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Diversity of condensed tannin structures affects rumen *in vitro* methane production in sainfoin (*Onobrychis viciifolia*) accessions

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Abstract

Sainfoin is a non-bloating temperate forage legume with a moderate-to-high condensed tannin (CT) content. This study investigated whether the diversity of sainfoin accessions in terms of CT structures and contents could be related to rumen *in vitro* gas and methane (CH_4) production and fermentation characteristics. The aim was to identify promising accessions for future investigations. Accessions differed ($P < 0{\cdot}0001$) in terms of total gas and CH_4 productions. Fermentation kinetics (i.e. parameters describing the shape of the gas production curve and half-time gas production) for CH_4 production were influenced by accession ($P \leq 0{\cdot}038$), but not by PEG. Accession, PEG and time affected ($P < 0{\cdot}001$) CH_4 production, but accession and PEG interaction showed only a tendency ($P = 0{\cdot}08$). Increase in CH_4 due to PEG addition was not related to CT content. Further analysis of the relationships among multiple traits (nutritional composition, CT structure and CH_4 production) using principal component analysis (PCA) based on optimally weighted variables revealed differences among accessions. The first two principal component axes, PC1 (57·6%) and PC2 (18·4%), explained 76·0% of the total variation among accessions. Loading of biplots derived from both PCAs made it possible to establish a relationship between the ratio of prodelphinidin: procyanidin (PD:PC) tannins and CH_4 production in some accessions. The PD:PC ratio seems to be an important source of variation that is negatively related to CH_4 production. These results suggested that sainfoin accessions collected from across the world exhibited substantial variation in terms of their effects on rumen *in vitro* CH_4 production, revealing some promising accessions for future investigations.

Keywords: sainfoin accessions, methane, condensed tannins, prodelphinidin

Introduction

Sainfoin (*Onobrychis viciifolia*) is a perennial forage legume that is widely adapted to warm-temperate and dryland areas of Europe, Asia and the west of North America (Hayot Carbonero *et al.*, 2011a). It can be grown as a pure pasture or mixed with companion grasses such as perennial ryegrass (*Lolium perenne*), meadow fescue (*Festuca pratensis*) and timothy (*Phleum pratense*) (Haering *et al.*, 2008; Liu *et al.*, 2010). It leads to higher intakes than lucerne when consumed by sheep (Khalilvandi-Behroozyar *et al.*, 2010) and other ruminants without causing bloat (Mueller-Harvey, 2009). In addition, sainfoin also has potential for control of parasitic worms (Hoste *et al.*, 2006), for reducing methane emissions (McMahon *et al.*, 1999) and nitrogen losses as urine (Scharenberg *et al.*, 2007; Aufrere *et al.*, 2008; Theodoridou *et al.*, 2010) from ruminants. Although it is understood that the condensed tannins (CT) in sainfoin are responsible for these beneficial effects, it is far from clear which particular sainfoin tannins are responsible. A survey of the currently available sainfoin germplasm (Stringano *et al.*, 2012) revealed that (i) sainfoin contains a complex set of different tannin molecules and (ii) different sainfoin

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accessions vary enormously in their tannin compositions.

A number of studies have screened many plants and plant extracts for their potential to decrease CH_4 production when used as feed additives, as supplementary feeds or as sole feeds for ruminants (Patra *et al.*, 2006; Bodas *et al.*, 2008; Garcia-Gonzalez *et al.*, 2008). These studies have tended to focus on the contents of plant secondary metabolites such as essential oils, saponins or CT. Among these, CT have received much attention for their ability to modify ruminal fermentation patterns. However, CT contents and structures differ substantially not only between plant species but also between cultivars, varieties, accessions, and also between the different plant parts (Lees *et al.*, 1993; Theodoridou *et al.*, 2010; Bodas *et al.*, 2012).

Some previous studies reported variation in CT structures from a few sainfoin accessions (Koupai-abyazani *et al.*, 1993; Marais *et al.*, 2000). However, a recent evaluation of a large sainfoin germplasm collection from the EU 'HealthyHay' project found substantial variation in tannin sizes (i.e. the mean degree of polymerization, mDP), monomeric flavanol compositions that give rise to different prodelphinidin:procyanidin ratio (PD:PC) and stereochemistry at the heterocyclic C-ring (*cis:trans* flavanol ratio) and CT contents among accessions (Stringano *et al.*, 2012). This sort of variation could account for some of the contradictory reports on the beneficial effects of sainfoin (reviewed by Mueller-Harvey, 2006; Theodoridou *et al.*, 2011; Scharenberg *et al.*, 2009). To our knowledge, such a large CT-containing forage legume germplasm collection has not yet been evaluated for possible CT structure–activity relationships in terms of ruminal *in vitro* CH_4 production and fermentation characteristics. The objective of this study was to investigate whether variations in CT structures and contents within a large sainfoin germplasm collection affect rumen *in vitro* CH_4 production and fermentation characteristics. The aim was to identify promising sainfoin accessions suitable for future investigations and to provide novel guidelines for plant breeders interested in developing new varieties with optimized tannin compositions.

Materials and methods

Forage sample collection and preparation

This study was part of the EU funded 'HealthyHay' project (http://legumeplus.eu/). Sainfoin plants had been grown since 2007, and during June and July 2008, a collection of 360 sainfoin accessions was evaluated for their agronomic performances at Cambridge National Institute of Agricultural Botany (NIAB, Cambridge, UK). A subset of 46 accessions was selected for laboratory studies based on morphology, vigour, disease resistance and flower colour (Hayot Carbonero, 2011b). The accessions, a collection of plant material from a particular location (Aubry *et al.*, 2005), originated from Central Europe, Eastern Europe, North America and Asia (Table 1).

Seeds of each accession were initially tested for germination for 2 weeks in the greenhouse. Accessions with sufficient germination rate (> 25%) were then sown in the field in May 2007. After initial plantings, up to 30% of the seedlings were found dead in the field. Therefore, a second strategy was proposed and used in which the seedlings were grown for longer in the greenhouse (1·5 months instead of 2 weeks) in large permeable Jiffy pots (Jiffy Products Ltd., Winchester Hampshire, UK) using a *Rhizobium* species inoculum (either the UK1 strain isolated from an *Onobrychis viciifolia* Cotswold Common cultivar or the 6862 USDA strain from the US culture collection) obtained from Legume Technology Ltd., UK. The plants were then transferred individually to the field.

Each accession was sown in rows in replicate plots (plot size = 1·5 m^2, row spacing = 0·25 m) in May 2008. The top layer of the soil was a slightly stony clay loam, and the subsoil was characterized by a permeable brown slightly stony clay loam. Sainfoin accessions were harvested when about 50% of stems showed open flowers on the lowest half of the flower stem. All accessions were harvested between 9 June and 1 August 2008. The plants were harvested at 5 cm height above the ground using hand shears. For more detailed information, readers are referred to Hayot Carbonero (2011b). Sample collection and preparation were carried out as described previously (Stringano *et al.*, 2012). In brief, plant material was then weighed, packed into Nalgene low-density polyethylene bags and frozen at −20°C. The frozen samples were subsequently transported to the freeze-drying facility at the Archaeological Trust in York, UK. The freeze-dried material was first ground to < 8 mm (knife mill P-15, Retsch, Haan, Germany) and then to < 1 mm using a Wiley mill (T. Peppink and Zn., Machinefabriek, Amsterdam, the Netherlands). Subsequently, the ground material was passed through a sample divider, which generated appropriate subsamples. Subsamples were used at Wageningen University (the Netherlands) for the *in vitro* experiments and had been used at the University of Reading (UK) for the tannin analysis. Subsamples were stored in a dark and cool (4°C) place until analysis.

Experimental design

All sainfoin accessions were first characterized for their CT structures and contents (Stringano *et al.*, 2012).

Table 1 List and origins of sainfoin germplasm accessions.

