JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Condensed Tannins and Flavonoids from the Forage Legume Sulla (*Hedysarum coronarium*)

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ABSTRACT: The condensed tannin concentrations and composition and the characterization of the phenolic constituents in the leaves of the forage legume sulla (*Hedysarum coronarium*), a biennial forage legume found in temperate agricultural regions, were studied. The colorimetric butanol−HCl assay was used for the quantitation of the seasonal condensed tannin concentrations in the leaves of sulla. Fractionation of extracts on Sephadex LH-20 using step elution with aqueous methanol, followed with aqueous acetone or gradient elution with water, aqueous methanol, and aqueous acetone, gave condensed tannin and flavonoid fractions. The chemical characteristics of the purified condensed tannin fractions were studied by acid-catalyzed degradation with benzyl mercaptan and electrospray ionization mass spectrometry (ESI-MS). Thiolysis revealed that epigallocatechin was the major extender unit (15−75%) while gallocatechin was the major terminal unit (50−66%), thus indicating the extractable sulla condensed tannin fraction as the prodelphinidin type. Condensed tannin oligomers to polymers obtained from Sephadex LH-20 gradient fractions ranged between 2.9 and 46 mDP. The homo- and heterogeneous oligomer ions in condensed tannin gradient fractions detected by ESI-MS ranged from 2 to 10 DP and are consistent with the values obtained by thiolysis (2.9−6.9 DP). Lower molecular weight phenolics, including flavonoids and phenolic acids, were characterized by liquid chromatography atmospheric pressure chemical ionization mass spectrometry (LC-APCI/MS) and ESI/MS/MS on a linear ion trap. The flavonoids extracted with aqueous acetone and methanol from sulla leaves and identified included kaempferol, rutin, quercetin-7-*O*-α-L-rhamnosyl-3-*O*-glucosylrhamnoside, quercetin-3-*O*-α-L-rhamnosyl-7-*O*-glucoside, kaempferol-3-*O*-β-D-glucoside-dirhamnoside, genistein-7-*O*-β-D-glucosyl-6″-*O*-malonate, formononetin-7-*O*-β-D-glucoside-6″-*O*-malonate, and afrormosin and the phenolic acid chlorogenic acid.

KEYWORDS: *Hedysarum coronarium*, sulla, thiolysis, ESI-MS, LC-MS/MS, APCI, butanol-HCl, condensed tannins, flavonoids

INTRODUCTION

The most widely grazed legume forages in New Zealand by ruminants are lucerne and white clover, but the major problem is that these forages have high leaf protein contents compared to that of conventional ryegrass pasture. Rapid fermentation in the rumen leads to protein loss and increased urinary nitrogen excretion to the environment.[1] In addition, these legumes increase the occurrence of bloat, which can be lethal to the animal. To improve performance in ruminants and reduce environmental pollution, nonconventional forages are urgently required. One such approach may be to incorporate forage legumes that contain condensed tannins in farming operations.[2] The condensed tannin concentration of leguminous forages vary from >1 to 12% dry matter (DM).[3] Although condensed tannins are often considered antinutritional, some studies have shown that condensed tannins from leguminous forages such as *Lotus corniculatus* in low to moderate concentrations (3−4% DM) have beneficial effects on ruminants.[4] The effects on ruminants depend on the concentration, chemical structure, and other factors such as different ruminant species, physiological state of the animal, and composition of the diet.[5]

The biennial forage legume sulla (*Hedysarum coronarium*) is native to Eurasia and the Mediterranean region and can tolerate arid environmental extremes.[6] In addition to medium to higher (3−12% DM) condensed tannin concentrations, sulla has a higher nutritive value with a higher crude protein content (14−18% DM), nonstructural carbohydrates (18−25% DM), and lower dietary fiber[7] than white clover or lucerne. It is highly palatable with high voluntary feed intake and high dry matter yields.[8] The potential benefits attributed to sulla condensed tannins have included an increase in the absorption of essential amino acids, live weight gain, milk protein concentration and yield, reduced methane emissions,[9,10] milk and cheese characteristics,[11] improved lamb carcass characteristics and meat quality,[12] primed γδ T cells (Schreurs, personal communication), and reduced nematode burdens in lambs and enhanced host immune response.[13] However, some studies suggest that improvement of protein utilization in sheep may be determined by the chemical structure of the condensed tannins. The high to medium concentration of condensed tannins from sulla act in a manner similar to that for lower concentrations of condensed tannin from *L. corniculatus* by increasing non-ammonia nitrogen outflow from the rumen and increasing the absorption of the essential amino acids from the small intestine.[3] An in vitro study performed by Tanner et al.[14] on purified condensed tannin quantitatively reduced protein

Received: April 13, 2011
Accepted: July 22, 2011
Revised: July 21, 2011
Published: July 22, 2011

foams in a dose-dependent manner, but no significant difference between the compositions of the condensed tannins was observed. In vitro foam production experiments are commonly used in bloat investigations. There is conflicting evidence of the effect of sulla in vivo on nematode worm burdens in sheep, with high efficacy[15] or no significant effect reported.[16] The in vitro data for extractable sulla condensed tannin fractions suggested an effect in several nematode assays.[17] Despite the higher nutritive value of sulla, it has not been widely adopted in farming systems.

