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In Situ Analysis and Structural Elucidation of Sainfoin (*Onobrychis viciifolia*) Tannins for High-Throughput Germplasm Screening

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A rapid thiolytic degradation and cleanup procedure was developed for analyzing tannins directly in chlorophyll-containing sainfoin (*Onobrychis viciifolia*) plants. The technique proved suitable for complex tannin mixtures containing catechin, epicatechin, gallocatechin, and epigallocatechin flavan-3-ol units. The reaction time was standardized at 60 min to minimize the loss of structural information as a result of epimerization and degradation of terminal flavan-3-ol units. The results were evaluated by separate analysis of extractable and unextractable tannins, which accounted for 63.6−113.7% of the *in situ* plant tannins. It is of note that 70% aqueous acetone extracted tannins with a lower mean degree of polymerization (mDP) than was found for tannins analyzed *in situ*. Extractable tannins had between 4 and 29 lower mDP values. The method was validated by comparing results from individual and mixed sample sets. The tannin composition of different sainfoin accessions covered a range of mDP values from 16 to 83, procyanidin/prodelphinidin (PC/PD) ratios from 19.2/80.8 to 45.6/54.4, and *cis*/*trans* ratios from 74.1/25.9 to 88.0/12.0. This is the first high-throughput screening method that is suitable for analyzing condensed tannin contents and structural composition directly in green plant tissue.

KEYWORDS: Tannins; thiolysis; *in situ* analysis; green plants; germplasm screening; *Onobrychis viciifolia*; sainfoin

INTRODUCTION

Most condensed tannins tend to occur as complex mixtures of homo- and heteropolymers in plants. They contain variable proportions of different flavan-3-ols as terminal or extension units (**Figure 1**) (*1*). Although several rapid colorimetric assays exist for tannin analysis, they are non-specific and yield no or little information on tannin structures in terms of monomeric composition or mean degree of polymerization (mDP), which may be important parameters for biological activities (*1*). Although structural information can be obtained with physicochemical techniques, tannins need to be extracted first and then purified by chromatography for characterization by nuclear magnetic resonance (NMR) (*2*), mass spectrometry (MS) (*3*−*5*), or chemical degradation (*2*, *6*−*9*) techniques. This is not only a slow process, but extraction and fractionation can also lead to considerable losses (*7*, *10*, *11*).

Tannins with molecular weights of up to 7000 Da have been detected (*5*, *12*), and at lower masses, matrix-assisted laser desorption/ionization−time-of-flight (MALDI−TOF) MS can also reveal the presence of individual compounds within complex tannin mixtures. However, MALDI−TOF MS is not a quantitative technique and does not yield diastereochemical information, which could indeed be important for their biological activities. In contrast, chemical degradation can provide quantitative and qualitative information on tannins in terms of structural features, e.g., flavan-3-ol units in extension and terminal positions and the mDP (**Figure 1**). Tannins up to ca. 55 000 Da have been described using thiolytic degradation (*6*, *13*).

Two types of degradation reactions are most widely used: thiolysis with benzyl mercaptan (BM) (*10*) and phloroglucinolysis with phloroglucinol (*7*, *9*). Although phloroglucinol is odorless, it is a less powerful nucleophile and tends to generate lower yields than BM (*7*). As a result, much higher molecular weights have been reported with BM than phloroglucinol (*6*, *9*, *13*).

Degradation reactions are usually applied to crude extracts after removal of chlorophyll or to purified tannin fractions (*7*, *13*). However, recent interest has also focused on quantifying unextractable tannins because some fruits, in fact, contain more unextractable than extractable tannins and may also possess nutritional or health effects (*14*, *15*). Indeed, thiolysis has been applied directly to the plant residue after extraction of soluble tannins to determine unextractable tannins (*7*, *8*, *16*). In addition, a few reports applied thiolysis directly to freeze-dried plant tissues, e.g., apples (*10*, *17*), coffee pulp (*18*), and plums (*19*). Apple, plum, and coffee pulp tannins are pure procyanidins (PCs), which contain catechin (C) and epicatechin (EC) as terminal units and EC as

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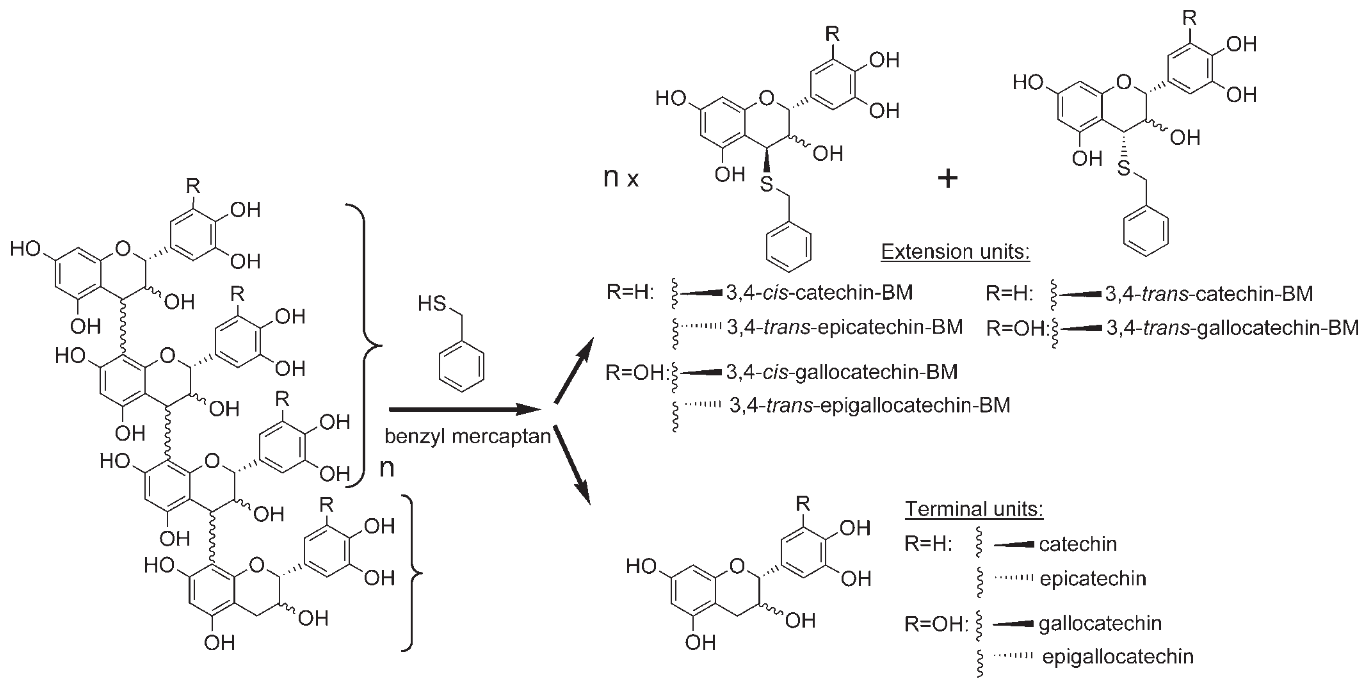

Figure 1. Thiolytic degradation of sainfoin tannins. Extension units are released as flavan-3-ol benzyl mercaptan (BM) adducts. Terminal units are released as the free flavan-3-ols. Note that catechin and gallocatechin extension units are derivatized to the 3,4-*cis* and 3,4-*trans* BM adducts, whereas epicatechin and epigallocatechin are derivatized to the 3,4-*trans* BM adducts only.

extension units. These particular tannins yielded readily identifiable reaction products in HPLC chromatograms after thiolysis from these matrices.

Current methods for characterizing tannins in green plants require an initial extraction, removal of chlorophyll with halogenated solvents, and removal of sugars and other impurities by column chromatography (*20–22*) before being subjected to chemical degradation (*9*, *20*, *23–26*). The thiolysis reaction releases the terminal units as the free flavan-3-ols [C, EC, gallocatechin (GC), and epigallocatechin (EGC)] and the extension units as their BM derivatives (**Figure 1**). Here, we describe a new approach for analyzing tannins directly in freeze-dried sainfoin, which is a chlorophyll-containing forage legume, to facilitate rapid screening of germplasm collections for plant-breeding programs.