No.	NIAB accession number	Species	Variety	Country of origin
1	1005R2	*O. viciifolia* Scop.	Perly	n.a.
2	1007R2	*O. viciifolia* Scop.		China
3	1012R2	*O. viciifolia* Scop.	Ambra	Italy
4	1012R1	*O. viciifolia* Scop.	Ambra	Italy
5	1013R2	*O. viciifolia* Scop.	Somborne	n.a.
6	1017R1	*O. viciifolia* Scop.	Teruel	Spain
7	1017R2	*O. viciifolia* Scop.	Teruel	Spain
8	1019R2	*O. viciifolia* Scop.	Jaja	Poland
9	1026R1	*O. viciifolia* Scop.	Buciansky	Slovakia
10	1026R2	*O. viciifolia* Scop.	Buciansky	Slovakia
11	1028R1	*O. viciifolia* Scop.	Simpro	France
12	1041R2	*O. viciifolia* Scop.	Camaras	Romania
13	1043R2	*O. viciifolia* Scop.	Bivolari	Romania
14	1071R2	*O. viciifolia* Scop.	Hampshire Common	n.a.
15	1077R2	*O. viciifolia* Scop.	Nova	n.a.
16	1103R2	*O. viciifolia* Scop.	Korunga	Turkey
17	1104R2	*O. viciifolia* Scop.		Turkey
18	1110R2	*O. viciifolia* Scop.	CPI63750	Turkey
19	1113R2	*O. viciifolia* Scop.	CPI63753	Spain
20	1123R2	*O. viciifolia* Scop.	CPI63763	Turkey
21	1127R2	*O. viciifolia* Scop.	CPI63767	USA, Washington
22	1156R2	*O. viciifolia* Scop.	Dukorastushchii	Former Soviet Union
23	1157R1	*O. viciifolia* Scop.	Miatiletka	Former Soviet Union
24	1157R2	*O. viciifolia* Scop.	Miatiletka	Former Soviet Union
25	1163R2	*O. viciifolia* Scop.	Giant	England
26	1165R1	*O. viciifolia* Scop.	Rees 'A'	England
27	1165R2	*O. viciifolia* Scop.	Rees 'A'	England
28	1169R2	*O. viciifolia* Scop.	CPI63810	Lithuania
29	1179R1	*O. viciifolia* Scop.	CPI63820	Spain
30	1197R2	*O. viciifolia* Scop.	CPI63838	Norway
31	1199R2	*O. viciifolia* Scop.	CPI63840	Former Soviet Union
32	1200R2	*O. viciifolia* Scop.	CPI63841	Germany
33	1210R2	*O. viciifolia* Scop.	Premier	Switzerland
34	1213R2	*O. viciifolia* Scop.	CPI63854	Switzerland
35	1220R2	*O. viciifolia* Scop.	247	Morocco
36	1230R2	*O. viciifolia* Scop.	Visnovsky	Czech Rep., Central
37	1253_04R2	*O. viciifolia* Scop.	Tu86-43-04	Turkey, Hakkari
38	1253_03R1	*O. viciifolia* Scop.	Tu86-43-03	Turkey, Hakkari
39	1256R1	*O. viciifolia* Scop.	Wkt 10	Turkey, Afyon
40	1260R2	*O. viciifolia* Scop.	X93234	China, Xinjiang
41	1261R2	*O. viciifolia* Scop.	Line 108	Armenia
42	1261R1	*O. viciifolia* Scop.	Line 107	Armenia
43	1262R1	*O. viciifolia* Scop.	Cotswold Common	Great Britain
44	1262R2	*O. viciifolia* Scop.	Cotswold Common	Great Britain
45	1264R1	*O. antasiatica*	Sisiani local	Armenia
46	1264R2	*O. antasiatica*	Sisiani local	Armenia

List of accessions were published previously in Stringano *et al.* (2012). Here, it is shown for ease of understanding. Reproduced with permission from Elsevier. R1 and R2 refer to two replicates of the same accession collected from different plots. n.a. = not available.

Then about 0·5 g (on air DM basis) of finely ground (< 1 mm) freeze-dried sample of each accession was weighed into duplicate bottles (250 mL Schott bottle, Mainz, Germany) per accession within run and with triplicate runs at different times. All accessions ($n = 46$) were incubated with polyethylene glycol (PEG, MW 6000 Daltons), a tannin-binding agent, at 1:1 (w/w, substrate:PEG) and without PEG. The PEG treatments were referred as +PEG (with PEG) and -PEG (without PEG).

Experimental procedures and sampling

All animal handling procedures were approved by the Animal Care and Use Committee of Wageningen University and accorded with the Dutch legislation on the use of experimental animals. Cumulative gas production was measured using a fully automated time-related gas production system (Cone *et al.*, 1996). Substrates were weighed into 250-mL volume bottles (Schott bottle, Mainz, Germany) and incubated with buffered rumen fluid. Rumen fluid was obtained from three rumen-cannulated lactating Holstein–Friesian dairy cows fed grass–maize silage and concentrate-based total mixed ration. The cows received grass and maize silage in the morning and afternoon and 7–8 kg of concentrate (160 g kg^{-1} DM starch, 200 g kg^{-1} DM CP, 38 g kg^{-1} DM crude fat and 80 g kg^{-1} DM ash) according to their requirements. Rumen fluid was collected prior to morning feeding and obtained by a suction method using a long plastic tube strainer which was inserted into the rumen cannula and creating a vacuum at the other end of the tube connected to a 1-L plastic bottle. Rumen fluid was transferred into pre-warmed and carbon dioxide (CO_2)-flushed thermos flasks, transported quickly to the laboratory, pooled and filtered through two layers of cheesecloth into a flask flushed with CO_2.

Rumen fluid was mixed with a buffered mineral solution at 1:2 ratio (v/v) as described by Cone *et al.* (1996) under constant stirring and continuous flushing with CO_2, while maintained in a water bath (HAAKE SWB25, Thermo Electron Corporation, Karlsruhe, Germany) at 39°C. Subsequently, 60 mL of buffered rumen fluid was dispensed into pre-warmed fermentation bottles that contained substrate (sainfoin) sample and pre-flushed with CO_2. The bottles were directly incubated at 39°C in a water bath shaking at 40–50 movements per minute. The experiment was repeated three times (runs) on different periods (days). During each run, four bottles containing only buffered rumen fluid were included as blanks. Total gas production for each bottle was corrected for the blank.

The buffer–mineral solution contains, per litre, 8·75 g $NaHCO_3$, 1·0 g NH_4HCO_3, 1·43 g Na_2HPO_4, 1·55 g KH_2PO_4, 0·15 g $MgSO_4{\cdot}7H_2O$, 0·017 g $CaCl_2{\cdot}2H_2O$, 0·015 g $MnCl_2{\cdot}4H_2O$, 0·002 g $CoCl_2{\cdot}6H_2O$, 0·012 g $FeCl_3{\cdot}6H_2O$, 0·3 g $Na_2S{\cdot}9H_2O$ and 1·25 mg resazurin.

Methane measurement and calculation

Methane composition in the headspace of the fermentation bottle was measured by gas chromatography (GC; GC8000Top, CE Instruments, Milan, Italy). To allow CH_4 sampling from the headspace, fermentation bottles were modified as described by Pellikaan *et al.* (2011). The bottles were fitted with a glass extension and sealed with a screw cap fitted with an air-tight septum (XLB-11 Septa 7/16 GRACE, Breda, the Netherlands). The screw caps were furnished with a small aperture to allow a fine needle to pass. Ten microlitre (10 μL) aliquots of the bottle headspace gas were sampled through this opening at 0, 2, 4, 6, 8, 10, 12, 24, 32 and 48 h of incubation using a gas-tight syringe (Gastight® # 1701 Hamilton 1701N, 10 μL Syringe, point style 5, Bonaduz, Switzerland) and directly injected into the GC split injector port. The GC was fitted with a flame ionization detector (FID) and stainless steel column (6 m length, 0·53 mm i.d., 25 μm film thicknesses, and packed with PoraPack Q 50-80 mesh (GRACE, Breda, the Netherlands). The temperatures of the injector, column and detector were maintained at 150°C, 60°C and 150°C respectively. The carrier gas was nitrogen, and the pressure for nitrogen, hydrogen and air was set at 100, 50 and 100 kPa, respectively.

