Polyphenols, Condensed Tannins, and Other Natural Products in *Onobrychis viciifolia* (Sainfoin)

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An acetone/water extract of the fodder legume *Onobrychis viciifolia* afforded arbutin, kaempferol, quercetin, rutin, afzelin, the branched quercetin-3-(2^G-rhamnosylrutinoside), the amino acid L-tryptophan, the inositol (+)-pinitol, and relatively high concentrations of sucrose (ca. 35% of extractable material). Acid-catalyzed cleavage of the condensed tannins with phloroglucinol afforded catechin, epicatechin and gallocatechin as the terminal and extender units, but epigallocatechin was only present in extender units. The condensed tannins in *O. viciifolia* presumably consist of hetero- and homopolymers containing both procyanidin and prodelphinidin units. Comparison of data from the present study and the literature suggests that sainfoin tannins have a highly variable composition with cis:trans ratios ranging from 47:53 to 90:10 and delphinidin:cyanidin ratios from 36:64 to 93:7. The composition of terminal and extender units in sainfoin tannins seems to be cultivar specific.

Keywords: *Onobrychis viciifolia; sainfoin; arbutin; sucrose; (+)-pinitol; tryptophan derivatives; flavonols; condensed tannins*

INTRODUCTION

Existing and predicted protein shortages in animal feeds require new or improved fodder legumes (MDC/MLC/MAFF/SOAFF, 1998). Global warming will further increase the demand for varieties that are better adapted to seasonal drought conditions (Cooper and Carleton, 1968; Mowrey and Volesky, 1993; Martiniello and Ciola, 1994; Morrill et al., 1998). Past efforts to produce improved fodder legumes have largely concentrated on lucerne and clover. At present, birdsfoot trefoil (*Lotus corniculatus*) is being investigated for use in grass-based ruminant production systems in New Zealand (Waghorn et al., 1987; Lowry et al., 1996). However, there are other promising species, such as sainfoin (*Onobrychis viciifolia*; Leguminosae), which have received little attention despite superior nutritional properties (John and Lancashire, 1981; Sheldrick et al., 1987; Mueller-Harvey and McAllan, 1992; Majak et al., 1995). Sainfoin gave a 50% improvement in net absorption of intestinal amino acids when compared with an isonitrogenous lucerne diet (Thomson et al., 1971; Waghorn et al., 1987). Voluntary intake in sheep and cattle is 20−24% higher than for grasses and 10−29% higher than for red clover or lucerne (Waghorn et al., 1990; Griggs and Matches, 1991; Karnezos et al., 1994). In addition, the efficiency with which the metabolizable energy (ME) in sainfoin is utilized for growth and fattening is higher than for grass of equal ME content (Thomson, 1982). As a result, large body weight gains of >400 g/day have been reported for growing lambs (Thomson et al., 1971).

Although it is thought that tannins are responsible for the excellent nutritive value of sainfoin, it is still unclear what distinguishes them from tannins in other fodder legumes (Tanner et al., 1994). The composition of sainfoin tannins varies with variety and growth stage and has a mixed procyanidin/prodelphinidin character (Czochanska et al., 1980; Koupai-Abyazani et al., 1993a,b; Lees et al., 1995). It is not clear if sainfoin tannins occur in the form of a mixed polymer containing catechin/epicatechin (CE) and gallocatechin/epigallocatechin (GE) units or if they are a mixture of two pure homopolymers, i.e., prodelphinidins and procyanidins.

Past phytochemical studies of sainfoin identified two unique 2-arylbenzofurans, sainfuran and methylsainfuran from roots, but not leaves, which are insect feeding deterrents (Russell et al., 1984). These compounds are structurally related to the pterocarpan, medicarpin, and the isoflavan, vestitol, which were found in sainfoin leaves, red clover, and lucerne (Dewick, 1977; Ingham 1978; Russell et al., 1984) together with the chalcone, isoliguiritigenin (Dewick, 1977). Sainfoin also contains 7,2′-dihydroxy-4′-methoxyisoflavanone, which was postulated to be metabolically related to the pterocarpan (Dewick, 1977) and condensed tannins (Koupai-Abyazani et al., 1993a). This study attempts to elucidate the chemical composition that may be responsible for the nutritional and veterinary benefits attributed to sainfoin (Hill, 1998).

MATERIALS AND METHODS

Instrumentation. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ at 300 and 75 MHz on a Bruker AVANCE DPX-300 instrument with TMS as internal standard. MS spectra and accurate mass estimations were carried out with a Kratos MS-80 mass spectrometer (MS) with double focusing in EI mode. Electrospray ionization mass spectra (ESI-MS) were deter-

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mined on a Micromass Quattro triple quadrupole instrument, fitted with an electrospray ionization source. Circular dichroism (CD) curves were recorded in methanol on a Jasco J-710 spectropolarimeter.

Chromatography. Thin-layer chromatography (TLC) was done on Al plates (3 × 7 cm) coated with Kieselgel 60F_{254} (Merck), 0.25 mm thickness. Free phenolic polymer fractions were assessed on 3 × 6.5 cm Cellulose F plates (Merck), 0.1 mm thickness in 5% acetic acid and sprayed with bis-diazotized benzidine and anisaldehyde−sulfuric acid. Preparative TLC was done on TLC plates (20 × 20 cm; 10−25 mg mixture/plate), coated with 1 mm layer of Kieselgel PF_{254} (Merck). Compounds were eluted with acetone. Microseparations were carried out on TLC Silica gel 60 PF_{254} (0.25 mm) charged with 1−3 mg of extract. TLC plates were sprayed with formaldehyde (40%)−sulfuric acid (2:98) or with anisaldehyde−sulfuric acid−ethanol (5:5:90) and developed at 120 °C. Two-dimensional paper chromatography (PC) was done on Whatman No. 1 paper (28.5 × 46 cm) in water-saturated butan-2-ol and acetic acid−water (2:98). Paper chromatograms were sprayed with bis-diazotized benzidine. Column chromatography (CC) was carried out on Sephadex LH-20 (various column lengths). Compounds were eluted at 0.5 mL/min, and fractions of 15 mL were collected. All evaporations were done at reduced pressure at 35 °C.

Derivatizations. Methylations were done with diazomethane for 48 h at −15 °C, while acetylations were performed in acetic anhydride/pyridine at 40 °C for 48 h.

Acid-Catalyzed Cleavage of the Tannin Fraction with Phloroglucinol as the Nucleophile. The substrate and phloroglucinol (1:1 ratio, m/m) were stirred in a 5% HCl−ethanol solution (25 mL) at room temperature (ca. 25 °C) under a nitrogen atmosphere (Foo et al., 1989). The reaction was monitored by TLC (silica gel, benzene−acetone−methanol, 6:4:1).

