Research Article

Received: 21 August 2012 Revised: 4 February 2013 Accepted article published: 28 February 2013 Published online in Wiley Online Library: 8 April 2013

(wileyonlinelibrary.com) DOI 10.1002/jsfa.6119

Exploring variation in proanthocyanidin composition and content of sainfoin (*Onobrychis viciifolia*)

Blasius N Azuhnwi,[a,b†] Beat Boller,[c] Frigga Dohme-Meier,[a] Hans D Hess,[a] Michael Kreuzer,[b] Elisabetta Stringano[d‡] and Irene Mueller-Harvey[d*]

Abstract

BACKGROUND: To maximise the potential benefits to ruminants from sainfoin, plant breeding should focus on developing varieties with predictable condensed tannin (CT) profiles. Little is known about whether and to what extent accession and environment influence sainfoin CT structures. We sought to investigate the likely extent of accession and environment effects on CT characteristics of sainfoin. Four single-flowering (Communis) accessions and two multiple-flowering (Bifera) accessions, grown at three sites and collected at two harvest times were used. Sainfoin CTs were characterised by thiolytic degradation and by high-performance liquid chromatography–gel permeation chromatography (HPLC-GPC). Also, CT concentration measured earlier by the HCl–butanol method was compared with that from thiolysis.

RESULTS: Thiolysis revealed that accession and harvest influenced most CT structural attributes. Bifera CTs eluted as single peaks (M_p < 6220 Da) in HPLC-GPC across the two harvests and two sites, whereas Communis generated two to three CT peaks, which included a peak ($M_p \leq$ 9066 Da) in the second harvest. A discrepancy was observed in CT concentrations measured by the two methods.

CONCLUSION: CTs from Bifera accessions had more stable and predictable characteristics across harvests and sites and this could be of interest when breeding sainfoin.

Keywords: sainfoin; accession; harvest; condensed tannins; thiolysis; molecular weight

INTRODUCTION

Sainfoin (*Onobrychis viciifolia*) is a perennial legume native to South Central Asia and was introduced to Central Europe in the 15th century.[1] Until the early 1950s, before the pre-eminence of commercial fertilisers in most farming systems in Europe and North America, sainfoin was a common forage legume and was also used as a ley in order to improve soil fertility. It is drought resistant and possesses beneficial nutritional, veterinary and environmental attributes.[2–5] For these reasons sainfoin has received renewed interest in Europe, Northern America and New Zealand.[6–8]

Condensed tannins (CT), or proanthocyanidins, are widespread plant polyphenols. They are considered to account for some positive nutritional and anthelmintic attributes of sainfoin and several other CT-containing forage legumes. However, attempts to relate responses from feeding tanniniferous forages to CT concentrations have not been particularly successful. This suggests that structural differences in the CT polymers need to be considered in order to explain the variable responses in feeding trials.[9] Differences in CT polymers stem from variations in their monomeric flavanol units (Fig. 1). Variations in monomer units refer to B-ring hydroxylation patterns and affect procyanidin/prodelphinidin (PC/PD) ratio; variation of the stereochemistry at the heterocyclic C-ring affects *cis*/*trans* ratios; and variations of interflavanol linkages and molecular weight (MW) are relevant to oligomers and polymers. These features affect the three-dimensional structure of each molecule and may also influence their biological activities.

Structural analysis is therefore needed in addition to concentration measurements in order to establish tannin structure–activity relationships.[10] Specifically for sainfoin, considerable variation has been reported in terms of CT

* *Correspondence to: Irene Mueller-Harvey, Chemistry and Biochemistry Laboratory, School of Agriculture, Policy and Development, University of Reading, 1 Earley Gate, PO Box 236, Reading RG6 6AT, UK. E-mail: i.mueller-harvey@reading.ac.uk*

† *Current address: IHAP INRA ENVT, 23 chemin des Capelles, BP 87614, 31076 Toulouse Cedex 03, France*

‡ *Present address: Sandoz Biopharmaceuticals and Oncology Injectables, EBEWE Pharma GmbH Nfg. KG, Unterach, Austria*

a *Agroscope Liebefeld-Posieux Research Station ALP, 1725 Posieux, Switzerland*

b *ETH Zurich, Institute of Animal Sciences, 8092 Zurich, Switzerland*

c *Agroscope Reckenholz-Tanikon Research Station ART, Zurich, Switzerland*

d *Chemistry and Biochemistry Laboratory, School of Agriculture, Policy and Development, University of Reading Reading, RG6 6AT, UK*

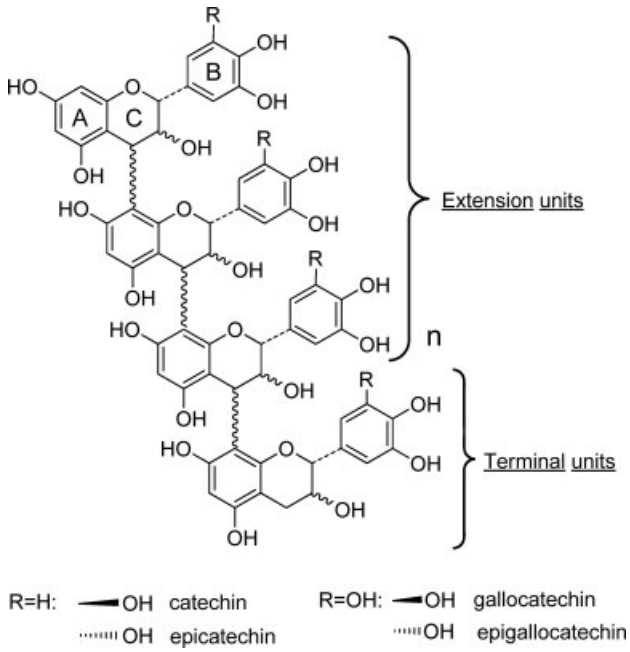

Figure 1. Example of a condensed tannin structure; R = H gives rise to procyanidin and R = OH to prodelphinidin type tannins.

composition and this appears to be caused by variety, plant organ, growth stage and season.[11–15] Previous studies focused on determining the importance of just one of these factors but none has investigated the combined effects of variety, site and harvest on sainfoin tannin composition. Considerable variation in the phenolic content among individual plants of the same sainfoin accession has also been reported.[16] It has been suggested[16] that future breeding efforts will need to develop varieties with relatively stable and thus predictable CT profiles in order to maximise the potential benefits that can be derived from these bioactive compounds.