The CH_4 concentration in the headspace was calculated by external calibration using a certified gas mixture containing a known composition of CH_4 (Linde Gas Benelux, Schiedam, the Netherlands). Peak areas were determined by automatic integration software for GC (Chrom-Card data system Version 2.4, 2006, Rodano Milan, Italy). The methane concentrations were plotted against time, and a modified nonlinear monophasic equation (Groot *et al.*, 1996) was fitted through the data points using the nonlinear least-squares regression procedure, PROC NLIN of SAS (SAS, 2010). Curve-fit parameter estimates or the final estimates of the model parameters describing the increasing CH_4 concentrations in time were then used to compute CH_4 concentrations at each individual valve opening. Cumulative CH_4 production was calculated as the sum of the increase in headspace CH_4 concentration between two successive valve openings and the amount of CH_4 vented from the bottle, using the following formula:

Total CH_4 production = headspace (HS) volume × HS CH_4 concentration + gas production × K × HS CH_4 concentration, where the total gas volume is

automatically recorded by the gas production system. K is a coefficient, and it is the ratio of CH_4 concentration in outflow gas to HS.

The cumulative CH_4 produced was corrected for the initial amount of OM weighed into individual bottles and reported in mL per g OM.

Analytical procedures

Analysis of chemical composition was carried out at the University of Reading (UK). Dry matter (DM) and ash were determined using AOAC 967·03 (1995) and AOAC 942·05 (1995) respectively. Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were determined using the ANKOM method (ANKOM^{220} Fibre Analyser, Ankom Corp., Fairport, NY, USA). Nitrogen content was determined using Kjeldahl method (ISO 5983, 2005), and CP was calculated by multiplication of total N content by 6·25. The gross energy content of samples was determined in an adiabatic bomb calorimeter (IKA C7000, IKA®-Werke GmbH and Co. KG, Staufen, Germany). Total water-soluble carbohydrates (TWSC) were hydrolysed in dilute sulphuric acid and analysed on an autoanalyser system (SFA-2000 instrument, Burkard Scientific, Uxbridge, UK) using the neocuproine/cupric sulphate reagent (Chemlab Instruments Ltd, Great Dunmow, UK).

Volatile fatty acid (VFA) concentration was determined by gas chromatography (GC; Fisons HRGC MEGA2, CI instruments, Milan, Italy) as described by Pellikaan *et al.* (2010). Total VFA concentration was corrected for the VFA concentration of blank (i.e. rumen fluid plus buffer) and expressed as mM g^{-1} OM.

Condensed tannin was analysed by thiolytic degradation as described by Gea *et al.* (2011). The HPLC data on monomeric flavanol composition in the CT provided information on the mean degree of polymerization (mDP), prodelphinidin:procyanidin (PD:PC) and *cis:trans* ratios for each sainfoin accession (Gea *et al.*, 2011).

$$\text{mDP} = \frac{\text{amount of extension and terminal flavanol units}}{\text{amount of terminal flavanol units}}$$

Prodelphinidin:procyanidin (PD:PC) ratio of tannins were calculated as follows:

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

The formula for *cis:trans* ratio was as follows:

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

where C = catechin; EC = epicatechin; GC = gallocatechin; and EGC = epigallocatechin.

Statistical analysis

Data per run were averaged before statistical analysis. Repeated measures of total gas and methane production data were analysed using PROC MIXED procedure in SAS with accession, PEG, time and their interactions (accession × PEG, and accession × PEG × time) as a fixed-treatment effect and bottle as a random effect using the repeated-measure procedure with time as a repeated variable (timely data obtained from the same bottle as a repeated-measure analysis).

Data were analysed according to a 46 × 2 factorial arrangement of treatment (46 accessions × 2 treatments, with or without PEG) using the following model:

$$Y_{ijk} = \mu + A_i + P_j + T_k + (A \times P)_{ij} + (A \times P \times T)_{ijk} + e_{ijk}$$

where Y_{ijk} is the dependent variable, μ is the overall mean, A_i is the effect of accession ($i = 46$), P_j is the effect of PEG ($j = 2$), T_k is the effect of time ($k = 4$); $(A \times P)_{ij}$ is the interaction of accession with PEG, and $(A \times P \times T)_{ijk}$ is interactions of accession with PEG and with time; e_{ijk} is the residual error term.

Data for VFA and kinetic parameter estimates (B1 and half-time) were based on one time point. Therefore, the model for these variables does not include time as a repeated variable, and data were subjected to ANOVA using the PROC GLM procedure in SAS.

Post hoc analysis was performed using the Tukey–Kramer multiple range tests for pairwise comparison with least-square means declared significant at a probability of $P \leq 0{\cdot}05$. Tendency was declared at $0{\cdot}06 \leq P < 0{\cdot}10$.

To understand the major patterns of variations among the sainfoin accessions and establish correlation among multiple traits (nutritional composition, tannin structure and CH_4 production), principal component analysis (PCA) was performed using the program CANOCO version 4.5 (Ter Braak and Šmilauer, 2002). To reduce the dimensionality of the data set, PCA was based on optimally weighted observed variables. The data were log-transformed to ensure normality, and the correlation matrix was used for the calculation after the data were mean-centred and standardized. The transformed new variables referred to as principal components (PCs) are the linear combinations of the original variables. Each PC is independent and orthogonal to each other. By plotting the PCs, the interrelationships between each accession in terms of nutritional or tannin characteristics and fermentation products were shown in a two-dimensional PCA correlation biplots. In the correlation biplots, variables with high positive correlations have acute

angles between their vectors. The length indicates the strength of the variable in relation to the displayed ordination (Ter Braak, 1994). The direction of each variable in the loading plot describes its relationship to the other variables and the position of individual accessions on the plot describes their classification along the two principal components.

Results

Nutritional composition and condensed tannin characteristics

The nutritional composition and CT characteristics of sainfoin accessions are shown in Table 2. Large variations were observed among the accessions. Total water-soluble carbohydrates (TWSC) showed a 3·0-fold variation (range from 47·9 to 142·8 g kg^{-1} DM) followed by a 2·2-fold variation for CP (range from 77 to 166 g kg^{-1} DM), ADF varied from 235·1 to 444·8 g kg^{-1} DM and NDF from 337·7 to 584·9 g kg^{-1} DM. Dry matter varied least from 885·3 to 952·3 g kg^{-1}).

Condensed tannin concentrations ranged from 5·7 g kg^{-1} freeze-dried sainfoin plant material (accession 1264R2) to 28·0 g kg^{-1} freeze-dried sainfoin plant material (accession 1256R1). There was also a considerable variation in CT composition as reported previously (Stringano *et al.*, 2012). This variation gave rise to prodelphinidin:procyanidin (PD:PC) ratio that ranged from 52·7:47·3 to 94·8:5·2 (accessions 1179R1 and 1256R1, respectively); to mean degrees of polymerization (mDP) that ranged from 12·0 in accession 1071R2 to 84·0 in accession 1127R2; and the proportion of *trans* varied from 12·0 to 34·0 in accessions 1077R2 and 1156R2, respectively.

In vitro gas and methane production

With respect to the total data set of the 46 sainfoin accessions, +PEG on average increased total gas (GP) by 7·8 mL g^{-1} OM at 6 h of incubation, which equates to a 5·6% increase relative to the GP produced by sainfoin −PEG treatment (Table 3). Similarly, the +PEG compared to −PEG treatment increased GP by 4·1% and 2·8% at 12 and 24 h. The highest increase in GP as a result of +PEG treatment was 17·0% and 11·0% (accession 1165R2 at 6 h and accession 1123R2 at 24 and 48 h, respectively), but addition of PEG had no statistical significant effect on GP at different time points (Table 3).

