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Facile Purification of Milligram to Gram Quantities of Condensed Tannins According to Mean Degree of Polymerization and Flavan-3-ol Subunit Composition

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Supporting Information

ABSTRACT: Unambiguous investigation of condensed tannin (CT) structure−activity relationships in biological systems requires well-characterized, high-purity CTs. Sephadex LH-20 and Toyopearl HW-50F resins were compared for separating CTs from acetone/water extracts, and column fractions analyzed for flavan-3-ol subunits, mean degree of polymerization (mDP), and purity. Toyopearl HW-50F generated fractions with higher mDP values and better separation of procyanidins (PC) and prodelphinidins (PD) but required a prepurification step, needed more time for large scale purifications, and gave poorer recoveries. Therefore, two gradient elution schemes were developed for CT purification on Sephadex LH-20 providing 146−2000 mg/fraction. Fractions were analyzed by thiolysis and NMR spectroscopy. In general, PC/PD ratios decreased and mDP increased during elution. ^{1}H NMR spectroscopy served as a rapid screening tool to qualitatively determine CT enrichment and carbohydrate impurities present, guiding fractionation toward repurification or ^{1}H−^{13}C HSQC NMR spectroscopy and thiolysis. These protocols provide options for preparing highly pure CT samples.

KEYWORDS: *proanthocyanidins, Sephadex LH-20, Toyopearl HW-50F, nuclear magnetic resonance, thiolysis, batch chromatography*

INTRODUCTION

As a class of plant polyphenols, condensed tannins (CTs; *syn.* proanthocyanidins) (Figure 1) have received considerable attention due to their human health benefits,[1] their ability to improve the economic impact of livestock production in terms of farming efficiency and animal health, and can thus contribute to environmental and sustainability aspects of agriculture.[2−5] Due to this potentially significant impact of CTs on food security, studies of these effects require accurate information on CT content and composition of plants or isolated CTs to elicit optimal ruminant productivity and minimize environmental impact. Through this work, potential forage targets may be identified to optimize their content and composition.

It is generally accepted that the beneficial effects CTs have on ruminant productivity stem from their interactions with proteins, since CTs in forages can modulate protein utilization in ruminants, impact the forage ensiling process, and exert positive downstream environmental effects. Interest is growing in using CTs isolated from plants for in vitro studies that evaluate protein-binding, fermentation, anthelmintic, antimicrobial, anti-inflammatory, and immunological effects.[6−10] In addition, some nutritional and even feeding trials with small animals could also be performed if gram quantities of CTs were available.[1]

The understanding of the modulations during rumen digestion and the ensiling process has made significant progress in the past, but we still lack a basic understanding of how CT−protein interactions translate into improved livestock production, which is partly due to the difficulty of obtaining highly pure and well-characterized CTs. Several procedures have been developed for securing purified CTs, and most purifications commence with extraction from the plant material with an aqueous solvent mixture of common protic solvents (methanol or ethanol) or acetone/water mixtures. These initial extracts are often purified using either Sephadex LH-20[11−13] or Toyopearl HW-50F columns,[14−16] although some studies also used sequential exposure to a combination of Sephadex LH-20, Toyopearl TSK HW-50F, Amberlite XAD7HP, or poly(styrene/divinylbenzene) (PS/DVB) polymer resins.[14,16−19] Water or methanol/water mixtures are often used as a first eluent to remove carbohydrates and low molecular weight phenolics from the resins, after which the CTs are eluted with 70% acetone/water from Sephadex LH-20 columns.[20] However, depending on the plant source, this aqueous acetone fraction may still contain >50% of contaminants on a mass basis and is rarely suitable as a "CT" standard. For this reason, Grabber et al.[21] stressed the importance of establishing the actual CT content in the standards used for colorimetric assays via either separate analysis such as thiolysis[22] or NMR[23] as CT content will otherwise be overestimated.

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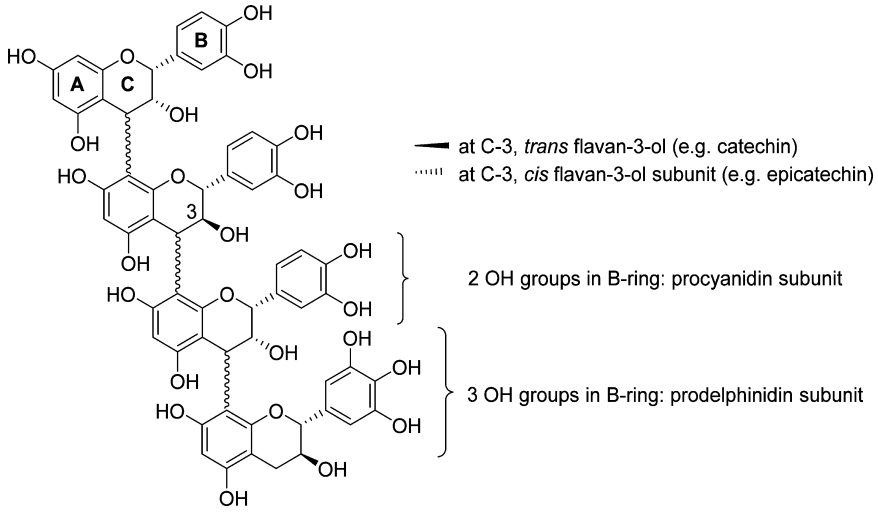

Figure 1. Example of a condensed tannin (*syn.* proanthocyanidin) structure.

Indeed, a variety of materials have been suggested as reference standards for colorimetric assays. These include CTs purified from the commercially available quebracho extracts or apples[14] or commercially available delphinidin and cyanidin chlorides. However, it has been shown that all of these polyphenols vary greatly in their UV−vis absorbance yields.[14,24] Moreover, most plant materials typically contain mixtures of procyanidins (PC) and prodelphinidins (PD), which also generate different absorbance yields, and readings depend on the PC/PD ratios of their CTs.[25] Therefore, it is important that CT content be measured using a purified CT sample of known and high purity from the plant material undergoing analysis as the reference standard.[21,24−27] However, it is a major undertaking to purify CTs and determine their purity as the removal of impurities can be challenging and time-consuming. Proteins, carbohydrates, and lipids are strong CT-binders.[14,17,28] The novice researcher will find an array of different methods but little guidance on the advantages and disadvantages of various chromatographic resins, column conditions, or sample sizes that can be loaded to achieve high CT purities and yields. Moreover, few publications report the CT yields that can be expected from a particular purification setup or from a specific plant material.[13]

In this study, we first compared and contrasted two of the most widely used resins for the purification of CTs, Sephadex LH-20 and Toyopearl HW-50F, with the overall goal of selecting one resin for purification of gram quantities of CT samples. After selection of the preferred resin, we investigated different solvent elution schemes to purify CTs from plant sources with a wide range of composition[29] to enable structure−activity relationship studies.[6−10] The composition and purities in these fractions were analyzed through a combination of NMR spectroscopy and thiolytic degradation. This communication compiles the results of several studies, developed in two separate laboratories, in an effort to provide researchers with a choice of easy-to-use CT purification methods.

MATERIALS AND METHODS

Plant Samples. The tannin composition, collection, and processing of plant materials has been previously described.[9,21,22,29−31] These include aerial parts of the sainfoin (*Onobrychis viciifolia* Scop.; 'Cotswold Common')[22] and NIAB sainfoin accessions (1123, 1127, 1165r1),[30] white clover (*Trifolium repens* L.) flowers,[9] aerial parts of birdsfoot trefoil (*Lotus corniculatus* L.), big trefoil (*L. pedunculatus* Cav.), and crown vetch (*Securigera varia* (L.) Lassen).[9,21] *Tilia inflorescentia* flowers (a mixture of *Tilia cordata* P. Mill., *T. platyphyllos* Scop., and *T. vulgaris* Hayne) were from Flos (Mokrsko, Poland),[31] and two batches of pelleted leaf meal of sericea lespedeza (*Lespedeza cuneata* (Dum. Cours.) G. Don) were from Sims Brothers Seed Company (Union Springs, AL). Weeping willow (*Salix babylonica* L.) catkins were collected in Emmer Green (Reading, U.K.). Goat willow (*S. caprea* L.) leaves were collected from the University of Wisconsin Arboretum (Madison, WI). High tannin "Mediterranean" birdsfoot trefoil, aerial parts, was grown from combined seed sown from high tannin accessions (PI 235525, France; PI 246720, Spain; PI 249753, Greece; PI 260268, Ethiopia; PI 273937, Ethiopia; PI 273938, Ethiopia) that were provided by the National Genetic Resources Program as described.[32] Black currant leaves (*Ribes nigrum* L.) were collected from Carandale Farm LLC (Oregon, WI).