While sulla has been evaluated as an alternative forage legume in numerous in vivo trials, the phenolic chemistry has not been studied in detail. The butanol−HCl colorimetric assay has been utilized to quantify condensed tannin concentrations in sulla diets, but no data on the composition of the condensed tannin fraction or other phenolic components are available. Condensed tannins, also known as proanthocyanidins, are oligomers and polymers of flavan-3-ol units. The flavan-3-ol units in several leguminous forages consist of procyanidin (PC) monomers, catechin (C, 2,3-trans) and epicatechin (EC, 2,3-cis) and/or prodelphinidin (PD) monomers, gallocatechin (GC, 2,3-trans), and epigallocatechin (EGC, 2,3-cis) isomers. Many polymers are mixtures containing prodelphinidin and procyanidin units in differing ratios.

Other plant phenolics such as flavonoids, phenolic acids, and their derivatives may affect animal performance. Flavonoids are a large group of lower molecular weight compounds that are further divided into different subclasses of flavanols, flavones, flavanones, flavan-3-ols, isoflavones, neoflavones, and anthocyanidins. Low-molecular-weight phenolics, including flavonoids and phenolic acids, were characterized by liquid chromatography coupled with photodiode array and mass spectrometry.[18]

A detailed investigation of the chemical composition of phenolics and condensed tannins in sulla leaves has not been reported, even though they have superior nutritional properties. In order to fully understand the effects of these compounds upon ruminants, it is necessary to identify their chemical structures. The aim of the present study was to characterize the chemical composition of condensed tannin fractions and other major flavonoids from sulla leaves, in order to develop a better understanding of the nutritional and health benefits in ruminants.

MATERIALS AND METHODS

Chemicals. Analytical grade acetone and dichloromethane and HPLC grade methanol and acetonitrile and ascorbic acid were obtained from BDH, Auckland, New Zealand. Catechin, epicatechin, gallocatechin, epigallocatechin, and dihydroquercetin were obtained from Sigma, St. Louis, MO, USA. Benzyl mercaptan was obtained from Merck, Darmstadt, Germany. Sephadex LH-20 was obtained from Pharmacia, Uppsala, Sweden.

Plant Material. Fresh sulla (*Hedysarum coronarium* cv Grasslands Aokau) plants were collected in different seasons from Aorangi, an AgResearch farm near Palmerston North, New Zealand, in Dec 2001 (summer), Aug 2002 (spring), and Jan 2003 (summer). These samples were kept frozen at −20 °C prior to extraction.

Quantitation of Condensed Tannins by the Butanol−HCl Assay. A three-step extraction procedure was performed according to the method of Terrill et al.[19] for freeze-dried, ground sulla leaves to determine the free, protein-bound, and fiber-bound condensed tannin concentrations. Measurements were performed in duplicate and a mean value reported as a portion (g/kg) of the weight of the dry matter. A purified condensed tannin fraction from sulla was used as a standard for calibration.

Extraction and Isolation. Frozen sulla leaves (1.5 kg) were extracted with water/acetone (3:7 v:v; 3 L) containing ascorbic acid (1 g/L) in a VCM62 Varning blender (AB Hallde Maskiner, Kista, Sweden) for 30 min and strained through cheesecloth to remove plant material. The extract was concentrated in vacuo (40 °C) to remove acetone and the aqueous solution defatted with dichloromethane. The aqueous layer was then concentrated in vacuo to remove residual dichloromethane and partitioned with ethyl acetate.

Step Fractionation. The fractionation on Sephadex LH-20 was carried out according to the method of Meagher et al.[20] for condensed tannin extract (CTE) from fresh-frozen sulla leaves collected in spring and summer. The condensed tannin extract (5 g) was dissolved in aqueous methanol (1:1, 30 mL), loaded onto an SR 25 × 300 Sephadex LH-20 column (Pharmacia, Uppsala, Sweden), and equilibrated with aqueous methanol (1:1). Four fractions SF1−SF4 (150 mL each) were obtained after elution with aqueous methanol (1:1). Elution with aqueous acetone (3:7) yielded two fractions, SF5 (150 mL) and SF6 (350 mL), which were concentrated in vacuo and freeze-dried.

