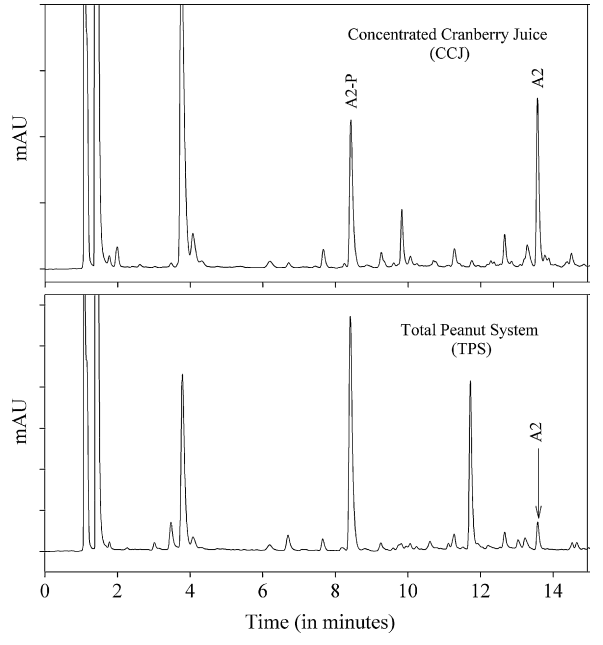

Fig. 3. Analytical HPLC Chromatograms (280 nm) for CCJ and TPS at 135 min reaction time. P = phloroglucinol adduct.

3.2. Isolation and characterization of peaks of interest

The semi-preparative HPLC method described above was used to isolate approximately 15 mg of each peak from CCJ. The purified products then underwent characterization to confirm the preliminary identification.

3.2.1. Mass spectrometry

Isolates from the CCJ system first underwent MS analysis. The positive ion mass spectrum of peak 9 gave an m/z of 577.4. TOF MS ES+ elemental composition analysis (ECA) gave a mass of 577.1360, which is a difference of 2.4 ppm from the calculated mass of 577.1346 for A2 ($C_{30}H_{25}O_{12}$). Enhanced product ions (EPI) on a triple quadrapole with ion trap gave major (M+H) daughter peaks at m/z 451.1, 437.1, 425.1, 311.1, 299.1, and 287.0. The positive ion mass spectrum of peak 6 gave an m/z of 701.2. ECA gave a mass of 701.1529, which is a difference of 3.2 ppm from the calculated mass of 701.1506 for A2-P ($C_{36}H_{29}O_{15}$). EPI gave (M+H) daughter peaks at m/z 575.1, 561.1, 549.2, 533.2, 435.2, 423.2, and 287.1. Fig. 4 is a modification of published schematics [16,17] showing the combined A2 and A2-P pathways for the daughter ions. These data are consistent with peaks 6 and 9 being A2-P and A2, respectively.

3.2.2. Nuclear magnetic resonance (NMR) experiments

Isolates from the CCJ system then underwent NMR analysis. ^{1}H NMR and ^{13}C NMR of peak 9 were consistent with previously published reports on A2 [7,18–20]. HSQC and HMBC further confirmed the identification of this peak as A2.

The NMR data of peak 6 was very similar to that of A2, with a couple of expected differences. ^{1}H NMR of peak 6, when compared to A2, showed a loss of peaks equivalent to two hydrogens at 2.80–2.97 ppm corresponding to the two C-4 hydrogens on the F ring. A new peak integrating to one hydrogen was found at 4.67 ppm. HMBC correlation to C-2 and C-3 of the F-ring, along with HSQC data allowed for the identification of this peak as the C-4 hydrogen on the F-ring. At ambient temperature, the region of 5.90–6.11 ppm integrated to five hydrogens instead of three hydrogens as it did in A2. The splitting patterns and chemical shifts were similar to those seen in A2, however no peaks were seen to account for the increased integration value, which should have appeared as a singlet integrating to two hydrogens in this region to account for the hydrogens on the phloroglucinol moiety. This is likely due to rapid rotation about the bond between the C-4 on the F-ring and C-1 on the G-ring. In order to limit this rotation, the probe temperature was reduced to 274 K for ^{1}H NMR, HSQC and HMBC. The reduced temperature ^{1}H NMR revealed two broad singlets at 6.08 and 5.90 ppm each integrating to one hydrogen. It is expected that further reducing the probe temperature would eliminate rotation at the C-4 F-ring and C-1 G-ring bond, and thus sharpen the phloroglucinol singlets further. Additionally, the ^{13}C NMR of A2-P contained a multiplet at 95.30–95.70 ppm that could not be deconvoluted, and C-4 on the C-ring was lost in the solvent peak, therefore these assignments were assigned using HSQC and HMBC data. Table 2 shows the full ^{1}H NMR and ^{13}C NMR assignments for both A2 and A2-P. Table 3 shows the HMBC correlations. NMR data, when combined with other data, confirmed the identity of peak 6 as A2-P and peak 9 as A2.