Materials. Acetone (AR grade) and methanol (HPLC grade) were purchased from ThermoFisher Scientific Ltd. (Loughborough, U.K.) or Sigma (St. Louis, MO), deionized water was purified in an Option 3 water purifier (ELGA Process Water, Marlow, U.K.) or ultrapure water (Milli-Q Plus system, Millipore, Watford, UK and Billerica, MA), and dichloromethane was obtained from Sigma (St. Louis, MO). Sephadex LH-20 was obtained from GE Healthcare (Little Chalfont, U.K. and Marlborough, MA), and Toyopearl HW-50F from Hichrom Ltd. (Theale, Berks., U.K.).

Condensed Tannin Analysis. CT content (purity) and composition were determined by thiolytic degradation with benzylmercaptan.[22,31] Where necessary, a combination of NMR (providing CT composition)[23] and HCl−butanol−acetone colorimetric assay (providing CT content/purity[21] relative to previously analyzed reference standard CT samples of the same plant material) were used.

General Extraction Procedure for Crude Plant Extracts. The freeze-dried and cyclone-milled (≤1 mm) (UDY Corporation, Fort Collins, CO) plant samples were extracted with acetone/water (7:3, v/v) using a magnetic stirrer for 30−40 min at room temperature, in some cases multiple times (i.e., 3 times), with solvent volume/sample weight ratios of 6 to 16. The solutions were filtered under vacuum and concentrated on a rotary evaporator (<35 °C) to remove acetone. The resulting aqueous layer was extracted two or three times and phase-separated in a separatory funnel with an equal volume of dichloromethane. The aqueous phase was concentrated on a rotary evaporator to remove any traces of dichloromethane and freeze-dried, and then these crude extracts were stored at −20 °C.

CT Purification Methods. Four different protocols were evaluated for the generation of high purity CTs at the milligram to gram scale. All purified CT samples were stored at −20 °C.

Method 1: "Standard Column" Toyopearl HW-50F Chromatography. Four sainfoin accessions (25 g; 1123, 1127, 1165r1, Cotswold Common)[33] were extracted with acetone/water (7:3, v/v; 200 mL) as

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described above, yielding the crude plant extracts (yield: 15.6%, 15.3%, 22.0%, 15.8%, respectively). Samples of the crude extracts (2 g each) were dissolved in ultrapure water (20 mL) and separated on a Toyopearl HW-50F column (230 × 30 mm). The column was rinsed first with water (3 × 100 mL; giving Toyopearl fractions **T1**−**T3**), then with methanol/water (3 × 100 mL; 1:1, v/v, yielding **T4**−**T6**), and CTs were eluted with acetone/water (7:3, v/v; 3 × 100 mL), providing fractions **TF7**−**TF9**, and finally with acetone to give fraction **TF10** (100 mL). All fractions were concentrated on a rotary evaporator and freeze-dried.

Method 2: "Standard Column" Chromatography on Toyopearl HW-50F versus Sephadex LH-20. Prepurification: The Cotswold Common sainfoin accession (25 g)[22] was extracted with acetone/water (7:3, v/v; 400 mL) as described above (yield: 24.1%). A Sephadex LH-20 column (120 × 30 mm i.d.) equilibrated with methanol/water (1:1, v/v) was conditioned with water (600 mL) just before use. The crude extract (6.0 g) was dissolved in water (40 mL) and loaded onto the column. The column was washed with water (5 L) until there was a negative reaction to carbohydrates with the phenol test.[34] Then a single, partially purified CT fraction was eluted with acetone/water (7:3, v/v; 2 L). Acetone was removed with a rotary evaporator, and the aqueous phase freeze-dried (yield: 477 mg). The extraction and purification were repeated to generate sufficient material by pooling both sets of acetone/water fractions for the subsequent column purification comparison.

Chromatography. Two aliquots of the partially purified, pooled CT mixture (379.5 mg each) were then dissolved in water (10 mL) and loaded onto either a Toyopearl HW-50F column or a Sephadex LH-20 column of identical sizes (185 mm length × 30 mm i.d.). Columns were rinsed with water (100 mL), and fractions were eluted with water (3 × 100 mL; giving Toyopearl fractions **T1**−**T3** or Sephadex fractions **S1**−**S3**), methanol/water (3 × 100 mL; 1:1, v/v, yielding **T4**−**T6** or **S4**−**S6** fractions), acetone/water (100, 50, 50, 100 mL, 7:3, v/v, yielding **T7**, **T8a**, **T8b**, **T9** or **S7**, **S8a**, **S8b**, **S9** fractions), and acetone (100 mL; **T10** or **S10** fractions).

For Methods 3 and 4, the following fraction designations are used: For pre-elution washes, **F0**; for the 3:7, 1:1, and 7:3 (or 4:1) acetone/water fractionation, **F1**, **F2**, and **F3**, respectively.

Method 3: "Wide Column" Sephadex LH-20 Chromatography. A slurry of Sephadex LH-20 (50 g) in water was poured into a glass column (400 × 65 mm i.d.) equipped with a sintered-glass frit, resulting in a resin bed of 70 × 65 mm i.d. Plant samples (50 g) were extracted with acetone/water (7:3, v/v; 500 mL) by stirring at room temperature for 40 min, filtering, and extracting in a separatory funnel with dichloromethane (250 mL). The upper, aqueous phase was concentrated on a rotary evaporator to remove trace volatile organic solvents and centrifuged to remove insoluble material. The aqueous phase containing ca. 10 g of crude plant extract was stored in a freezer overnight and thawed the next day, diluted with deionized water (1 L), and filtered, and then water (1 L) was added. This solution was transferred to a separatory funnel above the glass column. The CT solution was allowed to flow, slowly initially, along the inside of the column without disturbing the resin bed, and then rapidly once sufficient liquid was above the column, onto the Sephadex LH-20 resin (ca. 40 mL/min). Then 2 L of deionized water was added to the funnel and the column rinsed until the eluent was clear (flow rate: 40−50 mL/min). Acetone/water (3:7, v/v, 1 L) was added to the separatory funnel, and the first 200 mL of eluent discarded. The CTs were collected at 15 mL/min with the next 500 mL giving **F1** (vanillin/HCl was used to test for CTs in eluent).[26] **F2** was similarly eluted with acetone/water (1:1, v/v, 1 L), where the first 200 mL of eluent was discarded and CTs were collected at 25 mL/min with the next 300 mL. The column was reconditioned with water (2 L) at 25 mL/min. Columns could be reused approximately 10 times without losing separation efficiency as monitored by thiolysis of each fraction.