Source of Plant Material. *Onobrychis viciifolia* (var. Cotswold Common) was sown in September 1995 on an alkaline, free-draining soil at Broadwell near Lechlade, Gloucestershire, U.K., and harvested by hand on May 1, 1996 from a 30 cm stand at 5 cm above ground, air-dried at r.t., and ground (<1 mm). The expected yield of the Cotswold Common variety of sainfoin is 7 t/ha (Ian Wilkinson, Cotswold Seeds Ltd., personal communication). General agronomic details on sainfoin cultivation have been reported (Hill, 1998). Seeds of the pure landrace, Cotswold Common, are being maintained by Cotswold Seeds Ltd., Moreton-in Marsh, Gloucestershire, U.K.

Extraction and Fractionation. Leaf and stem samples (1.658 kg) were extracted with chloroform (5 × 9 L) at r.t. to remove chlorophyll. The plant material was air-dried and extracted with acetone−water (7:3; 6 × 9 L) for 48 h at 25 °C. Extracts were combined and concentrated under reduced pressure at 35 °C. Water was removed by freeze-drying which yielded a yellow-green powder (488.0 g, 29.4% of total leaf and stem material). The dried material was extracted for 24 h at r.t. with methanol (5 × 2.5 L) in order to separate monomeric and polymeric compounds. The methanol extracts were combined and solvent removed at r.t. yielding a brown-yellow powder (441.0 g) of phenolic monomers. Part of this material (42.0 g) was dissolved in 100 mL of the lower phase of water−butan-2-ol−hexane (5:4:1) and placed into the first of a 20 tube Craig countercurrent distribution setup. This was repeated 5 times. After 2-dimensional PC, the contents of the tubes were combined into 3 fractions, which were dried in a stream of air to give fractions 1 (tube 1, 131.7 g), 2 (tubes 2−13, 60.5 g), and 3 (tubes 14−20, 12.8 g).

The residue (47.0 g) remaining from the methanol extraction was extracted further with water yielding a red-brown powder after freeze-drying (38.4 g, 7.8% of the total acetone−water extract). Two-dimensional PC showed that this fraction had a high concentration of polymeric material. The remainder (8.6 g) did not dissolve in any solvent and was discarded as it did not give recognizable products when cleaved with phloroglucinol/H^+.

1. Isolation of Monomeric Compounds. Fraction 2 (21.0 g) was further fractionated on Sephadex LH-20 (150 × 5 cm column) with ethanol−water (65:35) at a flow rate of 30 mL/h. This resulted in 14 fractions after TLC in benzene−acetone−methanol (5:4:1): fraction A1 (tubes 16−48; 1.3 g), A2 (tubes 49−56; 0.233 g), A3 (tubes 57−68; 0.543 g), A4 (tubes 69−92; 7.6 g), A5 (tubes 93−100; 2.0 g), A6 (tubes 101−112; 1.1 g), A7 (tubes 113−128; 0.128 g), A8 (tubes 129−132; 2.3 g), A9 (tubes 133−148; 0.65 g), A10 (tubes 149−210; 1.3 g), A11 (tubes 211−225; 0.358 g), A12 (tubes 226−240; 0.053 g), A13 (tubes 241−250; 0.138 g), A14 (tubes 251−280; 0.031 g).

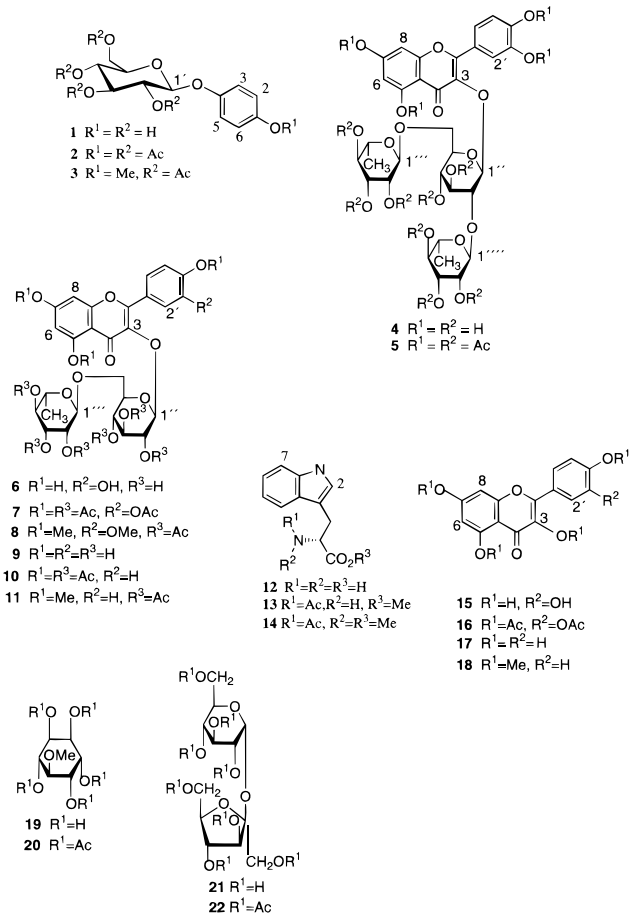

Figure 1. Structure of polyphenols and other natural products from sainfoin: arbutin (**1**); flavonol glycosides **4**, **6**, and **9**; tryptophan (**12**); flavonols **15** and **17**; (+)-pinitol (**19**); sucrose (**21**).

Fraction 1 (21.0 g) was separated under the same conditions as fraction 2 and yielded 11 fractions: B1 (tubes 1−48; 0.242 g), B2 (49−60; 6.2 g), B3 (61−84; 9.4 g), B4 (85−100; 1.7 g), B5 (101−112; 0.552 g), B6 (113−155; 0.566 g), B7 (156−175; 0.104 g), B8 (176−200; 0.278 g), B9 (201−215; 0.140 g), B10 (216−230; 0.083 g), B11 (231−260; 0.041 g).