3.3. Molar extinction coefficient determination

The molar extinction coefficients were determined in methanol for A2-P and A2 isolated from the CCJ system. Since A2 is a dimer of epicatechin units, and the molar absorptivity at 280 nm (ε_{280}) = 3988 M^{-1} cm^{-1} for epicatechin in methanol [12], it was estimated that A2 and A2-P would have $\varepsilon_{280} \approx 8000\,M^{-1}\,cm^{-1}$. With this assumption, a concentration of 100 μM for A2 and

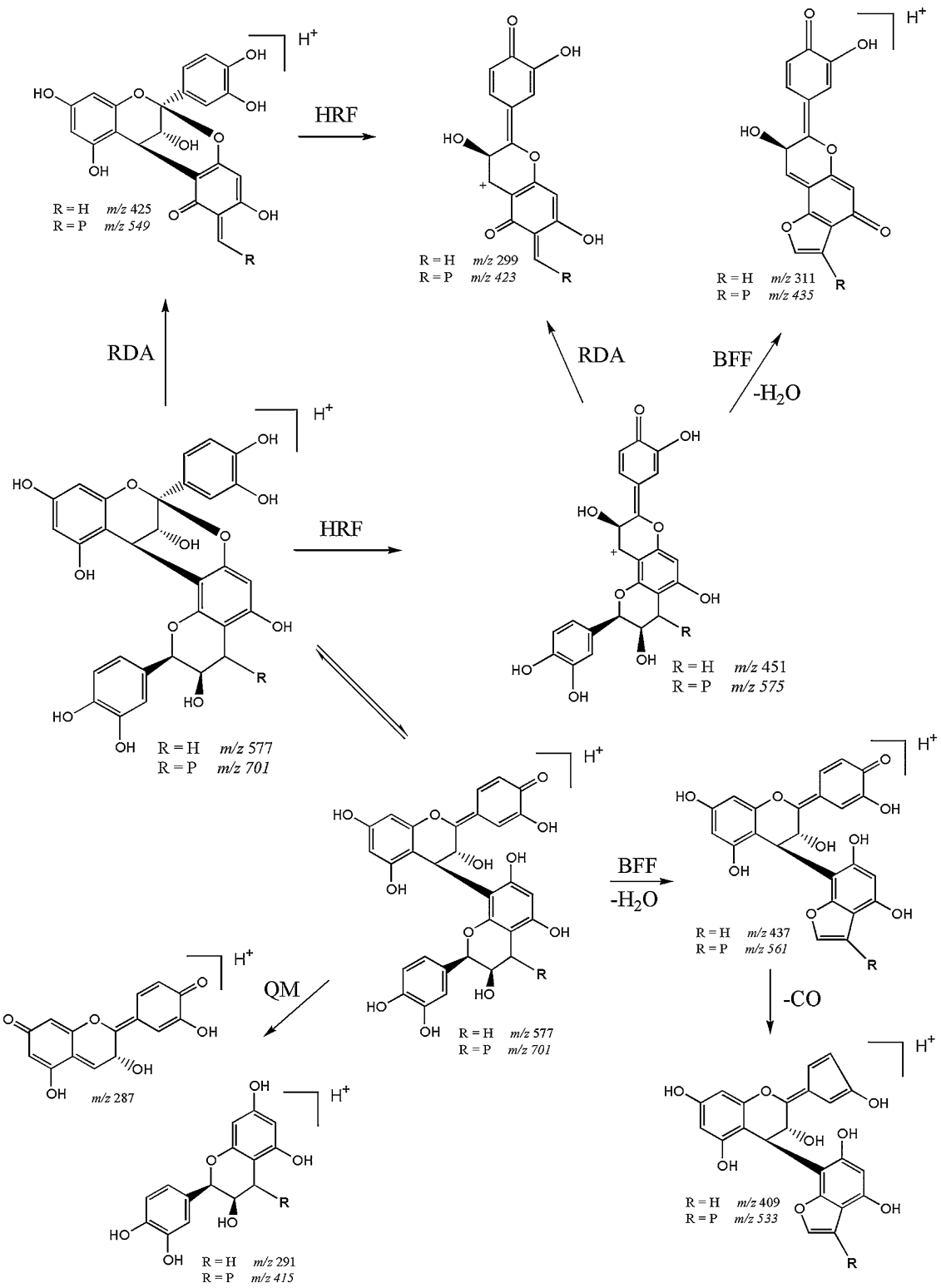

Fig. 4. Fragmentation pathways for A2 and A2-P. P = phloroglucinol, BFF = benzofuran forming, HRF = heterocyclic ring fission, QM = quinone methide fission, and RDA = retro-Diels-Alder. Modified from [16].

A2-P would produce an absorbance near 0.80. A2 and A2-P were isolated from CCJ in triplicate as described above and dissolved in methanol to the above concentration. It was determined that for A2-P, $\varepsilon_{280} = 8003\,M^{-1}\,cm^{-1}$, and for A2, $\varepsilon_{280} = 5857\,M^{-1}\,cm^{-1}$. These data were used to calculate the relative molar response and corrected relative mass response in Table 1.

3.4. Kinetic studies

Previously published kinetic studies of the acid-catalyzed cleavage of PAC using excess phloroglucinol as the trapping reagent found 20 min to be the optimal reaction time to obtain 90% of the maximal PAC products measured in a grape-based system [12]. It was anticipated that the optimal reaction conditions for A2 and