Gradient Fractionation. Fractions were collected by gradient fractionation according to the method of Meagher et al.[20] The condensed tannin extract (17 g) was fractionated onto an SR 25 × 1000 Sephadex LH20 column (Pharmacia, Uppsala, Sweden) equilibrated with water. Fractions were obtained by eluting with water (GF1, 100 mL; GF2, 500 mL), followed by aqueous methanol (3/1, GF3; 1:1, GF4; 1:3, GF5; 500 mL each), followed by methanol (GF6, 250 mL; GF7, 250 mL; GF8, 250 mL; GF9, 450 mL) and finally with aqueous acetone (3;7, GF10, 600 mL; 3:7, GF11, 600 mL). Fractions were concentrated in vacuo and freeze-dried.

The SF1 fraction was further fractionated on a Sephadex LH-20 matrix by using the gradient fractionation method. The fractions were obtained by eluting with water followed by aqueous methanol and finally with aqueous acetone (3:7, SF9, 500 mL). Fractions were concentrated in vacuo and freeze-dried.

Thiolysis of Condensed Tannins. Thiolysis was carried out by a method based on that described by Meagher et al.[20] Condensed tannin samples were freeze-dried and dried over P_2O_5 prior to weighing. A condensed tannin solution (4 mg/mL in methanol) was prepared for each fraction. A subsample (50 μL) was placed in a vial, and to this was added hydrochloric acid in methanol (3.3:96.7 v:v; 50 μL) and benzyl mercaptan in methanol (5:95 v:v; 100 μL). The solution was heated to 40 °C for 30 min in a heating block and left to cool to room temperature. An internal standard (IS), dihydroquercetin in water (100 μL, 5.2×10^{-2} mg/mL solution), was added and a 20 μL subsample analyzed immediately by RP-HPLC. Concentrations of terminal flavan-3-ol units and the extender flavan-3-ol thiol adducts from full thiolysis were estimated by integration at 280 nm relative to dihydroquercetin as the internal standard. Responses relative to dihydroquercetin determined from standards were 0.26 and 0.07, respectively, for terminal procyanidin (PC) and prodelphinidin (PD) flavan-3-ol units. For the extender flavan-3-ol thiol adducts response factors of 0.25 and 0.06, respectively, were determined for procyanidin and prodelphinidin benzyl thioethers relative to dihydroquercetin, in accordance with the published values of Gu et al.[21]

Electrospray Mass Spectrometry of Condensed Tannins. Data were acquired using a Shimadzu LC-MS QP8000 with ESI/MS in scan mode (m/z 250−1400) and detection in the negative ion as outlined previously.[20]

APCI Mass Spectrometry of Phenolics. The flavonoid fractions were subjected to APCI-MS performed on a Shimadzu LC-MS QP8000 α LC system coupled to a single quadrupole equipped with an API source using a chemical ionization (APCI) interface in full scan mode (m/z 100−900) with detection in the positive ion mode. The capillary

Table 1. Condensed Tannin Concentration (w/w DM) by the Butanol−HCl Colorimetric Assay[a]

sample	season	year	total	extractable	protein bound	fiber bound	ref
leaves + soft stems	summer	2003	8.4	6.9	1.4	0.1	
leaves + soft stems	spring	2002	5.5	4.2	1.3	ND	
leaves + soft stems	summer	2001	7.7	5.3	2.0	ND	
leaves	summer	1995	12.5	8.4	4.1	ND	22
stem	summer	1995	3.3	1.3	2.0	ND	22
flower	summer	1995	13.1	7.8	5.3	ND	22
whole plant	summer	1995	7.2	4.1	3.1	ND	23
whole plant	summer	1994	7.2	3.7	3.3	2.0	10
whole plant	autumn	1994	5.1	3.5	1.4	0.2	10
whole plant	spring	1994	8.4	7.3	0.9	0.2	10

[a] ND = not determined, gaps under references are for the experimental data.

temperature was 250 °C, the vaporizer temperature 400 °C, and drying gas (N_2) flow rate 2.5 L/min. The column used was Phenomenex RP (C-18) 150 mm × 4.6 mm i.d. 5 μm: the injection volume was 40 μL, the flow rate was 1 mL/min and the mobile phases were (A) water containing formic acid (0.1%, v/v) and (B) acetonitrile containing formic acid (0.1%, v/v). The gradient was established as follows: 0−5% B, 0−5 min; 5−10% B, 5−10 min; 10−25% B, 10−25 min; 25−35% B, 25−40 min; 35−45% B, 40−48 min; 45−97% B, 48−53 min; linear 97% B, 53−58 min; 97−5% B, 58−65 min. The UV absorbance was monitored between 240 and 420 nm using a Shimadzu PDA detector.

LC/MS/MS of Phenolics. The LC-MS/MS data were acquired on a Surveyor HPLC connected to a [-LTQ] linear ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an electrospray interface operated in positive ion mode. The column used was a 150 mm × 2.1 mm i.d., 5 μm, Phenomenex RP (C-18): the injection volume was 10 μL, the flow rate was 0.2 mL/min, and the mobile phase and gradients were as above. The analysis was performed under the following settings: temperature of the heated capillary set at 275 °C, spray voltage of +4.5 kV, and normalized collision of 35%. The mass spectrometer was programmed to perform a full MS scan (m/z 100−900) followed by three MS/MS scans on the most intense ions from the MS1 spectrum run with the following dynamic exclusion settings: repeat count 2, repeat duration 0.5 min, exclusion duration 2.0 min. An aqueous acetonitrile solution (50/50 v/v) containing flavonoid fractions (100 μg/mL) was used in the analysis.