Fraction A1 (50 mg) was acetylated and purified by preparative TLC (benzene−acetone, 8:2) to yield a white solid, which was identified as 1,2′,3′,4′,6′-penta-*O*-acetylarbutin (**2**; R_f 0.5, 11.2 mg) (Arend et al., 2000) (refer to Figure 1 for the structures of compounds **1**−**22**). ^{1}H NMR ($CDCl_3$) (δ_H): 7.02 (4H, s, H-2, -3, -5, -6), 5.05 (1H, d, J = 8.0 Hz, H-1′), 5.25−5.35 (2H, m, H-2′, H-3′), 5.19 (1H, t, J = 9.5 Hz, H-4′), 3.85 (1H, ddd, J = 2.5, 5.5 and 9.5 Hz, H-5′), 4.31 (1H, dd, J = 5.5 and 12.5 Hz, H-6′), 4.18 (1H, dd, J = 3.0 and 12.0 Hz, H-6′), 2.31 (3H, s, OAc), 2.10 (3H, s, OAc), 2.09 (3H, s, OAc), 2.07 (3H, s, OAc), 2.05 (3H, s, OAc).

Fraction A5 (100 mg) was acetylated and purified by TLC (benzene−hexane−acetone−ethyl acetate, 4:3:2:1, 2×) to give three bands at R_f 0.53 (10.1 mg), 0.33 (27.8 mg), and 0.25 (6.2 mg). The R_f 0.53 band gave the peracetate **5** of quercetin-3-(2^G-rhamnosyl-rutinoside) (**4**) (Buttery and Buzzell, 1975). ^{1}H NMR ($CDCl_3$) (δ_H): A-ring, 7.32 (1H, d, J = 2.0 Hz, H-8), 6.85 (1H, d, J = 2.0 Hz, H-6); B-ring, 7.96 (1H, dd, J = 2.0 and 8.0

Hz, H-6′), 7.92 (1H, d, J = 2.0 Hz, H-2′), 7.36 (1H, d, J = 8.0 Hz, H-5′); glucose, 5.58 (1H, d, J = 8.0, H-1″), 5.32 (1H, t, J = 10.0 Hz, H-3″), 4.88 (1H, t, J = 10.0 Hz, H-4″), 3.83 (1H, dd, J = 8.0 and 10.0 Hz, H-2″), 3.55−3.615 (1H, m, H-5″), 3.54 (1H, dd, J = 3.0 and 11.0 Hz, H-6″), 3.21 (1H, dd, J = 5.8 and 11.0 Hz, H-6″); rhamnose, 5.10 (1H, dd, J = 2.0 and 4.0 Hz, H-2‴), 5.07 (1H, dd, J = 4.0 and 10.0 Hz, H-3‴), 4.94 (1H, t, J = 10.0 Hz, H-4‴), 4.49 (1H, d, J = 2.0 Hz, H-1‴), 3.62 (1H, dd, J = 6.0 and 9.0 Hz, H-5‴), 1.02 (3H, d, J = 6.3 Hz, H-6‴); rhamnose, 5.45 (1H, dd, J = 3.5 and 10.0 Hz, H-3⁗), 5.09 (1H, dd, J = 2.0 and 3.5 Hz, H-2⁗), 5.05 (1H, t, J = 10.0 Hz, H-4⁗), 4.94 (1H, d, J = 2.0 Hz, H-1⁗), 4.43 (1H, dd, J = 6.5 and 10.0 Hz, H-5⁗), 0.905 (3H, d, J = 6.3 Hz, H-6⁗); aromatic OAc, 2.495 (3H, s), 2.355 (3H, s), 2.345 (3H, s), 2.32 (3H, s); aliphatic OAc, 2.15 (3H, s), 2.125 (3H, s), 2.10 (3H, s), 2.07 (3H, s), 2.02 (3H, s), 2.015 (3H, s), 1.94 (3H, s), 1.93 (3H, s).

The R_f 0.33 band comprised the peracetate **7** of rutin (**6**). ^{1}H NMR ($CDCl_3$) of **7** (δ_H): A-ring, 7.33 (1H, d, J = 2.0 Hz, H-8), 6.85 (1H, d, J = 2.0 Hz, H-6); B-ring, 7.97 (1H, dd, J = 2.0 and 8.0 Hz, H-6′), 7.92 (1H, d, J = 2.0 Hz, H-2′), 7.36 (1H, d, J = 8.0 Hz, H-5′); glucose, 5.435 (1H, d, J = 8.0, H-1″), 5.295 (1H, t, J = 10.0 Hz, H-3″), 5.19 (1H, dd, J = 8.0 and 10.0 Hz, H-2″), 4.96 (1H, t, J = 10.0 Hz, H-4″), 3.50−3.70 (1H, m, H-5″), 3.50−3.70 (1H, m, H-6″) and 3.28 (1H, dd, J = 6.0 and 12.0 Hz, H-6″); rhamnose, 5.10 (1H, dd, J = 2.0 and 3.5 Hz, H-2‴), 5.09 (1H, dd, J = 4.0 and 8.0 Hz, H-3‴), 4.96 (1H, dd, J = 8.0 and 11.5 Hz, H-4‴), 4.53 (1H, d, J = 2.0 Hz, H-1‴), 3.55−3.70 (1H, m, H-5‴), 1.075 (3H, d, J = 6.3 Hz, H-6‴); aromatic OAc, 2.46 (3H, s), 2.365 (3H, s), 2.355 (3H, s), 2.315 (3H, s); aliphatic OAc, 2.16 (3H, s), 2.11 (3H, s), 2.04 (3H, s), 1.975 (3H, s), 1.96 (3H, s).

The R_f 0.25 band comprised the peracetate **10** of afzelin (**9**). ^{1}H NMR ($CDCl_3$) of **10** (δ_H): A-ring, 7.318 (1H, d, J = 2.0 Hz, H-8), 6.85 (1H, d, J = 2.0 Hz, H-6); B-ring, 8.06 (2H, d, J = 9.0 Hz, H-2′ and H-6′), 7.24 (1H, d, J = 9.0 Hz, H-3′ and H-5′); glucose, 5.45 (1H, d, J = 8.0, H-1″), 5.30 (1H, t, J = 9.5 Hz, H-3″), 5.173 (1H, dd, J = 8.0 and 10.0 Hz, H-2″), 4.975 (1H, t, J = 10.0 Hz, H-4″), 3.52−3.70 (1H, m, H-5″), 3.52−3.70 (1H, m, H-6″), 3.22 (1H, dd, J = 6.0 and 11.0 Hz, H-6″); rhamnose, 5.12 (1H, dd, J = 2.0 and 3.5 Hz, H-2‴), 5.11 (1H, dd, J = 3.5 and 8.0 Hz, H-3‴), 4.97 (1H, dd, J = 8.0 and 10.0 Hz, H-4‴), 4.51 (1H, d, J = 2.0 Hz, H-1‴), 3.52−3.70 (1H, m, H-5‴), 1.07 (3H, d, J = 6.3 Hz, H-6‴); aromatic OAc, 2.467 (3H, s), 2.358 (3H, s), 2.332 (3H, s); aliphatic OAc, 2.15 (3H, s), 2.112 (3H, s), 2.044 (3H, s), 2.039 (3H, s), 1.98 (3H, s), 1.97 (3H, s).