Methane production differed among accessions and was affected by time of incubation ($P < 0·0001$; Table 4). Addition of PEG has significant effects on CH_4 production. The average cumulative CH_4 production with −PEG treatment was 17·1 ± 4·2 mL g^{-1} OM (10·9–27·9 mL g^{-1} OM), 29·7 ± 4·5 mL g^{-1} OM (19·8–39·4 mL g^{-1} OM), 43·3 ± 4·8 mL g^{-1} OM (31·3–53·6 mL g^{-1} OM) and 53·5 ± 5·7 mL g^{-1} OM (42·6–66·5 mL g^{-1} OM) at 6, 12, 24 and 48 h, respectively. Addition of PEG resulted in a general but small increase in CH_4 production for most accessions (average increases of 11·1, 9·2, 6·8 and 5·0% were observed at 6, 12, 24 and 48 h, respectively). However, the interaction between accession and PEG showed only a tendency ($P = 0·08$) to affect CH_4 (Table 4), while GP was not affected (Table 3). The CH_4 produced after 24 h averaged 18·7% and 19·5% of total gas with and without PEG, respectively.

The half-time parameters of fermentation kinetics were affected by accession and PEG for GP ($P < 0·001$; Table 3) and only by accession ($P = 0·038$) for CH_4 (Table 4). The highest half-time for GP was observed with accession 1199R2 for both +PEG and −PEG (16·1 and 16·5 h, respectively) treatments. Accession 1264R1 yielded the lowest half-time (5·8 h) without PEG and accession 1026R2 (5·3 h) with PEG (Table 3). Without PEG, accession 1005R2 required the least time to produce 50% of its final CH_4 (Table 4). However, taken together there was no accession × PEG interaction effect on GP ($P = 1·00$; Table 3) and CH_4 ($P = 0.185$; Table 4) half-times.

Correlation among nutritional composition, CT structure, *in vitro* CH_4 production and other fermentation products

Figure 1 presents the principal component analysis (PCA) ordination as a two-dimensional correlation biplots. Principal component analysis was made based on the fermentation product data without PEG (−PEG). The first two principal component axes, PC1 (57·6%) and PC2 (18·4%), explained 76·0% of the total variation in the data. Interestingly, there was significant correlation among tannin composition and nutritional parameters with CH_4 production in some sainfoin accessions. Four groups of variables with positive or negative correlations could be identified. Group I consists of variables that include nutritional (NDF, ADF) and CT composition (PD:PC) parameters. Group II explains the CP content of the accessions. Group III describes contributions by mDP, TWSC and the C2:C3 acid ratio, while Group IV comprises GP, CH_4, TVFA, C2, C3, C4 and total isoacids data. Based on the dispersion of the accessions on the loading plot, several groups could be identified. Sainfoin accession represented by number 4, 6, 10, 13, 16, 20 and 46 (Figure 1) fell into Group I. There was a strong negative correlation of fibre (NDF and ADF) with GP and CH_4 production for this group. The PD:PC ratio was

Table 2 Forage composition, condensed tannin (CT) content and CT composition of selected sainfoin accessions.

NIAB accession number	DM (g kg^{-1})	Ash (g kg^{-1} DM)	TWSC (g kg^{-1} DM)	ADF (g kg^{-1} DM)	NDF (g kg^{-1} DM)	CP (g kg^{-1} DM)	CT	mDP	% PD	% *trans*
1005R2	929·0	60·5	100·7	333·9	496·6	128·0	12·5	26·4	70·5	24·5
1007R2	917·4	44·5	124·5	364·7	508·6	91·0	11·4	35·0	81·8	23·1
1012R1	911·9	61·1	83·4	281·0	423·3	161·0	12·9	27·4	75·5	19·5
1012R2	931·0	50·4	74·2	407·0	560·8	106·0	8·7	30·9	79·3	25·6
1013R2	925·8	56·8	118·5	316·8	493·1	117·0	15·3	29·8	71·0	27·3
1017R1	919·0	52·0	99·7	375·9	510·0	112·0	9·1	27·2	72·3	24·2
1017R2	919·9	60·4	84·2	292·9	499·1	147·0	12·9	29·6	71·4	17·6
1019R2	917·5	51·0	142·8	313·6	476·6	90·0	14·6	21·6	77·9	23·6
1026R1	923·4	56·9	86·7	349·5	488·5	119·0	9·3	23·0	75·6	28·6
1026R2	918·4	60·8	101·5	295·2	499·8	128·0	13·6	27·3	80·2	26·5
1028R1	926·8	55·4	78·4	350·5	487·4	141·0	7·9	32·6	72·0	21·3
1041R2	921·3	38·4	116·9	346·4	550·1	77·0	11·6	38·4	79·1	14·9
1043R2	937·8	48·0	114·2	308·5	503·2	118·0	12·9	21·3	84·2	22·5
1071R2	938·0	59·6	82·5	284·8	453·9	142·0	10·5	12·0	67·8	20·5
1077R2	936·9	48·6	74·2	444·8	571·3	109·0	7·1	74·3	78·7	12·0
1103R2	938·0	42·7	105·1	358·3	511·7	112·0	9·0	18·2	78·8	20·8
1104R2	931·1	49·1	77·7	357·2	507·3	110·0	7·0	49·5	78·9	30·8
1110R2	923·7	55·6	79·9	339·7	496·6	129·0	7·8	20·7	80·5	31·3
1113R2	929·8	58·9	93·4	361·1	500·6	116·0	8·2	82·6	78·6	25·9
1123R2	926·4	47·9	79·3	366·7	504·2	116·0	8·8	20·4	80·6	29·9
1127R2	930·6	51·3	80·7	366·2	517·9	113·0	9·0	84·0	82·8	29·6
1156R2	923·6	55·1	90·9	336·2	558·2	127·0	8·5	19·4	78·6	34·0
1157R1	923·3	43·2	90·0	343·3	469·6	124·0	9·6	26·5	78·0	26·9
1157R2	924·6	51·3	104·1	343·4	561·1	125·0	11·6	32·8	80·9	28·5
1163R2	937·0	55·9	88·5	388·4	509·9	117·0	12·4	27·8	64·8	23·7
1165R1	920·5	56·0	121·0	242·3	452·2	166·0	15·6	19·4	65·7	23·5
1165R2	917·5	52·6	113·8	248·0	365·3	141·0	20·1	21·8	67·6	19·6
1169R2	923·6	49·6	122·1	243·3	490·6	148·0	20·1	29·7	77·9	18·8
1179R1	932·7	57·8	95·6	362·6	584·9	142·0	11·0	17·9	52·7	16·8
1197R2	932·1	52·2	82·9	357·3	550·4	118·0	9·0	32·9	80·4	23·8
1199R2	920·6	55·4	73·0	392·6	529·2	122·0	8·9	28·1	77·9	19·8
1200R2	909·7	58·1	99·3	282·6	403·0	162·0	9·1	18·5	72·9	18·7
1210R2	917·1	55·3	113·3	267·1	460·6	166·0	11·7	24·1	72·6	20·8
1213R2	909·4	65·3	96·2	260·4	387·5	144·0	17·3	27·4	80·1	19·0

Table 2 (continued)