MATERIALS AND METHODS

Reagents. Hydrochloric acid (36%), acetone [analytical reagent (AR) grade], dichloromethane (HPLC grade), and methanol (HPLC grade) were obtained from ThermoFisher Scientific (Loughborough, U.K.). (±)-Dihydroquercetin (98%) and ampelopsin were obtained from Apin Chemicals (Abingdon, U.K.). BM (98%), (+)-C, (−)-EC, (−)-GC, (−)-EGC, and Procyanidin B2 were obtained from Sigma-Aldrich (Poole, U.K.). Sephadex LH-20 was obtained from GE Healthcare (Little Chalfont, U.K.). Deionized water was obtained from a Milli-Q system (Millipore, Watford, U.K.).

Sainfoin Samples: Development of the *in Situ* Thiolysis Method. Sainfoin (Cotswold Common; second cut) was harvested on July 24, 2007 from Hartley Farm (near Seven Springs, Cheltenham, Gloucestershire, U.K.), manually separated from weeds, and freeze-dried. It was ground in an impeller SM1 cutting mill (Retsch, Haan, Germany) to pass one sieve (< 8 mm), then ground to pass another sieve (< 1 mm), and stored. Before use, small batches were prepared by ball-milling an aliquot of this bulk sample for 10 min in a Pulverisette 5 (Fritsch, Idar-Oberstain, Germany). Ball-milled batches of this bulk sample were used for developing the direct thiolysis method, and the data are shown in **Tables 1** and **3** and **Figures 2–5**.

Sainfoin Samples: Tannin Composition in Whole Plants, 70% Aqueous Acetone Extracts, and Plant Residues. In addition to the above Cotswold Common sample, six sainfoin lines, Nova [National Institute of Agricultural Botany (NIAB) code 1077R2], Visnovsky (1230R2), Giant (1163R2), Line 108 (1261R2), Sisiani Local (1264R1), and CPI63753 (1113R2), were harvested in June 2008 from the EU "HealthyHay" germplasm collection at NIAB, Cambridge, U.K. These samples were passed through 8 and 1 mm sieves but were not ball-milled. The data are shown in **Tables 2** and **4**.

Preparation of Extractable and Unextractable Tannin Samples. Sainfoin (5 g) was extracted once with acetone/water (80 mL; 7:3, v/v) containing ascorbic acid (80 mg) for 1 h. Chlorophyll was removed from the acetone/water solution by extraction with dichloromethane. Acetone was then removed on a rotary evaporator, and the aqueous phase was freeze-dried. Acetone was allowed to evaporate in the fume cupboard before freeze-drying the plant residue. The freeze-dried aqueous acetone extracts or plant residues were treated with BM as described below (see Thiolysis of Extractable Tannins and Thiolysis of *in Situ* Tannins).

Thiolysis of Extractable Tannins. Extracted tannins were added to methanol (4 mg/mL), dissolved by sonicating for 3 min, and filtered through a Cameo Teflon filter (0.45 μm; Anachem Instruments, Luton, U.K.). The solution (50 μL) was transferred into a 0.8 mL vial (Chromacol 08-CPV vial; ThermoFisher Scientific). Then, methanol (50 μL) acidified with concentrated HCl (3.3%; i.e., 3.3 mL of 37% HCl plus 100 mL of methanol) was added followed by BM in methanol (100 μL; 5:95, v/v). The vial was closed with a crimp top and incubated at 40 °C for 30 min (*6*, *25*). The reaction was stopped by placing the vial in an ice bath. Then, water (250 μL) was added, followed by the internal standard, dihydroquercetin in methanol (50 μL; 0.047 mg/mL). Samples were stored at −25 °C until HPLC analysis within 24 h.

Thiolysis of *in Situ* Tannins. Freeze-dried plant material (200 mg) and a stirring magnet (10 × 5×5 mm) were placed into a screw cap glass tube (Fisherbrand FB59557, 100 × 16 mm). Then, methanol (1.6 mL; HPLC grade) was added to the tube, followed by 3.3% HCl in methanol (800 μL), and then BM (80 μL; Sigma, Poole, U.K.) was added. The tube was capped immediately with a rubber Suba-Seal and placed in a water bath at 40 °C for 60 min. The contents were stirred continuously at 1000 rpm. The reaction was stopped by placing the tube in an ice bath for 10 min. The sample was centrifuged at 3000 rpm for 5 min, and the supernatant poured into a 50 mL conical flask containing 20 mL of water. Methanol (1 mL; HPLC grade) was added to the residue. The pellet was shaken vigorously and centrifuged again. The supernatant was added to

the conical flask. The combined solution was added to a previously prepared Sephadex LH-20 mini-cartridge (see Packing of Mini-cartridges) and eluted under gravity. The conical flask and the reservoir were rinsed with water (5 mL). Water (40 mL) was then added to the reservoir to remove sugars from the cartridge by first applying a slight pressure from the top of the reservoir and then a slight vacuum at the outlet of the VacMaster-10 glass chamber (Biotage AB, Uppsala, Sweden). The last drops were eluted under gravity, but care was taken, so that the cartridges did not run dry. Then, the stopcock taps were closed. The reservoirs and adaptors were removed from the top of the syringe barrels (see Packing of Mini-cartridges). A rack with new tubes was placed into the VacMaster-10 glass chamber; care was taken to ensure that each needle below the Sephadex LH-20 cartridges was inside a centrifuge tube. Methanol (2 mL) was added and left to soak the cartridge for 10 min. Then, the stopcock taps were opened to elute the thiolysis reaction products under gravity, and the eluate was collected in the test tube. Four more aliquots of methanol (2 mL each) were added to complete the elution (i.e., final volume of methanol was 10 mL). The tubes were capped, shaken, and stored at −25 °C until HPLC analysis within 7 days. Just before HPLC analysis, the internal standard, dihydroquercetin in methanol (100 μL; 0.384 mg/mL), was added to this methanol solution (10 mL). Samples were mixed and centrifuged for 3 min at 3000 rpm [Note that cartridges can be used twice. After the first use, they were rinsed with acetone/water (20 mL; 7:3, v/v) and then with methanol/water (20 mL; 1:1, v/v) and stored. Just before the second use, the cartridges were again conditioned with water (20 mL) under gravity].

Thiolysis of Mixed Samples. Mixed sainfoin samples were prepared for the purpose of comparing measured and calculated tannin parameters. Individual samples were analyzed first as above (see Thiolysis of *in Situ* Tannins), and then, samples were mixed using equal quantities from each accession and re-analyzed (see Thiolysis of *in Situ* Tannins). The results are shown in **Table 4**.

Packing of Mini-cartridges. Sephadex LH-20 (10 g; GE Healthcare, Little Chalfont, U.K.) was suspended in methanol/water (1:1; 100 mL) and gently dispersed by shaking overnight. A PTFE frit (Isolute 120-1073 L; Biotage AB, Uppsala, Sweden) was placed into an empty plastic syringe barrel (5 mL; Terumo BS05S04 syringe with Luer fitting; Industrial and Scientific Supplies, Ltd., Cambridge, U.K.). The barrel was inserted into the lid of a VacMaster-10, which was fitted with PTFE stopcocks (121-0009). The syringe barrel was filled with Sephadex LH-20 (ca. 0.5 g) and left to settle under gravity to reach the 2 mL mark exactly. A second PTFE frit was placed on top of the Sephadex LH-20 bed, which was then conditioned with 50% methanol. Just before use, a PTFE column adaptor (Isolute 120-1100) was inserted into the top of the syringe barrel and a reservoir tube (Isolute 120-1009F) was added. The Sephadex LH-20 mini-cartridge was then equilibrated with water (20 mL).

HPLC Analysis of Thiolysis Reaction Products. External standard solutions in methanol were prepared as follows. C (0.05 mg/mL), EC (0.10 mg/mL), EGC (0.22 mg/mL), and dihydroquercetin (0.047 mg/mL) were dissolved in methanol, and then, equal volumes from each standard were combined for the first standard solution. GC (0.01 mg/mL) and the internal standard, dihydroquercetin (0.047 mg/mL), were dissolved in methanol and then mixed in a 9:1 ratio for the second standard solution.