CT analysis poses methodological challenges[17–19] and progress in differentiating individual tannins has been hampered by technical difficulties.[20] Therefore, Waghorn[5] recommended the use of several complementary methods when studying CT-containing forages. Colorimetric methods such as the HCl–butanol assay have been used extensively for quantitative measurements and rely on acid-catalysed oxidative depolymerisation of CTs to yield anthocyanidins;[18] however, this assay does not yield information on CT structures. Phloroglucinolysis or thiolysis can provide more detailed compositional information on CTs.[19] These methods use acid-catalysed cleavage of the interflavanol linkages in the presence of a nucleophile. Subsequent analysis by high-performance liquid chromatography (HPLC) of the reaction products allows calculation of the average procyanidin/prodelphinidin ratio, *cis*:*trans* ratio, mean degree of polymerisation (mDP) of the CT polymers and CT concentration. Information on the MW distribution profile, however, cannot be obtained from thiolysis since all constituent polymers are cleaved and gel permeation chromatography (GPC) needs to be employed instead.[21] The literature on GPC analysis for the determination of CT MWs and distribution profiles was reviewed recently.[21]

A previous study[22] found significant differences in CT concentration between the Communis and Bifera types, and a significant accession type by harvest time interaction, but there was a noticeable absence of an accession type by site interaction. In the present study, we hypothesise that qualitative CT attributes (tannin polymer size, hydroxylation pattern, stereochemistry and MW profile) are influenced differently by accession, harvest and site. For this, six sainfoin accessions, grown at three sites in Switzerland and harvested at two different times, were used. Two different analytical techniques, namely thiolysis and HPLC-GPC, were employed. We also compared the results obtained here for CT concentration using thiolysis and those obtained using the HCl–butanol method reported previously.[22]

MATERIALS AND METHODS

Plant material, harvests and sites of cultivation

From the six sainfoin accessions, Moiry, Premier, and Sarzens represent three landraces, Wiedlisbach is an ecotype, and Perly as well as Visnovsky are two commercial cultivars (see Boller and Greene[23] for definition of landrace, ecotype and cultivar). The landraces and ecotype studied were of the Communis or single-flowering type, whereas the two commercial cultivars were of the Bifera or multiple-flowering type. A total of 15 accessions, including those investigated in detail in this study, were established between 13 and 20 April 2007 on 1.5 × 6.0 m plots in a randomised complete block design with three replications planted at each of the following three Swiss sites: Thun (altitude 565 m above sea level), Ellighausen (520 m) and Reckenholz (440 m). The three sites chosen lay on an axis of about 180 km distance, with Thun in the western lowlands, whereas Ellighausen and Reckenholz are in the eastern lowlands. The soil type at the three sites varied from eutric cambisol to gleyic cambisol and organic carbon content varied 13.5–16.5 g kg^{-1} dry matter of soil. Thirty days prior to harvests, total precipitation, as well as the sum of the positive average daily temperatures (growing degree day, °C d) differed between sites (42.5–77.9 mm and 476–511 °C d, respectively) and between harvests (39.6–92.5 mm and 426–568 °C d, respectively). The sowing, crop management, agronomic, and other climatic and soil details were described previously.[22] The entire aerial plant material was harvested always at the early flowering stage by cutting at a stubble height of about 5 cm in late May (23–29 May 2008; first harvest) and again after 42 days of regrowth (8–10 July 2008; second harvest). This was done to reflect usual agricultural practices for forages where sainfoin harvested during the first growth cycle is generally used as silage or hay because of higher yields and the regrowth from the second growth cycle tends to be used for grazing.[24] Representative samples from each plot (2 kg) were collected and put into plastic bags, transported on ice and subsequently stored at −20 °C until freeze-drying (Christ Delta 1–24 LSC, Osterode, Germany). Freeze-dried samples were ground to pass a 1 mm screen[16] using a knife-type mill (Brabender, Duisburg, Germany) and stored in translucent bottles at room temperature prior to analysis.

Tannin analysis by *in situ* thiolysis of plant samples

This was performed as described by Gea *et al*.[19] in duplicate directly on freeze-dried samples (200 mg) at 40 °C for 60 min. Flavanols and their benzylmercaptan adducts were identified by their UV spectra and retention times; no corrections were needed for free flavanols, as fully grown sainfoin plants contain negligible amounts.[19] Flavanol concentrations were either reported on a molar basis (μmol flavanol units g^{-1} sainfoin dry matter) or on a mass basis (g total flavanols kg^{-1} dry matter, which is equivalent to CT concentration in sainfoin plants). The only modification was

that argon was bubbled over the thiolysis solution for 30 s just after the addition of methanol (1.6 mL) and 3.3% HCl in methanol (800 μL) to the reaction tubes.

