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Influence of Alternative Liquid Chromatography Techniques on the Chemical Complexity and Bioactivity of Isolated Proanthocyanidin Mixtures

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Extracts rich in proanthocyanidins, which are implicated in multiple human health benefits, were comparatively separated using alternative separation methods [vacuum or open column liquid chromatography], separation matrices [Toyopearl, Sephadex, or silica gel], and degrees of subfractionation [8 or 12 subfraction series], to evaluate the influence of separation technique on the resolution of the chemical composition and the biological activity of separated proanthocyanidin mixtures in individual subfractions. Bioactivity was assessed using a DNA human topoisomerase II bioassay and structural composition by acid thiolysis (average degree of polymerization, DP) and HPLC-ESI/MS. The amount of parent fraction needed to inhibit 50% of topoisomerase II was 3.38 ng/mL with an average DP of 25.5. A 2^3 factorial analysis revealed that the vacuum and open column strategies for separation, when individually considered, did not yield significantly different results in terms of mass recovery, DP, or bioactivity; however, interactions with other factors such as matrix or subfraction series resulted in distinctive shifts in fraction profiles and biological activity. In general, Sephadex as a matrix permitted elution and separation of discrete, polymerized subfractions with potent inhibition against human topoisomerase II. Sephadex vacuum chromatography, Toyopearl open column chromatography, and Toyopearl vacuum chromatography separation techniques eluted highly polymerized proanthocyanidin mixtures, but the inhibitory bioactivity was attenuated as compared to the parent fraction, whereas Sephadex open column chromatography eluted highly polymerized subfraction mixtures that retained bioactive potential.

KEYWORDS: Proanthocyanidins; wild blueberry; *Vaccinium angustifolium* Ait.; Toyopearl HW-40; Sephadex LH-20; silica gel type G; topoisomerase II bioassay

INTRODUCTION

Proanthocyanidins, oligomeric and polymeric flavan-3-ols common in many plant-derived foods, are increasingly associated with human health benefits. These complex flavonoids have been linked to concentration-dependent free radical scavenging, inhibition of chemically induced lipid peroxidation (*1, 2*), inhibition of bacterial growth (*3*), protective cardiovascular effects (*4*), endothelium-dependent relaxing activity in blood vessels through nitric oxide production (*5*), and anticarcinogenic activity (*6–11*). Proanthocyanidin oligomers demonstrated immunomodulatory effects (*12*), whereas A-type proanthocyanidins from cranberry and blueberry have been shown to improve urinary tract health by impeding uropathogenic bacterial attachment (*13–15*). The structural complexity of the proanthocyanidin has been correlated to antioxidant activity, with increasing size typically associated with greater potency (*16–18*).

Effective separation of multimeric proanthocyanidin mixtures has been elusive, despite recent progress in analytical techniques. A significant obstacle to conclusive research on health benefits of these phytochemicals in foods is that proanthocyanidin mixtures may actually be changed in composition and/or bioactivity during the routine process of separating and purifying them for identification and analysis (*19*). The extraction/separation procedures are typically multistep and vary extensively between different laboratories (*11, 20*). Extraction method, species of plant, and sample tissue selection may influence the degree of polymerization for isolated proanthocyanidins, which may or may not be indicative of polymerization levels in situ and which may effectively alter bioactive potential (*11*). Degradation of compounds through processing may also occur (*11, 21, 22*), although loss of bioactivity due to structural changes during extraction/fractionation has not been adequately analyzed.

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Some proanthocyanidin mixtures resist separation and may be adsorbed onto a specific solid matrix used for chromatography (*23, 24*). Separation of proanthocyanidins up to decamers using HPLC has been reported (*18, 25–27*), but the isolation of complex proanthocyanidin polymeric mixtures remains difficult due to their elution either as a mass of intermingled flavonoids (*28–30*) or elution as mixtures throughout the chromatographic separation process (*18*). Characterization of polyphenol polymers in grape, wine, and some other fruits using HPLC-ESI/MS, thiolysis, and NMR methods has been previously reported (*31*); however, the isolation of higher molecular weight proanthocyanidins has not yet been successful due to the increasing numbers of isomers that develop as polymerization increases (*11–18, 32*). Alternative supports used in chromatographic separation of proanthocyanidins and other flavonoids may have a significant influence on the integrity and nature of separated compounds. Sephadex LH-20 chromatography has been used to separate condensed and hydrolyzable tannins from simple phenolic compounds (*33–36*), but the series of solvents has varied widely. Toyopearl HW-40 and Sephadex matrices can effectively partition the lower and higher molecular weight flavonoids (*29, 37, 38*), and silica gel support has been used to separate organic compounds (*39, 40*). Although the chemical properties of Toyopearl HW-40, Sephadex LH-20, and silica gel type G column packing materials are known, the full influence each may exert on the integrity of high molecular weight flavonoids, including proanthocyanidins, has not been fully assessed.

This study comparatively evaluated alternative column support matrices (Toyopearl, Sephadex, or silica gel), chromatography methods (vacuum or open column liquid chromatography), and solvent gradients (resulting in 8 or 12 subfractions) during the separation of proanthocyanidin-rich fractions from wild blueberry fruits. Subfractions isolated using alternative separation treatments were evaluated in terms of phytochemical composition using HPLC-ESI/MS and acid thiolysis and in terms of biological activity using a chemoprevention bioassay. A 2 (*3*) factorial analysis evaluated the influence of the three variables (matrix, chromatography method, and solvent series) on the chemical composition and bioactivity of separated proanthocyanidin-rich subfractions.

MATERIALS AND METHODS

Chemicals and Materials. A 10× Tris acetate−-EDTA (TAE) buffer, acetone, ethanol, and methanol were obtained from Sigma-Aldrich Co. (St. Louis, MO). All other topoisomerase II assay chemicals were obtained from TopoGen, Inc. (Columbus, OH). Toyopearl HW-40 was obtained from Tosohaas (Bioseparation Specialists, Montgomeryville, PA), and Sephadex LH-20, silica gel type G 10–40 μm, and $CaSO_4$ binder were from Sigma-Aldrich Co.

Plant Material. Whole individually quick-frozen wild blueberry fruits (*Vaccinium angustifolium* Ait.) were obtained from the Wild Blueberry Association of North America (WBANA), Bar Harbor, ME. The blueberries were a composite of fruit from different growing regions including Prince Edward Island, New Brunswick, Nova Scotia, and Maine, and the composite sample was made in fall 2004. The fruit was stored at −80 °C until extraction at room temperature in 70% aqueous acetone.

Preparation of Crude Extract. Frozen wild blueberries (1.5 kg) were homogenized in a Waring blender with 1 L of 70% aqueous acetone for 5 min. The resulting slurry was filtered through eight layers of cheesecloth. The remaining pulp and seeds were homogenized with 500 mL of 70% aqueous acetone and again subsequently filtered through eight layers of cheesecloth. This procedure was repeated two more times, removing the purple pigment from the pulp and seeds. The resulting crude extract was concentrated by rotary evaporation at 40 °C until all acetone was removed. A sample of the crude extract was lyophilized for later chemical analysis and bioactivity testing.