Table 2
^{1}H NMR and ^{13}C NMR assignments.

Ring	Carbon number	Chemical shift of carbon		Chemical shift of proton			
		A2-P	A2	A2-P	Splitting (*J* value (Hz))	A2	Splitting (*J* value (Hz))
A	5[a]	151.3	154.5				
	6	95.5	96.7	6.05	d (2.09)	6.04	d (2.42)
	7	156.3	158.2				
	8	97.5	98.3	5.96	d (2.09)	5.97	d (2.42)
B	1	131.6	131.9				
	2	114.9	115.7	7.16	d (1.69)	7.19	d (2.13)
	3	145.5	146.1				
	4	144.7	146.7				
	5	114.8	115.8	6.84	d (8.41)	6.85	d (7.03)
	6	119.0	115.8	7.05	dd (8.13, 1.69)	7.06	dd (8.37, 2.13)
C	2	99.2	100.4				
	3	67.3	66.0	4.42	d (4.52)	4.34	d (3.27)
	4	28.3	29.1	4.34	d (2.83)	4.24	bs
	4α	105.1	104.8				
	8α[a]	153.4	153.1				
D	5	151.4	152.4				
	6	95.4	96.7	6.06	s	6.14	s
	7	156.1	156.6				
	8	106.6	107.5				
E	1	130.6	131.6				
	2	115.9	120.9	7.29	s	7.29	d (2.05)
	3[a]	145.6	146.1				
	4[a]	144.7	146.7				
	5	115.1	120.4	6.82	d (8.08)	6.84	d (7.18)
	6	120.4	116.8	7.00	d (8.13)	7.06	dd (8.20, 2.05)
F	2	78.3	82.2	5.32	s	4.95	s
	3	71.3	67.6	4.10	m	4.15	d (3.32)
	4	37.5	30.8	4.61	s	2.80/2.94	dd (17.36, 4.65)
	4α	104.5	102.9				
	8α[a]	151.7	151.0				
G	1[a]	103.5					
	2, 4, 6[a]	156.3					
	3, 5	95.2		6.02	bs		
	3, 5	95.6		5.85	bs		

[a] Assigned based on HMBC data and previously published assignments [18,19].