CT isolation and analysis by HPLC–gel permeation chromatography (HPLC-GPC)

The first and second harvests of two Bifera (Visnovsky and Perly) and two Communis (Sarzens and Premier) accessions from two sites (Thun and Ellighausen) were chosen. Samples (25 g) were extracted with acetone–water (7:3, v/v; 200 mL) in the presence of ascorbic acid (0.25 g; Fisher Scientific, Loughborough, UK) for 40 min at room temperature[21] and filtered through a 125 mm filter paper No. 22 (Whatman International Ltd, Maidstone, UK). Acetone was removed in a rotary evaporator at $<40\,^{\circ}C$ and dichloromethane (200 mL, HPLC grade; Fisher Scientific, Loughborough, UK) was added to remove chlorophyll. The upper aqueous phase containing CT was concentrated on a rotary evaporator at $<40\,^{\circ}C$ and freeze-dried to yield the crude CT extract. A slurry of Sephadex LH-20 (16 g; GE Healthcare Bio-Sciences AB, Uppsala, Sweden) in 64 mL of 50% aqueous methanol was prepared and then poured into a column (40 cm × 2 cm) to obtain a column height of 24.2 cm and a bed volume of 76.0 cm^3. The column was equilibrated with 240 mL Millipore water before use. The crude CT extract (800 mg) was dissolved in water (20 mL) in an ultrasonic bath for 10 min. The extract was filtered through a 0.45 μm Teflon (PTFE) syringe filter (Savillex, Minnetonka, MN, USA) and applied to the Sephadex LH-20 column. The column was eluted first with deionised water (240 mL) aided by gentle pressure with a manual, double-spray bellow pump (Fisher Scientific, Loughborough, UK), then with 50% aqueous methanol (240 mL), followed by 70% aqueous acetone (240 mL). The aqueous methanol fraction yielded low quantities of CT dimers and trimers, which were of low purity and therefore discarded (unpublished results). The aqueous acetone eluate was collected, concentrated at $<40\,^{\circ}C$ and freeze-dried to yield a fluffy white CT fraction, which was kept at $-20\,^{\circ}C$ until GPC analysis. The Sephadex column was washed with acetone (200 mL) and then 50% methanol (240 mL) before using it again. The CT fractions were dissolved in water and analysed by HPLC-GPC using two serially connected PolarGel-L columns (Polymer Laboratories, Church Stretton, UK) as described previously.[21] Briefly, two serially connected PolarGel-L columns (300 mm × 7.5 mm, 8 μm) were eluted with dimethylformamide containing 5% water, 1% acetic acid and 0.25 M LiBr at 0.7 mL min^{-1} and $50\,^{\circ}C$. MWs were determined with a calibration curve that included polyphenols and three condensed tannin standards (see Table 6, 2nd calibration, and Figure 7 of Stringano *et al.*[21]), which covered an MW range from 290 to 8318 Da.

Calculation of CT composition

The following variables were calculated[19] from the monomeric flavanols (molar concentrations) obtained from the thiolysis reaction to describe tannin composition: mean degree of polymerisation (mDP), procyanidin/prodelphinidin (PC/PD) and *cis*:*trans* ratios:

$$\text{mDP} = \frac{\text{Sum of extension and terminal flavanol units [moles]}}{\text{Sum of terminal flavanol units [moles]}}$$

$$\text{PC/PD} = \frac{\text{Molar percentage of catechin} + \text{epicatechin units}}{\text{Molar percentage of gallocatechin} + \text{epigallocatechin units}}$$

$$\textit{cis/trans} = \frac{\text{Molar percentage of epicatechin} + \text{epigallocatechin units}}{\text{Molar percentage of catechin} + \text{gallocatechin units}}$$

HPLC-GPC analysis using the Cirrus GPC Offline 3.2 software provided three MW parameters: M_p (peak molecular weight), M_n (number average molecular weight and M_w (weight average MW) (for further details see http://pslc.ws/mactest/sec.htm).[21] The polydispersity index (PDI) was calculated as the ratio of M_w/M_n.

Statistical analysis

The CT composition data were analysed by the procedure MIXED of SAS (version 9.1, SAS Inst. Inc., Cary, NC, USA) with site, harvest, accession and their two-way and three-way interactions as fixed effects and replicate as random effect. Means were separated using Fischer's LSD and all statistical tests were at the $\alpha = 0.05$ level of significance. Owing to the complexity of isolating confounding effects among three factors (site, harvest and accession) only second-degree interactions are reported. The relationship between CT concentration as measured by the thiolysis and the HCl–butanol method of Terrill *et al.*[25] and reported earlier[22] was established by regression analysis using a quadratic equation, which gave the best fit to the data.

RESULTS AND DISCUSSION

The CT composition of different sainfoin accessions and changes during plant growth have been described previously.[11–13,19,26,27] However, to our knowledge, no information exists on the combined effects of accession and environment with respect to sainfoin CTs.

CT terminal and extension units

Table 1 gives the concentration of extension and terminal flavanols in CTs for the sainfoin accessions. The terminal units were characterised by catechin and epicatechin only, and no gallocatechin and epigallocatechin were detected. Terminal units accounted on average for ~3% of all flavanol units in tannin polymers. The remaining 97% extension units contained all four flavanols, i.e. epigallocatechin (48%), epicatechin (28%), gallocatechin (15%) and catechin (6%). The flavanol extension units of the Wiedlisbach accession, and to some extent of the Sarzens accession, increased most consistently, i.e. by more than 50% from the first to second harvest across all three sites. Most earlier studies of sainfoin tannins were done on purified extracts and reported the presence of all four flavanols[12,13] or just three[11,26,27] as terminal units. However, recent *in situ* studies[19,28,29] found between one and four of the flavanols as terminal units amongst 37 different sainfoin accessions, which demonstrates that sainfoin CTs exhibit considerable structural variation.