Samples (10 μL) were injected into a HPLC system connected to a Luna C18(2) column (3 μm; 150 × 4.6 mm; 00F-4251-EO; Phenomenex, Macclesfield, U.K.) fitted with a guard column (KJO-4282). The Gilson HPLC system (Anachem Instruments, Luton, U.K.) consisted of a 234 autoinjector, two 306 pumps, a UVD340S diode array detector (Dionex, Macclesfield, U.K.), and a personal computer with Chromeleon version 6.8 software. The flow rate was 0.75 mL min^{-1} using 1% acetic acid in water (solvent A) and HPLC-grade methanol (solvent B). The following gradient program was employed: 0−5 min, 20% B; 5−40 min, 20−70% B linear; 40−45 min, 70−90% B linear; 45−50 min, 90% B; 50−55 min, 90−20% B linear; and 55−60 min, 20% B. Terminal and extension units were identified by their retention times and ultraviolet−visible (UV−vis) spectra recorded between 220 and 595 nm (see the Supporting Information for UV−vis spectra of terminal flavan-3-ols and their BM adducts). Peak areas at 280 nm were integrated and quantified using response factors relative to dihydroquercetin (*27*): 0.30 for terminal units of C or EC, 0.06 for terminal units for GC or EGC, 0.26 for extension BM adducts of C or EC, and 0.06 for extension BM adducts of GC or EGC.

Calculation of Tannin Composition. Structural information was obtained from the HPLC chromatograms as follows: mDP was calculated according to (*11*)

$$\mathrm{mDP} = \frac{\text{amount of extension and terminal flavanol units (mol)}}{\text{amount of terminal flavanol units (mol)}}$$

Procyanidin/prodelphinidin (PC/PD) ratios of tannins were calculated according to

$$\mathrm{PC/PD} = \frac{\text{percentage of C} + \text{EC units}}{\text{percentage of GC} + \text{EGC units}}$$

The formula for *cis*/*trans* ratios was

$$cis/trans = \frac{\text{percentage of EC} + \text{EGC units}}{\text{percentage of C} + \text{GC units}}$$

Synthesis of BM Adducts. Retention times of flavan-3-ol BM adducts were established with synthesized standards and confirmed by liquid chromatography−mass spectrometry (LC−MS) analysis. Dihydroquercetin was reacted with BM at 0 °C in the presence of $NaBH_4$ for 45 min followed by 50% aqueous acetic acid at room temperature for 12 h to yield the 3,4-*cis*- and 3,4-*trans*-C BM adducts in the ratio of 4:1 as described (*28*), except that the reaction mixture was not poured into water after 12 h. Ampelopsin was dissolved in ethanol and reacted with $NaBH_4$ for 30 min before adding 50% aqueous acetic acid as above. This yielded the 3,4-*cis*- and 3,4-*trans*-GC BM adducts. PC B2 was reacted with BM at 50 °C for 40 min and yielded the 3,4-*cis*- and 3,4-*trans*-EC BM adducts in the ratio of 1:4 (*29*, *10*).

Analysis by LC−MS. Flavan-3-ols and their BM adducts were identified by LC−MS analysis on a μTOF Bruker Daltonik (Bremen, Germany) instrument by comparing retention times with injected standards. The stereochemistry at C-4 was determined by comparing the retention times of BM adducts with published retention times (*20*). MS spectra were recorded in the negative ionization mode between *m*/*z* 100 and 1200 using the following conditions: capillary voltage, 4000 V; nebulizer gas pressure, 100 kPa; drying gas, 10 mL min^{-1}; and dry heater temperature, 160 °C. An Intersil ODS2 column (GL Sciences, Torrance, CA; 250 × 4.6 mm, 5 μm particle size, 150 Å pore size) was kept at 35 °C. The flow rate was 0.5 mL min^{-1} using solvent A (1% acetic acid in water) and solvent B (methanol) with the following gradient profile: 0 min, 20% B; 5 min, 20% B; 40 min, 70% B; 43 min, 90% B; 48 min, 90% B; 51 min, 20% B; and 60 min, 20% B. UV detection was at 280 nm.

Time Course Study of Thiolysis with Purified Tannins and *in Situ* Sainfoin Tannins: a) Tannin Purification. Cotswold Common (24.9 g) was extracted with acetone/water (400 mL; 7:3, v/v) containing ascorbic acid (400 mg) by stirring for 40 min. The solution was filtered, and acetone evaporated at <35 °C. The aqueous residue was extracted 3 times with dichloromethane (300 mL), and the organic phase was discarded. The aqueous phase was then concentrated on a rotary evaporator at <35 °C and freeze-dried. The freeze-dried extract (105.3 mg) was dissolved in water (10 mL) by sonication and applied to a Sephadex LH-20 column (1.3 g; 1×6.8 cm). The column was washed with water (200 mL), and tannins were eluted with acetone/water (30 mL; 7:3, v/v). Acetone was evaporated at <35 °C, and the purified tannins were freeze-dried. **b) Thiolysis**. Purified (4.2 mg) were dissolved in methanol (1 mL) and thiolysed (see Thiolysis of Extractable Tannins). The reaction was stopped after 0.5, 1, 4, 7, and 24 h. Sainfoin (Cotswold Common) (200 mg) was subjected to thiolysis in duplicate as described above (see Thiolysis of *in Situ* Tannins). The reaction was stopped after 0.5, 1, 5, and 24 h (**Table 1** and **Figures 3−5**).

***In Situ* Thiolysis under Air and Argon.** The *in situ* thiolysis reaction was carried out in triplicate as described above (see Thiolysis of *in Situ* Tannins). For the argon experiments, methanol (1.6 mL) and 3.3% HCl in methanol (800 μL) were added to the tube as described above. Tubes were capped immediately with a rubber Suba-Seal, and a short needle was inserted. Argon was bubbled over the solution using a long needle for 30 s, and then, BM (80 μL) was added. The reaction was continued at 40 °C (see Thiolysis of *in Situ* Tannins).

Table 1. Time Course of Flavan-3-ol Composition (%) in Purified Tannins and in Freeze-Dried Sainfoin (Cotswold Common var.) Plant as Determined by Thiolysis with BM [Standard Deviation (SD) in Parentheses; $n = 2$]

	fraction in terminal units (%)				fraction in extension units (%)			
time (min)	GC[a]	EGC[b]	C[c]	EC[d]	GC	EGC	C	EC
				Purified Tannins				
30	0.93 (0.222)	1.52 (0.420)	2.74 (0.231)	3.24 (0.008)	9.41 (0.181)	49.5 (0.33)	3.51 (0.027)	29.2 (0.52)
65	0.81 (0.178)	1.25 (0.161)	2.72 (0.111)	3.21 (0.121)	6.70 (1.271)	50.7 (1.03)	3.60 (0.249)	31.0 (0.35)
240	1.42 (0.203)	0.79 (0.095)	3.28 (0.577)	2.50 (0.392)	5.42 (0.468)	50.2 (3.93)	3.74 (0.027)	32.6 (2.63)
420	1.10 (0.335)	ND[e]	3.04 (0.072)	1.89 (0.095)	4.58 (0.123)	52.0 (0.41)	3.27 (0.146)	34.1 (0.94)
1440	ND	ND	2.86 (0.147)	1.03 (0.075)	6.02 (0.443)	44.1 (0.11)	2.69 (0.037)	43.3 (0.52)
				In Situ Sainfoin Tannins				
30	2.55 (0.375)	1.93 (0.075)	4.14 (0.329)	4.24 (0.441)	8.87 (0.759)	40.5 (1.04)	4.44 (0.422)	33.3 (1.00)
60	2.45 (1.319)	1.21 (0.110)	3.75 (0.772)	3.53 (1.130)	7.56 (0.116)	44.0 (3.86)	4.54 (0.747)	33.0 (0.10)
300	0.76 (0.051)	0.38 (0.146)	1.50 (0.223)	0.83 (0.266)	6.97 (0.366)	61.1 (0.22)	2.48 (0.058)	25.9 (0.48)
1440	0.49 (0.162)	0.20 (0.074)	0.78 (0.223)	0.27 (0.116)	7.70 (0.910)	64.6 (2.04)	2.23 (0.299)	23.8 (1.41)

[a] GC = gallocatechin. [b] EGC = epigallocatechin. [c] C = catechin. [d] EC = epicatechin. [e] ND = not detected.

RESULTS

Developing the Sample Cleanup Procedure. Preliminary experiments showed that not all of the phloroglucinol reaction products could be assigned because the HPLC chromatograms were too complex. Therefore, thiolytic degradation was evaluated with BM instead. Preliminary experiments also revealed that these sainfoin varieties contained negligible amounts of free C, and other free flavan-3-ols were not detected. C concentrations ranged from 0 to 8.9 mg/100 g of freeze-dried samples. The terminal unit concentrations obtained after thiolytic degradation were, therefore, not corrected for free flavan-3-ols (*10*). The thiolytic reaction mixture was applied to several different mini-cartridges, but Sephadex LH-20 resulted in fewer and smaller contaminant peaks than C18 cartridges. Contaminant peaks, denoted by x, interfered especially with peaks 6, 7, 8, and 9 (*trans*- and *cis*-GC BM, *trans*-C BM, and EGC BM adducts, respectively) after elution from the C18 cartridge (**Figure 2a**). However, only minor contaminant peaks were obtained after cleanup on the Sephadex LH-20 cartridge, and all peaks were detectable (**Figure 2b**). It is of note that both the 3,4-*trans* and 3,4-*cis* isomers of the C and GC BM adducts were separated. The identities of the free terminal flavan-3-ols and the flavan-3-ol BM adducts of the extension units were confirmed by LC−MS and with synthesized authentic compounds.