Fractionation Procedures. The initial fractionation procedure was adapted from that of Schmidt et al. (*15*). Crude extract (350 mL) was placed on a 210 mm × 70 mm column of 240 g of Toyopearl HW-40 (T) (Tosohaas) (bed volume of approximately 3.23×10^6 mm^3). Fractionation by vacuum (40 psi) liquid chromatography (VC) used the following series of solvents: fraction 1, 800 mL of distilled water; fraction 2, 800 mL of 50% aqueous methanol; fraction 3, 800 mL of 100% methanol; fraction 4, 800 mL of 100% acetone; and fraction 5, 800 mL of 50% aqueous acetone. After the fifth fraction, all color was removed from the column. Fraction 1, which contained sugars, was discarded, and fractions 2–5 were individually concentrated by rotary evaporation at 40 °C and lyophilized. Fractions were stored at −80 °C until further fractionation or bioassay.

Subfractionation Procedures. On the basis of previous work (*15*), fraction 5 (F5), a fraction composed almost entirely of oligomeric and polymeric proanthocyanidins, was selected for further fractionation. F5 was further fractionated using either vacuum column liquid chromatography (VC) or open column liquid chromatography (OC) with Sephadex LH-20 (S), silica gel type G (10–40 μm) (SG), or Toyopearl HW-40 (T) as the chromatography bed, using solvent series that resulted in either 8 or 12 subfractions. S, SG, and T have each been routinely used as column matrices for plant tissue or in vitro culture extracts containing proanthocyanidins (*15, 41*).

Sephadex LH-20. A 200 mg sample of F5 was dissolved in 25 mL of 25% aqueous ethanol and fractionated using OC on a 173 mm × 18 mm S column (bed volume of 1.76×10^5 mm^3). The same amount of F5 was also fractionated using VC on a 150 mm × 70 mm column of S (bed volume of 2.31×10^6 mm^3). All columns were prewashed with 35% aqueous ethanol and primed with 25% aqueous ethanol. All experimental methods used either an 8-fraction solvent series (8 SF) or a 12-fraction solvent series (12 SF) (**Figure 1**); the subfractions were eluted with 3 times the column volume of each column. The 8 SF series was as follows: 1a, 25% aqueous ethanol; 2a, 50% aqueous ethanol; 3a, 100% ethanol; 4a, 100% methanol; 5a, 25% aqueous acetone; 6a, 50% aqueous acetone; 7a, 75% aqueous acetone; and 8a, 100% acetone. The 12 SF series was as follows: 1b, 25% aqueous ethanol; 2b, 50% aqueous ethanol; 3b, 75% aqueous ethanol; 4b, 100% ethanol; 5b, 25% aqueous methanol; 6b, 50% aqueous methanol; 7b, 75% aqueous methanol; 8b, 100% methanol; 9b, 25% aqueous acetone; 10b, 50% aqueous acetone; 11b, 75% aqueous acetone; and 12b, 100% acetone. All subfractions were reduced by rotary evaporation, lyophilized, and stored at −80 °C until bioassay and quantification procedures were performed.

Toyopearl HW-40. A 195 mg sample of F5 was fractionated using OC on a 160 mm × 18 mm T column (bed volume of 1.63×10^5 mm^3). Again, the same amount of F5 was also fractionated using VC on a 160 mm × 70 mm column of T (bed volume of 2.46×10^6 mm^3). All columns were prewashed with 35% aqueous ethanol and primed with 25% aqueous ethanol. All experimental methods used either an 8 SF or 12 SF series as described above; the fractions were eluted with 3 times the column volume of each column (**Figure 1**). All fractions were reduced by rotary evaporation, lyophilized, and stored at −80 °C until bioassay and quantification procedures were performed.

Silica Gel. A 116 mg sample of F5 was mixed with 8 g of dry silica gel (SG) and 2.0 mL 100% methanol. A 50 g quantity of pure dry SG was placed in a 160 mm × 70 mm glass chromatography column (bed volume of approximately 2.46×10^6 mm^3) and primed with 100% acetone. The F5:SG mixture was added to the glass column, and cotton was placed on top of the column along with a glass stopper. Only VC was performed with SG because preliminary trials of OC with SG resulted in very poor solvent elution. The 12 SF series was not performed because the solvent type and order were analogous to the 8 SF series. Subfractions were eluted with 75 mL fractions as follows: 1a, 100% acetone; 2a, 75% aqueous acetone; 3a, 50% aqueous acetone; 4a, 25% aqueous acetone; 5a, 100% methanol; 6a, 100% ethanol; 7a, 50% aqueous ethanol; and 8a, 25% aqueous ethanol. All subfractions were reduced by rotary evaporation, lyophilized, and stored at −80 °C until bioassay and quantification procedures were performed.

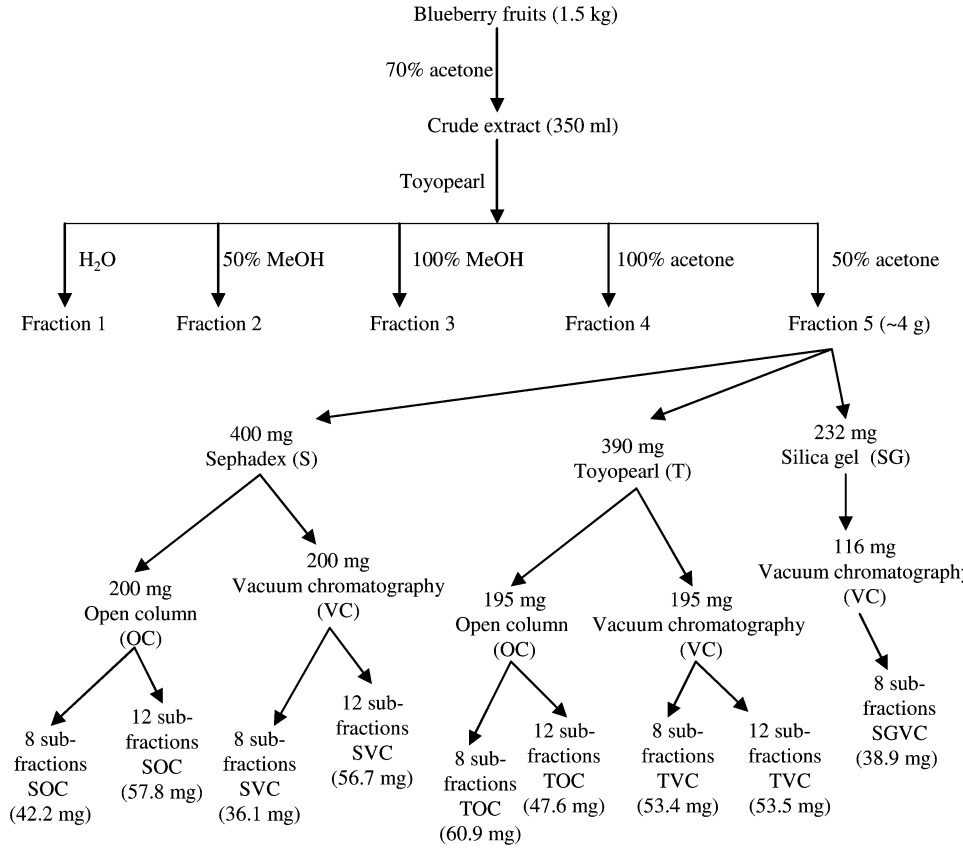

Figure 1. Sequential fractionation of blueberry fruit extract into series of 8 or 12 proanthocyanidin subfractions using three different column matrices (Sephadex, Toyopearl, or silica gel) with open column chromatography or vacuum chromatography. Individual subfraction yield data are presented in **Tables 1** and **2**.