Fraction A9 (232.7 mg) was methylated and separated by preparative TLC (benzene−acetone−methanol, 5:4:1) to yield four fractions: A9.1 (R_f 0.10−0.20, 11 mg), A9.2 (R_f 0.20−0.30, 15 mg), A9.3 (R_f 0.38−0.50, 14 mg), A9.4 (R_f 0.60−0.80, 10 mg).

Fraction A9.1 was acetylated and purified by TLC (benzene−hexane−acetone−ethyl acetate, 4:3:2:1) to give two fractions. Fraction A9.1.1 gave *N*-acetyl-L-tryptophan methyl ester (**13**, R_f 0.14, 7.2 mg), and fraction A9.1.2, *N*-acetyl-*N*-methyl-L-tryptophan methyl ester (**14**, R_f 0.2, 1.8 mg) (Mandava et al., 1974).

Fraction A9.2 was acetylated and purified by TLC (benzene−hexane−acetone−ethyl acetate, 4:3:2:1) to give 2″,2‴,3″,3‴,4″,4‴-hexa-*O*-acetyl-3′,4′,5,7-tetra-*O*-methylrutin (**8**, R_f 0.25, 10.2 mg). ^{1}H NMR ($CDCl_3$) of **8** (δ_H): A-ring, 6.53 (1H, d, J = 2.5 Hz, H-8), 6.38 (1H, d, J = 2.5 Hz, H-6); B-ring, 7.74−7.69 (2H, second-order spectrum, H-2′ and H-6′), 7.01 (1H, d, J = 9.0 Hz, H-5′); glucose, 5.85 (1H, d, J = 8.0, H-1″), 5.29 (1H, t, J = 9.5 Hz, H-3″), 5.18 (1H, dd, J = 8.0 and 10.0 Hz, H-2″), 5.005 (1H, dd, J = 9.5 and 10.0 Hz, H-4″), 3.60−3.75 (1H, m, H-5″), 3.595 (1H, dd, J = 3.0 and 11.5 Hz, H-6″) and 3.40 (1H, dd, J = 5.5 and 11.5 Hz, H-6″); rhamnose, 5.115 (1H, dd, J = 4.0 and 10.0 Hz, H-3‴), 5.08 (1H, dd, J = 2.0 and 4.0 Hz, H-2‴), 4.95 (1H, t, J = 10.0 Hz, H-4‴), 4.52 (1H, d, J = 2.0 Hz, H-1‴), 3.60−3.75 (1H, m, H-5‴), 1.05 (3H, d, J = 6.3 Hz, H-6‴); aromatic OMe, 4.03 (3H, s), 3.995 (3H, s), 3.98 (3H, s), 3.92 (3H, s); aliphatic OAc, 2.11 (3H, s), 2.08 (3H, s), 2.065 (3H, s), 2.03 (3H, s), 1.995 (3H, s), 1.95 (3H, s).

Fraction A9.3 was acetylated and purified by TLC (benzene−acetone, 8:2) to give 2″,2‴,3″,3‴,4″,4‴-hexa-*O*-acetyl-4′,5,7-tri-*O*-methylafzelin (**11**) as a white amorphous solid (R_f 0.40, 9.2 mg). ^{1}H NMR ($CDCl_3$) of **11** (δ_H): A-ring, 6.53 (1H, d, J = 2.0 Hz, H-8), 6.37 (1H, d, J = 2.0 Hz, H-6); B-ring, 8.065 (2H, d, J = 9.5 Hz, H-2′ and H-6′), 7.02 (2H, d, J = 9.5 Hz, H-3′ and H-5′); glucose, 5.80 (1H, d, J = 8.0 Hz, H-1″), 5.28 (1 H, t, J = 10.0 Hz, H-3″), 5.25 (1H, dd, J = 8.0 and 10.0 Hz, H-2″), 5.08 (1H, t, J = 9.5 Hz, H-4″), 3.55−3.75 (1H, m, H-5″), 3.55−3.75 (1H, m, H-6″), 3.36 (1H, dd, J = 5.0 and 12.0 Hz, H-6″); rhamnose, 5.13 (1H, dd, J = 4.0 and 10.5 Hz, H-3‴), 5.10 (1H, dd, J = 2.0 and 4.0 Hz, H-2‴), 4.95 (1H, t, J = 10.0 Hz, H-4‴), 4.49 (1H, d, J = 2.0 Hz, H-1‴), 3.55−3.75 (1H, m, H-5‴), 1.055 (3H, d, J = 6.3 Hz, H-6‴); aromatic OMe, 3.985 (3H, s), 3.91 (3H, s), 3.90 (3H, s); aliphatic OAc, 2.12 (3H, s), 2.085 (3H, s), 2.06 (3H, s), 2.035 (3H, s), 1.99 (3H, s), 1.96 (3H, s).

Fraction A9.4 was acetylated and purified by preparative TLC (benzene−hexane−acetone−ethyl acetate, 4:3:2:1) to give 2′,3′,4′,6′-tetra-*O*-acetyl-1-*O*-methylarbutin (**3**) as a white amorphous solid (R_f 0.6, 6.9 mg). ^{1}H NMR ($CDCl_3$) (δ_H): 6.96 (2H, d, J = 9.0 Hz, H-2 and H-6), 6.83 (2H, d, J = 9.0 Hz, H-3 and H-5); glucose, 4.97 (1H, d, J = 8.0 Hz, H-1′), 5.24−5.34 (2H, m, H-2′, H-3′), 5.18 (1H, t, J = 9.5 Hz, H-4′), 4.31 (1H, dd, J = 5.5 and 12.5 Hz, H-6′), 4.18 (1H, dd, J = 2.5 and 12.5 Hz, H-6′), 3.82 (1H, ddd, J = 2.5, 5.5 and 9.5 Hz, H-5′), 3.79 (3H, s, OMe), 2.10 (3H, s, OAc), 2.09 (3H, s, OAc), 2.06 (3H, s, OAc), 2.05 (3H, s, OAc). MS: m/z 331 (34.2%) [M − 4-methoxyphenol]$^+$, 124 (40.5%), 109 (80.2%).

Fraction A10 (50.0 mg) was acetylated and purified by TLC (benzene−acetone, 8:2) to give a further portion (R_f 0.33, 12.1 mg) of 2″,2‴,3′,3″,3‴,4′,4″,4‴,5,7-deca-*O*-acetylrutin (**7**).