Method 4: "Batch Chromatography" with Sephadex LH-20. General Purification Protocol. The crude CT extract (4.5−19 g) obtained from the acetone/water (7:3 v/v) extraction was dissolved in 1:1 methanol/water (30 mL of solvent per g of extract), and the resulting mixture was briefly stirred to dissolve the material. Sephadex LH-20 (10 g per g of extract) was added in small portions, while stirring with a spatula, until the mixture reached the consistency of wet sand. The extract-laden resin was transferred to a coarse sintered-glass Buchner filter funnel (600−1500 mL) equipped with a filter paper. The resin was stirred with a mixture of methanol/water (1:1, v/v; 5 mL/g Sephadex LH-20). The suspension was allowed to settle for 10 min and then vacuum filtered. This process of resin washing was repeated two additional times using methanol/water, and the filtrates were combined to provide the pre-elution fraction (**F0**). The resin was then washed in the same manner consecutively (3 times each with 5 mL/g Sephadex LH-20 per washing) with solutions of acetone/water of decreasing polarity (3:7; 1:1; 7:3) to give fractions **F1**−**F3**, respectively. Fractions were concentrated on a rotary evaporator (<35 °C) to remove the volatile organic solvent, and the resulting aqueous phases were freeze-dried. The purity of the fractions was qualitatively assessed by NMR spectroscopy,[23] and this analysis led to the selection of the higher CT content fractions for thiolytic degradation analysis or the lower CT content fractions for a second purification, prefixed with the label **P2**, leading to production of fractions **P2F0**, **P2F1**, **P2F2**, and **P2F3**.

Specific Example. Crude extract (12 g) from black currant leaves (50 g) was dissolved in 1:1 methanol/water (300 mL), and the resulting mixture was briefly stirred to dissolve the material. Sephadex LH-20 (120 g) was added in small portions, stirring with a spatula. After transferring the CT-adsorbed resin to a 1500 mL coarse sintered-glass Buchner filter funnel containing a filter paper, the resin was processed as described above in the general purification protocol using methanol/water (1:1 v/v; 3 × 650 mL) and acetone/water (3:7; 1:1; and 7:3 v/v; 3 × 650 mL each) to afford, after freeze-drying, **F1** (336 mg), **F2** (2000 mg), and **F3** (692 mg, 98% CT). Repurification of the combined **F2** and **F3** fractions produced **P2F0** (532 mg), **P2F1** (186 mg), **P2F2** (913 mg, >99% CT), and **P2F3** (440 mg, >99% CT). A parallel, smaller scale purification of crude black currant leaf extract (4.5 g), which did not require a second purification, produced **F0** (1670 mg), **F1** (212 mg), **F2** (508 mg, >99% CT), and **F3** (643 mg, 96% CT).

NMR Spectroscopy. ^{1}H NMR, ^{13}C NMR, and ^{1}H−^{13}C HSQC NMR spectra were recorded on an Avance 360 (^{1}H 360.13 MHz, ^{13}C 90.55 MHz) instrument equipped with XWINNMR software (Bruker Corporation, Billerica, MA). Spectra were recorded in D_2O/acetone-d_6 (4:1) and were referenced to the residual signals of acetone-d_6 (2.04 ppm for ^{1}H and 29.8 ppm for ^{13}C spectra). For ^{1}H−^{13}C HSQC experiments, spectra were obtained using 128 scans (acquisition time 18 h 30 min each) with the standard Bruker pulse program (invietgpsi) with the following parameters: Acquisition: TD 1584 (F2), 768 (F1); SW 11.0 ppm (F2), 160 ppm (F1); O1 1800.65 Hz; O2 7244.38 Hz; D1 = 1.00 s; CNST2 = 145. Acquisition time: F2 channel, 200 ms, F1 channel 2.65 ms. Processing: SI = 1024 (F2, F1), WDW = QSINE, LB = −0.76 Hz (F2), 0.30 Hz (F1); PH_mod = pk; Baseline correction ABSG = 5 (F2, F1), BCFW = 1.00 ppm, BC_mod = quad (F2), no (F1); Linear prediction = no (F2), LPfr (F1). Sample sizes used for these spectra ranged from 10 to 15 mg, providing NMR sample solutions with concentrations of 20−30 mg/mL.

RESULTS AND DISCUSSION

The purification of CTs poses numerous challenges. First, CTs are most effectively extracted with aqueous acetone but these extracts also contain sugars, phenolic acids, cinnamic acids, flavonoids, amino compounds, proteins, nucleic acids, and lipids.[35−39] Second, CTs occur in homo- and heterogeneous mixtures[31] that can include a wide range of molecular weights, PC/PD and *cis*/*trans* ratios, plus a mixture of A- or B-type interflavan linkages. Finally, large polymers can be particularly difficult to isolate as they tend to adsorb strongly to plant materials[40] and column packings.[41] Toyopearl HW-50F and Sephadex LH-20 are the most widely used resins for their purification,[11−17,40] but the purities of the eluted CTs are variable and are often not reported. Thus, we began our studies

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Table 1. Yields and Composition of Condensed Tannin (CT) Fractions Purified from Crude Sainfoin Extracts on a Standard Toyopearl HW-50F Column (Method 1)[a]

sainfoin accession	fraction no.	fraction yield (mg)[b]	CT content (g/100 g fraction)[cd]	mDP[cd]	PC/PD[cd]	*cis/trans*[cd]
NIAB 1123 (CPI 63763)	TF7	36 (18)	12.6 (3.0)	5.2 (0.5)	39.3/60.7 (4.4)	79.3/20.7 (6.1)
	TF8	91 (25)	54.6 (4.0)	7.7 (0.1)	24.1/75.9 (0.1)	74.8/25.2 (0.9)
	TF9	19 (2)	43.8 (1.1)	9.2 (0.1)	20.7/79.3 (0.2)	73.0/27.0 (1.8)
	TF10	7 (4)	49.4 (1.5)	22.2 (1.5)	12.5/87.5 (0.1)	75.8/24.2 (0.9)
NIAB 1127 (CPI 63767)	TF7	11 (7)	3.30 (0.01)	6.4 (1.2)	100.0/0.0 (0.0)	63.3/36.7 (7.0)
	TF8	93 (10)	50.9 (1.0)	6.6 (0.3)	28.3/71.7 (1.6)	73.4/26.6 (1.6)
	TF9	25 (6)	43.4 (1.1)	25.5 (2.6)	11.0/89.0 (0.1)	77.6/22.4 (0.4)
	TF10	7 (0)	59.2 (3.6)	95.0 (8.8)	11.5/88.5 (0.1)	81.6/18.4 (0.4)
NIAB 1165r1 (Rees "A")	TF7	39 (20)	3.64 (0.55)	1.9 (0.2)	68.9/31.1 (0.3)	60.4/39.6 (0.1)
	TF8	221 (52)	60.1 (9.8)	8.6 (0.5)	39.6/60.4 (0.3)	76.9/23.1 (0.5)
	TF9	54 (30)	56.0 (0.7)	18.2 (0.1)	19.1/80.9 (0.3)	78.0/22.0 (3.7)
	TF10	11 (8)	46.0 (1.0)	22.7 (0.2)	17.5/82.5 (0.3)	77.0/23.0 (1.5)
Cotswold Common	TF7	53 (13)	37.0 (2.4)	4.5 (0.1)	55.3/44.7 (0.8)	78.6/21.4 (1.3)
	TF8	147 (20)	54.3 (0.3)	9.5 (0.5)	40.4/59.6 (1.6)	76.0/24.0 (1.0)
	TF9	35 (5)	43.6 (3.2)	27.6 (8.5)	22.4/77.6 (0.9)	77.7/22.3 (2.4)
	TF10	21 (8)	44.6 (0.1)	60.9 (8.7)	22.9/77.1 (0.3)	79.6/20.4 (1.1)

[a]Abbreviations used: % PD: molar percentage of procyanidins/prodelphinidins; *cis/trans*: molar percentages of *cis/trans* flavan-3-ols. [b]n = 2 (chromatographic runs). [c]n = 3 (analytical replicates by thiolysis). [d]Fractions were analyzed by thiolysis with benzylmercaptan (standard deviations in parentheses).