Table 1. Percent Mass Recovery, Average Degree of Polymerization (DP), and Percent Topoisomerase II Inhibition of the Eight-Subfraction Series Using Open Column or Vacuum Column Chromatography Techniques with Sephadex or Toyopearl[a]

	SF1	SF2	SF3	SF4	SF5	SF6	SF7	SF8
				Sephadex Open Column Chromatography (SOC)				
mass	4.0 ± 2.5	8.9 ± 2.7	0.8 ± 1.2	3.5 ± 1.8	5.1 ± 1.8	12.5 ± 1.3	5.9 ± 5.3	1.5 ± 2.1
DP	9.3 ± 3.3	11.2 ± 1.4	4.0 ± 1.0	8.0 ± 1.2	13.3*	29.0 ± 5.0	43.1 ± 10.5	INC
% inh	69.5 ± 11.1	53.8 ± 3.2	65.2 ± 8.1	63.7 ± 5.6	61.7 ± 4.0	63.7 ± 17.6	98.6 ± 2.8	65.2 ± 8.1
				Sephadex Vacuum Chromatography (SVC)				
mass	4.2 ± 0.5	4.3 ± 1.5	3.3 ± 3.4	0.5 ± 0.5	3.7 ± 3.9	17.3 ± 7.0	2.44 ± 0.3	0.4 ± 0.5
DP	21.2 ± 0.2	8.91 ± 0.2	5.6 ± 0.4	9.2 ± 0.2	10.1 ± 3.2	15.8 ± 0.0	11.75 ± 4.6	13.9 ± 5.3
% inh	35.6 ± 7.2	97.2 ± 8.2	64.6 ± 3.1	98.8 ± 2.7	78.5 ± 0.0	97.9 ± 1.3	55.1 ± 3.0	45.3 ± 1.8
				Toyopearl Open Column Chromatography (TOC)				
mass	1.9 ± 2.6	5.9 ± 2.2	0.1 ± 0.0	0.3 ± 0.3	0.6 ± 0.7	50.6 ± 7.5	1.5 ± 2.0	0.0 ± 0.0
DP	59.2 ± 12.3	11.2 ± 5.6	6.3 ± 2.4	3.6 ± 1.6	6.4 ± 2.4	9.0 ± 1.9	23.4 ± 2.4	8.2 ± 8.8
% inh	87.4 ± 0.5	51.6 ± 26.4	86.9 ± 14.2	79.2 ± 3.6	59.9 ± 3.6	96.6 ± 0.4	75.4 ± 5.8	97.5 ± 8.5
				Toyopearl Vacuum Chromatography (TVC)				
mass	2.4 ± 0.5	4.7 ± 4.3	1.1 ± 0.1	0.1 ± 0.2	2.5 ± 0.5	27.7 ± 1.8	14.5 ± 12.8	0.4 ± 0.6
DP	9.1 ± 0.4	6.6 ± 2.4	INC	3.4 ± 0.9	6.6 ± 0.3	7.6 ± 1.6	20.5 ± 1.0	NR
% inh	51.2 ± 12.1	88.0 ± 37.7	67.5 ± 5.4	63.7 ± 5.7	32.3 ± 2.1	79.7 ± 7.4	60.2 ± 1.7	NR

[a] Solvents: SF1, 25% aq EtOH; SF2, 50% aq EtOH; SF3, 100% EtOH; SF4, 100% MeOH; SF5, 25% aq acetone; SF6, 50% aq acetone; SF7, 75% acetone; SF8, 100% acetone. Mass, percent mass recovery (starting, 200 mg for Sephadex or 195 mg for Toypearl); DP, average degree of polymerization; % inh, percent inhibition; *, subfraction tested three times with only one result; INC (inconclusive), samples produced results that could not be analyzed; NR (not run), sample was not analyzed.

Thin Layer Chromatography. Thin layer chromatography was performed using silica thin layer chromatography plates on aluminum, 200 μm thickness, pore size = 60 Å (Sigma-Aldrich Co.). Ethyl acetate, methanol, and water were used as the mobile phase in a ratio of 79:11:10, respectively. The plates were heated at 100 °C for 10 min after spraying with vanillin−HCl or dichromate reagent (*15*). Proanthocyanidins and sugars were monitored with these sprays.

Acid Thiolysis. In this study, a careful acid thiolysis was performed, based on a modification of the procedure by Guyot et al. (*28*), to determine the average DP of selected subfractions. A 2.0 mg sample of each subfraction was dissolved in 1.0 mL of 100% MeOH solution, and 1.25 mg of epicatechin/catechin was dissolved in 1 mL of 100% MeOH. The following components were added to a vial that was subsequently placed in a 40 °C water bath for 30 min: 50 μL of the prepared sample, 50 μL of concentrated HCl (3.3% v/v)−methanol solution, and 100 μL of benzyl mercaptan (5% v:v)−methanol solution. After 30 min, the glass vials were kept at room temperature for an additional 10 h and then placed in a −20 °C freezer until HPLC analysis. This careful sequence of steps (incubation in a 40 °C water bath for 30 min, transfer to room temperature for an additional 10 h,

Table 2. Percent Mass Recovery, Average Degree of Polymerization (DP), and Percent of Topoisomerase II Inhibition of the 12-Subfraction Series of Treatments Using Open Column or Vacuum Column Chromatography with Sephadex or Toyopearl[a]

	SF1	SF2	SF3	SF4	SF5	SF6
			Sephadex Open Column Chromatography (SOC)			
mass	2.1 ± 0.7	6.0 ± 0.8	10.7 ± 1.7	1.4 ± 1.3	0.2 ± 0.2	0.8 ± 0.1
DP	4.5 ± 1.2	6.3 ± 0.8	1.2 ± 0.3	6.3 ± 0.4	18.5 ± 1.2	7.3 ± 4.7
% inh	74.9 ± 3.5	62.5 ± 9.1	52.3 ± 15.9	49.9 ± 3.5	99.2 ± 13.2	45.5 ± 16.4
			Sephadex Vacuum Chromatography (SVC)			
mass	5.7 ± 2.2	9.2 ± 7.7	3.1 ± 4.5	1.5 ± 0.1	0.3 ± 0.4	11.4 ± 1.6
DP	11.3 ± 3.7	10.5 ± 1.5	8.9 ± 1.8	6.4 ± 3.0	6.5 ± 0.3	INC
% inh	35.7 ± 1.5	98.9 ± 6.0	61.4 ± 0.1	80.8 ± 8.2	74.6 ± 9.6	INC
			Toyopearl Open Column Chromatography (TOC)			
mass	1.7 ± 1.2	5.6 ± 2.7	3.7 ± 0.5	1.1 ± 0.1	3.8 ± 2.4	0.8 ± 0.4
DP	2.8 ± 1.1	10.2 ± 2.1	6.3 ± 5.3	4.6 ± 1.1	5.5 ± 1.0	4.7 ± 0.1
% inh	71.0 ± 10.7	70.4 ± 4.9	72.3 ± 17.4	85.6 ± 3.0	48.5 ± 0.5	96.8 ± 5.2
			Toyopearl Vacuum Chromatography (TVC)			
mass	1.9 ± 1.3	6.8 ± 3.2	2.9 ± 2.0	0.0 ± 0.0	0.3 ± 0.0	0.5 ± 0.4
DP	8.8 ± 0.8	7.4 ± 0.9	5.0 ± 0.9	4.7 ± 2.3	31.7 ± 0.9	9.7 ± 9.2
% inh	51.3 ± 1.2	84.8 ± 6.0	83.4 ± 5.3	71.6 ± 3.8	90.7 ± 4.9	83.1 ± 6.4