RESULTS AND DISCUSSION

Seasonal Variation in Extractable Condensed Tannins. Sulla leaves were collected in two consecutive summer seasons (2001 and 2003) and the following spring (2002). The extractable, protein-bound, and fiber-bound condensed tannin concentrations were determined by the butanol−HCl colorimetric method. The results from the present study were compared with literature results (Table 1). The literature values reported are for sulla plant samples also harvested at the AgResearch farm Aorangi, Palmerston North, New Zealand, in 1994 and 1995. Sulla leaves harvested in summer contained condensed tannin concentrations of 7.7 and 8.4% w/w DM, which were higher than those found in the spring, 5.5% w/w DM. The condensed tannins ranged between 3.3 and 13.1% w/w DM, as observed by other researchers, with summer collection[15] reported to contain lower concentrations, 3.3% w/w DM, while leaves and flowers contained higher concentrations during summer.[22] Variation is also dependent on the parts of the plant harvested. As the season changes, the sulla leaf to stem ratio decreased from 8:1 in autumn to 1:1 in spring.[23] The condensed tannin concentration showed a corresponding increase from autumn through summer, suggesting a relationship with maturity. The ratio of extractable to protein-bound condensed tannins varies from 84:16 to 54:46 in the leaves and flowers, but in the stems, the ratio was low (60:40). Higher concentrations of extractable condensed tannins can be detrimental to ruminants because of their ability to inactivate microbial enzymes, but this was not observed for protein-bound condensed tannin. However, the high concentration of protein-bound condensed tannins found in sulla may result in high volatile fatty acid (VFA) production. Protein-bound CT can enhance animal performance by increasing nitrogen supply in the small intestines.[5] A higher intake was observed in ruminants, although the condensed tannin concentration was in the undesirable range >6%.[24]

Extraction and Fractionation. The aqueous and organic layers were concentrated in vacuo and subsequently freeze-dried to yield a condensed tannin extract (CTE) and an ethyl acetate extract (1.5−3%; w/w) of the CTE. The defatted aqueous acetone (3:7) extract of the leaves of sulla contained ca. 2.17% of ethyl acetate extractable materials as compared with ca. 1.7% in *L. pedunculatus* and ca. 4.8% in *L. corniculatus*.[25] Due to the lower yield, the ethyl acetate fraction was not analyzed further. The aqueous layer (condensed tannin extract) was further analyzed for condensed tannins and lower molecular weight phenolic compounds.

For the purification of condensed tannins by step fractionation, the lower molecular weight phenolics are removed first by elution with aqueous methanol (1:1; SF1−4), and the adsorbed condensed tannin-enriched fraction is obtained last by elution with aqueous acetone (3:7; SF5−6). In the present study all fractions eluting from Sephadex LH-20 were analyzed by RP-HPLC with PDA detection at 280 nm, the UV absorption maximum for condensed tannins (data not shown). In addition, a portion of the sulla condensed tannin extract was not adsorbed on Sephadex LH-20 but eluted in the early aqueous methanol (1:1) fraction. A similar effect was observed for *L. pedunculatus*.[20] This fraction (SF1) was rechromatographed on Sephadex LH-20 and eluted with water followed by aqueous methanol (1:1) and finally with aqueous acetone (3:7). Elution with aqueous methanol (1:1) did not yield a high-molecular-weight condensed tannin fraction, in contrast to *L. pedunculatus*, which contained such a fraction (44 mDP). However, subsequent elution with aqueous acetone (3:7) afforded a high-molecular-weight condensed tannin fraction (46 mDP). In *L. pedunculatus* this step provided a condensed tannin fraction of 18 mDP.[20]

The fractionation method produced a linear gradient of fractions (GF6−GF9) which contain monomers (DP 1) and oligomers (DP 2−8) by ESI/MS. The aqueous acetone fractions (GF10 and GF11) were shown to contain medium-molecular-weight condensed tannins (15 and 21 mDP), as determined by thiolysis. RP-HPLC-PDA detection also showed the presence of condensed tannins (broad absorption peak at 280 nm) in the initial water fraction (GF1).