Figure 2 also shows that the composition of acetone/water extractable tannins resembled the composition of the measured *in situ* tannins. However, the composition of the residual tannins, which remained after two 70% aqueous acetone extractions, differed noticeably and especially in terms of the relative EC BM adduct concentration. Because the terminal units were difficult to detect, it can be concluded that residual tannins had a high mDP (> 80; **Figure 2d**).

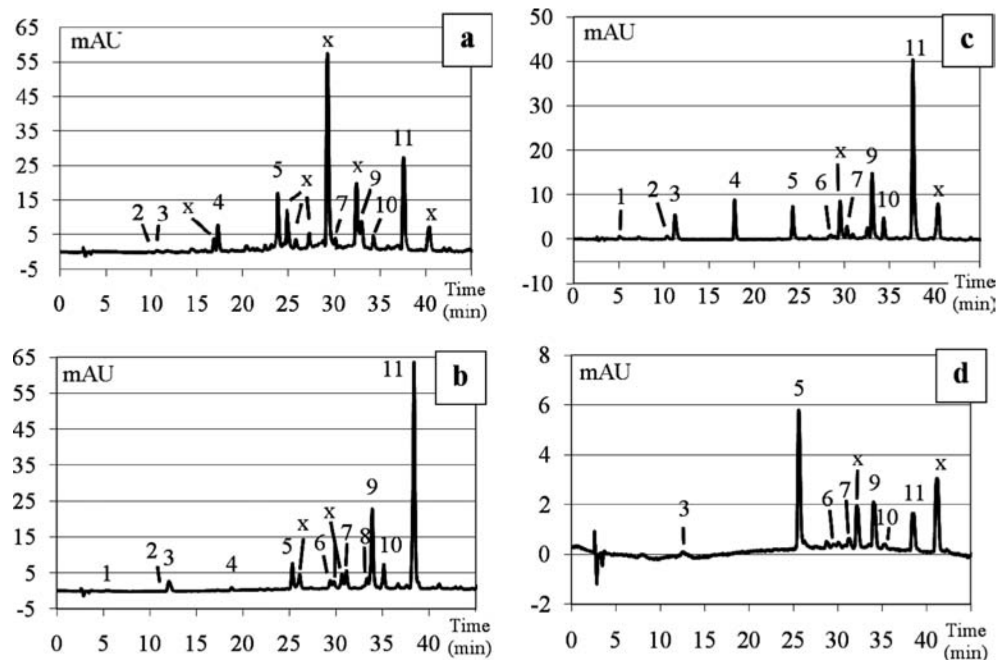

Figure 2. HPLC of thiolysis reaction products that were obtained using freeze-dried sainfoin (Cotswold Common var.) and passed through either (a) C18 or (b) Sephadex LH-20 cartridges. HPLC of thiolysis products that were obtained from the (c) acetone/water (7:3) extract and (d) extracted residue (thiolytic reaction mixtures of panels c and d were passed through Sephadex LH-20 cartridges). Peak assignments: 1, GC; 2, EGC; 3, C; 4, EC; 5, internal standard (dihydroquercetin); 6, *trans*-GC BM; 7, *cis*-GC BM; 8, *trans*-C BM; 9, EGC BM; 10, *cis*-C BM; 11, EC BM; and x, unknown contaminant.

Development of the Screening Method: Time Course Study. It is well-known that the acid conditions during thiolysis of proanthocyanidins can epimerize terminal *cis* to *trans* flavan-3-ols (*16*, *30*). Therefore, a kinetic study investigated changes in flavan-3-ol concentrations over a 24 h period. The apparent increase of "C" and "GC" concentrations after 65 min was caused by the decline of terminal EC and EGC units (**Figure 3b**) [Note that C-2 epimerization of EC and EGC leads to *ent*-C and *ent*-GC; however, under the chosen HPLC conditions, C and GC were not separated from their corresponding *ent* isomers (*16*, *30*)]. Although extension units did not epimerize, the EGC BM adduct concentration decreased from 17 to 8 μg/mL within 24 h (**Figure 3a**).

In situ thiolysis of sainfoin tissue also revealed epimerization of terminal EC after 60 min, but this was less obvious with EGC. Overall, terminal unit concentrations decreased less sharply (**Figure 3d**) compared to purified tannins (**Figure 3b**), which can be attributed to the fact that losses were masked by the ongoing thiolytic degradation of plant tissue tannins (**Figure 3c**). Over a 24 h period, *in situ* thiolysis generated an increase in extension units (**Figure 3c**), and this was particularly noticeable for EGC BM concentrations (from 7.7 to 43.5 μg/mL). This indicated that flavan-3-ol units were released more slowly from the plant tissue than from purified tannins.

The gradual change in flavan-3-ol concentrations over 24 h had the following effects: loss of terminal units (**Figure 3b**) caused apparent mDP changes from 12 to 26 of the purified tannins (**Figure 4a**). However, the mDP of *in situ* tannins changed from 8 to 61 (**Figure 4d**) because of the combined effect of the decreasing terminal unit and increasing extension unit concentrations. The apparent PD content of purified tannins decreased (**Figure 4b**) as a result of relatively greater EGC BM loss relative to other BM adducts (**Figure 3a**). However, the apparent PD content of *in situ* tannins increased (**Figure 4e**) because of the 5.6-fold increase of EGC BM adduct concentration (**Figure 3c**). The apparent *cis* content decreased because of the simultaneous degradation and isomerization of flavan-3-ols, which was especially noticeable for terminal units.

Table 1 illustrates in more detail the effect of these changes on the percent flavan-3-ol composition of purified and *in situ* tannins. Given that a slightly longer reaction time improved the release of EGC BM units (from 40.4 to 44.8%), we standardized the reaction to 60 min for the *in situ* thiolysis of freeze-dried sainfoin samples. This decision was taken, despite the fact that longer reaction times yielded higher tannin concentrations: a 24 h reaction resulted in a 3.5-fold higher tannin content (**Figure 5**) but caused a loss of structural information.

Comparison of *in Situ*, Extractable, and Residual Tannins. **Table 2** shows that *in situ* tannins had a higher mDP than 70% aqueous acetone extractable tannins. The mDP of the residual tannins was estimated to be above 80 but could not be measured accurately, because the terminal unit peaks were within the baseline noise (see **Figure 2d**). PD contents of *in situ* and extractable tannins were similar for two of the accessions, Cotswold Common (54 versus 55% PD) and Nova (79 versus 75% PD), but differed for Visnovsky (81 and 66% PD). In contrast, the *cis* contents of *in situ* and extractable tannins were similar for Visnovsky (75 and 77% *cis*) but differed for Cotswold Common (77 and 85% *cis*) and Nova (88 and 67% *cis*). Residual tannins had higher *cis* contents than *in situ* tannins, and this was particularly the case for Visnovsky tannins (87 versus 75% *cis*).