	SF7	SF8	SF9	SF10	SF11	SF12
			Sephadex Open Column Chromatography (SOC)			
mass	2.1 ± 0.8	7.3 ± 7.1	2.3 ± 1.8	22.6 ± 7.9	2.3 ± 0.5	0
DP	7.2 ± 1.0	10.7 ± 3.1	11.9 ± 3.9	29.2 ± 0.5	43.5 ± 0.4	
% inh	79.8 ± 0.7	35.4 ± 2.0	87.4 ± 2.9	88.6 ± 11.8	67.6 ± 6.1	
			Sephadex Vacuum Chromatography (SVC)			
mass	1.5 ± 1.3	3.3 ± 3.9	1.4 ± 0.2	14.7 ± 1.2	3.8 ± 3.7	0.8 ± 0.4
DP	10.7 ± 1.5	9.1 ± 2.6	10.2 ± 1.5	18.6 ± 1.8	13.7 ± 3.5	14.3 ± 1.2
% inh	89.8 ± 15.4	73.6 ± 9.3	77.6 ± 10.0	47.5 ± 3.0	51.4 ± 13.2	29.3 ± 12.0
			Toyopearl Open Column Chromatography (TOC)			
mass	2.7 ± 1.3	7.7 ± 4.7	2.4 ± 1.2	13.7 ± 4.2	4.4 ± 3.0	0
DP	5.5 ± 6.1	9.1 ± 1.3	4.3*	15.6 ± 0.4	11.6 ± 1.0	
% inh	60.6 ± 1.6	40.6 ± 4.5	58.1 ± 0.2	95.3 ± 2.2	80.4 ± 16.3	
			Toyopearl Vacuum Chromatography (TVC)			
mass	2.3 ± 1.0	2.0 ± 0.1	1.0 ± 0.1	33.0 ± 3.41	2.8 ± 0.1	0
DP	6.6 ± 0.5	5.7 ± 0.8	6.2 ± 0.4	31.7 ± 4.5	24.5 ± 18.4	
% inh	87.5 ± 3.2	59.8 ± 2.8	87.4 ± 2.9	55.8 ± 1.4	49.1 ± 22.7	

[a] Solvents: SF1, 25% aq EtOH; SF2, 50% aq EtOH; SF3, 75% aq EtOH; SF4, 100% EtOH; SF5, 25% aq MeOH; SF6, 50% aq MeOH; SF7, 75% aq MeOH; SF8, 100% MeOH; SF9, 25% aq acetone; SF10, 50% aq acetone; SF11, 75% aq acetone; SF12, 100% acetone. Mass, percent mass recovery (starting, 200 mg for Sephadex or 195 mg for Toyopearl); DP, average degree of polymerization; % inh, percent inhibition; *, subfraction tested three times with only one result; INC (inconclusive), samples produced results that could not be analyzed.

followed by placement at −20 °C) was taken to avoid secondary rearrangements involving inversion of the stereochemistry of the pyran ring at C-2 in flavan-4-phloroglucinol adducts and dehydration to [1]benzofuro[2,3-*c*]chromenes (*42*). The subsequent HPLC analysis was performed using an Agilent system (Agilent Technologies Inc., Wilmington, DE) with autosampler, diode array detector (DAD, 280 nm), and 250 mm × 4 mm × 5 μm Supelcosil LC-18 reversed-phase column (25 °C) (Supleco, Bellefonte, PA). The elution solvents consisted of 2% acetic acid in H_2O (A) and 100% MeOH (B). The linear gradient started at 15% B and increased to 80% B in 45 min and continued for 5 min at 80% followed by 15% B to equilibrate the column with a constant flow rate of 1 mL/min.

HPLC-ESI/MS Analysis. A 5 mg sample of each of the subfractions from the Toyopearl HW-40, Sephadex LH-20, or silica gel type G columns was dissolved in 1 mL of aqueous methanol (1:1 v/v) and analyzed using HPLC-ESI/MS analysis on an LCQ Deca XP mass spectrometer (Thermo Finnigan Corp., San Jose, CA), MS version 1.3 SRI, electrospray ionization (ESI) in the positive ion mode (*m*/*z* 150–2000). The HPLC-ESI/MS has a photodiode array (PDA) detector version 1.2 set at 200–600 nm, autosampler version 1.2, and Xcalibur software for data processing. The spray voltage was 10 kV, and the capillary temperature was 250 °C. Analysis was performed according to procedures developed by Yousef et al. (*41*) with minor modifications. Briefly, the HPLC separation was carried out on a C18 reversed-phase column (2.1 × 150 mm, VYDAC, catalog no. 201 SPS215). The mobile phase solvents consisted of 95% dd H_2O, 5% acetonitrile with 0.1% formic acid (A) and 95% acetonitrile, 5% H_2O with 0.1% formic acid (B). A step gradient of 0, 30, 60, 90, and 0% of solvent B was used at 3, 30, 45, 50, and 60 min, respectively, with 200 μL/min flow rate and 10 μL injection volumes. The column was equilibrated with solvent A for 10 min between samples at the same flow rates.

Topoisomerase II Bioassay. Topoisomerase inhibitors constitute a class of chemopreventive agents that inhibit carcinogenesis via their antiproliferative or cell-differentiating action. Many chemopreventive agents are found to be topoisomerase II catalytic inhibitors, which has provided a useful strategy to select chemopreventive agents that may be effective at the stages of promotion and progression. Especially, topoisomerase II has emerged as a chemotherapeutic target for a diverse group of antitumor agents (*43, 44*). Whereas differentiated cells express very low levels of topoisomerase II, highly proliferative tumor cells often express 25–300 times higher levels than those of quiescent cells (*45*). Several assays have been developed to evaluate the ability of a compound to modulate biochemical events presumed to be linked to carcinogenesis (*46*). Resveratrol was one of the potent chemopreventive agents with effective inhibition of catalytic activity on mammalian topoisomerase II (IC_{50} of 15 μg/mL) (*43*).

In this study, examination of the ability of an extract to provide inhibition against human topoisomerase II was carried out using a topoisomerase drug screening kit (TopoGen, Inc., Columbus, OH). The reaction was carried out in a 20 μL mixture, containing 13 μL of purified distilled water, 2 μL of 10× assay cleavage buffer, 1 μL of pRYG DNA, 2 μL of sample compound (50 μg/mL 5% aqueous methanol to