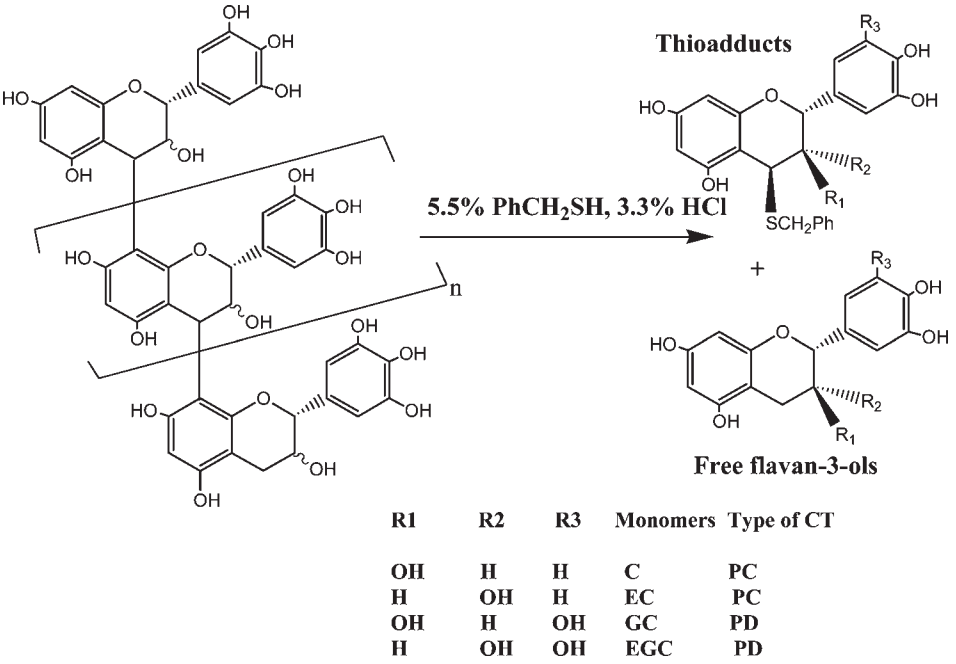

Figure 1. Acid-catalyzed thiolysis degradation of CT polymer using benzyl mercaptan to yield free flavan-3-ols and thioether derivatives.

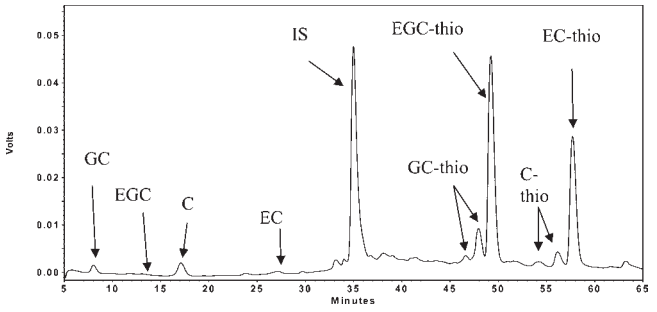

Figure 2. HPLC-UV chromatogram recorded at 280 nm for elution of free flavan-3-ols and thioyl ether (thio) derivatives obtained through thiolysis reactions for a 70% acetone fraction.

Thiolysis of the Proanthocyanidins. This is the first report on the evaluation of condensed tannin fractions from sulla. The thiolysis reaction products (Figure 1) were analyzed using RP-HPLC to determine the nature of each individual units in the polymer, the procyanidin to prodelphinidin ratio, and the mDP (Figure 2). These results are reported along with the composition of condensed tannin fractions from other forage legumes in Table 2. Sulla condensed tannin fractions from both step and gradient fractionations were subjected to thiolytic cleavage. The mean composition of the terminal units can be determined from the ratio of the released monomers, the mean composition of the extender units in the polymer chain from the ratio of benzyl thioether adducts and the mDP from the ratio of monomer to extender units. In the step procedure (SF5−6) gallocatechin was the dominant terminal unit (50−66%) while epigallocatechin was the dominant extender unit (15−75%), characteristic of prodelphinidin predominant condensed tannin fractions. In contrast, prodelphinidin predominant *L. pedunculatus* fractions had catechin as the dominant terminal unit[20] and Marais et al.[26] reported that the forage legume sainfoin condensed tannins had variable prodelphinidin to procyanidin ratios. Thus, the 2,3-cis stereochemistry was predominant in sulla as observed in the case for some forage legume species.

The mean degree of polymerization of gradient fractions (Table 2) of sulla condensed tannins ranged from 2.9 to 21. The prodelphinidin to procyanidin overall ratio remained fairly constant (from 73:27 to 88:12), in contrast to extender unit composition; terminal units were dominated by gallocatechin and ranged from 50 to 59% for the oligomer to polymer fraction. Condensed tannin fractions from *L. corniculatus* grown in Sweden have shown considerable variation, as prodelphinidin to procyanidin ratio ranged from 16:84 to 33:67,[27] and for *L. corniculatus* grown in New Zealand the ratios ranged from 27:73 to 50:50.[28,29]

These thiolysis results suggest that sulla contains extractable condensed tannins with a considerable variation of chain lengths, which are prodelphinidin type and are dominated by cis stereochemistry. The terminal units are comprised predominantly of gallocatechin, whereas for most of the forage legumes investigated in New Zealand, catechin was dominant.