A2-P would differ from B-type PACs, therefore kinetic studies were performed on CCJ PACs. In order to optimize the reaction time for the two A-type PACs, CCJ PAC concentrations of 2.5 and 5 g/L were tested using the published method [12], which called for 50 g/L phloroglucinol. The results showed the A-type PACs reached a maximum considerably later than 20 min (Fig. 5). Additionally, it was found that higher phloroglucinol equivalents resulted in an apparent increase in product stability. The large error bars seen in Fig. 5 for both A2 and A2-P at 300 min are due to this variation in phloroglucinol equivalents.

To determine the maximum yield and stability of the desired products, phloroglucinol concentrations were varied from 5 to 100 g/L with a constant PAC concentration of 5 g/L. Combining the results of these studies, it was concluded that a concentration of 5 g/L crude CCJ PAC, 100 g/L phloroglucinol and 10 g/L ascorbic acid in 0.1N methanolic HCl at 50 °C was found to be optimal for a CCJ system. A reaction time 135 min was optimal to obtain >90% of the maximum of both A2 and A2-P. Therefore this time point was used for quantification of these PACs.

3.5. Testing analytical method with TPS

Based on the above work, it was concluded that the analytical method provided single product peaks for both A2 and A2-P in the CCJ system and therefore could be used to quantify these PACs. The method was then applied to another A-type system to determine if terminal and extension A2 PACs were purely eluted in a system known to contain additional A-type PACs. Previously published work provided evidence for six A-type dimers in peanuts [11]. Therefore, TPS was chosen to test the analytical method. An analytical sample of TPS was prepared as described above, which then underwent HPLC analysis. The results showed peaks at both

Table 3
HMBC correlations, H → C.

Ring	Carbon number	A2-P[a]	A2[a]
A	6	A-5, A-7, C-4α	A-5, A-7, A-8, C-4α
	8	A-6, C-4α	A-6, A-7, C-4α
B	2	B-6, B-3, C-2	B-1, B-4
	5	B-1, B-4	B-1, B-3, B-4
	6	B-4, B-5	B-3, B-5, C-2
C	3		C-2, C-4α, D-8
	4	C-2, C-3, C-4α	C-3
D	6	D-5, F-4α	D-5, D-7, D-8, F-4α
E	2	E-3, E-6, F-2	E-5, F-2
	5	E-1, E-5	E-1, E-3, E-4
	6	E-2, F-2	E-5
F	2	E-1, E-2, E-6, F-3	E-1, E-2, E-6, F-4
	3	F-4α	
	4	D-5, F-2, F-3, F-4α, G-2/G-4	D-5, F-2, F-3, F-4α
G	3, 5	G-1, G-3/G-5, G-2/G-4/G-6	n/a
	3, 5	G-3/G-5	n/a

[a] Letter–number indicates ring letter and carbon number.