NIAB accession number	DM (g kg^{-1})	Ash (g kg^{-1} DM)	TWSC (g kg^{-1} DM)	ADF (g kg^{-1} DM)	NDF (g kg^{-1} DM)	CP (g kg^{-1} DM)	CT	mDP	% PD	% *trans*
1220R2	885·3	57·7	128·5	235·1	337·7	159·0	6·4	24·9	83·1	27·2
1230R2	934·0	48·9	121·6	309·2	503·2	125·0	10·7	15·8	80·8	24·8
1253_03R1	936·1	48·5	77·9	352·0	550·1	123·0	9·2	15·7	85·2	22·2
1253_04R2	914·8	57·1	80·3	383·1	517·0	123·0	10·0	14·7	85·1	23·3
1256R1	942·3	49·8	47·9	377·4	559·8	100·0	28·0	17·6	94·8	31·6
1260R2	928·8	53·8	67·4	397·4	529·7	120·0	8·0	26·4	83·9	26·3
1261R1	914·3	59·0	84·3	287·1	411·4	151·0	7·1	46·1	79·4	18·8
1261R2	952·3	49·8	77·2	411·4	553·3	108·0	9·7	44·8	74·3	23·2
1262R1	935·9	52·5	84·6	315·8	448·9	149·0	12·0	25·8	67·8	20·4
	916·6	65·3	84·0	291·0	432·1	144·0	15·7	38·1	66·7	20·0
1264R1	916·4	53·5	102·8	295·5	411·6	148·0	10·0	60·0	78·3	21·5
1264R2	924·8	53·2	96·2	354·6	490·0	125·0	5·7	58·5	80·9	21·9
Mean	924·9	53·6	94·4	332·4	491·9	127·0	11·3	31·5	76·7	23·4
Minimum	885·3	38·4	47·9	235·1	337·7	77·0	5·7	12·0	52·7	12·0
Maximum	952·3	65·3	142·8	444·8	584·9	166·0	28·0	84·0	94·8	34·0
s.d.	11·0	5·7	19·0	49·5	55·8	20·5	4·1	16·8	7·0	4·6

Results for mDP,% PD and% trans were published previously as Supplementary Info (Table S1) in Stringano *et al.* (2012). Reproduced with permission from Elsevier. R1 and R2 refer to two replicate samples of the same accession collected from different plots.CT = condensed tannins (g/kg freeze-dried sainfoin plant material); mDP = mean degree of polymerization; PD = mean proportion of prodelphinidin in tannin molecules (% PD = 100 – mean proportion of PC); *trans* = mean proportion of *trans* flavanols units in tannin molecules (% *trans* = mean proportion of *cis*).

Table 3 *In vitro* gas production and fermentation kinetics of sainfoin accessions incubated with (+PEG) and without polyethylene glycol (−PEG).

NIAB accession (A) number	Total gas (mL g^{-1} OM)								Kinetic parameter estimates			
	6 h		12 h		24 h		48 h		B1		Half-time (h)	
	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG
1005R2	145·1	153·0	194·3	201·2	235·4	240·8	264·1	268·4	0·95	0·95	6·6	6·0
1007R2	144·2	149·3	193·0	197·5	235·9	241·2	268·2	275·4	0·87	0·80	7·5	7·8
1012R1	155·2	163·9	207·3	214·7	250·6	256·0	280·8	284·4	0·95	0·95	6·5	5·8
1012R2	127·5	135·0	171·8	180·6	213·3	222·6	246·8	255·8	0·80	0·87	9·1	8·5
1013R2	146·9	165·6	196·2	215·0	238·3	254·2	268·7	280·8	0·90	0·95	6·9	5·5
1017R1	137·6	148·5	183·4	193·8	223·7	232·8	253·8	261·7	0·90	0·85	7·3	6·6
1017R2	148·9	154·7	198·6	203·3	239·5	242·0	267·5	268·0	1·00	1·00	6·3	5·7
1019R2	148·7	158·7	195·7	205·7	233·4	242·9	259·1	268·0	0·95	0·95	5·9	5·4
1026R1	133·2	146·7	178·3	193·6	217·1	232·0	245·3	258·4	0·90	0·95	7·0	6·1
1026R2	142·3	148·1	188·1	192·7	225·3	227·3	250·7	250·0	0·95	1·00	6·1	5·3
1028R1	126·7	138·7	169·8	184·8	206·4	222·3	232·4	247·9	0·97	0·97	6·8	6·2
1041R2	130·9	135·4	176·7	179·7	216·9	215·5	246·7	239·9	0·93	0·95	7·6	6·1
1043R2	132·0	149·3	177·9	196·1	216·7	232·6	244·2	256·5	0·95	1·00	6·9	5·6
1071R2	138·0	144·1	187·1	191·9	228·9	230·5	258·6	256·5	1·00	1·00	7·1	6·1
1077R2	109·9	115·0	149·3	155·6	186·4	193·0	216·3	222·2	0·85	0·90	9·4	8·6
1103R2	133·7	139·6	180·0	185·1	220·5	222·7	250·5	248·8	0·90	0·95	7·5	6·3
1104R2	152·0	157·6	202·3	205·8	248·4	249·8	284·7	285·1	0·85	0·80	8·4	8·3
1110R2	145·9	140·8	195·8	188·9	241·6	231·2	277·8	262·8	0·87	0·90	8·6	7·5
1113R2	148·2	153·0	196·6	199·8	241·1	242·4	276·7	276·4	0·85	0·80	8·6	8·1
1123R2	125·7	140·4	168·8	187·3	208·6	230·7	240·0	265·6	0·80	0·83	8·6	9·0
1127R2	135·4	145·5	182·4	192·5	226·3	234·6	261·8	267·1	0·83	0·87	9·4	7·7
1156R2	149·5	152·4	198·7	200·9	248·1	244·6	292·0	278·9	0·70	0·85	12·2	8·0
1157R1	142·5	149·3	190·8	198·5	235·8	243·1	271·8	278·2	0·85	0·85	9·1	8·5
1157R2	139·6	145·8	187·4	193·9	232·5	238·8	269·2	275·2	0·85	0·80	9·6	9·2
1163R2	124·0	139·0	168·3	184·9	212·0	228·0	249·6	263·1	0·80	0·80	11·8	9·4
1165R1	153·4	165·6	204·5	217·3	248·7	260·1	281·4	290·6	0·90	0·93	7·2	6·4
1165R2	147·8	173·4	198·4	219·3	243·5	259·1	277·8	289·6	0·90	0·80	8·0	6·0
1169R2	157·2	169·7	212·1	223·2	264·0	272·9	306·2	313·1	0·80	0·80	9·5	8·4
1179R1	105·3	115·3	148·9	158·6	191·3	199·5	225·9	232·4	0·90	0·90	11·2	9·8
1197R2	138·8	119·9	187·9	162·8	237·9	203·6	282·6	236·7	0·70	0·87	13·2	9·8
1199R2	131·6	135·4	180·4	183·5	232·1	234·1	280·5	281·3	0·70	0·70	16·5	16·1
1200R2	144·1	161·2	195·3	213·4	245·3	263·6	287·5	305·7	0·80	0·80	10·7	9·8
1210R2	166·4	169·3	220·7	221·4	265·8	262·1	297·6	288·7	0·95	1·00	6·5	5·5
1213R2	144·8	151·9	196·5	203·9	240·0	247·1	270·7	277·0	0·97	0·95	7·1	6·6
1220R2	166·9	172·4	219·6	223·8	262·6	266·1	292·3	295·8	0·95	0·90	6·0	5·9
1230R2	142·8	161·2	196·0	213·5	242·3	257·0	275·6	287·4	0·95	0·93	7·7	6·4
1253_03R1	134·4	142·7	182·4	190·8	225·5	232·4	258·1	263·1	0·90	0·90	8·2	7·4
1253_04R2	136·0	150·9	185·9	200·2	229·5	242·4	261·3	273·0	0·95	0·90	7·7	6·9
1256R1	141·5	152·7	190·5	202·7	229·7	241·3	255·6	265·9	1·05	1·05	6·2	5·7
1260R2	139·7	131·6	177·5	177·8	211·4	218·8	237·6	249·5	0·80	0·90	6·6	7·9
1261R1	154·4	150·0	206·1	201·6	249·0	243·7	279·0	272·2	0·95	1·00	6·5	6·5
1261R2	127·2	135·0	172·3	181·7	214·0	222·5	246·8	252·5	0·90	0·90	8·9	7·6
1262R1	143·8	148·3	191·7	198·4	231·5	238·9	259·1	266·1	1·00	1·00	6·5	6·3
1262R2	137·1	150·3	185·4	198·6	226·2	237·8	255·2	264·4	0·97	0·93	7·1	6·0
1264R1	159·6	155·3	208·9	206·5	249·0	249·3	276·6	279·5	0·90	0·95	5·8	6·6
1264R2	135·7	150·1	183·6	200·1	227·1	242·8	260·7	273·5	0·90	0·95	8·4	6·8