Fraction A11 (50.0 mg) was acetylated and purified by TLC (benzene−hexane−acetone−ethyl acetate, 4:3:2:1, 2×) to give two fractions. Fraction A11.1 yielded 2″,2‴,3″,3‴,4′,4″,4‴,5,7-nona-*O*-acetylafzelin (**10**; R_f 0.25, 15.5 mg), and fraction A11.2 yielded an additional crop of **7** (R_f 0.33, 9.1 mg).

Fraction A12 yielded the free phenol, quercetin (**15**), as a lightgreen amorphous solid (Billeter et al., 1991). Acetylation of fraction A12 (30.0 mg), followed by TLC (benzene−acetone, 8:2), gave 3,3′,4′,5,7-penta-*O*-acetylquercetin (**16**) as a white amorphous solid (R_f 0.5, 15.7 mg).

Fraction A13 (20 mg) was methylated and purified by TLC (benzene−acetone, 8:2) to give the permethyl ether **18** of kaempferol (**17**) as a yellow amorphous solid (R_f 0.5, 7.2 mg) (Batterham and Highet, 1964).

Fraction B3 (100 mg) was acetylated and purified by preparative TLC (benzene−acetone, 8:2) to give two fractions. Fraction B3.1 (R_f 0.63, 11.3 mg) gave the peracetate **20** of (+)-pinitol (**19**) as a colorless oil (Angyal and Odier, 1983). Fraction B3.2 (R_f 0.38, 18.4 mg) comprised the peracetate **22** of sucrose (**21**) as a colorless oil (Buckingham, 1984). Compounds **20** and **22** were also obtained after acetylation of fraction A4.

All remaining fractions were examined by TLC and shown to contain no compounds other than the ones described above.

2. Purification and Cleavage of the Polymeric Fraction. A portion (13.9 g) of the water-soluble polymeric material was fractionated on Sephadex LH-20 (150 × 4 cm; flow rate 30 mL/h) in ethanol−water (50:50) which gave 8 fractions: C1 (tubes 1−60, 2.907 g), C2 (61−80, 1.242 g), C3 (81−112, 0.587 g), C4 (113−148, 0.240 g), C5 (149−160, 0.059 g), C6 (161−212, 0.737 g), C7 (213−264, 0.193 g), C8 (265−328, 0.180 g). Fraction C9 was eluted with acetone−water (50:50) yielding 1.373 g.

The acid-catalyzed cleavage products of the tannins (456 mg of fraction C3 with 410 mg of phloroglucinol; 382 mg of fraction C9 with 310 mg of phloroglucinol) were purified on Sephadex LH-20 columns (3 × 100 and 75 cm, respectively) with ethanol (30 mL/h). This gave fractions C3.1 (tubes 1−6, unreacted phloroglucinol), C3.2 (7−20), C3.3 (21−33), C3.4 (34−45), C3.5 (46−68), and C3.6 (69−97) and fractions C9.1 (tubes 1−11, unreacted phloroglucinol), C9.2 (12−33), C9.3 (34−44), C9.4 (45−54), C9.5 (55−82), and C9.6 (83−112). Owing to very limited yields and to reduce the risk of decomposition these fractions were directly acetylated.

After acetylation, fractions C3.2−C3.6 and C9.2−C9.6 were further purified by TLC (benzene−acetone, 8:2). Fractions C3.2

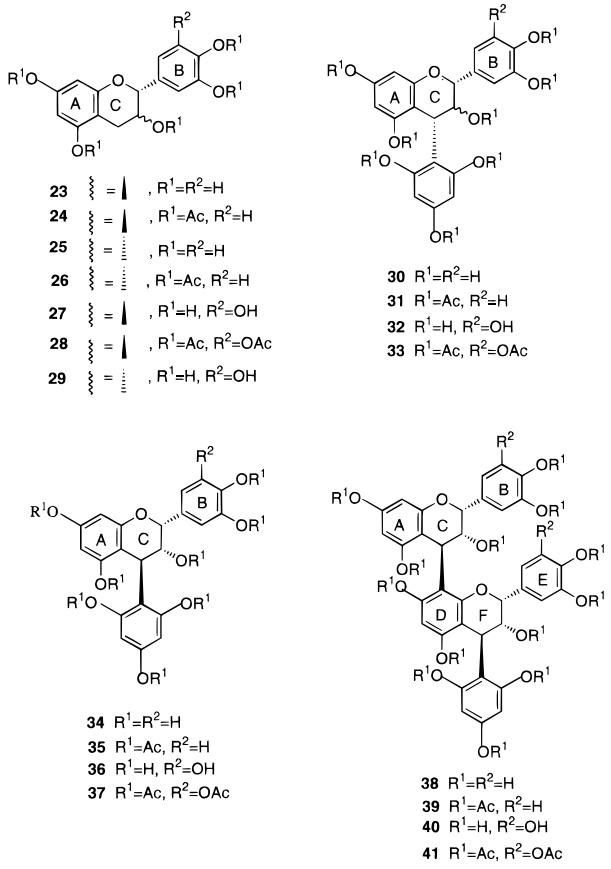

Figure 2. Structures of the sainfoin tannin degradation products: flavan-3-ols **23**, **25**, **27**, and **29**; 4-arylflavan-3-ols **30**, **32**, **34**, and **36**; 4-arylbiflavan-3-ols **38** and **40**.

and C9.2 gave the peracetates **24** and **26** of (+)-catechin (**23**) (1.9 and 8.5 mg, respectively) (refer to Figure 2 for the structures of compounds **23**−**41**) and (−)-epicatechin (**25**) (2.2 and 12.4 mg) and the peracetate **28** of (+)-gallocatechin (**27**) (1.8 and 6.2 mg); fractions C3.3 and C9.3 gave the peracetate **32** of (+)-gallocatechin-(4α→2)-phloroglucinol (**31**) (10.9 and 9.1 mg). Fractions C3.4 and C9.4 gave the peracetates **30** and **34** of (+)-catechin-(4α→2)-phloroglucinol (5.8 and 7.8 mg) and (−)-epicatechin-(4*β*→2)-phloroglucinol (**33**) (2.0 and 68.7 mg). Fractions C3.5 and C9.5 gave the peracetate **36** of (−)-epigallocatechin-(4*β*→2)-phloroglucinol (10.2 and 43.1 mg), and fractions C3.6 and C9.6 gave a mixture of the peracetates **38** and **40** of the dimeric phloroglucinol adducts **38** and **40** (3.5 and 2.8 mg). The structures of all these compounds were unequivocally assigned by comparison of ^{1}H NMR and CD data with those of authentic samples from our collection of reference compounds.