Electrospray Mass Spectrometry of Condensed Tannins. The composition of the condensed tannin fractions was also investigated by ESI/MS. Mass spectrometry can provide useful information on the composition of condensed tannin oligomer mixtures in the form of ion masses corresponding to sets of oligomers of the same DP and composition. The low-molecular-weight condensed tannin oligomers were detected as singly charged ions, but the high-molecular-weight condensed tannin oligomers were detected as doubly and triply charged ions. The step fraction of sulla (SF6) harvested in summer was poorly ionized (mDP 26 from thiolysis), and no ions for the CT polymers were obtained. Hetero-oligomers containing both PC and PD ions were detected from the step fraction (SF6), as shown in Table 3. They were singly charged species m/z 577 $(PC)_2^-$, 593 $(PCPD)^-$, 609 $(PD)_2^-$, 881 $(PC)_2(PD)^-$, 897 $(PC)(PD)_2^-$, and 913 $(PD)_3^-$ and doubly charged species m/z

Table 2. Condensed Tannin Composition of LH-20 Fractions by Thiolysis Reaction Products for the Mean Degree of Polymerization, Percent Contributions of Terminal and Extender Flavan-3-ol Units, and Ratio of Prodelphinidin (PD) to Procyanidin (PC) Units

			amt of terminal (%)				amt of extender (%)					
legume		mDP[a]	GC[b]	EGC[c]	C[d]	EC[e]	GC	EGC	C	EC	PD[f]:PC[g]	ref
					Step Fractions							
H. coronarium												
spring 2002	SF5	12	56	17	26	0	20	67	3	13	84:16	
	SF6	13	66	7	25	3	19	66	3	12	84:16	
summer 2001	SF5	14	53	17	27	3	20	66	3	11	85:15	
	SF6	26	52	22	24	3	16	72	2	10	87:13	
	SF9	46	63	7	28	1	74	15	1	10	89:11	
L. pedunculatus	SF5	12	26	14	46	14	16	68	4	14	80:20	29
	SF7	44	25	11	51	13	13	72	2	13	84:16	29
	SF9	18	23	13	47	17	6	76	3	15	72:21	20
L. corniculatus	SF5	8.7	2	2	75	21	5	39	3	53	40:60	29
					Gradient Fractions							
H. coronarium	GF6	2.9	54	12	30	5	23	53	8	17	73:27	
	GF7	4.1	51	11	32	6	21	55	7	18	73:27	
	GF8	5.0	50	16	29	5	23	54	5	18	75:25	
	GF9	6.9	51	13	32	4	19	59	5	17	76:24	
	GF10	15	59	13	26	2	20	67	3	11	86:14	
	GF11	21	59	12	27	2	14	75	2	9	88:12	

		amt of terminal (%)				amt of extender (%)					
legume	mDP[a]	GC[b]	EGC[c]	C[d]	EC[e]	GC	EGC	C	EC	PD[f]:PC[g]	ref
Onobrychis viciifolia	~5	21	0	32	47	9	32	6	53	36:64	26
	~5	28	0	33	38	32	37	18	13	60:40	26
	56−93	0	20	40	40	ND[h]	ND	ND	ND	77:23	26

[a] mDP = mean degree of polymerisation. [b] GC = gallocatechin. [c] EGC = epigallocatechin. [d] C = catechin. [e] EC = (epi) catechin. [f] PD= prodelphinidin. [g] PC = procyanidin. [h] ND = not detected, gaps under references are for the experimental data.

Table 3. Observed *m/z* Values of CT Ions in Negative ESI-MS Analysis of Sulla Polymer Fractions

DP	ion	step fraction	gradient fraction
2	$[M-H]^-$	577, 593, 609	577, 593, 609
3	$[M-H]^-$	881, 897, 913	865, 881, 897, 913
4	$[M-H]^{2-}$	600, 608	576, 584, 592, 600, 608
5	$[M-H]^{2-}$	744, 752, 760	720, 728, 736, 744, 756, 760
6	$[M-H]^{2-}$	888, 896, 904	881, 888, 896, 912
7	$[M-H]^{2-}$	1052, 1064	ND[a]

[a] ND - not detected.

600 $(PC)(PD)_3^{2-}$, 608 $(PD)_4^{2-}$, 744 $(PC)_2(PD)_3^{2-}$, 752 $(PC)_2(PD)_4^{2-}$, 760 $(PD)_5^{2-}$, 888 $(PC)_3(PD)_3^{2-}$, 896 $(PC)_2(PD)_4^{2-}$, 904 $(PC)(PD)_5^{2-}$, 912 $(PD)_6^{2-}$ 1052 $(PC)(PD)_6^{2-}$, and 1064 $(PD)_7^{2-}$. Similar ions were detected in the sulla fractions (SF5 and SF6) harvested in spring with 12−13 mDP by thiolysis.