Table 3 (continued)

	s.e.m.	*P*-value[1]		*P*-value[2]	
A	4·48	< 0·0001	A	< 0·0001	< 0·0001
PEG	0·93	< 0·0001	PEG	0·272	0·0004
Time	1·87	< 0·0001	A × PEG	1·000	1·000
A × PEG	6·34	0·750			
A × PEG × Time	12·68	1·000			

R1 and R2 refer to two replicate samples of the same accessions collected from different plots. s.e.m. = standard error of the means. [1]P-value for total gas; [2]P-value for kinetics parameter estimates.

the most important factor among the CT properties considered and showed a negative correlation with the total gas, CH_4 and total VFA produced for accessions in Group I.

The CP content tended to show a positive association with fermentation characteristics such as total isoacids. On the other hand, the TWSC showed a weak relationship with gas production characteristics, although there was a strong positive correlation with the C2:C3 ratio. The dietary CP content was negatively related to mDP, TWSC and C2:C3 ratio. Accessions numbered 24, 32 and 42 were close to the origin and therefore cannot reliably be described as falling into this group. The *trans:cis* ratio was associated with the neutral axis, and this might suggest that no correlation (either positive or negative) exists.

Discussion

Before the 1950s, sainfoin was grown much more widely in Europe because of its positive contributions to ecosystem services, i.e., enhancing soil fertility, attracting pollinating insects and also to animal nutrition and health (Mueller-Harvey, 2009). While several forage legumes have been the subject of intensive plant improvement programmes, sainfoin has not yet been targeted in Europe although several opportunities have been identified (Doyle *et al.*, 1984; Hayot Carbonero, 2011b). This study was part of the EU 'HealthyHay' project, which sought to characterize and identify promising candidates and lay foundations for future sainfoin improvement programmes that will contribute towards developing sustainable ruminant feeding strategies. Therefore, accessions evaluated in the present study were not restricted to currently grown cultivars or varieties, but included also accessions that were collected from different germplasm collections around the world (Table 1). The sainfoin collection that was established at the National Institute of Agricultural Botany (Cambridge, UK), therefore, was representative of the currently available genetic diversity. This germplasm collection has been evaluated for agronomic performance (Hayot Carbonero, 2011b), tannin contents and composition (Stringano *et al.*, 2012). The present study used the *in vitro* gas production technique for screening this germplasm collection for inhibitory properties of the different accessions on ruminal CH_4 production.

Nutritional analysis

The CP and fibre contents of most accessions were consistent with previous findings (Liu *et al.*, 2008). On average, the NDF and CP contents in this previous study ranged from 435 to 488 g kg^{-1} DM and 148 to 160 g kg^{-1} DM, respectively, for the first harvest (April–September). The NDF in NIAB collection ranged from 338 to 585 g kg^{-1} DM, whereas the CP contents in the NIAB collection ranged from 77 to 166 g kg^{-1} DM and met the minimum required level (80 g kg^{-1} DM) for optimal microbial activity in the rumen (Annison and Bryden, 1998) except for accession 1041R2 which was marginal at 77 g CP per kg DM.

Correlations between tannin content, composition and fermentation products

Generally, tannins have been associated with less CH_4 production because (i) they reduce fibre degradation by complexing with lignocellulose, which in turn prevents microbial fermentation, or (ii) by directly inhibiting cellulolytic micro-organisms or a combination of both (McSweeney *et al.*, 2001). To date, it is not known whether these effects are related to CT content or CT structures. The current study used PEG as a CT neutralizing agent (Silanikove *et al.*, 2001), which was expected to increase CH_4 production. Although PEG did increase CH_4 production, the increase was not related to CT content in all accessions (Table 4 and Figure 2). Several accessions with relatively high CT concentrations did not yield low CH_4 (−PEG treatment). In contrast, accessions 1012R2, 1077R2, 1104R2 and 1157R1 contained low CT concentrations (Table 2) but produced high CH_4 in +PEG treatment (Table 4). This suggested that CT concentration was

Table 4 *In vitro* methane production and fermentation kinetics of sainfoin accessions incubated with (+PEG) and without polyethylene glycol (−PEG).

NIAB accession (A) number	Methane production (mL g^{-1} OM)								Kinetic parameter estimates			
	6 h		12 h		24 h		48 h		B1		Half-time (h)	
	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG
1005R2	21·5	25·1	38·2	37·7	50·9	50·0	57·8	59·4	1·65	1·00	9·7	11·6
1007R2	17·1	17·0	32·4	31·3	46·9	45·8	55·9	55·6	1·57	1·40	12·3	12·3
1012R1	20·0	20·5	35·1	34·7	49·4	49·8	58·7	61·8	1·35	1·20	11·4	15·1
1012R2	12·9	17·9	26·8	32·9	40·1	47·3	47·2	56·7	1·70	1·43	11·3	12·6
1013R2	18·9	19·2	32·8	34·1	47·0	48·6	57·4	57·9	1·30	1·40	14·3	11·3
1017R1	18·4	19·1	31·7	33·4	45·3	47·8	55·6	57·8	1·25	1·35	15·4	12·2
1017R2	19·1	18·8	32·7	33·5	46·4	48·5	56·2	58·8	1·25	1·35	12·3	12·8
1019R2	16·4	19·6	26·5	36·1	38·3	49·5	49·2	56·4	1·05	1·65	19·2	19·7
1026R1	17·1	18·0	29·3	34·6	42·0	47·8	51·5	54·9	1·20	1·65	13·3	11·0
1026R2	16·2	17·3	28·4	31·4	41·1	45·2	50·4	54·3	1·30	1·40	13·0	12·9
1028R1	15·5	16·9	29·7	30·9	42·9	44·2	50·8	52·5	1·53	1·47	12·0	11·6
1041R2	13·3	15·6	26·9	30·6	41·2	44·0	50·3	51·0	1·57	1·65	12·8	10·7
1043R2	15·6	16·4	28·2	31·2	42·0	44·4	52·5	51·5	1·25	1·60	14·7	10·6
1071R2	14·9	16·4	28·0	31·1	42·5	46·4	53·2	56·8	1·35	1·40	14·6	13·5
1077R2	11·2	13·9	24·3	27·1	38·2	40·6	46·4	49·2	1·70	1·50	13·4	12·5
1103R2	13·3	15·0	26·0	29·6	41·1	43·8	51·9	52·6	1·45	1·55	15·2	13·0
1104R2	22·7	35·2	35·3	46·8	49·6	59·0	63·0	70·1	0·95	0·70	13·9	13·4
1110R2	20·6	16·8	33·9	29·7	48·6	43·4	60·9	53·6	1·10	1·30	16·0	13·4
1113R2	17·5	19·6	29·7	29·9	44·3	41·2	57·7	51·4	1·10	0·90	19·1	22·4
1123R2	17·0	21·5	29·2	33·4	41·0	46·3	48·9	57·6	1·30	1·00	11·3	18·0
1127R2	20·7	23·5	31·3	34·5	42·6	46·0	52·0	55·6	0·97	0·93	14·6	13·7
1156R2	22·6	44·0	35·8	59·6	50·7	75·5	64·1	89·3	1·00	0·85	17·1	12·9
1157R1	15·8	28·7	24·1	40·3	33·9	52·6	43·5	63·3	0·80	0·85	16·7	14·9
1157R2	19·3	21·5	32·2	33·9	47·0	48·2	60·3	61·6	1·10	0·95	17·6	20·9
1163R2	24·6	27·0	34·0	35·6	44·3	44·7	54·0	53·1	0·75	0·65	18·3	14·3
1165R1	19·9	19·9	30·0	32·7	40·8	46·6	49·7	58·3	0·97	1·10	15·8	16·0
1165R2	19·2	18·6	31·7	30·6	46·5	45·8	60·1	61·4	1·05	1·05	19·5	15·9
1169R2	27·9	30·1	38·9	41·8	50·9	54·0	62·0	64·5	0·75	0·85	17·3	13·5
1179R1	22·1	18·3	33·7	29·4	46·1	42·3	56·6	54·6	0·95	0·95	14·7	19·8
1197R2	21·3	23·8	33·9	34·1	47·8	45·4	59·7	55·5	1·00	0·87	15·7	16·1
1199R2	26·4	25·3	39·4	36·3	53·6	48·4	66·5	59·5	0·90	0·85	16·4	17·1
1200R2	20·9	18·1	34·6	30·2	50·0	45·6	63·0	61·0	1·10	1·05	16·0	13·5
1210R2	15·9	14·9	28·5	28·8	42·5	44·3	52·2	54·8	1·47	1·50	13·5	13·9
1213R2	14·0	17·8	27·9	30·4	41·6	44·1	49·8	55·0	1·65	1·20	13·4	14·4
1220R2	15·1	15·2	31·3	33·6	46·7	48·4	55·1	55·8	1·73	1·97	13·1	12·6
1230R2	18·3	15·7	30·7	32·7	44·0	47·9	54·7	55·7	1·15	1·78	14·3	11·6
1253_03R1	13·1	13·7	25·5	26·8	39·4	41·3	49·1	50·9	1·50	1·57	15·3	14·8
1253_04R2	13·2	13·8	26·0	27·4	40·3	42·2	49·7	51·7	1·57	1·57	14·7	13·7
1256R1	13·2	13·1	24·5	26·0	37·5	40·3	47·2	50·1	1·43	1·53	16·6	14·6
1260R2	11·0	13·3	19·8	26·1	31·3	39·5	42·6	48·4	1·20	1·55	14·4	14·7
1261R1	13·0	13·3	25·4	27·3	40·1	42·6	50·7	52·5	1·50	1·63	15·3	14·0
1261R2	12·7	13·2	24·1	27·4	37·3	40·6	46·8	47·7	1·50	1·80	16·5	12·3
1262R1	12·7	13·2	25·7	27·1	39·3	41·3	47·9	49·9	1·63	1·70	16·2	13·0
1262R2	11·7	13·6	24·0	27·4	38·7	42·0	48·6	51·1	1·63	1·63	15·0	13·5
1264R1	10·9	13·3	24·0	27·2	40·3	43·3	50·6	53·8	1·80	1·63	14·7	14·5
1264R2	12·8	12·0	24·8	25·9	39·1	40·6	49·8	49·3	1·43	1·73	16·0	13·2