The tannin composition of the plant residues was variable. Residual Visnovsky and Cotswold Common tannins had the highest percent PD content, but residual Nova tannins were pure PCs because only EC BM adducts were detected. Residual tannin contents ranged from 0.02 to 0.16% and represented 3−11% of the *in situ* tannins as determined by the 60 min thiolysis reaction. Extractable tannins accounted for 53−103% of the

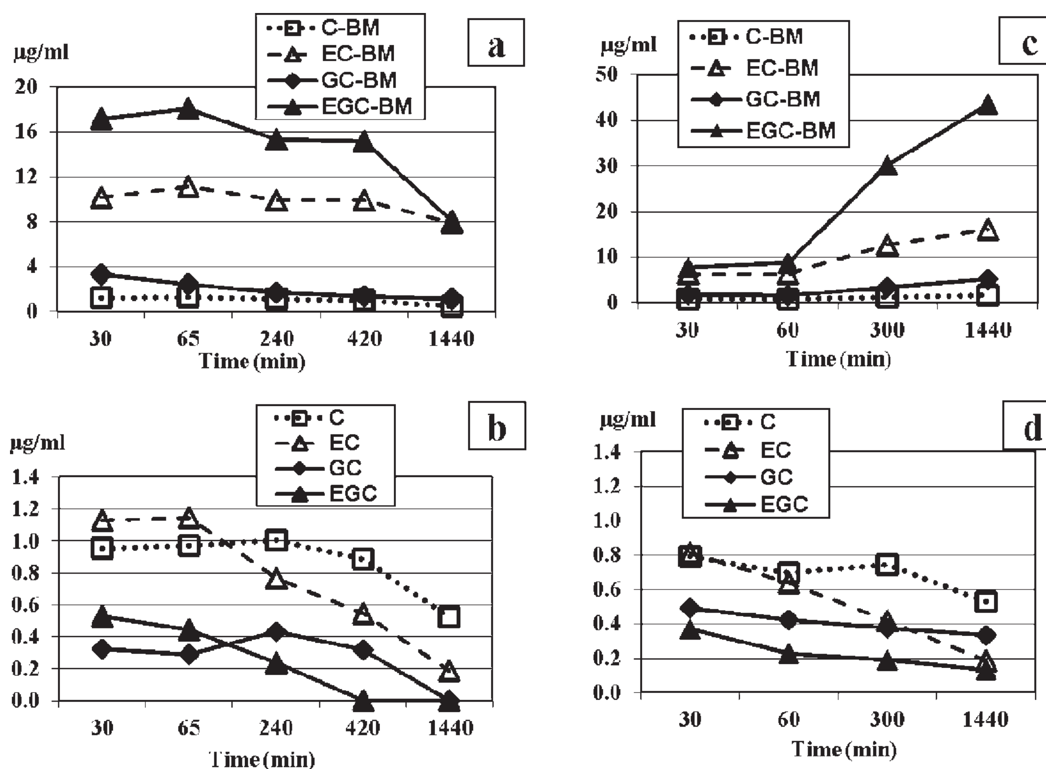

Figure 3. Time course study of thiolytic degradation using purified tannins (a and b) or freeze-dried sainfoin (Cotswold Common var.) tissue (c and d) samples. Panels a and c depict changes of extension units, and panels b and d depict changes in terminal units in the reaction solution (μg/mL). Abbreviations: C, catechin; EC, epicatechin; GC, gallocatechin; EGC, epigallocatechin; and BM, benzyl mercaptan adduct.

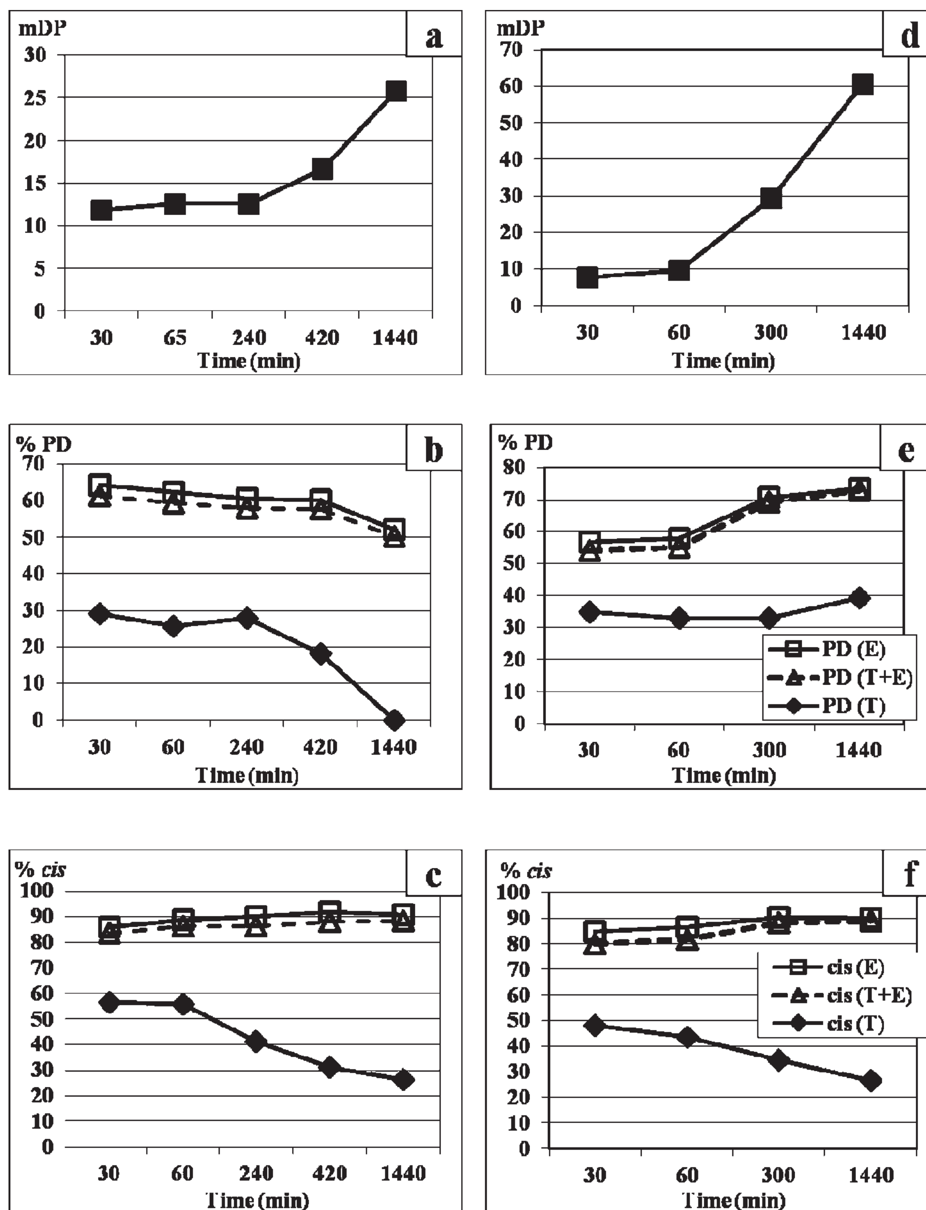

Figure 4. Effect of the thiolysis reaction time on the composition of extractable tannins (a−c) and *in situ* sainfoin (Cotswold Common var.) tannins (d−f). Abbreviations: mDP, mean degree of polymerization; % PD, proportion of prodelphinidin units; % *cis*, proportion of *cis* flavan-3-ol units; T, terminal units; and E, extension units.

in situ tannins. The sum of residual and extractable tannins accounted for 63.6, 113.7, and 81.6% of the *in situ* tannins in Visnovsky, Cotswold Common, and Nova, respectively.

DISCUSSION

Minimizing Epimerization Side Reactions. Various authors have used several different conditions for the thiolytic degradation of tannins (*7−10*, *18*, *19*, *30*, *31*), and epimerization can be an important side reaction under some conditions (*30*). As shown in **Figure 3**, epimerization was detected among terminal flavan-3-ols but not among extension units, and this agreed with previous reports (*30*, *31*). It especially affected the terminal *cis* flavan-3-ols, EC and EGC, which were converted into the corresponding *trans* flavan-3-ols (*ent*-C and *ent*-GC) (*7*, *16*, *30*, *32*).

Our preliminary experiments showed that PC B2 was converted to the EC BM adduct within 40 min at 50 °C but 50% of the terminal (−)-EC had apparently epimerized to *ent*-C [Note that enantiomeric C and *ent*-C co-eluted on this HPLC column]. Further tests showed that 17.1% of pure EC epimerized within 2 min at 90 °C, 9.21% of pure EC epimerized within 10 min at 60 °C, but only 1.67% of pure EC epimerized within 30 min at 40 °C. These results agreed with previously reported epimerization percentages (*31*, *33*). Therefore, we selected a reaction temperature of 40 °C and a reaction time of 60 min for two reasons: (i) tannin content and composition in terms of mDP and percent PD and percent *cis* contents were relatively stable within the first hour (**Table 1** and **Figures 4** and **5**), and (ii) this period proved convenient for a screening method. The same combination of temperature and time was used previously (*18*) for the direct thiolysis of coffee pulp. Obviously, if analysis of the total tannin content was to be the objective, the reaction would need to be optimized near the plateau, which occurred between 4 and 8 h for the different flavan-3-ol BM adducts.