Tannin polymer size, hydroxylation pattern and stereochemistry

The mean degree of polymerisation (mDP) reflects the average polymer size of tannins and ranged from 23.9 to 45.9 (Table 2). Mean polymer size was significantly affected by accession ($P < 0.001$) and site ($P < 0.001$) but not harvest ($P = 0.35$). There were also significant interactions between these factors (Table 2). The observed polymer sizes are comparable with those found in

Table 1. Concentration of terminal and extension units (± standard deviation, $n = 3$) in condensed tannins measured by *in situ* thiolysis of six sainfoin accessions, which were harvested at three sites and two harvest times (μmol flavanol units g^{-1} sainfoin dry matter)

		Terminal units				Extension units							
		Catechin		Epicatechin		Gallocatechin		Epigallocatechin		Catechin		Epicatechin	
Site	A[a]	1st har.[b]	2nd har.	1st har.	2nd har.	1st har.	2nd har.	1st har.	2nd har.	1st har.	2nd har.	1st har.	2nd har.
Thun	Mo	0.3 ± 0.05	0.5 ± 0.29	0.3 ± 0.05	0.3 ± 0.18	2.7 ± 0.16	4.2 ± 1.91	10.8 ± 1.68	4.9 ± 2.81	1.2 ± 0.13	2.7 ± 1.32	7.6 ± 1.61	5.3 ± 2.63
	Sa	0.5 ± 0.15	0.8 ± 0.09	0.2 ± 0.12	0.5 ± 0.23	2.3 ± 0.68	5.8 ± 0.18	7.9 ± 3.95	22.9 ± 9.11	0.7 ± 0.11	2.1 ± 0.23	5.6 ± 1.67	11.5 ± 2.39
	Pr	0.2 ± 0.04	0.9 ± 0.12	0.1 ± 0.06	0.6 ± 0.15	2.0 ± 0.32	5.2 ± 0.56	5.5 ± 1.15	22.7 ± 3.41	1.1 ± 0.60	2.6 ± 0.38	3.3 ± 0.47	10.8 ± 1.61
	Wi	0.4 ± 0.15	1.0 ± 0.20	0.3 ± 0.05	0.5 ± 0.12	3.4 ± 0.59	7.1 ± 1.18	10.6 ± 2.76	30.7 ± 5.82	0.9 ± 0.47	3.0 ± 1.28	6.9 ± 1.07	13.4 ± 2.11
	Vi	0.3 ± 0.09	0.4 ± 0.12	0.1 ± 0.06	0.1 ± 0.08	1.9 ± 0.58	2.9 ± 0.95	6.9 ± 4.42	8.6 ± 3.96	0.4 ± 0.15	0.8 ± 0.26	2.5 ± 0.63	2.9 ± 0.89
	Pe	0.2 ± 0.01	0.4 ± 0.10	0.2 ± 0.10	0.4 ± 0.09	2.1 ± 0.26	3.3 ± 0.80	5.6 ± 2.12	8.2 ± 3.14	0.7 ± 0.38	2.0 ± 1.20	4.1 ± 0.40	3.9 ± 1.58
Ellighausen	Mo	0.5 ± 0.03	0.9 ± 0.09	0.3 ± 0.06	0.6 ± 0.03	3.5 ± 0.03	7.9 ± 1.09	8.3 ± 0.01	25.9 ± 0.94	2.3 ± 0.33	4.0 ± 0.35	9.6 ± 0.49	17.3 ± 0.76
	Sa	0.5 ± 0.17	0.7 ± 0.08	0.3 ± 0.13	0.2 ± 0.06	2.8 ± 0.90	4.6 ± 0.59	8.5 ± 1.96	18.0 ± 3.85	1.5 ± 0.63	1.5 ± 0.23	6.3 ± 1.91	9.3 ± 1.42
	Pr	0.6 ± 0.20	0.6 ± 0.13	0.3 ± 0.11	0.2 ± 0.05	2.8 ± 1.27	3.9 ± 0.63	10.4 ± 4.29	11.3 ± 2.25	2.0 ± 0.56	1.5 ± 0.31	7.3 ± 2.23	7.2 ± 1.35
	Wi	0.5 ± 0.07	1.0 ± 0.20	0.3 ± 0.07	0.2 ± 0.09	2.9 ± 0.76	8.1 ± 1.80	13.6 ± 2.53	28.2 ± 3.00	1.6 ± 0.44	1.8 ± 0.17	7.5 ± 1.18	13.2 ± 1.72
	Vi	0.4 ± 0.06	0.4 ± 0.11	0.2 ± 0.03	0.1 ± 0.02	2.4 ± 0.43	2.7 ± 0.58	13.4 ± 3.40	8.5 ± 3.60	1.1 ± 0.13	0.7 ± 0.20	4.2 ± 0.96	2.9 ± 0.97
	Pe	0.4 ± 0.07	0.6 ± 0.21	0.3 ± 0.05	0.1 ± 0.05	3.7 ± 1.14	3.8 ± 1.15	15.3 ± 2.50	13.0 ± 5.98	1.7 ± 0.25	1.4 ± 0.45	8.2 ± 1.40	7.9 ± 3.04
Reckenholz	Mo	0.4 ± 0.14	0.5 ± 0.18	0.2 ± 0.13	0.2 ± 0.04	3.0 ± 0.96	3.2 ± 0.74	8.3 ± 2.85	8.7 ± 5.20	1.2 ± 0.48	1.4 ± 0.48	6.1 ± 2.22	7.1 ± 2.68
	Sa	0.4 ± 0.16	0.8 ± 0.15	0.2 ± 0.11	0.2 ± 0.10	1.7 ± 0.60	6.3 ± 0.75	7.3 ± 2.35	15.7 ± 2.28	1.1 ± 0.56	2.4 ± 0.78	5.4 ± 1.76	9.2 ± 0.73
	Pr	0.5 ± 0.06	0.5 ± 0.13	0.2 ± 0.05	0.1 ± 0.06	3.4 ± 0.96	3.5 ± 0.50	12.6 ± 4.01	5.6 ± 1.01	1.3 ± 0.24	1.3 ± 0.31	7.1 ± 1.32	4.6 ± 0.95
	Wi	0.6 ± 0.16	1.2 ± 0.22	0.2 ± 0.05	0.6 ± 0.14	2.7 ± 0.96	5.9 ± 1.11	10.7 ± 3.00	25.1 ± 5.80	1.1 ± 0.35	2.4 ± 0.31	6.6 ± 1.85	14.3 ± 0.93
	Vi	0.2 ± 0.07	0.6 ± 0.20	0.1 ± 0.03	0.2 ± 0.12	1.9 ± 0.68	4.2 ± 1.09	5.2 ± 2.22	11.1 ± 4.85	0.5 ± 0.33	1.3 ± 0.62	2.1 ± 0.90	7.1 ± 2.23
	Pe	0.4 ± 0.11	0.5 ± 0.08	0.1 ± 0.02	0.2 ± 0.03	2.3 ± 0.82	4.1 ± 0.64	10.7 ± 4.05	11.4 ± 1.23	0.7 ± 0.19	1.6 ± 0.21	5.5 ± 1.53	6.5 ± 1.07

[a] Accessions - Mo – Moiry; Pe – Perly; Pr – Premier; Sa – Sarzens; Vi – Visnovsky; Wi – Wiedlisbach.
[b] 1st harvest: 23 – 29 May 2008; 2nd harvest (or regrowth): 8 – 10 July 2008.