Table 4 (continued)

	s.e.m.	*P*-value[1]		*P*-value[2]	
A	1·85	< 0·0001	A	< 0·0001	0·038
PEG	0·39	< 0·0001	PEG	0.661	0·110
Time	0·55	< 0·0001	A × PEG	1·000	0·185
A × PEG	2·62	0·080			
A × PEG × Time	5·23	1·000			

R1 and R2 refer to replicate samples of the same accession collected from different plots. s.e.m. = standard error of the means. [1]P-value for methane production; [2]P-value for kinetics parameter estimates.

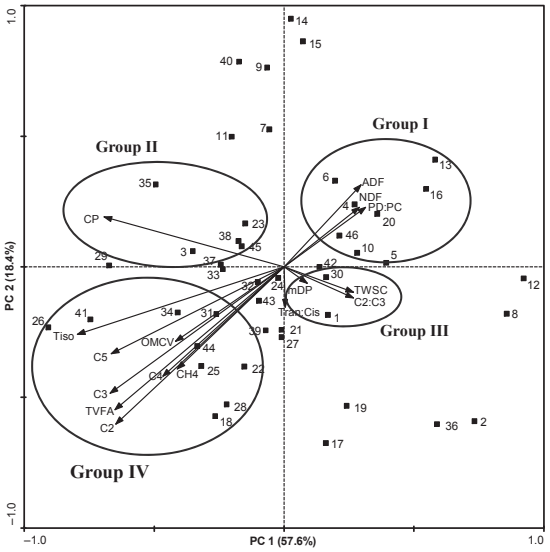

Figure 1 Principal component analysis (PCA) ordination diagram showing the position of each sainfoin accession in relation to the measured feed chemical composition, tannin composition, CH_4, GP and VFA. The numbers in the panel correspond to the sainfoin accessions as presented in Table 1. ADF = acid detergent fibre; CP = crude protein; mDP = mean degree of polymerization; NDF = neutral detergent fibre; PD:PC = ratio of prodelphinidin:procyanidin in CTs; TVFA = total volatile fatty acids (C2 + C3 + C4 + C5 + Tiso); *trans:cis* = ratio of *trans*- and *cis*-flavanols in CTs; TWSC = total water-soluble carbohydrate; Tiso = total isoacids (isobutyrate + isovalerate); C2 = acetate; C3 = propionate; C4 = butyrate; C5 = valerate; CH_4 = methane and OMCV = gas production.

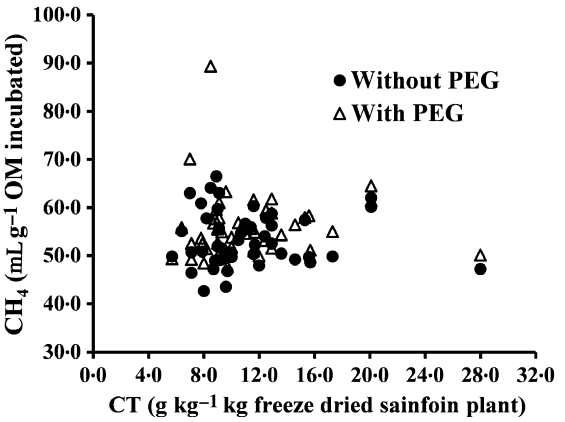

Figure 2 Relationship between condensed tannin (CT) content and *in vitro* methane production of sainfoin accessions incubated without (•) and with (Δ) polyethylene glycol (PEG) for 48 h.

not obviously responsible. Other accessions provided further evidence. With accession 1156R2, the +PEG treatment produced the highest CH_4 increase at 6, 12, 24 and 48 h, but this accession had a relatively low CT content (8·5 g CT per kg freeze-dried sainfoin plant material). Accession 1256R1 with a relatively high CT content (28 g CT per kg freeze-dried sainfoin plant material) did not produce much CH_4 in the presence of PEG (+PEG). Most surprisingly, for accession 1165R2 (20·1 g CT per kg freeze-dried sainfoin plant material), no difference in CH_4 production was observed between −PEG and +PEG treatments. It is possible that CT structural features and the presence of other plant components, which may have interacted with CT, may have modified their activities. It is well known that CT interacts with proteins, cell walls, fibres and other compounds (Le Bourvellec *et al.*, 2007; Bindon and Kennedy, 2011; Dobreva *et al.*, 2011).

Gas production from a variety of feeds incubated *in vitro* is closely related to VFA production and is related to carbohydrate fermentation (Getachew *et al.*, 2002). The VFA produced during enteric fermentation is a primary source of energy for ruminants. Besides, the type of VFA produced in the rumen determines CH_4 production. The formation of acetate in the rumen promotes CH_4 production, whereas propionate production and methanogenesis involve processes that compete for hydrogen (Moss *et al.*, 2000). Therefore, quantification of VFA concentrations produced from fermentation of sainfoin accessions provides useful

Table 5 Volatile fatty acid production of sainfoin accessions incubated with (+PEG) and without polyethylene glycol (−PEG).NIAB accession (A) number.