Table 2. Yield and Composition of Condensed Tannin (CT) Fractions Purified from a Crude Sainfoin ('Cotswold Common' var.) Extract on Standard Columns of Either Sephadex LH-20 or Toyopearl HW-50F (Method 2)[a]

columns and eluents	fraction	fraction yield (mg)	CT content (g/100 g fraction)[c]	mDP[c]	PC/PD[c]	*cis/trans*[c]
Sephadex LH-20						
water	S1	40.4	2.8 (0.1)	12.4 (0.3)	77.5/22.5 (1.5)	96.7/3.3 (0.4)
	S2	4.8	1.8 (0.1)	4.6 (0.2)	80.3/19.7 (1.9)	94.1/5.9 (1.3)
	S3	2.6	1.2 (0.2)	4.6 (0.3)	81.6/18.4 (3.2)	95.5/4.5 (0.5)
methanol/water (1:1, v/v)	S4	4.8	2.0 (0.1)	5.7 (0.3)	79.9/20.1 (0.1)	95.6/4.4 (0.8)
	S5	13.6	4.5 (0.4)	6.1 (0.4)	74.1/25.9 (3.4)	94.7/5.3 (0.1)
	S6	10.9	2.2 (0.3)	7.4 (1.1)	78.8/21.2 (3.1)	94.3/5.7 (1.4)
acetone/water (7:3, v/v)	S7	118.7	84.5 (7.7)	10.3 (0.1)	46.5/53.5 (0.2)	85.5/14.5 (0.3)
	S8a	105.5	101.8 (2.0)	7.4 (0.1)	49.8/50.2 (0.2)	79.4/20.6 (0.1)
	S8b	32.6	88.1 (1.1)	8.0 (0.1)	42.3/57.7 (0.4)	79.4/20.6 (0.1)
	S9	18.0	80.4 (0.9)	10.0 (0.5)	35.4/64.6 (0.8)	81.5/18.5 (0.2)
acetone	S10	3.2	68.1 (5.9)	14.9 (0.7)	33.0/67.0 (0.4)	82.8/17.2 (0.1)
recovery		355.1				
Toyopearl HW-50F						
water	T1	28.0	15.2 (0.3)	38.2 (2.7)	31.5/68.5 (0.1)	91.5/8.5 (0.3)
	T2	4.5	0.4 (0.1)	-[b]	100.0/0.0 (0.0)	100.0/0.0 (0.0)
	T3	1.5	nd			
methanol/water (1:1, v/v)	T4	2.5	nd			
	T5	4.9	4.2 (0.4)	4.9 (0.4)	89.1/10.9 (4.5)	85.4/14.6 (2.0)
	T6	6.1	1.5 (0.1)	9.5 (2.1)	100.0/0.0 (0.0)	93.5/6.5 (0.1)
acetone/water (7:3, v/v)	T7	80.0	85.7 (4.0)	8.0 (0.1)	56.5/43.5 (0.3)	84.5/15.5 (0.1)
	T8a	83.9	122.2 (17.1)	9.4 (0.4)	40.4/59.6 (0.1)	81.3/18.7 (0.2)
	T8b	16.2	90.5 (8.3)	23.4 (1.0)	15.2/84.8 (0.1)	85.6/14.4 (0.1)
	T9	37.5	73.8 (13.7)	53.7 (3.7)	10.8/89.2 (0.2)	88.1/11.9 (0.5)
acetone	T10	3.0	73.1 (0.6)	45.8 (1.4)	14.0/86.0 (0.1)	88.5/11.5 (0.1)
recovery		268.1				

[a]Abbreviations used: mDP: mean degree of polymerization; PC/PD: molar percentages of procyanidins/prodelphinidins; *cis/trans*: molar percentages of *cis/trans* flavan-3-ols. [b]nd: mDP value could not be calculated as peaks of terminal units were too small to be detected. [c]Fractions were analyzed by thiolysis with benzylmercaptan (n = 3; SD in parentheses).

to directly compare these resins in a side-by-side purification. We first tested the hypothesis that Toyopearl HW-50F enables better separation and purification of CTs than Sephadex LH-20.

Method 1: Toyopearl HW-50F Chromatography for CT Separations. Table 1 shows the CT content and composition of fractions eluted from a Toyopearl HW-50F column using extracts from four sainfoin accessions. The 70% acetone fractions (**TF7**−**TF9**) yielded masses of 11−53, 91−221, and 19−54 mg, respectively, with pure acetone (**TF10**) eluting only 7−21 mg. The % CT content in these fractions varied considerably and increased from **TF7** (3−37% CT) to **TF8** (51−60% CT), but decreased slightly in **TF9** (43−56% CT)

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and **TF10** (45−59% CT). It can be clearly seen (Table 1) that aqueous acetone and acetone eluted CTs according to polymer size with mDP values of **TF7**−**TF10** fractions of 2−6, 7−10, 9−28, and 22−95, respectively. The latter fraction is of particular interest, as large molecular weight CTs are difficult to purify and are often obtained only in milligram quantities. Sainfoin CTs consist of complex mixtures[33] and Toyopearl HW-50F achieved some separation into CTs rich in PC and PD subunits. Molar percentages of PD increased from **TF7** (0−60%), **TF8** (60−76%), **TF9** (78−89%), to **TF10** (77−89%). The most likely explanation for this separation stems from our observations that many plants tend to synthesize PC as smaller polymers and PD as larger polymers, although exceptions exist.[42] An alternative explanation for the decreasing PC/PD ratios and increasing mDP with higher acetone content in the eluent could be due to hydrogen bonding. Smaller CTs containing a preponderance of PC subunits would likely desorb faster from the hydrophilic surface of Toyopearl HW-50F whereas CTs with higher PD content/molecular weight require the disruption of a larger number of hydrogen bonds with the resin before they can elute. Molar percentages of *trans*-flavan-3-ols varied most in the first fraction, i.e., from 21 to 40% (**TF7**), and much less in subsequent fractions (23−27% in **TF8**, 22−27% in **TF9**, and 18−24% in **TF10**). It can be concluded that fractionation on a Toyopearl HW-50F column achieved good CT separations based on molecular weights and PC/PD ratios, but that other approaches were needed to increase the quantity and purity of the isolated CTs. Next, we evaluated standard length columns packed with Sephadex LH-20 and Toyopearl HW-50F and stepwise solvent elutions in a side-to-side comparison, after an initial prepurification step.

Method 2: Sephadex LH-20 versus Toyopearl HW-50F Chromatography. Partially purified CTs, rather than crude acetone/water (7:3) plant extracts, were required before direct comparison of the Sephadex LH-20 and Toyopearl HW-50F columns could be conducted. Unless a prior purification of the crude plant extract was performed on Sephadex LH-20, the subsequent Toyopearl HW-50F column purification suffered from slow flow rates and poor sample recoveries. Identical quantities of this partially purified CT sample were then applied to equally sized Sephadex LH-20 and Toyopearl HW-50F columns. The mass recoveries from the Sephadex LH-20 and Toyopearl HW-50F columns were 94% and 71%, respectively, of the applied sample (Table 2). Similar percentages of the extract, based on the recovered material, were obtained from the Sephadex LH-20 and Toyopearl HW-50F columns with each of the eluents: water eluted 14% and 13%, aqueous methanol eluted 8% and 5%, aqueous acetone eluted 77% and 81%, respectively, and acetone eluted just 1% from either column (Table 2). These solvents also eluted fractions with comparable CT content from both columns. Water and aqueous methanol eluted fractions with just 1−5% CT from the Sephadex LH-20 and 0−15% CT from the Toyopearl HW-50F column. We note that the first water fraction from both columns contained an unexpected group of CTs with mDP values of 12 and 38. It is possible that these CTs were either glycosylated or may have formed water-soluble complexes with other materials and then simply been washed off the column as complexes that did not extensively interact with the resin but these were not investigated further.

The first three aqueous acetone fractions **7**, **8a**, and **8b** had the highest CT contents, i.e. 85−100%. However, acetone on its own also eluted CTs but of lower purities, 68% and 73% CT for **S10** and **T10**, respectively. Our results indicate that acetone also elutes lipids from Sephadex LH-20, which suggests that CTs, just like gallotannins, are strong phospholipid binders.[43] NMR studies from the USDA laboratory revealed that lipid materials elute from Sephadex LH-20 late in these elution schemes as evidenced by carbon−carbon double bond C−H cross peaks[44] present in the $^{1}H-^{13}C$ HSQC NMR spectra of these fractions. CTs eluted from Sephadex LH-20 with decreasing PC/PD ratios, i.e. from 78/22 to 33/67, but mDP values also increased slightly in the acetone-containing fractions. In contrast, fractions from the Toyopearl HW-50F column generated fractions with distinctly different PC/PD ratios that decreased from 100/0 to 14/86 and mDP values that increased from 5 to 46. In addition, CTs also separated according to PCs or PDs on the Toyopearl HW-50F column; for example, the **T6** and **T8a** fractions had similar mDP values (∼9.5) but very different PC/PD ratios (100/0 and 40/60).