Table 2. Mean degree of polymerisation, ratio of procyanidins to prodelphinidins, ratio of *cis* to *trans* flavanols, and concentration (g kg^{-1} DM) of condensed tannins (CT) as measured by *in situ* thiolysis of six sainfoin accessions grown at three sites and collected at two harvest times ($n = 3$)

		mDP[b]		Procyanidin (%)		Prodelphinidin (%)		*cis* (%)		*trans* (%)		CT concentration (g kg^{-1} DM[c])	
Site	A[a]	1st har.[d]	2nd har.	1st har.	2nd har.	1st har.	2nd har.	1st har.	2nd har.	1st har.	2nd har.	1st har.	2nd har.
Thun	Mo	40.5	23.9	40.9	49.5	59.1	50.5	81.4	57.5	18.6	42.5	7.3	5.7
	Sa	26.5	32.7	41.3	34.9	58.7	65.1	78.4	78.9	21.6	21.1	5.5	13.7
	Pr	37.4	29.6	38.8	34.7	61.2	65.3	73.1	79.7	26.9	20.3	4.0	13.7
	Wi	34.3	36.5	37.7	32.2	62.3	67.8	79.2	80.1	20.3	19.9	8.0	17.8
	Vi	36.0	36.6	28.4	26.8	71.6	73.2	76.4	72.9	23.6	27.1	3.4	5.0
	Pe	31.3	25.8	41.1	36.1	58.9	63.9	76.3	68.5	23.7	31.5	4.1	5.8
Ellighausen	Mo	30.7	38.9	51.8	40.2	48.2	59.8	74.3	77.3	25.7	22.7	7.8	17.7
	Sa	26.3	38.7	43.1	34.2	56.9	65.8	76.3	80.1	23.7	19.9	6.2	10.8
	Pr	24.3	31.8	44.8	38.5	55.2	61.6	76.7	75.7	23.3	24.3	7.5	7.7
	Wi	34.8	45.9	37.4	30.8	62.6	69.2	81.2	79.2	18.8	20.8	8.4	16.5
	Vi	39.9	36.3	27.0	26.5	73.0	73.5	81.7	74.3	18.3	25.7	7.0	4.8
	Pe	41.3	40.0	36.0	37.8	64.0	62.2	80.5	77.8	19.5	22.2	9.5	8.4
Reckenholz	Mo	32.9	29.6	41.1	44.7	58.9	55.3	75.8	74.3	24.2	25.7	6.0	6.6
	Sa	30.1	35.0	43.9	36.5	56.1	63.5	80.6	72.5	19.4	27.5	5.0	10.9
	Pr	36.3	26.1	37.0	41.5	63.0	58.5	79.0	66.2	21.0	33.8	7.9	4.8
	Wi	32.2	26.6	38.1	38.0	61.9	62.0	80.3	80.6	19.7	19.4	6.8	15.6
	Vi	37.0	29.1	28.3	38.5	71.7	61.5	73.6	73.9	26.4	26.1	3.2	7.6
	Pe	39.6	35.7	34.7	35.8	65.4	64.2	82.9	74.6	17.1	25.5	5.7	7.6

Statistics	mDP	Procyanidin/prodelphinidin ratio	*cis*:*trans* ratio	CT concentration
SEM[e]	*0.509*	*0.013*	*0.076*	*0.30*
Site	<0.001	0.46	0.15	<0.001
Harvest	0.34	0.004	<0.001	<0.001
Accession	<0.001	<0.001	<0.001	<0.001
Site × Accession	<0.001	<0.001	0.09	<0.001
Har. × Accession	<0.001	<0.001	0.08	<0.001
Site × Harvest	<0.001	<0.001	0.11	<0.001

[a] Accessions: Mo, Moiry; Pe, Perly; Pr, Premier; Sa, Sarzens; Vi, Visnovsky; Wi, Wiedlisbach.
[b] mDP, mean degree of polymerisation.
[c] Dry matter.
[d] 1st harvest: 23–29 May 2008; 2nd harvest (or regrowth): 8–10 July 2008.
[e] Standard error of the mean.

other sainfoin accessions harvested at similar stages, with mDP of 11–31,[28] 12–84[29] and 24–50.[14] The two Bifera accessions – Visnovsky and Perly – showed decreases in mDP from the first to the second harvest across all three sites, while no consistent pattern was found for the Communis accessions. This accounted for the significant two-way interactions observed for mDP. This observation points to a better predictability for tannin polymer sizes of Bifera accessions. This may be related to the fact that Bifera accessions produce two or more harvests that can flower, while the Communis accessions only flowered at the first harvest. Theodoridou *et al.*[15] also reported higher mDP values, i.e. 20 additional flavanol units, in the first compared to the second growth cycle for the multiple-flowering Perly accession. Seed producers who seek to harness the nutritional and anthelmintic potential of sainfoin might wish to consider this when developing appropriate breeding programmes.