Minimizing Degradation Side Reactions. Although longer reaction times generated higher tannin yields (**Figure 5**), they

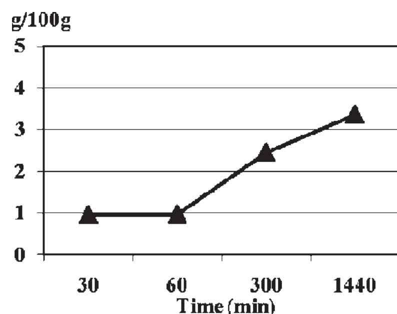

Figure 5. Effect of the thiolysis reaction time on the measured tannin content of freeze-dried sainfoin (Cotswold Common var.) tissue (g of tannin/100 g of sainfoin).

Table 2. Tannin Composition Resulting from Thiolysis of Whole Plant, 70% Acetone Extract, and Residue (SD in Parentheses; n = 3 for Plants and Residues and 6 for Extracts)

sainfoin sample	mDP[a]	PC/PD[b]	*cis*/*trans*[c]	% CT[d]
NIAB 1230R2 (Visnovsky)				
plant	15.8 (1.98)	19.2/80.8 (0.93)	75.2/24.8 (2.20)	1.18 (0.174)
extract	11.4 (1.94)	34.4/65.6 (5.32)	77.9/22.1 (5.39)	0.62 (0.088)
residue	ND[e]	14.0/86.0 (1.07)	87.0/13.0 (1.81)	0.13 (0.037)
Cotswold Common				
plant	30.0 (2.95)	45.6/54.4 (2.55)	76.7/23.3 (4.07)	1.33 (0.218)
extract	10.3 (0.67)	44.6/55.4 (0.60)	84.8/15.2 (1.25)	1.50 (0.032)
residue	ND	22.9/77.1 (0.25)	81.2/18.8 (1.00)	0.16 (0.004)
NIAB 1077R2 (Nova)				
plant	74.3 (3.42)	21.3/78.7 (2.47)	88.0/12.0 (1.65)	0.76 (0.087)
extract	45.7 (3.51)	25.1/74.9 (3.75)	66.5/33.5 (5.40)	0.60 (0.037)
residue	ND	100[f]/0 (0.00)	100/0 (0.00)	0.02 (0.001)

[a] mDP = mean degree of polymerization. [b] PC/PD = ratio of procyanidin and prodelphinidin tannins. [c] *cis*/*trans* = ratio of EC plus EGC and C plus GC monomer units. [d] % CT = condensed tannin content (g/100 g of dry matter). [e] ND = not detected. [f] only EC BM is detected in this residue.

also compromised information on the structural composition of tannins, because disappearing terminal units resulted in an artificially high mDP after 4 h with the purified tannins and after 60 min with the *in situ* tannins (panels **a** and **d** of **Figure 4**). Thiols, such as BM and mercaptoacetic acid, are known to degrade flavan-3-ols by promoting cleavage of the heterocyclic C ring (*7*, *34*, *35*). While some authors employed nitrogen or argon to reduce oxidative degradation during thiolysis and phloroglucinolysis (*7*, *8*, *36*−*38*), Wang and Helliwell (*32*) reported that nitrogen lowered the pH and, thus, actually increased flavan-3-ol losses. Therefore, we tested whether an argon atmosphere would limit terminal unit losses. **Table 3** showed that performing the reaction under argon had no effect on the final result. Indeed, BM is a good antioxidant (*39*), and the results indicated that an inert atmosphere was not necessary. Several other authors also chose not to use an inert gas for thiolytic degradations (*10*, *17*).

Figure 3 indicates some surprising differences in the rate by which the various extension units were released. The concentration of EGC units increased 5.6-fold (from 7.71 to 43.5 μL/mL). The concentration of EC units increased 2.5-fold (from 6.37 to 16.0 μL/mL). The concentration of GC units increased 3.1-fold (from 1.68 to 5.17 μL/mL). The concentration of C units increased 1.8-fold (from 0.84 to 1.50 μL/mL). This finding suggested that either initial access of the BM reagent to EGC extension units was more difficult or that there were different types of tannins. Indeed, a comparison of the extractable and residual tannin compositions appeared to support the latter explanation; residual tannins tended to have higher PD and *cis*

Table 3. Comparison of *in Situ* Thiolysis of Sainfoin (Cotswold Common var.) Carried out in an Ambient Air Atmosphere or under Argon (SD in Parentheses; n = 3)

	mDP[a]	PC/PD[b]	*cis*/*trans*[c]	% CT[d]
air	30.0 (2.95)	45.6/54.4 (2.55)	76.7/23.3 (4.07)	1.33 (0.218)
argon	29.8 (3.34)	48.4/51.6 (0.96)	76.0/24.0 (2.44)	1.31 (0.182)
significance (95% level)	ns[e]	ns	ns	ns

[a] mDP = mean degree of polymerization. [b] PC/PD = ratio of procyanidin and prodelphinidin tannins. [c] *cis*/*trans* = ratio of EC plus EGC to C plus GC monomer units. [d] % CT = condensed tannin content (g/100 g of dry matter). [e] ns = not significant (pairwise t test).

contents (**Table 2**) (with the exception possibly of the residual Nova tannins). Hernes and Hedges (*37*) noted previously that terminal units were released more readily than extension units and surmised that depolymerization of internal extension units was inhibited until the ends were released. According to Guyot et al. (*6*), highly polymerized tannins required slightly longer reaction times than smaller molecular-weight tannins. Results from this *in situ* assay could provide an alternative explanation; i.e., EGC units bind most strongly to plant constituents.

Method Validation Using *in Situ* Tannin Analysis of Mixed Plant Samples. The validity of the *in situ* method was investigated further by comparing the results from contrasting individual samples to those prepared from mixtures of these samples. This approach was chosen to take into account unextractable tannins, which would not have been included if the samples had been spiked with extractable tannins. The individual samples covered a range of tannin properties: mDP varied from 28 to 83; percent PD contents varied from 65 to 79; and percent *trans* contents varied from 12 to 26. **Table 4** shows that the theoretical (calculated) averages were not significantly different from the actual (measured) values of the sample mixtures in terms of mDP and percent PD, percent *trans*, and tannin contents.

When the results from **Tables 2** and **4** are taken into account, this *in situ* method for sainfoin tannins yielded average relative standard deviation (RSD) values of 8.2% for mDP, 2.3% for PD content, 3.0% for *cis* content, and 11.3% for tannin content. In comparison, Guyot et al. (*10*) reported the following RSD values for the direct analysis of apple: 6.0% for mDP, 3.9% for EC (*cis*) content, 16.3% for C (*trans*) content, and 6.2% for tannin content. The RSDs reported here are higher for mDP and percent tannin content, which can be attributed to the fact that sainfoin tannins are much more complex than apple tannins and contain C, EC, GC, and EGC in extension and terminal units. In contrast, apple tannin extension units contain only EC and the terminal units contain mostly EC plus a small percentage of C.

Method Validation through a Comparison of *in Situ* and Extractable Tannins. Three sainfoin accessions covering a range of tannins with mDP from 15.8 to 74.3 were examined for their *in situ* and extractable tannin compositions. Larger molecular-weight tannins were apparently more difficult to extract than smaller tannins, because the mDP values of extracted tannins were 4−29 units lower than the mDP values of *in situ* tannins (**Table 2**). Extractable tannins had up to 66% lower mDP values, and this could explain why Bate-Smith (*40*) reported that sainfoin tannins were particularly difficult to extract. In contrast, the mDP of residual coffee pulp tannins were not particularly high (*18*), which could be explained by the fact that these tannins, whether extractable or not, had comparatively low mDP values of 5.6−9.1. While there was some indication that residual grape tannins had higher mDP than extractable tannins, this was not the case for all of the tested berry and fruit tannins (*16*). It is of particular note that, despite the loss of larger tannins, the PC/PD