Toyopearl HW-50F also yielded CTs with different compositions in the water and aqueous methanol fractions: the CTs in **T1** consisted of 69% PD with a high mDP of 38, but the CTs in **T2** had only epicatechin (EC) subunits and presumably a very high mDP as no terminal units could be detected. In contrast, the Sephadex LH-20 water and aqueous methanol fractions (**S1**−**S6**) had relatively similar PC/PD ratios and mDP values.

Aqueous acetone and acetone (**T7**−**T10**) eluted CTs from Toyopearl-HW-50F with mDP-values of 8−54, but the corresponding Sephadex LH-20 **S7**−**S10** fractions had mDP values of 7−15. Flavan-3-ol subunit composition of CTs from the Toyopearl **T2** and **T5**−**T10** fractions had decreasing molar percentages of EC (100−13%) and increasing epigallocatechin (EGC) percentages (10−78%). The **T2** fraction consisted of PC homopolymers that contained only EC subunits, the **T6** fraction had both catechin (C) and EC subunits, whereas the water (**T1**) and acetone (**T7**−**T10**) fractions contained all four flavan-3-ol subunits. In comparison, the CTs in the Sephadex LH-20 water and methanol/water fractions (**S1**−**S6**) had EC and EGC as extension units and EC and C as terminal units. The acetone fractions (**S7**−**S10**) contained all four flavan-3-ols as terminal and extension units.

This comparison revealed that several highly pure CT fractions could be eluted from both columns provided that the crude plant extract was first partially purified over a Sephadex LH-20 column. Toyopearl HW-50F proved to be superior to Sephadex LH-20 in separating complex CT mixtures according to their mDP and PC/PD ratios. However, separations took ca. 5 h in order to obtain between 3 and 120 mg of CTs and, therefore, alternative approaches were needed to obtain larger quantities of CTs.

Methanol/water (1:1) is often used as the first eluent to remove carbohydrates and low molecular weight phenolics, and CTs are then eluted with 70% acetone/water.[20] However, depending on the plant source, this aqueous acetone fraction may still contain considerable quantities of contaminants. On the basis of these results, we chose Sephadex LH-20 and our two laboratories worked separately with this resin to find a suitable method to deliver gram quantities of CTs of high purity.

Method 3: Wide Column Sephadex LH-20 Chromatography. Preliminary experiments showed that most contaminants in crude plant extracts could be removed by elution with water or methanol/water mixtures (1:9 or 3:7) using conventional columns (ca. 30 mm i.d.), but slow flow rates required >8 h for separations. Therefore, we experimented next with

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Table 3. Fractionations of Crude Extracts from Seven Plant Species on a Short, Wide Sephadex LH-20 Column (Method 3)[a]

plant species	fraction, F (no. of runs)	crude extract applied to column (g)	fraction yield (g)	CT content (g/100 g fraction)[b]	mDP[b]	PC/PD[b]	*cis*/*trans*[b]
birdsfoot trefoil	F1 (n = 5)	10	0.400	19.9 (2.2)	3.8 (0.5)	83.2/16.8 (0.7)	76.8/23.2 (2.2)
	F2 (n = 4)		~0.800	77.7 (5.2)	12.7 (2.0)	67.0/33.0 (2.5)	91.0/9.0 (1.0)
	F3 (n = 3)[b]			7.7 (3.6)	30.2 (3.4)	73.8/26.2 (8.4)	95.5/4.5 (1.4)
big trefoil	F1 (n = 4)	10	~0.800	53.9 (8.6)	5.4 (0.4)	35.3/64.7 (2.1)	67.3/32.7 (2.7)
	F2 (n = 4)		~1.200	104.2 (8.1)	17.8 (1.1)	24.9/75.1 (0.5)	84.4/15.6 (0.2)
sericea lespedeza	F1 (n = 1)	8.5	0.282	42.1	4.9	7.6/92.4	65.3/34.7
	F2 (n = 1)		0.321	82.6	11.3	7.7/92.3	75.2/24.8
	F3 (n = 1)[c]		0.920	69.7	25.0	5.6/94.4	80.8/19.2
Crownvetch	F1 (n = 5)	13.7	0.276 (0.093)	23.2 (11.8)	11.7 (1.5)	25.2/74.8 (4.4)	86.7/13.3 (1.4)
	F2 (n = 5)		0.174 (0.074)	63.5 (11.3)	13.6 (1.3)	26.3/73.7 (0.7)	88.1/11.9 (0.4)
	F3 (n = 1)[b]		0.004	43.0	20.3	25.7/74.3	89.3/10.7
Tilia sp	F1 (n = 9)	10	0.640 (0.113)	63.1 (3.2)	3.0 (0.2)	95.6/4.4 (1.1)	90.7/9.3 (0.3)
	F2 (n = 9)[b]		0.807 (0.113)	95.0 (2.8)	8.1 (0.4)	96.8/3.2 (1.1)	95.9/4.1 (0.3)
weeping willow	F1 (n = 2)	nd	0.146 (0.033)	32.7 (10.6)	2.9 (0.1)	74.7/25.3 (0.9)	56.4/43.6 (1.3)
	F2 (n = 2)		0.222 (0.070)	79.2 (25.7)	8.4 (0.1)	68.5/31.5 (2.1)	63.0/37.0 (7.5)
	F3 (n = 1)[b]		0.023	52.1	13.6	58.2/41.8	68.8/31.2
sainfoin (Cotswold Common var.)	F1 (n = 2)	nd	0.177 (0.075)	34.0 (4.6)	2.9 (0.2)	30.8/69.2 (3.8)	66.3/33.7 (0.6)
	F2 (n = 2)		0.193 (0.071)	104.0 (13.5)	9.5 (1.2)	35.3/64.7 (0.2)	80.4/19.6 (1.8)
	F3 (n = 1)[b]		0.035	88.8	25.4	31.2/68.8	84.3/15.7

[a]Abbreviations used: mDP: mean degree of polymerization; PC/PD: molar percentages of procyanidins/prodelphinidins; *cis*/*trans*: molar percentages of *cis*/*trans* flavan-3-ols; nd: not determined as the crude extract was rotary evaporated and the resulting aqueous phase was directly applied to the column. Note: the birdsfoot trefoil, big trefoil, and sericea lespedeza extracts were also subjected to Method 4 purification (Table 4). Condensed tannins (CT) in fractions were analyzed by thiolysis with benzylmercaptan (SD in parentheses). Fraction 1 (F1) was obtained by elution with acetone/water (3:7, v/v), fraction 2 (F2) with acetone/water (1:1, v/v), and fraction 3 (F3) with acetone/water (7:3 or 8:2, v/v). [b]Acetone/water (8:2, v/v). [c]Acetone/water (7:3, v/v).

column dimensions that are typically used in flash column chromatography[45] and found that a shorter, but wider Sephadex LH-20 column (70 × 65 mm i.d.) enabled faster elution times. This approach expedited cleanup of 8−14 g quantities of crude extracts. After applying the crude extracts in water, this column was then rinsed with copious quantities of water (ca. 2 L) and CTs were eluted with acetone/water (3:7, v/v) in **F1** and with acetone/water (1:1, v/v) in **F2** within 4 to 5 h. As an example, this fast chromatography of crude birdsfoot trefoil and big trefoil extracts yielded 400 mg and 800 mg in **F1** and 800 mg and 1200 mg in **F2**, respectively, in a single run (Table 3). Crude extracts from other plants yielded **F1** of 146−640 mg and **F2** of 174−321 mg. Elution with acetone/water (**F3;** 8:2, v/v) tended to yield just a few milligrams with most samples (4−35 mg), but the sericea lespedeza extract, having particularly high molecular weight prodelphinidins,[46] gave 92 mg. Repeat fractionations on the same column gave coefficients of variation for mass recoveries in **F1** and **F2**, respectively, for crown vetch (n = 5) of 34% and 44%; for weeping willow (n = 2) of 23% and 31%; for sainfoin of 42% and 37% and for *Tilia* sp. (n = 9) of 18% and 14% (the latter was an 8:2 acetone/water fraction).