All but one of the samples contained more prodelphinidins than procyanidins; indeed, high prodelphinidin contents are characteristic of sainfoin tannins.[11–14,19,26] Significant accession, harvest and site interactions precluded the emergence of any definite patterns. The Visnovsky accession was the only exception: averaged over two harvests, it had a comparatively higher percentage of prodelphinidins at all three sites than the other accessions. There were accession ($P < 0.001$) and harvest ($P = 0.004$) effects, but no ($P = 0.46$) site effect, on the PC:PD ratio.

The *cis*-isomers, epicatechin and epigallocatechin, dominated in sainfoin CTs, giving *cis*:*trans* ratios that ranged from 83:17 to 59:41. Such a variation agrees with that found in other sainfoin studies.[12–14,28] Accession ($P < 0.001$) and harvest ($P < 0.001$) effects, but again no site ($P = 0.15$) effect, were observed for the *cis*:*trans* ratio.

Molecular weight profiles of the extracted tannins

The GPC analysis of CT MWs poses considerable challenges and was recently reviewed and improved.[21] This study found that reasonably close agreement could be obtained between CT MWs calculated from thiolysis data and GPC separations as long as polyphenols and tannins were used to generate the calibration curve.[21] Commercially available polymer standards, such as polyethylene glycol, polystyrene and polymethyl methacrylate

Table 3. Molecular weights (M_p, M_n and M_w values) of tannins extracted from four sainfoin accessions harvested across two sites and at two harvest times as determined by HPLC-GPC. For comparison, the calculated MWs of CT in the sainfoin plants are also shown

			M_p[c] (Da)			M_n[d] (Da)			M_w[e] (Da)				
Site	A[a]	Har.[b]	1st peak[h]	2nd peak	3rd peak	1st peak[h]	2nd peak	3rd peak	1st peak[h]	2nd peak	3rd peak	PDI[f]	MW calc.[g]
Thun	Sa	1st		5860	926		4887	925		5658	925	1.1; 1.0	10 792
		2nd	9066	3641	427	9154	2697	428	9289	3265	428	1.0; 1.2;1.0	11 101
	Pr	1st		4759	617		3456	614		4595	617	1.3; 1.0	12 000
		2nd	8977	3330	593	8968	2550	589	9132	3025	591	1.0; 1.2; 1.0	10 803
	Vi	1st		3826			2940			3718		1.3	9 230
		2nd		4352			3041			4052		1.3	7 763
	Pe	1st		6220			4376			5905		1.4	12 838
		2nd		4332			2539			4573		1.8	11 931
Ellighausen	Sa	1st		5860	611		3800	565		5301	576	1.4; 1.1	8 039
		2nd	8800	2986	617	8992	2560	604	9157	2945	608	1.1; 1.2; 1.0	9 859
	Pr	1st	8714	3232	476	8729	2618	475	8865	3074	475	1.0; 1.2; 1.0	7 734
		2nd	8542	3396	611	8615	2669	606	8764	6117	609	1.0; 1.2; 1.0	11 655
	Vi	1st		4902	491		3462	488		4521	488	1.3; 1.0	11 030
		2nd		4142			3175			4093		1.3	8 964
	Pe	1st		6097			3878			5598		1.4	7 132
		2nd		4951			3555			4951		1.4	9 551

[a] Accessions: Pe, Perly; Pr, Premier; Sa, Sarzens; Vi, Visnovsky.
[b] 1st harvest: 23–29 May 2008; 2nd harvest (or regrowth): 8–10 July 2008.
[c] Peak-average molecular weight.
[d] Number-average molecular weight.
[e] Weight-average molecular weight.
[f] Polydispersity index.
[g] Calculated molecular weight (calculated from the direct, *in situ* thiolytic degradation of plants using mDP and procyanidin/prodelphinidin ratio parameters).
[h] Standard deviations ($n = 3$) for CTs of 2436 to 8318 Da (calculated) ranged from 120 to 137 for M_p, 101 to 130 for M_n and 114 to 144 for M_w values.[21]

standards, resulted in large and unpredictable overestimations. The M_p values measured in the present study ranged from 427 to 9066 Da (Table 3) and overlapped with the previously observed range, i.e. 307–7165 Da (M_p values measured) *versus* 290–8318 Da (MW calculated).[21]

Table 3 reports the presence or absence of MW peaks of tannins extracted from samples comprising four accessions, two sites and two harvests. The fact that the HPLC-GPC profiles varied considerably between CTs from different accessions indicated once again the heterogeneous nature of sainfoin CTs. M_p values ranged between 427 and 9066 Da and revealed up to three distinct peaks. The peaks could be broadly classified as follows: peak 1 with M_p between ~8500 and 9100, peak 2 with M_p between ~3000 and 6200 and peak 3 with $M_p < 1000$ Da. The polydispersity index (PDI) ranged from 1.0 to 1.8, but most values were close to 1, which indicated a normal distribution of MWs in these peaks. Most previous studies, which used nuclear magnetic resonance and GPC, reported MW ranges of 1152–3300 Da.[12,26,27] However, one study[30] isolated and purified sainfoin CT by gel chromatography on Sephadex G-50 and LH-20 media and described MWs of tannins ranging between 17 000 and 28 000 Da.

A wide variation in sainfoin CT MWs was previously attributed to season and cultivar.[11] We found similar MW ranges at the Thun and Ellighausen sites. This suggests that site may have little influence on the MW profiles of the accessions tested in the present study. However, more sites will need to be studied to confirm this result, especially as this finding contrasts with the significant site effect on tannin polymer sizes (i.e. mDP values)