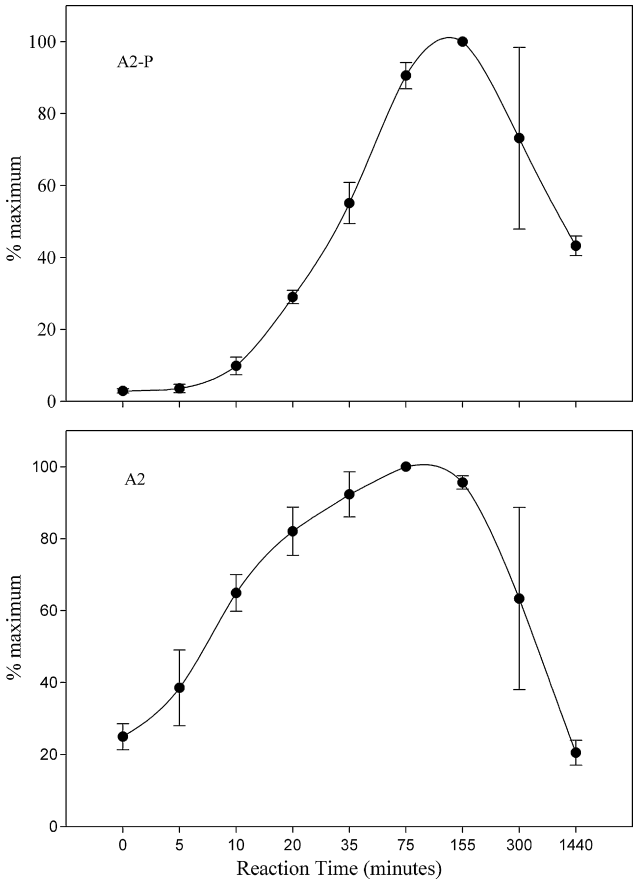

Fig. 5. Kinetics of A2-P and A2 ($n = 4$), error bars are standard deviation.

$k = 7.5$ and 12.6 that correspond to A2-P and A2, respectively in the CCJ system. Co-injection of CCJ and TPS resulted in clean co-elution of A2-P and A2 in CCJ with the $k = 7.5$ and 12.6 peaks of TPS.

TPS was optimized using the same kinetic studies as CCJ. The TPS studies indicated a reduction in the yield of A2-P (~8% reduction) and A2 (~12% reduction) at higher phloroglucinol equivalents (100 g/L vs. 50 g/L phloroglucinol). Additionally, the stability of A2 and A2-P did not depend on concentration, as was seen in CCJ. Therefore a concentration of 50 g/L phloroglucinol [12] was used for TPS. As seen in the CCJ system, a reaction time 135 min was optimal to obtain >90% of the maximum of both A2 and A2-P. Therefore this time point was used for quantification of these PACs. The CCJ and TPS kinetic studies suggest that reaction conditions should be optimized for any PAC system under investigation to ensure maximum yield of A2 PACs. The TPS study also revealed four additional peaks that presented similar A2/A2-P kinetics, which could potentially correspond to the additional A-type PACs previously reported [11].

Approximately 2 mg of the $k = 7.5$ peak was isolated from the TPS. There was an insufficient amount of the $k = 12.6$ peak for isolation. The other four potential A-type peaks, while present in significant quantities, were not isolated or characterized at this time. ^{1}H NMR was performed on the peak at $k = 7.5$ isolated from the TPS and the results showed a pure product that was consistent with the CCJ data, allowing for the confirmation of this peak as A2-P in TPS.

Table 4
Quantity of A2-P and A2 in CCJ and TPS ($n = 9$), errors are standard deviation.

System	Mass A2-P (mg/g)[a,b]	% A2-P[b,c]	Mass A2 (mg/g)[a]	% A2[c]
CCJ	209.4 ± 4.9	21%	135.7 ± 4.7	14%
TPS	404.9 ± 14.4	40%	29.1 ± 0.9	3%

[a] Mass of A2 per gram of crude PAC powder.
[b] Not including phloroglucinol (G-ring) moiety.
[c] Percent A2 found in crude PAC powder (w/w).

3.6. Quantitative application of A2 method

The kinetically optimized analytical methods were performed ($n = 9$) to quantify the A2-P and A2 in CCJ and TPS (Table 4). As previously mentioned, the identity of the peak at $k = 12.6$ in TPS was not confirmed as being A2, so the quantity listed in Table 4 is tentative.

4. Conclusion

Methods for the identification, isolation and purification of two A-type PACs were presented here. Isolates were extensively characterized and molar extinction coefficients were determined for the terminal A2 dimer and phloroglucinol adduct of the A2 extension dimer. Kinetic studies were conducted and reaction conditions were optimized for the A2 units in both CCJ and TPS. The end result is an analytical method that can be used for the quantification of terminal and extension A2 dimers in CCJ and TPS.

Using this analytical method to study A-type PACs, and comparing the kinetics of the reaction with previous studies [14], suggests that reaction kinetics need to be optimized for each PAC under investigation. Given the variation in PAC subunit composition, interflavonoid bond location and molecular mass distribution, this conclusion is somewhat expected.

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