Table 3 presents the CT contents and compositions from wide column separations of seven crude plant extracts obtained by five individual researchers. Purities of these CT samples ranged from 20 to 63% CT (**F1**), from 64 to 100% CT (**F2**), and from 8 to 89% CT (**F3**). The mDP value ranges increased during elution; for **F1** (3−12), **F2** (8−18), and **F3** (14−30). Thus, CTs can be quickly purified to a high degree directly from 10 g quantities of crude plant extracts and with moderate resolution into CT fractions of low and intermediate mDPs yielding up to 1.2 g of CTs. Use of the shorter, wider column enabled higher flow rates, minimizing on-column diffusion, thus increasing separation efficacy and recovery. This purification technique is comparable to flash chromatography that was first described by Still et al.,[45] wherein the authors reported that separation efficacy and recovery benefitted from a high eluent flow rate as it minimized on-column diffusion. An alternative explanation for how CTs may be fractionated during stepwise elution was proposed by Putman and Butler,[41] who pointed out that a sudden change in solvent composition coupled to fast flow rates may give rise to conformational change or aggregation of CTs and lead to desorption from C_{18}−HPLC column surfaces. The present and other studies[13] demonstrated that CTs with mDP values of 900−34200 Da can be separated on Sephadex LH-20 when using different solvents (Table 3), which suggests that chromatographic separation of CTs is based on both adsorption and molecular size.[47]

Method 4: Batch Chromatography on Sephadex LH-20. We also describe an alternative elution scheme using a batch chromatography approach to obtain gram quantities of highly pure CTs. This batch chromatography method uses Sephadex LH-20 and an elution scheme of (1:1) methanol/water (**F0**), followed by 3:7 (**F1**), 1:1 (**F2**), and finally 7:3 (**F3**) acetone/water mixtures. Here, the crude extracts were adsorbed onto the resin, and the resin washed successively, and in portions, with each eluent over 30 min. The wash solutions were removed using vacuum filtration. Total separation time was 2.5 h.

To evaluate this method versus Methods 2 and 3, replicate purifications (n = 2) of crude sainfoin extract (2 g each) were performed. The resulting fractions were evaluated for CT composition and purity. The sainfoin fraction (**F1−F3**) purity (20−96%) and the mDP (7−22) increased as the proportion of acetone in the eluent increased. These fractions provided similar molar % PD (86−88) as the molar % *trans*-flavan-3-ols decreased slightly (20−14). Similar trends in mDP, % PD, and

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Table 4. Condensed Tannin (CT) Composition from Thiolysis Analysis of Replicate Fractionations (n = 1−3) of Plant Materials Purified Once by Batch Sephadex LH-20 Chromatography (Method 4)[a]

	plant species	fraction, F (no. of runs)	crude extract applied to column (g)	fraction yield (g)	CT content (g/100g fraction)	mDP[c]	PC/PD	*cis/trans*
1	birdsfoot trefoil[b]	F2 (n = 2)	12, 16	0.98, 1.23	76, nd	16.1	61.8/38.2 (7)	93/7 (2)
2		F3 (n = 2)		0.37, 0.19	79 (4)	30.6	61.0/39.0 (5)	97.0/3.0 (4)
3	big trefoil	F2 (n = 2)	12, 19	1.60, 1.50	nd, 86	12.7	20.6/79.4 (4)	76.8/23.2 (4)
4		F3 (n = 2)		0.50, 0.40	96 (11)	19 (1)	16.0/84.0 (2)	81/19 (1)
5	sericea lespedeza (lot 1)	F2	9	0.51	63	nd	8.1/91.9	58.3/41.7
6		F3		0.35	nd	nd	5.8/94.2	55.3/44.7
7	sericea lespedeza (lot 2)	F2	11	0.52	71	9.3	6.5/93.5	75.4/24.6
8		F3		0.10	96	13.7	5.9/94.1	79.1/20.9
9	HT Mediterranean trefoil[b]	F2 (n = 3)	4.5, 17, 17	0.24, 1.20, 1.30	nd	nd	34.0/66.0 (5)	88.0/12.0 (4)
10		F3 (n = 3)		0.10, 0.10, 0.32	85 (2)	38.2	28.0/72.0 (8)	92.0/8.0 (2)
11	white clover flower	F2	12	1.10	75 (7)	nd	31.6/68.4	57.0/43.0
12		F3		0.18	93 (5)	nd	2.4/97.6	62.4/37.6
13	goat willow[b]	F2 (n = 2)	4.5, 9	0.83, 1.63	94 (1)	7.7(0.3)	46.3/53.7(0.6)	17.9/82.1 (0.2)
14		F3 (n = 2)		0.25, 0.49	90 (5)	12.1	45.0/55.0 (5)	20.0/80.0 (2)
15	black currant	F2 (n = 2)	4.5, 12	0.65, 2.00	87 (16)	7.7	8.0/92.0 (6)	15.9/84.1(0.9)
16		F3 (n = 2)		0.50, 0.64	97 (3)	14.9	1.5/98.5 (0.2)	20.0/80.0 (8)

[a]Abbreviations used: mDP: mean degree of polymerization; PC/PD: molar percentages of procyanidins/prodelphinidins; *cis/trans*: molar percentages of *cis/trans* flavan-3-ols. Note: Fractions 2 (F2) was eluted with acetone/water (1:1), and fraction 3 (F3) with acetone/water (7:3). Note: the birdsfoot trefoil, big trefoil, and sericea lespedeza extracts were also subjected to Method 3 purification (Table 3). [b]SD is from replicate fractionations and thiolysis with benzylmercaptan. [c]mDP from thiolysis analysis and NMR if necessary when mDP < 10, otherwise not determined.

Table 5. Condensed Tannin (CT) Composition from Thiolysis Analysis of Replicate Fractionations (n = 1−2) of Plant Materials Purified Twice (P2) by Batch Sephadex LH-20 Chromatography (Method 4)[a]

	plant species	fraction (no. of runs)	crude extract used in P1 (g)	fraction yield (g)	CT content (g/100 g fraction)	mDP	PC/PD	*cis/trans*
1	birdsfoot trefoil[b]	P2F2	12	0.30	78	15.0	64.8/35.2	91.3/8.7
2	big trefoil	P2F2	12	0.40	91	15.8	21.6/78.4	82.9/17.1
3		P2F3	12	0.20	87	17.9	20.7/79.3	83.9/16.1
4	sericea lespedeza (lot 1)	P2F2	9	0.14	87	11.0	6.9/93.1	78.3/21.7
5		P2F3	9	0. 19	89	14.4	6.4/93.6	80.6/19.4
6	HT Mediterranean trefoil[b]	P2F2 (n = 2)	17	0.35 (0.20)	98 (6)	17.0 (2.0)	33.6/66.4 (0.8)	87.0/13.0 (1.0)
7		P2F3 (n = 2)	17	0.18 (0.06)	>99	23.7 (0.7)	31.7/68.3 (0.3)	89.8/10.2 (0.2)
8	white clover flowers	P2F2	12	0.50	>99	11.5	1.1/98.9	67.9/32.1
9		P2F3	12	0.20	>99	17.7	1.0/99.0	71.2/28.8
10	black currant	P2F2	12	0.87	>99	10.4	2.8/97.2	16.7/83.3
11		P2F3	12	0.40	>99	15.3	2.7/97.3	19.8/80.2

[a]Abbreviations used: mDP: mean degree of polymerization; PC/PD: molar percentages of procyanidins/prodelphinidins; *cis/trans*: molar percentages of *cis/trans* flavan-3-ols. Results for rows 1, 4 and 5, 8 and 9, and 10 and 11 were from repurification of their respective, combined F2 and F3 fractions. Results for rows 2 and 3, and 6 and 7 were from repurification of their respective F2 fraction only. Note: Fractions P2F2 were eluted with acetone/water (1:1) and fractions P2F3 with acetone/water (7:3). [b]SD is from replicate fractionations (n = 2).