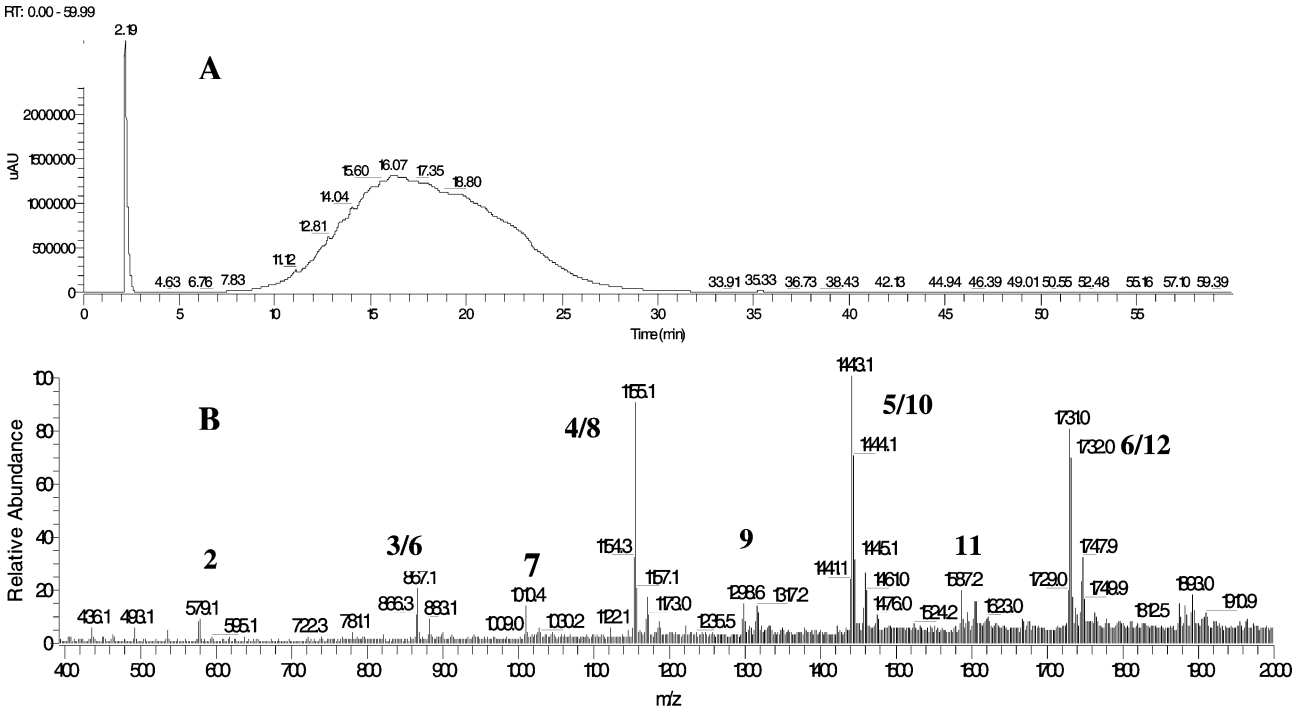

Figure 2. HPLC-ESI/MS chromatogram of F5, the highly polymerized parent fraction consisting of proanthocyanidin oligomers and polymers: (**A**) UV chromatogram with 280 nm absorption; (**B**) ESI/MS spectra (the large bold numbers indicate average degree of polymerization from dimers (2) to 12-mers, and peaks with two bold numbers indicate the presence of a doubly charged ion and the presence of both DP (i.e., 5/10-pentamers and decamers)].

make a final concentration of 5 μg/mL 5% aqueous methanol), and 2 μL of human topoisomerase II (2 units/μL). This mixture was incubated for 75 min at 37 °C, and after incubation, 2 μL of 10% SDS was added to stop the reaction. Proteinase K (0.6 μL) was added, and the mixture was incubated at 50 °C for 15 min. Loading buffer (0.25% bromophenol blue, 50% glycerol) (2 μL) was added to the mixture, and the DNA was extracted with 20 μL of chloroform isoamyl alcohol. The mixtures were centrifuged for 20 s at 6000 rpm. The extracted DNA (in the upper 15 μL of the mixture) was loaded into a 1% agarose gel and run in Tris−acetate−EDTA (TAE) buffer (pH 8.0) for 4 h at 85 V (Electrophoretic Gel System, E-C Apparatus Corp., St. Petersburg, FL). Relaxed pRYG and linear pRYG were used as markers in the system. After 4 h, the gel was stained in ethidium bromide (5% solution) and destained in distilled water for 10 min prior to image acquisition with the Kodak Image Station 440 CF (Eastman Kodak Co., New Haven, CT). Band analysis was obtained using Kodak 1D Image Analysis Software v. 3.5. The inhibitory activity was expressed as a percentage of the topoisomerase II uninhibited enzyme activity. A 5% aqueous methanol control was used as the negative control as all samples were dissolved in this aqueous solvent solution.

Statistical Analysis. SAS V9.1 for Windows (SAS Institute, Inc., Cary, NC) was used for all statistical analysis. A full 2 (*3*) factorial analysis was used to evaluate how methodological variables during proanthocyanidin separation influenced topoisomerase II inhibition, average DP, and mass recovery. Variables in the experimental design included matrix (Toyopearl HW-40 or Sephadex LH-20)m separation method (vacuum or open column chromatography), and subfraction (8 or 12).

RESULTS AND DISCUSSION

The HPLC-MS chromatogram of F5 illustrates the proanthocyanidin (PAC) complexity of the parent mixture F5 (**Figure 2**), containing 43.6 μg of gallic acid equivalent/mL. The UV chromatogram, at 280 nm absorption, showed a large hump of complex proanthocyanidins (**Figure 2A**). ESI/MS spectra (**Figure 2B**) showed an average DP ranging from dimers to 12-mers and also the presence of a doubly charged ion and the presence of both DP (i.e., 5/10, pentamers and decamers). The average DP was 25.5, and the concentration needed to provide 50% inhibition of human topoisomerase II was 3.38 ng/mL. As shown by the HPLC-ESI/MS chromatogram, the PACs eluted as one mass of intermingled flavonoids, which was consistent with previous reports for the isolation of complex PACs (*15, 18, 26, 27, 47*).

Variations in fractionation procedures and matrices resulted in different subfraction compositions, which in turn were reflected in differing capacity to inhibit topoisomerase II enzyme. Fractionation by SGVC produced two highly bioactive (**Figure 3A**) and highly polymerized subfractions (100% acetone and 75% aqueous acetone), but did not facilitate further separation of polymerized PACs. Using acetone on SGVC caused 47% of the mass to elute in the first subfractions (**Figure 3B**), and also the human topoisomerase II inhibitory activity was localized in those subfractions (>95%). Further HPLC-ESI/MS (**Figure 4A**) chemical analysis of the 100% aqueous acetone subfraction showed the presence of PACs, but subsequent subfractions had less PAC content as shown by the loss of UV signal for the 50% aqueous acetone subfraction (**Figure 4B**). This localization of the majority of bioactive polymeric PACs in the first two subfractions demonstrated the primary use of SGVC, which is the effective fractionation of lower molecular weight phenolics (*48, 49*) and organic compounds (*39*). SGOC proved to be unable to facilitate further separation of PACs, due to insufficient elution through the column. For comparison and separation of complex polymeric PACs, both SGOC and SGVC proved to be inadequate. As a result, the subsequent analyses describe separation treatments using only Toyopearl (T) and Sephadex (S) matrices.

A summary of the percent mass recovery, average DP, and human topoisomerase II inhibitory activity of subfractions separated by either open column or vacuum column chromatography on either Toyopearl or Sephadex matrices, using an

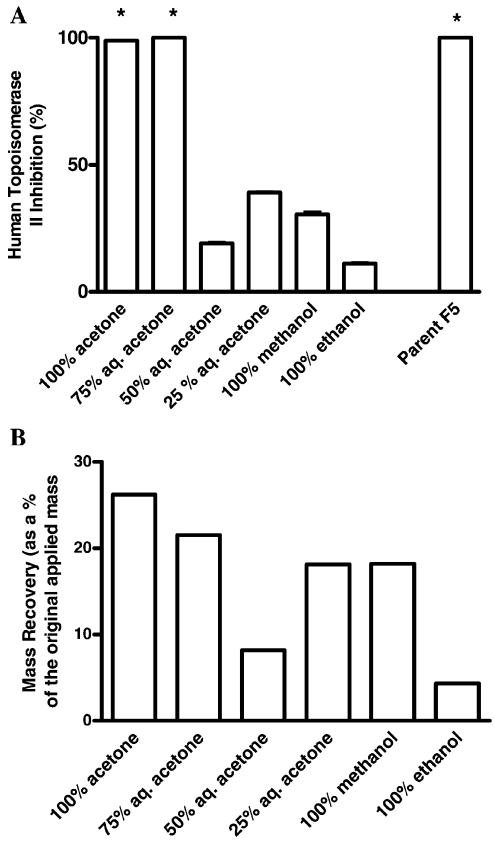

Figure 3. Topoisomerase II inhibition and mass recovery of SGVC subfractions: (**A**) human topoisomerase II inhibition from SGVC, with the subfraction eluents listed on the *x*-axis (the original highly polymerized parent fraction, F5, is listed last for comparison), *, $p < 0.01$; (**B**) mass recovery (as a percentage of the orignal applied mass) from SGVC, with the subfraction eluents on the *x*-axis.