RESULTS AND DISCUSSION

The acetone/water extract of the sainfoin (*Onobrychis viciifolia*) plants afforded a complex mixture of phenolic and nonphenolic material. This necessitated extensive enrichment and fractionation procedures using Craig countercurrent distribution, Sephadex LH-20 gel chromatography, and final purifications by derivatization and preparative TLC. Owing to the number of steps required for purification (cf. Materials and Methods), quantification of the constituents was not possible. The following classes of compounds were detected: the phenolic glucoside (arbutin) **1**; the nonreducing disaccharide (sucrose) **21**; the inositol [(+)-pinitol] **19**; the amino acid (L-tryptophan) **12**, two flavonols (kaempferol and quercetin) **17** and **15**; several flavonol glycosides **4**, **6**, and **9**; a mixture of procyanidin- and prodelphinidin-type condensed tannins.

4-(*β*-D-Glucopyranosyloxy)phenol. Arbutin was isolated from sainfoin and identified as two different derivatives, 1,2′,3′,4′,6′-penta-*O*-acetylarbutin (**2**) and 2′,3′,4′,6′-tetra-*O*-acetyl-1-*O*-methylarbutin (**3**). The first fraction from Craig countercurrent distribution yielded **3** in considerable quantity, whereas **2** was isolated from a mixture of several compounds after methylation and required extensive purification. The ^{1}H NMR spectrum of **3** showed the AA′BB′ spin system (J = 9.0 Hz) for the phenolic ring and a *β*-D-glucopyranosyl moiety. Comparison with published data (Ravn et al., 1990) confirmed an *O*-glucoside ($^3J_{1'-2'}$ = 7−8 Hz) rather than a *C*-glucoside ($^3J_{1'-2'} \sim$ 10.0 Hz). The ^{1}H NMR spectrum of the peracetate **2** showed the aromatic protons as a four proton singlet (*δ* 7.02, s). MS analysis yielded the phenolic ions at *m*/*z* 109 (80.2%) and 124 (40.5%) plus the 1-deoxyglucosyl ion at 331 (34.2%, M − 4-methoxyphenol). This also confirmed the existence of the C−O glycosidic link. Although these two compounds were not quantified accurately, they accounted for a considerable proportion of the phenolic constituents in sainfoin.

Carbohydrates. (+)-Pinitol (**19**) and sucrose (**21**) were identified by comparison of the ^{1}H NMR data of their *O*-acetyl derivatives **20** and **22** with those of the same derivatives of commercially available reference compounds. (+)-Pinitol is one of the most common inositols in plants and occurs in considerable concentrations in the leaves and wood of most gymnosperms and several other plant families (Dittrich and Korak, 1984). Sucrose was the main metabolite, comprising approximately 35% of the total extract. This may in part contribute to the high voluntary intake, which is typical of sainfoin (Karnezos et al., 1994). The water-soluble carbohydrate content in four different sainfoin varieties ranged from 41 to 101 g/kg (data not shown), which is in line with that of other fodder legumes, i.e., 51−109 g/kg (McDonald et al., 1991). Depending on the season, sucrose is the major water-soluble carbohydrate in many fodder legumes (McDonald et al., 1991).

Amino Acids. L-Tryptophan occurs widely in nature (Buckingham, 1994) and has also previously been found in sainfoin. The compound **12** was identified as derivatives **13** and **14** after methylation and acetylation by ^{1}H and ^{13}C NMR, MS, and CD comparisons with those of authentic samples prepared by similar derivatization of the commercial product (Mandava et al., 1974). A general doubling/broadening was clearly noticeable of the H-1, H-2, H-1′, and H-2′ resonances in the ^{1}H NMR spectrum of derivative **13** and may be ascribed to amide−iminol tautomerism. The mass spectrum of **13** gave prominent ions at *m*/*z* 130 (100%), which is typical of tryptophan derivatives (Jamieson and Hutzinger, 1970), and *m*/*z* 201 (75%) [M − COOMe]$^+$ together with the molecular ion at *m*/*z* 260 (7.1%). The CD spectrum showed a positive Cotton effect between 221 and 232 nm, which is in agreement with the chiroptical properties of authentic *N*-acetyl-L-tryptophan methyl ester. The ^{1}H NMR spectrum of **14** differed from that of **13** by the absence of the amide-H at *δ* 6.0 and presence of the N−Me group (*δ* 2.85 and 2.94). The H-1′ and H-2′ resonances of derivative **14** are conspicuously duplicated, presumably as a result of an induced diastereotopic effect between the stereogenic carbon and the amide nitrogen atom.

Flavonols. Two common flavonols, quercetin (**15**) and kaempferol (**17**), were obtained in relatively pure form. Quercetin was identified by ^{1}H NMR spectroscopy of the free phenol and as the per-*O*-acetate derivative, 3,3′,4′,5,7-penta-*O*-acetylquercetin (**16**) (Finger et al., 1991). Kaempferol (**17**) was identified after methylation as 3,4′,5,7-tetra-*O*-methylkaempferol (**18**) by ^{1}H NMR (Batterham and Highet, 1964).

Flavonol Glycosides. Several closely related glycosides were difficult to purify and tended to occur in varying proportions as mixtures across different fractions from the Craig countercurrent distribution. Two related flavonol disaccharides, afzelin (kaempferol-3-*O*-rutinoside) (**9**) and rutin (quercetin-3-*O*-rutinoside) (**6**) were identified after acetylation as the per-*O*-acetate derivatives, 2″,2‴,3″,3‴,4′,4″,4‴,5,7-nona-*O*-acetylafzelin (**10**) and 2″,2‴,3′,3″,3‴,4′,4″,4‴,5,7-deca-*O*-acetylrutin (**7**). They were also identified after methylation and acetylation as the hexa-*O*-acetyl methyl derivatives, 2″,2‴,3″,3‴,4″,4‴-hexa-*O*-acetyl-4′,5,7-tri-*O*-methylafzelin (**11**) and 2″,2‴,3″,3‴,4″,4‴-hexa-*O*-acetyl-3′,4′,5,7-tetra-*O*-methylrutin (**8**).

The ^{1}H NMR data of the rutin derivatives **7** and **8** were identical with those of similar derivatives of an authentic sample. The ^{1}H NMR spectra of **10** and **11** are almost identical with those of compounds **7** and **8** except that the B-ring protons resonate as an AA′BB′ spin system (J = 9.0 Hz) typical of a *p*-substituted aromatic ring.