% *trans* values were also observed in varying degrees in the 7:3 acetone/water fractions eluted in the purification of sainfoin using Methods 1 and 2 (Tables 1 and 2). Most of the mass (69.5%) was recovered in **F0**, while the higher purity CT fractions (**F2** and **F3**) accounted for 10.7 and 3.8% of total mass, respectively, with a 93% recovery of material from the initial 2 g applied to the column. This method performs well in comparison with column chromatography methods in regard to both separation and recovery of CTs.

Larger scale purifications of crude extracts (4.5−19 g) from a variety of plant sources were also performed using this method and yielded several fractions with >1 g of CTs (Table 4). NMR was used to screen fraction purity (vide infra), and purer fractions were directly analyzed by thiolytic degradation. Table 4 contains the CT purity and composition results for the higher CT-containing fractions. The % CT purity ranged from 63 to 97% CT, the mDP from 7.7 to 16.1 in **F2**, and from 12.1 to 38.2 in **F3** depending on the plant material. The molar % PD averaged ∼4% higher for the **F3** wash fractions and the molar % *trans*-flavan-3-ol subunits decreased an average of ∼4%. Samples that were less pure were directly repurified in a second purification step, **P2**, using a similar elution scheme. The purity and CT composition of the repurified fractions (Table 5) ranged from 78 to >99 and mDP values from 11 to 24. When **F2** fractions, which are depleted in longer polymers, were repurified (Table 5, rows 2, 3, 6, and 7), lower mDP values for **P2F3** fractions were obtained. **F3** fractions (Table 4) contained the longest polymers (mDP 12−38) with typical purities of >80% CT.

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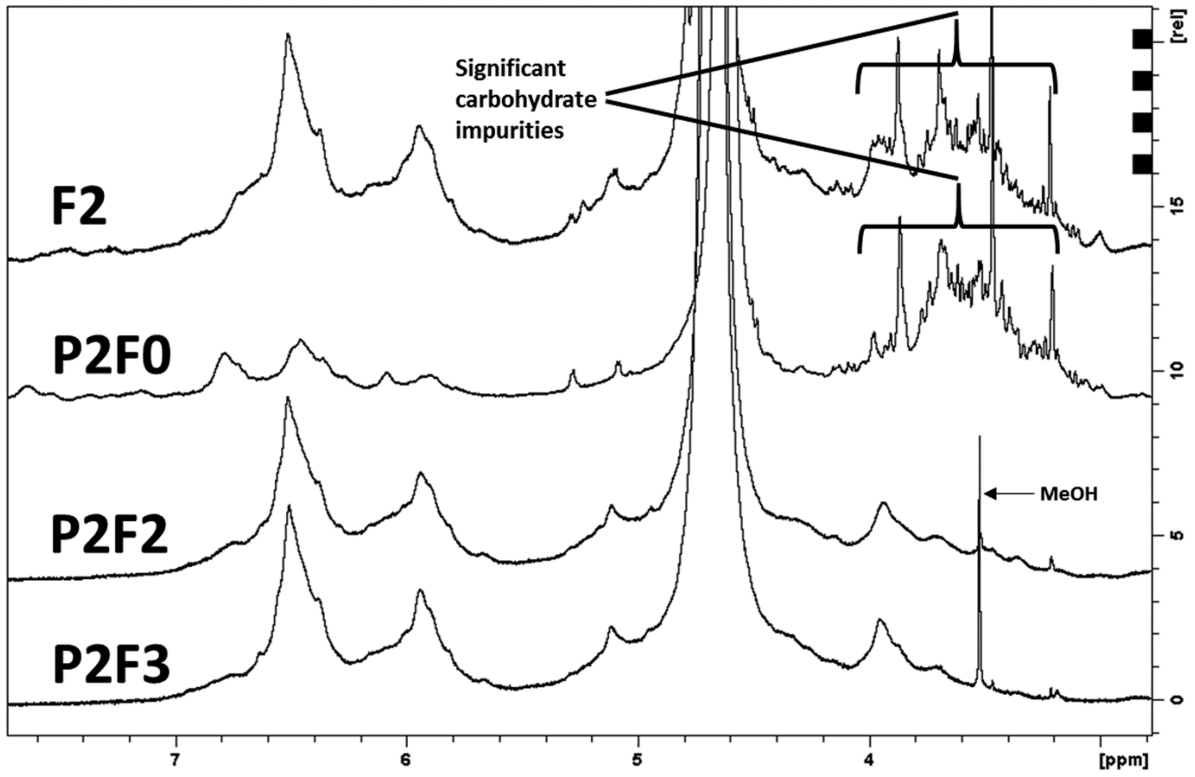

Figure 2. Stacked ^{1}H NMR (360 MHz) spectra of : (F2) material to be repurified by Method 4; (P2F0) the methanol/water (1:1) fraction; (P2F2) the acetone/water (1:1) fraction; and (P2F3) the acetone/water 7:3 fraction. NMR signals arising from carbohydrate impurities are noted for the beginning mixture (spectrum F2) and the methanol/water (1:1) fraction (spectrum P2F0).

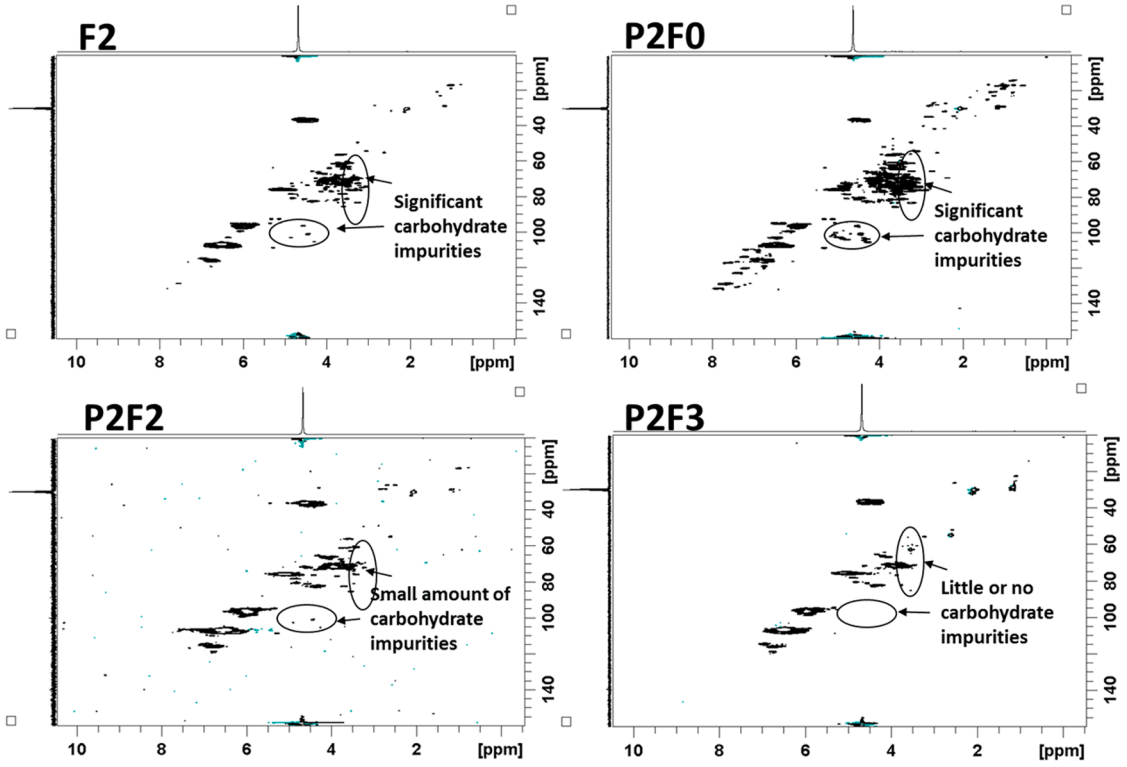

Figure 3. ^{1}H−^{13}C HSQC NMR spectra of fractions **F2**, **P2F0**, **P2F2**, and **P2F3**. Fraction **F2** is from a previous purification and provided fractions **P2F0**, **P2F2**, and **P2F3** on subsequent repurification. Fractions **P2F2** and **P2F3** were subjected to thiolysis degradation and were found to have condensed tannin purities of 72% and 95%, respectively.