Table 4. Comparison of *in Situ* Tannin Analysis of Individual and Mixed Sainfoin Samples (SD in Parentheses, $n = 3$; see **Table 2** for Abbreviations)

sainfoin samples (accession numbers)	mDP	PC/PD	*cis*/*trans*	% CT
NIAB 1163R2	27.8 (1.52)	35.2/64.8 (1.81)	76.3/23.7 (2.66)	1.24 (0.183)
NIAB 1261R2	44.8 (2.71)	25.7/74.3 (1.27)	76.8/23.2 (3.30)	0.71 (0.027)
mean (calculated)[a]	36.3	30.4/69.6	76.5/23.5	0.98
sample mixture (measured)[b]	31.3 (2.44)	29.7/70.3 (0.40)	74.9/25.1 (0.72)	0.77 (0.068)
significance (95% level)	ns	ns	ns	ns
p value	0.4192	0.8348	0.3662	0.3000
NIAB 1264R1	60.0 (1.68)	21.7/78.3 (0.46)	78.5/21.5 (3.10)	1.00 (0.093)
NIAB 1077R2	74.3 (3.42)	21.3/78.7 (2.43)	88.0/12.0 (1.65)	0.71 (0.084)
NIAB 1113R2	82.6 (6.79)	21.4/78.6 (1.06)	74.1/25.9 (1.02)	0.82 (0.073)
mean (calculated)[c]	72.3	21.5/78.5	80.2/19.8	0.84
sample mixture (measured)[d]	78.7 (4.35)	22.1/77.9 (0.35)	86.4/13.6 (1.14)	0.86 (0.028)
significance (95% level)	ns	ns	ns	ns
p value	0.3421	0.4785	0.1400	0.8842

[a] The mean represents the calculated average of tannin parameters from samples NIAB 1163R2 and 1261R2. [b] The sample mixture consisted of equal proportions of NIAB 1163R2 and 1261R2. [c] The mean represents the calculated average of tannin parameters from samples NIAB 1264R1, 1077R2, and 1113R2. [d] The sample mixture consisted of equal proportions of NIAB 1264R1, 1077R2, and 1113R2.

ratios of extractable tannins were similar to the measured *in situ* plant tannins for the Cotswold Common and Nova but differed for the Visnovsky accession (**Table 2** and panels **b** and **c** of **Figure 2**). Intriguingly, the *cis*/*trans* ratios were similar for the Visnovsky but differed for the Nova and Cotswold Common *in situ* and extractable tannins.

Residual tannins had apparently large mDP values, which were estimated to be above 80; however, their mDP could not be determined because the terminal units were too small to be quantified (**Figure 2d** and **Table 2**). Residual tannins from two sainfoin accessions also had higher PD contents than their extractable tannins (**Table 2**). This agrees with previous reports that larger tannin polymers tended to have a higher percentage of PD units (*11*, *41*, *42*). However, only EC extension units could be detected in the Nova residue, despite a very high mDP (74) of its *in situ* tannins (**Table 2**). These results, therefore, demonstrate considerable diversity among tannins in different sainfoin accessions.

Previous studies on sainfoin characterized several tannin trimers (*43*) and mixed tannin fractions with mDP of 4−8 (*2*, *24*). In addition, Marais et al. (*2*) indicated that another fraction with high mDP was present, but this was not investigated further. Two further studies described high-molecular-weight sainfoin tannins with up to 50 (*44*) and possibly 94 monomer units (*45*). Although sainfoin tannins have been described as "anomalous" (*44*, *46*, *47*), Foo et al. (*48*) attributed the high molecular weights to the ultracentrifugation method (*45*) or seasonal or varietal differences. This study has now revealed that sainfoin tannins are indeed highly complex mixtures, which can contain high-molecular-weight tannins, be difficult to extract, and differ greatly between accessions.

To conclude, this is the first report of a thiolytic degradation and cleanup method that can be applied directly to green plant tissue, such as sainfoin. Prior extraction with dichloromethane proved unnecessary for removing chlorophyll. The thiolysis reaction mixture required only a cleanup on a Sephadex LH-20 mini-cartridge before HPLC analysis. The method was designed for screening a sainfoin germplasm collection in terms of tannin structural composition and content (*49*). The method was validated by analyzing individual and mixed sample sets and comparing the composition of *in situ* to extractable tannins. Although both sets of tannins resembled each other, extraction caused losses of higher molecular-weight tannins. A comparison of several accessions revealed that sainfoin tannins differed in the complexity of their PC/PD and *cis*/*trans* ratios and extractabilities.

ABBREVIATIONS USED

BM, benzyl mercaptan; CT, condensed tannin content (g/100 g); C, catechin; EC, epicatechin; EGC, epigallocatechin; GC, gallocatechin; mDP, mean degree of polymerization; nd, not detected; PC, procyanidin; PD, prodelphinidin; SD, standard deviation.

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Supporting Information Available: UV−vis spectra of terminal flavan-3-ols (C, EC, GC, and EGC) and their BM adducts. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Mueller-Harvey, I. Unravelling the conundrum of tannins in animal nutrition and health. *J. Sci. Food Agric.* **2006**, *86*, 2010−2037.
(2) Marais, J. P. J.; Mueller-Harvey, I.; Brandt, E. V.; Ferreira, D. Polyphenols, condensed tannins, and other natural products in *Onobrychis viciifolia* (sainfoin). *J. Agric. Food Chem.* **2000**, *48*, 3440−3447.
(3) Foo, L. Y.; Lu, Y. R.; Howell, A. B.; Vorsa, N. The structure of cranberry proanthocyanidins which inhibit adherence of uropathogenic P-fimbriated *Escherichia coli in vitro*. *Phytochemistry* **2000**, *54* (2), 173−181.
(4) Hedqvist, H.; Mueller-Harvey, I.; Reed, J. D.; Krueger, C. G.; Murphy, M. Characterisation of tannins and *in vitro* protein digestibility of several *Lotus corniculatus* varieties. *Anim. Feed Sci. Technol.* **2000**, *87*, 41−56.
(5) Reed, J. D.; Krueger, C. G.; Vestling, M. M. MALDI−TOF mass spectrometry of oligomeric food polyphenols. *Phytochemistry* **2005**, *66*, 2248−2263.
(6) Guyot, S.; Marnet, N.; Drilleau, J. F. Thiolysis−HPLC characterization of apple procyanidins covering a large range of polymerization states. *J. Agric. Food Chem.* **2001**, *49*, 14−20.
(7) Matthews, S.; Mila, I.; Scalbert, A.; Pollet, B.; Lapierre, C.; Dupenhoat, C. L. M. H.; Rolando, C.; Donnelly, D. M. X. Method for estimation of proanthocyanidins based on their acid depolymerization in the presence of nucleophiles. *J. Agric. Food Chem.* **1997**, *45* (4), 1195−1201.
(8) Matthews, S.; Mila, I.; Scalbert, A.; Donnelly, D. M. X. Extractable and non-extractable proanthocyanidins in barks. *Phytochemistry* **1997**, *45* (2), 405−410.
(9) Taylor, A. W.; Barofsky, E.; Kennedy, J. A.; Deinzer, M. L. Hop (*Humulus lupulus* L.) proanthocyanidins characterized by mass

spectrometry, acid catalysis, and gel permeation chromatography. *J. Agric. Food Chem.* **2003**, *51*, 4101−4110.

(10) Guyot, S.; Marnet, N.; Sanoner, P.; Drilleau, J. F. Direct thiolysis on crude apple materials for high-performance liquid chromatography characterization and quantification of polyphenols in cider apple tissues and juices. *Methods Enzymol.* **2001**, *335*, 57−70.

(11) Labarbe, B.; Cheynier, V.; Brossaud, F.; Souquet, J. M.; Moutounet, M. Quantitative fractionation of grape proanthocyanidins according to their degree of polymerization. *J. Agric. Food Chem.* **1999**, *47*, 2719−2723.

(12) Es-Safi, N.-E.; Guyot, S.; Ducrot, P.-H. NMR, ESI/MS, and MALDI−TOF/MS analysis of pear juice polymeric proanthocyanidins with potent free radical scavenging activity. *J. Agric. Food Chem.* **2006**, *54*, 6969−6977.

(13) Le Bourvellec, C.; Guyot, S.; Renard, C. M. G. C. Non-covalent interaction between procyanidins and apple cell wall material. Part I. Effect of some environmental parameters. *Biochim. Biophys. Acta, Gen. Subj.* **2004**, *1672*, 192−202.

(14) Peréz-Jiménez, J.; Arranz, S.; Saura-Calixto, F. Proanthocyanidin content in foods is largely underestimated in the literature data: An approach to quantification of the missing proanthocyanidins. *Food Res. Int.* **2009**, *42*, 1381−1388.

(15) White, B. L.; Howard, R. L.; Prior, R. L. Release of bound procyanidins from cranberry pomace by alkaline hydrolysis *J. Agric. Food Chem.* **2010**, *58*, 7572−7579.

(16) Hellström, J. K.; Mattila, P. H. HPLC determination of extractable and unextractable proanthocyanidins in plant materials. *J. Agric. Food Chem.* **2008**, *56*, 7617−7624.