When the sulla condensed tannin extract was subjected to gradient fractionation on Sephadex LH20, a series of oligomer fractions were obtained (Table 3). The ESI/MS of the initial GF6 and GF7 fractions were consistent with singly charged species of *m/z* 289 $(PC)^-$, 305 $(PD)^-$, 577 $(PC)_2^-$, 593 $(PC)(PD)^-$, 609 $(PD)_2^-$, 865 $(PC)_3^-$, 881 $(PC)_2(PD)^-$, 897 $(PC)(PD)_2^-$, and 913 $(PD)_3^-$. The doubly charged species were evident in GF8 and GF9. They were tetramers 576 $(PC)_4^{2-}$, 584 $(PC)_3(PD)^{2-}$, 592 $(PC)_2(PD)_2^{2-}$, 600 $(PC)(PD)_3^{2-}$, and 608 $(PD)^{2-}$, pentamers 720 $(PC)_5^{2-}$, 728 $(PC)_4(PD)^{2-}$, 736 $(PC)_3(PD)_2^{2-}$, 744 $(PC)_2(PD)_3^{2-}$, 756 $(PC)(PD)_4^{2-}$, and 760$(PD)_5^{2-}$ and hexamers 881 $(PC)_3^{2-}$ $(PD)_3^{2-}$, 888 $(PC)_2(PD)_4^{2-}$, 896 $(PC)(PD)_5^{2-}$, and 912 $(PD)_6^{2-}$. In addition, doubly charged hexamer or triply charged nonamer (DP_9^{3-}), triply charged decamer (DP_{10}^{3-}), doubly charged heptamer (DP_7^{2-}), and doubly charged octamer (DP_8^{2-}) corresponding to *m/z* 912, 1008, 1056, and 1209 were detected respectively from GF10. These results indicate that sulla consists of homogeneous and heterogeneous condensed tannin oligomers. The validity of the mDP data by thiolysis of condensed tannin oligomer fractions was verified by comparison with ESI-MS spectra. The average mDP values determined from thiolysis were higher than the mass range estimates obtained from ESI-MS for fractions above mDP 5. In the ESI-MS as the DP increased, the number of isomers also increased. As an example, the series of procyanidin homo dimers (*m/z* 577) contain more than eight 4β/α-8-type and four 4β/α-6-type

Table 4. Identified Compounds, *m/z* of Parent Ions, *m/z* of Fragment Ions, and Retention Times As Determined by LC-DAD Using APCI-MS by LC-MS in Positive Ion Mode for Flavonoids in Sulla

identified compd	parent ion $[M + H]^+$	fragment ions $[A + H]^+$	retention times, min
quercetin-hexose-dirhamnoside	757	303	25.4
kaempferol-glucoside-dirhamnoside	741	287	27.83
methoxy-quercetin-glucoside-dirhamnoside	771	317	28.5
rutin (quercetin-3-*O*-rutinoside)	611	303	28.5, 29.2, 29.5
kaempferol-3-*O*-rutinoside	595	287	30.9
3-methoxy-quercetin-rutinoside	625	317	31.9, 32.3
isoquercitrin (quercetin-3-*O*-rhamnoside)	465	303	30.3
hyperoside	465	303	30.6
quercetin-3-glucoronide	479	303	31.2
kaempferol-3-glucoronide	463	287	33.5
formononetin-7-glucoside-6-*O*-malonate	517	269	41.6
afrormosin-4-glucoside-6-*O*-malonate	547	299	43.3
unknown malonate	579	331	36.8
unknown malonate	551	303	32.6
chlorogenic acid	355	ND[a]	16.4, 17.4

[a] ND = not detected.

isomers. As a result, higher DP polymers potentially have very large numbers of stereoisomers; this may reduce sensitivity, and the detection level was limited in ESI-MS.

The linear gradient fractionation provided a range of oligomers in comparison to the step fractionation of the extractable condensed tannin, which provided an understanding of the structural diversities existing in the condensed tannin from sulla. These results suggest that there is a considerable variation in mDP, but the prodelphinidin to procyanidin ratio and the cis to trans ratio of sulla condensed tannin fractions remained constant, with prodelphinidin being the predominant unit.