This procedure works well for rapid generation of high purity CT fractions in gram quantities from a variety of plant sources. There was no need for a prepurification step and the weights from the two fractions with the highest CT contents, **F2** and

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F3, constituted between 9% and 23% of crude extract used. The CT polymers can be fractionated according to length and mDP values are similar to the wide column procedure. Some degree of fractionation of PC/PD and *cis*/*trans*-flavan-3-ols was also observed. This approach, a time efficient method for purifying crude extracts of 2−19 g, provides up to 2.0 g quantities of high purity CTs/fraction.

Qualitative Purity Assessment of CT Samples by ^{1}H NMR Spectroscopy. We found that ^{1}H NMR spectroscopy is a very rapid screening method to assess, qualitatively, the purity of these chromatography fractions. Acquisition of a ^{1}H NMR spectrum of a sample is rapid and nondestructive. A variety of NMR solvents can be used in this screening. A 4:1 mixture of D_2O/acetone-d_6 works well, as carbohydrate signals, the major type of biomolecule impurity, provide sharp, easily distinguishable signals. For example, the ^{1}H NMR spectrum of the impure sample is given in Figure 2 (spectrum **F2**), and shows signals arising from carbohydrate impurities between 3 and 4 ppm. This fraction underwent repurification to give rise to three further fractions: fraction **P2F0**, eluted with 1:1 methanol/water; fraction **P2F2**, eluted with 1:1 acetone/water; and fraction **P2F3**, eluted with 7:3 acetone/water. Examination of the ^{1}H NMR spectra of these fractions (Figure 2) shows fraction **P2F0** containing a significant amount of the carbohydrate impurity whereas fractions **P2F2** and **P2F3** show little or no sharp signals in the carbohydrate region of the spectrum (3−4 ppm). The lack of observed carbohydrate signals in the ^{1}H NMR spectrum triggers analysis of the sample forward into a second purity screen using two-dimensional ^{1}H−^{13}C HSQC NMR (2D NMR) spectroscopy.

Visual Purity Assessment and Compositional Analysis of CT Samples by ^{1}H−^{13}C HSQC NMR (2D NMR) Spectroscopy. To illustrate the power of this method, the 2D NMR spectra of fractions **F2**, **P2F0**, **P2F2**, and **P2F3** are given in Figure 3 (a **P2F1** fraction was not eluted in this particular repurification scheme). The spectrum for the starting impure fraction **F2** clearly shows a wealth of carbohydrate impurities. The 2D NMR spectrum of fraction **P2F0** shows enhanced carbohydrate impurities, relative to the CT cross-peak signals in spectrum **F2**, indicating a large portion of the carbohydrates present in impure fraction **F2** were captured in the 1:1 methanol/water fraction of the second purification. Spectra of fractions **P2F2** and **P2F3** showed relatively low levels of impurities (non-CT attributed cross-peak signals). Although this second NMR screening method requires an overnight acquisition, it provides greater detail of the presence and identity of impurities still residing in the sample, some of which (carbohydrate- and lipid-derived) avoid detection by UV absorption-based detectors commonly used for analysis in CT chromatography. Once the CT sample passes this visual purity assessment, the sample is forwarded to thiolytic degradation, where quantitation of the structural features determined by 2D NMR (PC/PD and *cis*/*trans* ratios)[23] are confirmed and additional features (mDP, terminal and extension unit identification) and purity assessment of the sample can be determined.

In summary, conventional chromatography on a Toyopearl HW-50F column separated CTs according to polymer size (mDPs of 2−95) with acetone/water (7:3) and acetone elution. Some separation occurred of CTs rich in PC and PD subunits when methanol/water (1:1) and acetone/water (7:3) were used, but this method required a prepurification step, extended elution times and yielded CTs in smaller (mg) quantities. In contrast, fast eluent flow rates from a short, wide Sephadex LH-20 column readily removed contaminating carbohydrates, phenolic and other compounds from 10 g of crude plant extracts within 4−5 h. This required extensive rinsing with water before eluting the CTs in **F1** to give yields of 150−800 mg with purities of 20−63% CT (mostly oligomeric CTs). Elution of **F2** generated 190−1200 mg quantities of 60−100% CT (mostly polymeric CTs). The results presented are based on seven plant materials, obtained by five people conducting up to 10 separate fractionations on the same column. An alternative method, utilizing a different adsorption and solvent elution scheme, also enabled rapid purification of CTs without the need for a prepurification step and provided gram quantities of highly pure CTs (80 to >99%). The wide column and batch chromatography protocols (Methods 3 and 4) are simple to run and can be performed effectively by novice researchers. The purest fractions from the batch method provided larger quantities of CTs with higher mDP values (up to 38) (Table 5) than the wide Sephadex LH-20 column (up to 30) (Table 3). The decision on whether to use Method 3 (Sephadex LH-20 is reused) or Method 4 (Sephadex LH-20 is used once) will depend on laboratory resources. If CTs of more focused mDP ranges are required, smaller quantities of such Sephadex LH-20 fractions (e.g., 2 g) could then be applied to a Toyopearl HW-50F column to give 10−200 mg quantities of CTs with mDP values ranging from 2 to 95. Given several recent reports that linked CT size to bioactivity,[1,6−8,48−50] this approach will assist researchers in isolating a wide range of high purity CTs that cover oligomers to polymers for biological studies. Presented here are details of procedures we have used to obtain highly pure CTs and, in our opinion, which are the best methods to date to characterize these CT materials. We emphasize here that, given the diversity of CT structures (flavan-3-ol subunits, mDP, and interflavan linkages), we have not found a single, generic set of conditions that will provide large-scale optimal separation of CTs from all plant materials investigated in one operation. In some cases, repeating the purification protocol was necessary to achieve the desired purity. The approaches detailed here provide a set of methods which proved successful in obtaining substantial quantities of CTs, leading to the production of a library of purified CTs with diverse structural composition to investigate their structure−activity relationships. The inclusion of mass yields of purified CT fractions obtained from dried plant materials will allow researchers to project the quantity of dried plant material needed for extraction and purification to obtain targeted amounts of CTs for their proposed studies.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.7b03489.

Specific example for preparation of a crude plant extract; detailed description of "wide column" Sephadex LH-20 chromatography; re-purification of a sericea lespedeza CT fraction for demonstration of ^{1}H NMR spectroscopy as a CT purity screening tool; applied and recovered masses from chromatography of a crude sainfoin extract on standard Sephadex LH-20 or Toyopearl HW-50F columns; flavan-3-ol subunit composition of CT from sainfoin separated on standard Sephadex LH-20 or Toyopearl HW-50F columns; flavan-3-ol subunit compositions of CT fractions after "wide column" Sephadex

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LH-20 chromatography; weight, purity and composition of CT from thiolysis degradation of replicate purified fractions of sainfoin using Sephadex LH-20 batch chromatography; flavan-3-ol subunits composition from fractionations purified once and twice using Sephadex LH-20 batch chromatography; summary of conditions suited for isolating different CTs; set-up for "wide column" Sephadex LH-20 fractionation. (PDF)

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Notes

Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

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ABBREVIATIONS USED

C, catechin; CT(s), condensed tannin(s); EC, epicatechin; EGC, epigallocatechin; mDP, mean degree of polymerization; PC, procyanidins; PD, prodelphinidins; % CT, g CT × 100/100 g of fraction

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