A branched trisaccharide derivative was also isolated and identified as the per-*O*-acetyl derivative, 2‴,2⁗,3′,3″,3‴,4′,4″,4‴,4⁗,5,7-dodeca-*O*-acetyl-quercetin-3-(2^G-rhamnosylrutinoside) (**5**). The only other reported finding of this branched sugar is by Buttery and Buzzell (1975) in soyabean leaves. The ^{1}H NMR spectra of compounds **5** and **7** showed great similarities. This suggested that *O*-α-L-rhamnosyl-(1→6) glucosyl unit of **7** was also present in **4**. The second rhamnosyl unit showed identical coupling constants as that of the rutinose unit with slight variation in chemical shifts. The most noticeable difference was in the shift of the glucose H-2″ (δ 3.83) compared to that of compound **7** (δ 5.19) or compound **8** (δ 5.18). This suggested that the second rhamnosyl unit was probably linked to C-2 of the glucose. Long distance coupling ($^4J_{H-1‴''-H-2''}$) in a 2D COSY experiment confirmed the glucose-(2→O→1)-rhamnose linkage. The α-configuration of the rhamnose units was confirmed by the $^3J_{HH}$ coupling constants (2.0 Hz) between both sets of H-1‴ and H-2‴ (J = 2.0 and 2.0 Hz) and H-2‴ and H-3‴ (J = 4.0 and 3.5 Hz). Similarly a β-D-glucopyranosyl moiety was confirmed by $^3J_{HH}$ = 8.0 Hz between H-1″ and H-2″ and 10 Hz between H-2″ and H-3″.

A clear association in the 2D NOESY spectrum between H-2″ and H-6″ of glucose with H-1‴ and H-1⁗ of the rhamnose units, respectively, demonstrated the composition of the 2^G-rhamnosylrutinosyl unit. Furthermore, an association is discernible between glucose H-2″ and B-ring H-2′ and H-6′ protons, which confirms the *O*-glycosidic linkage at C-3.

Polymeric Proanthocyanidins. ^{1}H NMR of the residue from methanol extraction revealed three broad resonances typical of oligomeric or polymeric flavan-3-ols, e.g. A- and B-ring protons resonating at δ 7.3−5.5, H-2 and H-3 protons in the C-ring at δ 5.4−3.2, and the H-4 proton of terminal units at δ 3.0−2.2. This crude tannin fraction was extracted with water. The water-soluble tannins were eluted from Sephadex LH-20 with ethanol/water and then acetone/water yielding a total of nine fractions (see Materials and Methods). Fractions C3 (aqueous ethanol elution) and C9 (aqueous acetone elution) were studied in more detail.

Table 1. Electrospray Mass Spectrometry of Fraction C9 Eluted with Aqueous Acetone from Sephadex LH-20 (CE = Catechin or Epicatechin; GE = Gallocatechin or Epigallocatechin)

m/z values	rel abund (%)	composition
576.7	90.2	2 × CE
592.9	81.7	1 × CE + 1 × GE
608.8	100.0	2 × GE
864.8	61.4	3 × CE
880.8	50.0	2 × CE + 1 × GE
896.7	41.5	2 × GE + 1 × CE
912.7	29.7	3 × GE

Fraction C3 showed better resolved resonances, especially in ^{13}C NMR, than fraction C9. The latter had broad resonances in both ^{1}H and ^{13}C NMR, which suggested that the tannin was of high molecular weight with a coiled tertiary structure (Foo et al., 1982). Fraction C3 was, therefore, considered to be of lower molecular weight than fraction C9 or it implied that fraction C9 possessed a more complex tertiary structure, especially since it could only be obtained by washing the Sephadex column with a highly polar solvent (acetone/water).

Table 1 lists the *m/z* values of several ions obtained by ESI-MS from fraction C9. This relatively soft ionization technique yielded dimeric and trimeric ions. The data demonstrate that sainfoin has heteropolymers containing both catechin/epicatechin (C/E) and gallocatechin/epigallocatechin (GC/EG) units in the same tannin molecule, e.g. ions at *m/z* 592.9 (dimer of one C/E and one GC/EG unit), 880.8 (trimer of two C/E and one GC/EG), and 896.7 (trimer of one C/E and two GC/EG). Several homopolymeric ions, i.e., pure procyanidins (*m/z* 576.7, 864.8) or pure prodelphinidins (*m/z* 608.8, 912.7) were also measured. It is suggested that sainfoin tannins consist of heteropolymers and of homopolymers. However, the data cannot provide unambiguous evidence for the existence of homopolymers, as the homopolymeric ions could also be fragments derived from larger heteropolymers.

The phloroglucinol cleavage products were separated on Sephadex LH-20 and yielded the peracetates of the extender units after acetylation and TLC: the two 2,3-*trans*-3,4-*trans* isomers (+)-catechin-(4α→2)-phloroglucinol (**31**) and (+)-gallocatechin-(4α→2)-phloroglucinol (**33**) and the two 2,3-*cis*-3,4-*trans* isomers (−)-epicatechin-(4β→2)-phloroglucinol (**35**) and (−)-epigallocatechin-(4β→2)-phloroglucinol (**37**). (+)-Catechin (**23**), (−)-epicatechin (**25**), and (+)-gallocatechin (**27**) were present as terminal units and were identified as the peracetates **24**, **26**, and **28**. ^{1}H NMR confirmed the identities of the products by comparison with authentic reference compounds. Where applicable the absolute configuration at C-4 was confirmed by the Cotton effects between 220 and 240 nm in the CD curves of these compounds; a negative effect indicated α-substitution at C-4, and a positive effect, β-substitution (Botha et al., 1981).

In addition, two peracetylated dimers, (−)-epicatechin-(4β→8)-(−)-epicatechin-(4β→2)-phloroglucinol (**39**) and (−)-epigallocatechin-(4β→8)-(−)-epigallocatechin-(4β→2)-phloroglucinol (**41**), were isolated as a mixture but could not be separated by TLC. ^{1}H NMR of the

Table 2. Percentage of Flavan-3-ol Constituent Units (%), Ratios of Delphinidin:Cyanidin (D:C) and Cis:Trans Units in Condensed Tannin Fractions C3 and C9 from Sephadex LH-20 Chromatography

		fraction C3		fraction C9	
type of unit	type of flavan-3-ol	ratios	% of units	ratios	% of units
terminal	catechin		7.1		7.6
	epicatechin		8.3		11.2
	gallocatechin		6.0		4.9
	D:C ratio	28:72		21:79	
	cis:trans ratio	39:61		48:52	
extender	catechin		14.4		4.4
	epicatechin		10.1		40.6
	gallocatechin		25.3		6.7
	epigallocatechin		29.0		24.3
	D:C ratio	69:31		42:58	
	cis:trans ratio	50:50		85:15	
total			100.2		99.7

mixture showed that their two B-ring oxygenation patterns were easily discerned and both dimers had the 2,3-*cis*-3,4-*trans* stereochemistry (J = 2.0 Hz). A 2-D COSY spectrum revealed long distance coupling ($^5J_{HH}$) between H-6 of the A- and D-rings to H-4 of the C- and F-rings, respectively. The interflavanoid bonding was confirmed by long distance coupling ($^5J_{HH}$) between H-4 (C-ring) to H-6 (D-ring, singlet) and between H-4 (F-ring) to the *m*-coupled doublet of the phloroglucinol unit. The absolute configuration of the constituent monomeric units is derived from heterocyclic coupling constants and the high-amplitude positive Cotton effect at ca. 240 nm in the CD spectra of both **39** and **41**.