Characterization of Flavonoids. Twenty major flavonoid derivatives with attached sugars were separated by HPLC from the sulla Sephadex LH20 fraction GF3 to GF7. Identification of the individual compounds was performed by comparison of HPLC retention times and PDA UV/vis and APCI-MS spectrometric data with those of authentic standards or with published data.[18] APCI-LC-MS analysis in the positive ion mode was used to identify the molecular weight (M^+) of the flavonoid glycosides and molecular weight information on the aglycone moiety and to ascertain whether there were sugar conjugates attached as evidenced by $M^+ - 162$ or $M^+ - 146$ loss.[30] However, the exact nature of the sugar moiety cannot be ascertained solely by APCI-MS. The results obtained are summarized with the UV λ_{max} absorbance below and in Table 4. The gradient profile allowed separation of the compounds, where retention based on polarity followed the expected RP-HPLC pattern of flavanol glycoside malonate > flavonol glucoranide > flavonol monoglycosides > flavanol diglycosides > flavanol triglycosides > chlorogenic acid. Mass spectrometric analysis of the peaks eluting at R_t 25.4, 27.83, and 28.5 min identified three flavonol triglycoside protonated molecular ions $(M + H)^+$ present at *m/z* 757, 741, and 771, respectively. The APCI/MS data showed the protonated aglycone $(A + H)^+$ ions at *m/z* 303, 287, and 317, respectively, corresponding to quercetin, kaempferol,[30] and methoxy-quercetin[31] with the serial loss from the $(M + H)^+$ ion of two rhamnose units to yield intermediate ions $(I + H)^+$ and then further loss of a single hexose (glucoside) unit. This indicated that the hexose was attached to the aglycone moiety and the two rhamnose units were attached on the hexose rather than the aglycone. These fragmentations, although ascertained by APCI-MS, are comparable to those observed for authentic standards by ESI-MS.[32] They are tentatively identified as quercetin-glucoside-dirhamnoside, kaempferol-glucoside-dirhamnoside, and methoxy-quercetin-glucoside-dirhamnoside.

Six flavanol diglycosides, including three quercetin-3-*O*-rutinosides (rutin) at *m/z* 611 (R_t = 28.5, 29.2, and 29.5 min), kaempferol-3-*O*-rutinoside at *m/z* 595 (R_t = 30.9 min) and two 3-methoxy-quercetin-rutinosides at *m/z* 625 (R_t = 31.9 and 32.3 min) were tentatively identified. APCI-MS fragmentation patterns have shown that serial loss from the $(M + H)^+$ ion of *m/z* 146 and then at *m/z* 162 yields the corresponding aglycone. The hexose unit was directly attached to the aglycone, and the rhamnose unit position was uncertain. Two monoflavonol glycosides with UV λ_{max} values of 255 and 355 nm eluted at R_t 30.3 and 30.6 min, respectively; the mass spectra, which showed similar fragmentation patterns with a loss at *m/z* 162 from $(M + H)^+$ of *m/z* 465 yielded the $(A - H)^+$ ion at *m/z* 303. This indicated the presence of two quercetin hexosides (isoquercitrin and hyperoside).

Two peaks at R_t 31.2 and 33.5 min were tentatively identified as quercetin-3-glucoronide $(M + H)^+$ at *m/z* 479 and kaempferol-3-glucoronide $(M + H)^+$, *m/z* 463, with loss of glucoronic acid (*m/z* 176) to give the aglycones at *m/z* 303 and 287, respectively. Four different aglycones $(A + H)^+$ at *m/z* 269, 299, 331, and 303 were attached to a malonylhexose moiety (*m/z* 248, serial loss of 44 and 204 or 44, 42, and 162), as observed in red clover malonates,[33] which were identified from the fragmentation of the molecular ion $(M + H)^+$ at *m/z* 517, 547, 579, and 551 at R_t = 41.6, 43.3, 36.8, and 32.6 min, respectively. Along with UV data the aglycone $(A + H)^+$ was identified as formononetin (*m/z* 269, λ_{max} 250, 298 nm), as reported for floral red clover,[33] and quercetin (*m/z* 303, λ_{max} 255, 355 nm), as observed previously in tomato extracts.[34] UV spectra for the other two aglycones at *m/z* 299 and 331 were distorted due to the presence of an additional small peak. Chlorogenic acid eluted early at R_t = 16.4 and 17.4 min with $(M + H)^+$ at *m/z* 355 with a UV absorbance maximum at 325 nm comparable to that reported from cocoa.[35]

Thus, sulla extractable condensed tannins were found to be predominantly prodelphinidin-type, dominated by cis extender units and trans terminal units. Seasonality appeared to have no effect on composition, particularly in relation to prodelphinidin being the predominant unit. Numerous flavonoids extracted with aqueous acetone from sulla leaves were tentatively identified and may well contribute to the beneficial properties for ruminants. The higher nutritive value of sulla and the presence of both condensed tannins and flavonoids indicate incorporation into

pastoral agriculture systems to complement conventional forages and improve animal nutrition and health is worthwhile.

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ACKNOWLEDGMENT

We are grateful to the Nutrition and Behaviour Group at AgResearch Grasslands, particularly Suba Sivakumaran and Paul Spencer for their help and advice on the laboratory work and use of the analytical equipment. Finally, we thank Botswana College of Agriculture for providing funds to O.T.

ABBREVIATIONS

DM, dry matter; mDP, mean degree of polymerization; VFA, volatile fatty acids

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