eight-subfraction series (8 SF) is presented in **Table 1**. Highly bioactive subfractions were typically eluted late using the OC method except for two potent early subfractions from TOC (SF1 and SF3). Using the VC method with S, however, three discrete highly bioactive subfractions were eluted throughout the chromatographic process (SF2, SF4, and SF6 for SVC). Only SF2, 50% aqueous ethanol for TVC, presented significant levels of high topoisomerase inhibition. The average DP of these most bioactive subfractions ranged from 8 to 15, but the most active SOC subfraction had a DP of 43. In contrast, TOC had 50% of the total mass recovery in the 50% aqueous acetone subfraction (SF6), which retained high human topoisomerase II inhibition, but the average DP was lower and in the oligomer range (**Table 1**). Both TOC and TVC columns eluted significantly more mass in the 50% aqueous acetone subfraction (SF6) ($p < 0.05$). Distributing mass more evenly across subfractions increases the chance of compound separation, whereas localization of mass in one subfraction prevents separation. Using T as a matrix with the 8 SF series resulted in the majority of eluted mass to concentrate in SF6 (50% aqueous acetone) and SF7 (75% aqueous acetone) (**Table 1**).

In the 12 SF series, three subfractions, SOC (SF5; 25% aqueous methanol), SOC (SF10; 50% aqueous acetone), and TOC (SF10; 50% aqueous acetone), retained inhibitory values similar to the parent fraction F5 and high average DP values (**Table 2**). SVC (SF2; 50% aqueous ethanol) and TOC (SF6; 50% aqueous methanol) also retained high inhibitory values similar to the parent fraction F5, but the average DP was in the oligomer range. Using TVC also produced highly polymerized subfractions SF10 (50% aqueous acetone) and SF11 (75% aqueous acetone), but their bioactivities weres diminished (55.8 $\pm$ 1.4 and 49.1 $\pm$ 22.7, respectively). This decrease in inhibitory bioactivity could be attributed to the increased partitioning of compounds in the columns through the addition of subfractions, possibly separating phytochemicals that otherwise would act additively or synergistically to potentiate bioactivity.

Topoisomerase II is a target for several anticancer drugs through mechanisms such as the stabilization and accumulation of covalent complexes between topoisomerase II and DNA or by inhibiting the catalytic activity of the enzyme and interfering with the enzyme turnover; all of these mechanisms will result in cell death (*50*). Compounds that inhibit the catalytic activity of topoisomerase II are clinically useful in the treatment of cancer (*51*). Even though the in vitro assay may not have translational physiological meaning, these findings provide basic information for the potential chemopreventive use of active PAC mixtures, and further in vivo studies may lead to the elucidation of the role of DNA topoisomerase II inhibitors in carcinogenesis. PAC polymers are likely not absorbed due to their high molecular weight. Only their degradation products (either monomers or oligomers formed in the stomach or the aromatic acids formed in the colon) may be absorbed through the gut barrier. More data on PAC bioavailability are needed to conclusively determine their influence on human health (*52*).

The elution of PAC mixtures with higher average DP than the original parent fraction F5 (**Tables 1** and **2**) demonstrated the effective separation potential of these columns. The original parent F5 average DP was 25.5; the 75% aqueous acetone (SF7) on SOC using the 8 SF series was 43.1 $\pm$ 10.5, the 75% aqueous acetone (SF11) on SOC using 12 SF series was 43.5 $\pm$ 0.4, the 50% aqueous acetone (SF10) on TVC using the 12 SF series was 31.7 $\pm$ 4.5, the 25% aqueous methanol (SF5) on TVC using 12 SF series was 31.7 $\pm$ 0.9, and the 50% aqueous acetone (SF10) on SOC using 12 SF series was 29.2 $\pm$ 0.5. Only two of these subfractions, the 50% aqueous acetone (SF10) on SOC using 12 SF series (29.2 $\pm$ 0.5) and the 75% aqueous acetone (SF7) on SOC using the 8 SF series (43.1 $\pm$ 10.5), retained high (>85%) human topoisomerase II inhibitory values. The other separation techniques (SVC, TOC, TVC) eluted highly polymerized PACs, but the inhibitory bioactivity was lost as compared to the original parent subfraction (parent fraction F5, 100% inhibition). Differences in individual PAC constituents could not be distinguished due to the highly complex HPLC-ESI/MS chromatograms (data not shown); however, the decrease in inhibitory bioactivity was directly related to separation-induced changes.

In general, T as a matrix eluted well-resolved peaks of smaller flavonoids, including some anthocyanins such as cyanidin-3-glucoside ($[M^+]$ (*m*/*z*) 449), and less complex PACs such as a PAC dimer ($[M^+]$ (*m*/*z*) 579) and small amounts of heptamers ($[M^{+2}]$ (*m*/*z*) 1010) (**Figure 5A**). However, the high molecular weight PACs (SF4, 100% methanol), as seen in **Figure 5B**, still eluted together in an intermingled mass. Simpler PACs, such as dimers (2), trimers (3), and tetramers (4), and more complex PACs were present in the HPLC-ESI/MS chromatograms, indicating that this subfraction did not further separate PACs. The average DP for the 100% ethanol subfraction (for 8 SF) on TOC was 6.3 $\pm$ 2.4. The TOC had the higher average DP value for 25% aqueous ETOH, but upon closer examination of the HPLC-ESI/MS (**Figure 5A**) only dimers (2) and heptamers (7) were present. This result concurs with several studies in which T was used to effectively separate only dimers, trimers, and lower molecular weight oligomers of PACs (*53*–*55*).