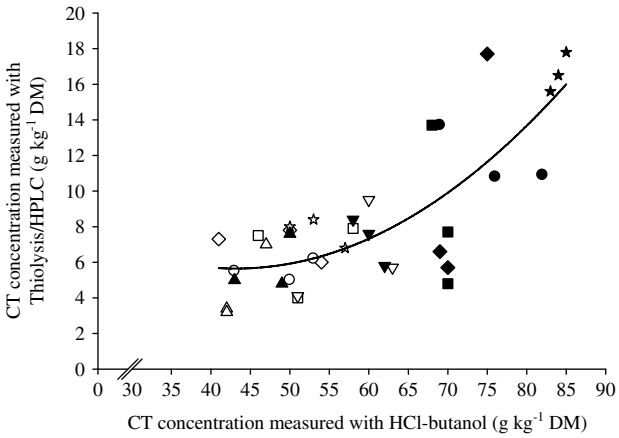

Figure 2. Relationship between CT concentrations, which were determined by *in situ* thiolytic degradation and HCl–butanol. Open symbols, first cut; closed symbols, second cut; ◆, Moiry; ●, Sarzens; ■, Premier; ★, Wiedlisbach; ▲, Visnovsky; ▼, Perly. Equation of fitted curve: $y = 16.7 - 0.51x + 0.006x^2$; $r^2 = 0.62$.

by the *in situ* thiolysis analysis (Table 2). The Bifera accessions – Visnovsky and Perly – had a slightly higher PDI than the Communis accessions. Tannins from Visnovsky and Perly tended to elute as single peaks, whereas the Communis tannins generated two to three peaks. Tannins from the Communis accessions at the second harvest contained large-MW CTs (peak 1 > 8000). Premier tannins from the Ellighausen site included peak 3 also at the first

harvest. In contrast, the Bifera tannins had relatively stable MW profiles. Visnovsky tannins revealed the smallest MW changes in peak 2 and Perly tannins exhibited slightly larger changes. The differences between Communis and Bifera accessions point to a possible genetic modulation of CT MW profiles in sainfoin as found previously for *Populus* CTs.[31] As discussed above for mDP values, this is another indication that some CT traits seem to be more predictable in Bifera accessions and this could be of interest for sainfoin breeding. Although Hayot Carbonero *et al*.[8] reported that current sainfoin lines do not fit into the original classification of single (Communis) and multiple (Bifera) flowering types, this classification proved useful here for tannin MW profiles.

Table 3 also lists the mean MWs that were calculated from the *in situ* thiolysis data. The tannins analysed directly in the plant samples were of much higher MW than the extracted tannins. Indeed, extracted tannins tend to have lower mDP values (between 4 and 29 lower) than tannins analysed *in situ*, because higher-MW tannins are more difficult to extract.[19]

Concentration of CT as measured by thiolysis and by the HCl–butanol method

According to the thiolysis method, CT concentrations ranged from 3.2 to 17.8 g kg^{-1} DM (Table 2). The significant two-way interactions observed precluded the establishment of a clear pattern of variation for the CT concentration amongst accessions but, in general, CT concentrations increased from the first to second harvest. This was particularly the case in the Wiedlisbach accession, which showed a more than twofold increase across all three sites. CT concentration in single-flowering accessions was previously shown[22] to increase markedly between the first and second harvest and may have been caused by higher temperatures recorded during regrowth, but this will require further proof. The CT concentrations obtained from the HCl–butanol method reported previously[22] were much higher: 41–85 g kg^{-1} DM (Fig. 2). These discrepancies therefore serve to highlight the fact that the *in situ* thiolysis and HCl–butanol assays are difficult to compare. Purity of CT standards and reaction yields appear to be some of the key issues. Although different, the thiolysis and HCl–butanol results were positively correlated ($r^2 = 0.62$; $P < 0.001$) and ranked accessions identically, with Wiedlisbach having the highest and Visnovsky the lowest CT concentration (Fig. 2). This correlation is higher than that reported by Stringano *et al*.[29] and the quadratic fit suggests that the two methods do not necessarily detect the same CT properties.

CONCLUSIONS

This preliminary study showed that accession, harvest and site influenced sainfoin tannin polymer size, B-ring hydroxylation pattern, stereochemistry and MW distribution profiles to varying extents. It would be interesting in further trials to ascertain these effects on more accessions, harvests and sites over many more growing seasons and, if confirmed, also to ascertain with *in vivo* feeding experiments whether these significant effects are biologically relevant in terms of nutritional and anthelmintic benefits. The more predictable and stable nature of the CT polymers seen here for Bifera compared to Communis accessions could be useful in future sainfoin breeding programmes. This study also highlighted differences that can result when determining CT concentrations by different methods.

ACKNOWLEDGEMENTS

This study was supported by a grant from the Swiss Grassland Society (Arbeitsgemeinschaft zur Förderung des Futterbaues, AGFF), Zurich, Switzerland, and also the Swiss Federal Department of Economic Affairs through its National Action Plan 'Diversity in Agriculture Campaign Plants'.