The terminal units accounted for 21.4% of the flavan-3-ol units in fraction C3 and for 23.7% in fraction C9 (Table 2). This suggested that the degree of polymerization (DP) is 4−5 in fraction C9, corresponding to a molecular weight range of 1152−1530. Since similar values were found for fraction C3, the difference between the fractions most likely resided in a difference of tertiary structure (see above). The relative occurrence of terminal units was similar in fractions C3 and C9, e.g. (−)-epicatechin (**25**) was the major unit (8.3 and 11.2% of the total number of flavan-3-ols, respectively), followed by (+)-catechin (**23**) (7.1 and 7.6%) and (+)-gallocatechin (**27**) (6.0 and 4.9%). However, these two fractions differed markedly in the composition of their extender units. Fraction C3 extender units consisted of (+)-gallocatechin (**27**) (25.3%), (−)-epigallocatechin (**29**) (29%), (+)-catechin (**23**) (14.4%), and (−)-epicatechin (**25**) (10.1%). In contrast, fraction C9 had a much higher proportion of the 2,3-*cis*-flavan-3-ols, (−)-epicatechin (**25**) (40.6%) and (−)-epigallocatechin (**29**) (24.3%), followed by (+)-gallocatechin (**27**) (6.7%) and (+)-catechin (**23**) (4.4%). The calculated delphinidin:cyanidin (D:C) ratios were 60:40 for fraction C3 (terminal units, 28:72; extender units, 69:31) and 36:64 for fraction C9 (terminal units, 21:79; extender units, 42:58).

The calculated ratios of 2,3-cis:2,3-trans were 47:53 for fraction C3 and 76:24 for fraction C9. Cis:trans ratios of terminal units were 39:61 (fraction C3) and 48:52 (fraction C9). Cis:trans ratios of extender units were 50:50 (fraction C3) and 85:15 (fraction C9). These results demonstrate that the two tannin fractions had clearly different compositions. The relationships of the flavanol units in fraction C9 were cis > trans and delphinidin < cyanidin, but in fraction C3 these were cis < trans and delphinidin > cyanidin.

Most previous studies (Table 3) of unfractionated, crude tannins from sainfoin reported more cis than trans and more delphinidin than cyanidin units. Sainfoin tannins appear to have a highly variable composition with cis:trans ratios ranging from 47:53 to 90:10 and D:C ratios from 36:64 to 93:7 (Table 3). The present study conducted on separate tannin fractions revealed that sainfoin possesses a mixture of different tannins. Furthermore, Koupai-Abyazani et al. (1993a,b) demonstrated that the composition varied between cultivars and growth stages. They found that tannins in older leaves had cis < trans and included newly synthesized polymers containing mostly gallocatechin. They concluded that tannin synthesis in sainfoin was a dynamic process, which resulted in a heterogeneous mixture of a large number of molecules of different monomer composition.

The composition of terminal and extension units also seems to be cultivar specific: Foo et al. (1982) found no gallocatechin in terminal units, the present study found no epigallocatechin, but Koupai-Abyazani et al. (1993b) detected all four types of flavan-3-ols. Similarly, Koupai-Abyazani et al. (1993b) found no catechin in extender units, but the present study detected all four types.

Reports on the molecular weights of sainfoin tannins generally range between 1470 and 3780 (Foo et al., 1982; Koupai-Abyazani et al., 1993b) with one major exception; Jones et al. (1976) reported tannins of very high molecular weights and as being extremely polydisperse, which was attributed later either to cultivar, seasonal effects, or an unusual tertiary structure (Foo et al., 1982). The present study confirmed the existence of an additional, highly polymerized fraction, which was soluble in acetone−water (7:3) and methanol but not in water, but this was not investigated further. These studies indicated a degree of polymerization (DP) of 6−12 (Koupai-Abyazani et al., 1993a). Our findings were 1152−1530 Da and a DP of 4−5 for both fractions (fractions C3 and C9). Perhaps such similarities are not surprising as the solubility of the tannins and the

Table 3. Ratios of Cis:Trans and Delphinidin:Cyanidin (D:C) Producing Units in Sainfoin Condensed Tannins and Their Molecular Weights (MW)

ref	cis:trans	D:C	terminal units	extender units	MW
Jones et al. (1976)	nd[c]	81:19	nd	nd	17 000−28 000
Czochanska et al. (1980)	87:13	77:23 to 82:18	nd	nd	nd
Foo et al. (1982)	90:10	77:23	no gc[a]	nd	3300
Koupai-Abyazani et al. (1993a)	77:23 to 88:12	68:32 to 85:15	all[b]	no c[a]	1470−3780
Koupai-Abyazani et al. (1993b)					
young plants	83:17	62:38	all	no c	1620−2070
old plants	48:52	93:7	all	no c	
present study					
fraction C3	47:53	60:40	no eg[a]	all	1152−1530
fraction C9	76:24	36:64	no eg	all	1152−1530

[a] c = catechin, gc = gallocatechin, eg = epigallocatechin. [b] All = all four flavan-3-ols. [c] nd = not determined.

techniques used for their isolation dictate what can be extracted and analyzed.

It may be of interest to note that the literature also reports large variations in the D:C ratios of *Lotus corniculatus* (Foo et al., 1982; Hedqvist et al., submitted for publication). Could it be that the existence of such mixtures of tannins in sainfoin and *L. corniculatus* are the key to their positive nutritional effects? Perhaps mixtures of tannins are better suited to capturing different proteins in the rumen, such as undegraded and partially degraded plant and microbial proteins. Further work is needed to investigate if the excellent nutritional properties of sainfoin are mainly due to the tannins, if these are somehow special, or if additional factors are also important such as the high concentration of sucrose or the particular location of two unique types of tannin cells in the abaxial and adaxial epidermis in *O. viciifolia* and the related *Hedysarum sulfurescens* (Skadhauge et al., 1997).

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