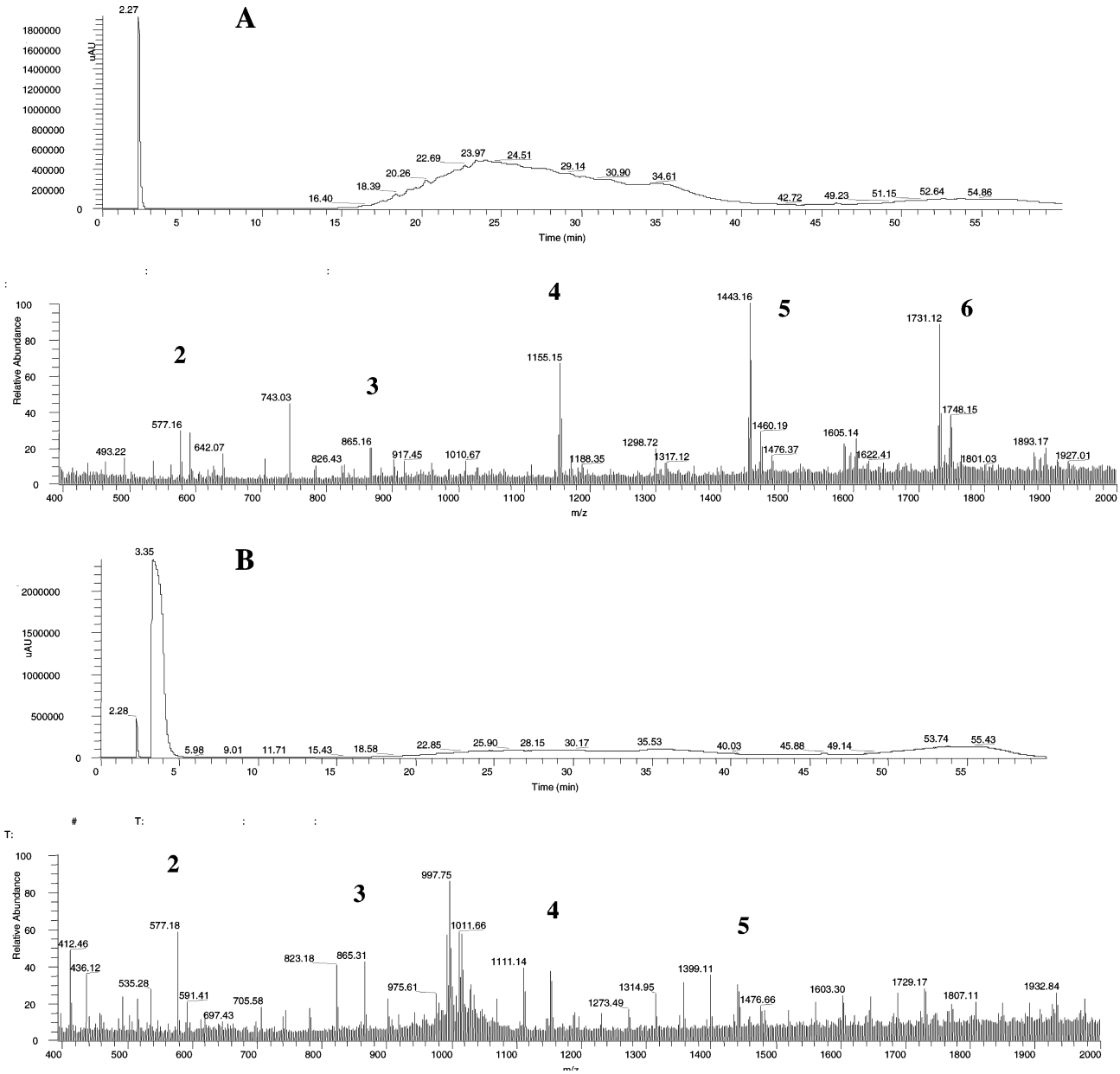

Figure 4. HPLC-ESI/MS chromatogram of SGVC subfractions: (**A**) 100% acetone subfraction from SGVC fractionation (numbers in bold correspond to the average DP); (**B**) 50% aqueous acetone subfraction from SGVC fractionation (numbers correspond to average DP).

T can be used effectively to remove sugars from fruit extracts (*41*) while also fractionating the complex fruit mixture into similar phenolic structural categories (i.e., smaller flavonoids and complex PACs). This continuous elution of PACs has been noted (*18, 30*) and demonstrates the highly complex nature of the PACs as well as the challenges that arise in the fractionation of polymerized PACs. Consequently, when these results are confirmed with mass recovery, T proved to be an inadequate matrix for separation of highly complex PACs, although it facilitated separation of lower molecular weight polyphenolics.

In contrast, S has commonly been used to fractionate PACs with success (*15, 26, 33, 34*), and the S columns in this study proved to be more useful in polymeric PAC distribution among all subfractions. The average DP for the 100% ethanol subfraction from SOC (**Figure 5C**) was 4.0 ± 1.0, and the HPLC-ESI/MS showed evidence of dimers (2), trimers (3), tetramers (4), and pentamers (5). As a result, the S subfractions contained a diverse mixture of PACs, and this mixture varied from subfraction to subfraction, whereas the T subfractions contained either smaller flavonoids or highly complex mixtures of PACs.

Results of the factorial statistical analysis for mass recovery in subfractions, %percent inhibition of topoisomerase II, and degree of polymerization are presented in **Table 3**. In the 2^3 factorial interaction comparison for mass recovery, the specific solvent mixture (subfraction) was the most influential to mass recovery for both the 8 SF ($p < 0.0001$) and 12 SF ($p < 0.0001$) columns, indicating that the solvents used for elution will determine the resulting subfraction composition. Matrix choice, on the other hand, had no significant effect (8 SF, $p = 0.0546$; 12 SF, $p = 0.963$). Similarly, there was not an effect of the method of separation, indicating that the selection of vacuum or open column did not significantly influence mass recovery (8 SF, $p = 0.463$; 12 SF, $p = 0.702$). Consequently, the interaction between matrix and separation method was not significant. Therefore, when the objective is to obtain the greatest mass recovery, the selection of the proper solvent mixture is