(17) Guyot, S.; Le Bourvellec, C.; Marnet, N.; Drilleau, J. F. Procyanidins are the most abundant polyphenols in dessert apples at maturity. *Lebensm.-Wiss. Technol.* **2002**, *35*, 289−291.

(18) Ramirez-Coronel, M. A. N.; Marnet, N.; Kolli, V. S. K.; Roussos, S.; Guyot, S.; Augur, C. Characterization and estimation of proanthocyanidins and other phenolics in coffee pulp (*Coffea arabica*) by thiolysis−high-performance liquid chromatography. *J. Agric. Food Chem.* **2004**, *52*, 1344−1349.

(19) Nunes, C.; Guyot, S.; Marnet, N.; Barros, A. S.; Saraiva, J. A.; Renard, C. M. G. C.; Coimbra, M. Characterization of plum procyanidins by thiolytic depolymerization. *J. Agric. Food Chem.* **2008**, *56*, 5188−5196.

(20) Meagher, L. P.; Lane, G.; Sivakumaran, S.; Tavendale, M. H.; Fraser, K. Characterization of condensed tannins from *Lotus* species by thiolytic degradation and electrospray mass spectrometry. *Anim. Feed Sci. Technol.* **2004**, *117*, 151−163.

(21) Renard, C. M. G. C.; Dupont, N.; Guillermin, P. Concentrations and characteristics of procyanidins and other phenolics in apples during fruit growth. *Phytochemistry* **2007**, *68*, 1128−1138.

(22) Stewart, J. L.; Mould, F.; Mueller-Harvey, I. The effect of drying treatment on the fodder quality and tannin content of two provenances of *Calliandra calothyrsus* Meissner. *J. Sci. Food Agric.* **2000**, *80*, 1461−1468.

(23) Foo, L. Y.; Lu, Y.; Molan, A. L.; Woodfield, D. R.; McNabb, W. C. The phenols and prodelphinidins of white clover flowers. *Phytochemistry* **2000**, *54*, 539−548.

(24) Koupai-Abyazani, M. R.; Muir, A. D.; Bohm, B. A.; Towers, G. H. N.; Gruber, M. Y. The proanthocyanidin polymers in some species of *Onobrychis*. *Phytochemistry* **1993**, *34*, 113−117.

(25) Sivakumaran, S.; Molan, A. L.; Meagher, L. P.; Kolb, B.; Foo, L. Y.; Lane, G. A.; Attwood, G. A.; Fraser, K.; Tavendale, M. Variation in antimicrobial action of proanthocyanidins from *Dorycnium rectum* against rumen bacteria. *Phytochemistry* **2004**, *65*, 2485−2497.

(26) Spencer, P.; Sivakumaran, S.; Fraser, K.; Foo, L. Y.; Lane, G. A.; Edwards, P. J. B.; Meagher, L. P. Isolation and characterisation of procyanidins from *Rumex obtusifolius*. *Phytochem. Anal.* **2007**, *18*, 193−203.

(27) Meagher, L. P.; Spencer, P.; Lane, G.; Sivakumaran, S.; Fraser, K. What do green tea, grapes seeds, and docks have in common? *Chem N. Z.* **2005**, No. September, 6−9.

(28) Steynberg, P. J.; Nel, R. J. J.; van Rensburg, H.; Bezuidenhardt, B. C. B.; Ferreira, D. Oligomeric flavanoids. Part 27. Interflavanyl bond formation in procyanidins under neutral conditions. *Tetrahedron* **1998**, *54*, 8153−8158.

(29) Kolodziej, H. Thiolysis of birch bark procyanidins: structural dependence in formation of 2,3-*cis*-3,4-*cis*-flavan-4-benzylthioethers from procyanidins. *Phytochemistry* **1990**, *29*, 1671−1674.

(30) Gu, L. W.; Kelm, M.; Hammerstone, J. F.; Beecher, G.; Cunningham, D.; Vannozzi, S.; Prior, R. L. Fractionation of polymeric procyanidins from lowbush blueberry and quantification of procyanidins in selected foods with an optimized normal-phase HPLC−MS fluorescent detection method. *J. Agric. Food Chem.* **2002**, *50*, 4852−4860.

(31) Fan, J. L.; Ding, X. L.; Tao, G. J. Study of components in the thiolysis media of procyanidin B3 with different thiolysis conditions by high performance liquid chromatography/electrospray ionization−mass spectrometry. *Chin. J. Anal. Chem.* **2006**, *34*, 251−254.

(32) Wang, H.; Helliwell, K. Epimerisation of catechins in green tea infusions. *Food Chem.* **2000**, *70*, 337−344.

(33) Prieur, C.; Rigaud, J.; Cheynier, V.; Moutounet, M. Oligomeric and polymeric procyanidins from grape seeds. *Phytochemistry* **1994**, *36*, 781−784.

(34) Betts, M. J.; Brown, B. R.; Shaw, M. R. Reaction of flavanoids with mercaptoacetic acid. *J. Chem. Soc. C* **1969**, 1178−1184.

(35) McGraw, G. W.; Steynberg, J. P.; Hemingay, R. W. Condensed tannins−a novel rearrangement of procyanidins and prodelphinidins in thiolytic cleavage. *Tetrahedron Lett.* **1993**, *34*, 987−990.

(36) Czochanska, Z.; Foo, L. Y.; Newman, R. H.; Porter, L. J. Polymeric proanthocyanidins. Stereochemistry, structural units, and molecular weight. *J. Chem. Soc., Perkin Trans. 1* **1980**, 2278−2286.

(37) Hernes, P. J.; Hedges, J. I. Determination of condensed tannin monomers in environmental samples by capillary gas chromatography of acid depolymerization extracts. *Anal. Chem.* **2000**, *72*, 5115−5124.

(38) Porter, L. J. Structure and chemical properties of the condensed tannins. In *Plant Polyphenols*; Hemingway, R. W., Laks, P. E., Eds.; Plenum Press: New York, 1992; pp 245−258.

(39) Brown, B. R.; Shaw, M. R. Reactions of flavanoids and condensed tannins with sulphur nucleophiles. *J. Chem. Soc., Perkin Trans. 1* **1974**, 2036−2049.

(40) Bate-Smith, E. C. Tannins of herbaceous Leguminosae. *Phytochemistry* **1973**, *12*, 1809−1812.

(41) Dueñas, M.; Sun, B. S.; Hernandez, T.; Estrella, I.; Spranger, M. I. Proanthocyanidin composition in the seed coat of lentils (*Lens culinaris* L.). *J. Agric. Food Chem.* **2003**, *51*, 7999−8004.

(42) Kennedy, J. A.; Taylor, A. W. Analysis of proanthocyanidins by high-performance gel permeation chromatography. *J. Chromatogr., A* **2003**, *995*, 99−107.

(43) Regos, I.; Urbanella, A.; Treutter, D. Idenficiation and quantification of phenolic compounds from the forage legume sainfoin (*Onobrychis viciifolia*). *J. Agric. Food Chem.* **2009**, *57*, 5843−5852.

(44) McAllister, T. A.; Martinez, T.; Bae, H. D.; Muir, A. D.; Yanke, L. J.; Jones, G. A. Characterization of condensed tannins purified from legume forages: Chromophore production, protein precipitation, and inhibitory effects on cellulose digestion. *J. Chem. Ecol.* **2005**, *31*, 2049−2067.

(45) Jones, W. T.; Broadhurst, R. B.; Lyttleton, J. W. The condensed tannins of pasture legume species. *Phytochemistry* **1976**, *15*, 1407−1409.

(46) Mangan, J. L. Nutritional effects of tannins in animal feeds. *Nutr. Res. Rev.* **1988**, *1*, 209−231.

(47) Waghorn, G. C.; John, A.; Jones, W. R.; Shelton, I. D. Nutritive value of *Lotus corniculatus* L. containing low and medium concentrations of condensed tannins for sheep. *Proc. N. Z. Soc. Anim. Prod.* **1987**, *47*, 25−30.

(48) Foo, L. Y.; Jones, W. T.; Porter, L. J.; Williams, V. M. Proanthocyanidin polymers of fodder legumes. *Phytochemistry* **1982**, *21*, 933−935.

(49) Hayot-Carbonera, C.; Mueller-Harvey, I.; Brown, T. A.; Smith, L. Sainfoin (*Onobrychis viciifolia*): A beneficial forage legume. *Plant Gen. Res.* **2011**, manuscript accepted.

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