REFERENCES

1 Delgado I, Salvia J, Buil I and Andrés C, The agronomic variability of a collection of sainfoin accessions. *Span J Agric Res* **6**:401–407 (2008).
2 Meyer DW and Badaruddin M, Frost tolerance of ten seedling legume species at four growth stages. *Crop Sci* **41**:1838–1842 (2001).
3 Aufrère J, Dudilieu M and Poncet C, *In vivo* and *in situ* measurements of the digestive characteristics of sainfoin in comparison with lucerne fed to sheep as fresh forages at two growth stages and as hay. *Animal* **2**:1331–1339 (2008).
4 Häring DA, Scharenberg A, Heckendorn F, Dohme F, Lüscher A, Maurer V, *et al.*, Tanniferous forage plants: agronomic performance, palatability and efficiency against parasitic nematodes in sheep. *Ren Agric Food Syst* **23**:19–29 (2008).
5 Waghorn G, Beneficial and detrimental effects of dietary condensed tannins for sustainable sheep and goat production: progress and challenges. *Anim Feed Sci Technol* **147**:116–139 (2008).
6 Rumball W and Claydon RB, Germplasm release: 'G35' Sainfoin (*Onobrychis viciifolia* Scop.). *NZ J Agric Res* **48**:127–128 (2005).
7 Piano E and Pecetti L, Minor legume species, in *Fodder Crops and Amenity Grasses*, ed. By Boller B, Posselt UK and Veronesi F. Springer, New York, pp. 477–500 (2010).
8 Hayot Carbonero C, Mueller-Harvey I, Brown TA and Smith L, Sainfoin (*Onobrychis viciifolia*): a beneficial forage legume. *Plant Gen Res* **9**:70–85 (2011).
9 Mueller-Harvey I, Unravelling the conundrum of tannins in animal nutrition and health. *J Sci Food Agric* **86**:2010–2037 (2006).
10 Yarnes CT, Boeckeln WJ and Salminen J, No simple sum: seasonal variation in tannin phenotypes and leaf-miners in hybrid oaks. *Chemoecology* **18**:39–51 (2008).
11 Foo YL, Jones WT, Porter LJ and Williams VM, Proanthocyanidin polymers of fodder legumes. *Phytochemistry* **21**:933–935 (1982).
12 Koupai-Abyazani MR, Muir AD, Bohm BA, Towers GHN and Gruber MY, The proanthocyanidin polymers in some species of *Onobrychis*. *Phytochemistry* **34**:113–117 (1993).
13 Koupai-Abyazani MR, McGallum J, Muir AD, Bohm BA, Towers GHN and Gruber MY, Developmental changes in the composition of proanthocyanidins from leaves of sainfoin (*Onobrychis viciifolia* Scop.) as determined by HPLC analysis. *J. Agric Food Chem* **41**:1066–1070 (1993).
14 Theodoridou K, Aufrère J, Andueza D, Le Morvan A, Picard F, Stringano E, *et al.*, Effect of plant development during first and second growth cycle on chemical composition, condensed tannins and nutritive value of three sainfoin (*Onobrychis viciifolia*) varieties and lucerne. *Grass Forage Sci* **66**:402–414 (2011).
15 Theodoridou K, Aufrère J, Niderkorn V, Andueza D, Le Morvan A, Picard F, *et al.*, *In vitro* study of the effects of condensed tannins in sainfoin on the digestive process in the rumen at two vegetative cycles. *Anim Feed Sci Technol* **170**:147–159 (2011).
16 Regos I, Urbanella A and Treutter D, Identification and quantification of phenolic compounds from the forage legume sainfoin (*Onobrychis viciifolia*). *J Agric Food Chem* **57**:5843–5852 (2009).
17 Stewart JL, Mould F and Mueller-Harvey I, The effect of drying treatment on fodder quality and tannin content of two provenances of *Calliandra calothyrsus* Meissner. *J Sci Food Agric* **80**:1461–1468 (2000).
18 Schofield P, Mbugua DM and Pell AN, Analysis of condensed tannins: a review. *Anim Feed Sci Technol* **91**:21–40 (2001).
19 Gea A, Stringano E, Brown RH and Mueller-Harvey I, *In situ* analysis and structural elucidation of sainfoin (*Onobrychis viciifolia*) tannins for high throughput germplasm screening. *J Agric Food Chem* **59**:495–503 (2011).
20 Stringano E, Cramer R, Hayes W, Smith C, Gibson T and Mueller-Harvey I, Deciphering the complexity of sainfoin (*Onobrychis viciifolia*) proanthocyanidins by MALDI-TOF mass spectrometry with a judicious choice of isotope patterns and matrices. *Anal Chem* **83**:4147–4153 (2011).

21 Stringano E, Gea A, Salminen JP and Mueller-Harvey I, Simple solution for a complex problem: proanthocyanidins, galloyl glucoses and ellagitannins fit on a single calibration curve in high performance-gel permeation chromatography. *J Chromatogr A* **1218**:7804–7812 (2011).
22 Azuhnwi BN, Boller B, Martens M, Dohme-Meier F, Ampuero S, Günter S, *et al.*, Morphology, tannin concentration and forage value of 15 Swiss accessions of sainfoin (*Onobrychis viciifolia* Scop.) as influenced by harvest time and cultivation site. *Grass Forage Sci* **66**:474–487 (2011).
23 Boller B and Greene SL, Genetic resources, in *Fodder Crops and Amenity Grasses*, ed. by Boller B, Posselt UK and Veronesi F. Springer, New York, pp. 13–37 (2010).
24 Butler TJ, Evers GW, Hussey MA and Ringer LJ, Flowering in crimson clover as affected by planting date. *Crop Sci* **42**:242–247 (2002).
25 Terrill TH, Rowan AM, Douglas GB and Barry TN, Determination of extractable and bound condensed tannin concentrations in forage plants. *J Sci Food Agric* **58**:321–329 (1992).
26 Foo LY and Porter JL, The phytochemistry of proanthocyanidin polymers. *Phytochemistry* **19**:1747–1754 (1980).
27 Marais JPJ, Mueller-Harvey I, Brandt EV and Ferreira D, Polyphenols, condensed tannins, and other natural products in *Onobrychis viciifolia* (sainfoin). *J Agric Food Chem* **48**:3440–3447 (2000).
28 Theodoridou K, Aufrère J, Andueza D, Pourrat J, Le Morvan A, Stringano E, *et al.*, Effects of condensed tannins in fresh sainfoin (*Onobrychis viciifolia*) on *in vivo* and *in situ* digestion in sheep. *Anim Feed Sci Technol* **160**:23–38 (2010).
29 Stringano E, Hayot Carbonero C, Smith LMJ, Brown RH and Mueller-Harvey I, Proanthocyanidin diversity in the EU 'HealthyHay' sainfoin (*Onobrychis viciifolia*) germplasm collection. *Phytochemistry* **77**:197–208 (2012).
30 Jones WT, Broadhurst RB and Lyttleton JW, The condensed tannins of pasture legume species. *Phytochemistry* **15**:1407–1409 (1976).
31 Scioneaux AN, Schmidt MA, Moore MA, Lindroth RL, Wooley SC and Hagerman AE, Qualitative variation in proanthocyanidin composition of *Populus* species and hybrids: Genetics is the key. *J Chem Ecol* **37**:57–70 (2011).