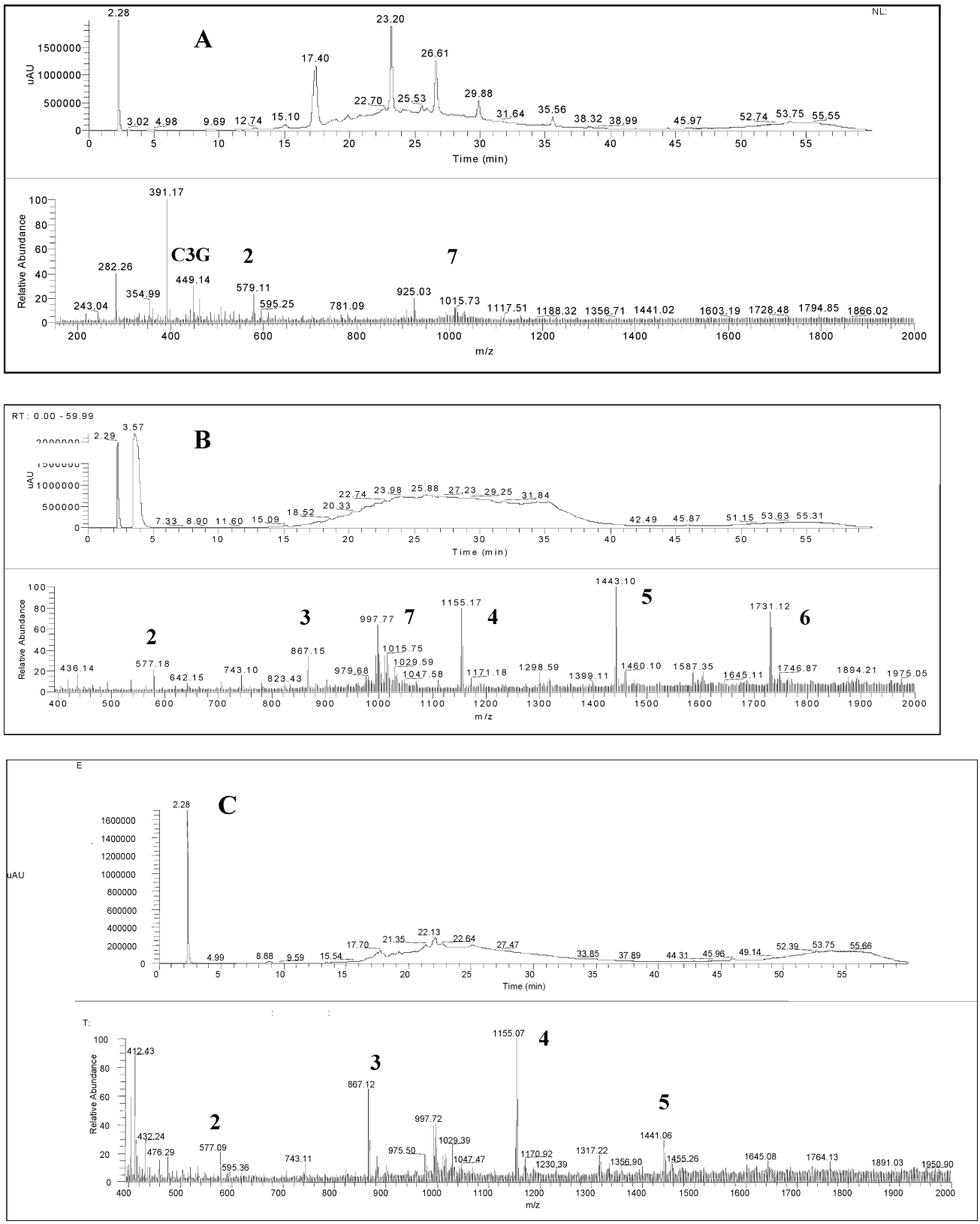

Figure 5. HPLC-ESI/MS chromatograms of T and S subfractions: (**A**) subfraction 3 eluted with 100% ethanol from the TOC column; (**B**) subfraction 4 eluted with 100% methanol from the TOC column; (**C**) subfraction 3 eluted with 100% ethanol from the SOC column. The large bold numbers indicate average degree of polymerization from dimers (2) to heptamers (7), and C3G indicates cyanidin-3-glucoside.

the most critical variable. The combination of matrix and subfraction also significantly affected mass recovery for the 8 SF series ($p < 0.0001$), but not for the 12 SF series, demonstrating the importance of solvent and matrix interaction when compounds with fewer eluting subfractions are chromatographically separated. Our results clearly indicate that the greater mass recovery was obtained by using 50% aqueous acetone solvent mixtures for both 8 SF and 12 SF columns (**Tables 1** and **2**).

Bioactivity, as determined by inhibition of topoisomerase II activity, was also most influenced by the specific solvent

Table 3. Factorial Interaction Comparisons (Matrix, Separation Method, and Subfractionation Series) for Percent Mass Recovery, Percent Topoisomerase II Inhibition, and Degree of Polymerization[a]

	% mass recovery		% inhibition		DP	
source	8 SF	12 SF	8 SF	12 SF	8 SF	12 SF
matrix	0.0546	0.9633	0.8400	0.0277	0.1687	0.0686
separation method	0.4632	0.7028	0.0562	0.8212	<0.0001	0.2028
subfraction	<0.0001	<0.0001	0.0007	<0.0001	<0.0001	<0.0001
matrix × separation	0.9723	0.3090	0.0008	0.2568	0.1521	<0.0001
matrix × subfraction	<0.0001	0.7749	0.0042	<0.0001	<0.0001	0.0349
separation × subfraction	0.1528	0.4067	<0.0001	<0.0001	0.0002	0.0299
matrix × separation × subfraction	0.0013	0.0104	0.0045	<0.0001	<0.0001	<0.0001

[a] Numbers in table are the probability p values. Matrix, Toyopearl (T) and Sephadex (S); separation method, vacuum chromatography (VC) and open column (OC); subfraction indicates the specific subfraction solvent mixtures used with the columns; SF, subfraction series; DP, degree of polymerization.

mixtures used (8 SF, $p < 0.0007$; 12 SF, $p < 0.0001$), and it was independent of the method of separation for both the 8 SF ($p = 0.056$) or 12 SF ($p = 0.821$) columns (**Table 3**). The matrix had no significant effect on the 8 SF column. However, when the number of subfractions was increased to 12 SF, the matrix became important in determining bioactivity ($p = 0.029$) as indicated by the significant matrix × subfraction interaction for both 8 SF ($p = 0.0042$) and 12 SF ($p < 0.0001$) columns. These results suggest that when subfractions in a solvent series are increased, the specific matrix must be considered due to its influential role in the separation. At any number of subfractions (8 SF or 12 SF), the method of separation by itself (open or vacuum column) seems to be irrelevant to maintenance of bioactive capacity, in terms of topoisomerase II inhibition; however, other mechanisms of bioactivity may differ in their response to separation methods. Some of the most active fractions were eluted with 50% aqueous acetone, 75% aqueous acetone, and 100% acetone.

The average DP was heavily influenced by the method of separation (OC or VC) ($p < 0.0001$) and the solvent mixtures ($p < 0.0001$) for the 8 SF column series (**Table 3**). However, when the number of subfractions was increased to 12 SF, only the influence of the solvent mixture remained significant ($p < 0.0001$). On the other hand, matrix selection had no effect on average DP for either the 8 SF column ($p = 0.168$) or the 12 SF column ($p = 0.0686$). This indicates that the separation method, up to 8 SF, is the most determining factor of the average DP. The greatest DP values were obtained with SOC 75% aqueous acetone for 12 SF. The S 12 SF columns eluted mixtures with significantly higher average DP ($p < 0.05$) as compared to the T 12 SF columns. Matrix × separation method interaction was significant only for 12 SF with regard to the average DP ($p < 0.0001$), indicative of increasingly important interaction between matrix and separation method for eluting highly polymerized compounds when the number of subfractions is increased (**Table 3**). The remaining interactions such as matrix × subfraction, separation method × subfraction, and matrix × separation method × subfraction influenced average DP independent of increasing number of subfractions from 8 to 12. The elution of increased average DP compounds with S columns using the 12 SF series suggested that this was the best technique for separating highly polymerized PACs.

In conclusion, using Sephadex (S) as a matrix effectively eluted subfractions that best retained human topoisomerase II inhibitory activity and were highly polymerized. Sephadex, using 12 SF, eluted mixtures with significantly higher average DP ($p < 0.05$) as compared to the Toyopearl 12 SF columns. The Toyopearl matrix successfully eluted low molecular weight flavonoids and some oligomers, suggesting T was not the best matrix for complex PAC separation. Open column or vacuum colum chromatography as an individual factor had no effect on bioactivity, mass recovery, or average DP. The Sephadex vacuum column chromatography (SVC), Toyopearl open column chromatography (TOC), and Toyopearl vacuum chromatography (TVC) separation techniques eluted highly polymerized PACs, but the inhibitory bioactivity was lost from the original parent F5 and the TVC mass recovery was limited to fewer subfractions. The Sephadex open column (SOC) eluted highly polymerized subfractions that retained topoisomerase II inhibition similar to the parent fraction. The best subfractionation method in terms of retention of bioactivity, mass recovery, and polymeric PAC compound separation was Sephadex open column (SOC) with 12 SF.

ABBREVIATIONS USED

PAC, proanthocyanidin; HPLC, high-performance liquid chromatography; HPLC-ESI/MS, high-performance liquid chromatography−electrospray ionization−mass spectrometry; SDS, sodium dodecyl sulfate; DP, degree of polymerization; pYRG, DNA with a highly susceptible topoisomerase II cleavage site; OC, open column liquid chromatography; VC, vacuum liquid chromatography; T, Toyopearl HW-40; S, Sephadex LH-20; SG, silica gel type G; SGVC, silica gel vacuum liquid chromatography; SOC, Sephadex open column liquid chromatography; SVC, Sephadex vacuum liquid chromatography; TOC, Toyopearl open column liquid chromatography; TVC, Toyopearl vacuum liquid chromatography; SF, subfraction.

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