NIAB accession (A) number	Total VFA (mM per OM)		Molar proportion (% of total VFA)											
			C2		C3		C4		C5		Total isoacids		C2:C3	
	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG
1005R2	6·5	6·7	65·7	64·3	18·5	19·6	10·0	9·9	1·7	1·9	4·3	4·5	3·5	3·3
1007R2	6·4	6·8	65·7	65·6	18·4	18·8	10·4	9·9	1·6	1·7	4·1	4·0	3·6	3·5
1012R1	6·8	6·4	65·6	65·0	18·7	19·3	9·7	9·3	1·8	1·9	4·5	4·5	3·5	3·4
1012R2	5·7	6·0	66·2	65·1	17·5	19·1	10·3	9·7	1·8	1·8	4·6	4·3	3·8	3·4
1013R2	6·2	6·2	65·2	64·2	18·7	19·6	10·4	10·2	1·7	1·8	4·3	4·3	3·5	3·3
1017R1	5·9	6·5	65·3	65·0	18·6	19·1	10·2	9·8	1·7	1·8	4·4	4·3	3·5	3·4
1017R2	6·2	6·5	64·9	64·8	18·7	19·1	10·3	9·9	1·8	1·8	4·7	4·4	3·5	3·4
1019R2	6·1	6·3	64·5	64·2	18·7	19·2	11·1	10·8	1·7	1·7	4·2	4·1	3·5	3·3
1026R1	6·0	6·0	64·5	63·6	19·1	19·5	10·3	10·4	1·8	1·9	4·6	4·6	3·4	3·3
1026R2	6·5	6·0	66·0	62·7	17·8	19·5	10·4	10·9	1·7	2·0	4·4	4·9	3·7	3·2
1028R1	6·4	6·6	65·5	65·0	18·4	18·6	10·0	9·9	1·8	1·9	4·6	4·6	3·6	3·5
1041R2	5·9	5·8	65·1	64·5	18·3	18·7	10·9	10·8	1·7	1·8	4·3	4·2	3·6	3·4
1043R2	6·0	6·0	64·8	63·3	18·4	19·4	11·0	10·9	1·8	1·9	4·5	4·6	3·5	3·3
1071R2	5·5	6·4	64·6	64·0	18·8	19·5	10·2	9·9	1·8	1·9	4·8	4·7	3·4	3·3
1077R2	5·6	6·4	64·9	64·3	18·5	19·1	10·3	10·0	1·8	1·9	4·7	4·6	3·5	3·4
1103R2	5·9	6·0	64·8	64·0	18·7	19·3	10·7	10·4	1·8	1·9	4·5	4·4	3·5	3·3
1104R2	7·4	7·4	66·3	65·6	18·3	18·8	9·9	9·7	1·7	1·9	4·2	4·0	3·6	3·5
1110R2	7·5	7·2	65·9	66·5	18·8	18·9	9·7	9·2	1·7	1·3	4·3	4·4	3·5	3·0
1113R2	7·4	7·5	66·4	65·7	18·1	18·6	9·8	9·8	1·7	1·8	4·2	4·1	3·7	3·5
1123R2	7·0	7·3	66·8	65·6	17·2	18·7	10·1	9·7	1·9	2·0	4·6	4·0	3·9	3·5
1127R2	7·1	7·2	65·7	65·6	18·6	18·7	10·0	9·8	1·8	1·9	4·4	4·0	3·5	3·5
1156R2	7·4	7·4	65·6	65·3	18·8	19·0	9·9	9·7	1·8	1·9	4·4	4·1	3·5	3·4
1157R1	7·1	7·3	65·5	65·3	18·6	18·9	10·0	9·7	1·8	1·9	4·5	4·2	3·5	3·5
1157R2	7·1	7·9	65·2	64·9	18·7	18·9	10·3	10·2	1·7	1·8	4·4	4·2	3·5	3·4
1163R2	7·6	7·0	65·2	65·0	18·9	18·8	10·2	10·1	1·7	1·8	4·3	4·2	3·5	3·4
1165R1	8·5	7·5	66·1	63·9	18·6	19·9	9·5	9·7	1·9	2·1	4·5	4·5	3·6	3·2
1165R2	7·4	7·3	66·1	63·8	18·4	19·5	9·8	10·2	1·7	1·9	4·3	4·6	3·6	3·3
1169R2	7·5	7·6	65·6	64·8	19·0	19·6	9·7	9·5	1·8	1·9	4·3	4·1	3·5	3·3
1179R1	7·6	7·8	65·2	64·7	18·8	19·1	9·9	9·9	1·8	1·9	4·6	4·5	3·5	3·4
1197R2	6·3	6·8	67·1	64·2	15·5	19·0	11·0	10·6	1·9	1·9	4·9	4·4	4·3	3·4
1199R2	7·6	7·3	65·9	65·7	18·5	18·7	9·8	9·6	1·8	2·0	4·4	4·1	3·6	3·5
1200R2	6·7	7·8	65·8	65·2	18·6	19·0	9·7	9·6	1·8	1·9	4·5	4·3	3·5	3·4
1210R2	7·7	8·0	64·0	62·8	19·1	20·2	10·9	10·6	1·9	2·0	4·6	4·4	3·3	3·1
1213R2	8·2	7·9	64·3	63·7	19·0	19·6	10·8	10·5	1·8	1·9	4·5	4·3	3·4	3·3
1220R2	8·0	8·2	63·9	63·8	18·9	19·3	11·0	10·6	2·0	2·0	4·8	4·3	3·4	3·3
1230R2	7·5	7·9	63·9	63·5	19·0	19·5	11·4	11·0	1·8	1·9	4·3	4·1	3·4	3·3
1253_03R1	8·3	7·7	64·4	63·5	18·8	19·6	10·8	10·5	1·9	2·0	4·6	4·3	3·4	3·2
1253_03R2	7·5	8·4	64·5	64·1	18·7	19·2	10·9	10·5	1·8	1·9	4·6	4·2	3·4	3·3
1256R1	7·6	7·8	64·9	63·9	19·0	19·6	10·3	10·2	1·8	2·0	4·4	4·3	3·4	3·3
1260R2	6·7	7·4	62·4	63·9	20·0	19·2	11·3	10·7	2·0	1·9	4·9	4·3	3·1	3·3
1261R1	8·5	8·1	64·1	64·0	19·3	19·3	10·6	10·5	1·9	1·9	4·6	4·3	3·3	3·3
1261R2	7·3	7·2	64·5	64·1	18·9	19·1	10·7	10·6	1·9	2·0	4·5	4·1	3·4	3·4
1262R1	7·6	7·8	64·5	64·1	18·9	19·1	10·8	10·6	1·8	1·9	4·5	4·2	3·4	3·4
1262R2	7·9	7·9	64·9	64·0	18·9	19·6	10·4	10·2	1·8	1·9	4·5	4·3	3·4	3·3
1264R1	7·6	7·9	64·2	63·7	18·8	19·3	11·0	10·8	1·9	2·0	4·7	4·3	3·4	3·3
1264R2	7·5	7·4	64·1	63·9	18·6	18·9	11·3	11·0	1·9	1·9	4·6	4·2	3·5	3·4

Table 5 (continued)

NIAB accession (A) number	Total VFA (mM per OM)		Molar proportion (% of total VFA)											
			C2		C3		C4		C5		Total isoacids		C2:C3	
	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG
s.e.m.														
A	0·03		0·41		0·01		0·10		0·06		1·93		0·03	
PEG	0·01		0·08		0·01		0·02		0·01		0·40		0·01	
A × PEG	0·04		0·58		0·02		0·14		0·09		2·73		0·04	
P-value														
A	< 0·0001		< 0·0001		< 0·0001		< 0·0001		< 0·0001		< 0·0001		< 0·0001	
PEG	< 0·0001		< 0·0001		< 0·0001		< 0·0001		< 0·0001		< 0·0001		< 0·0001	
A × PEG	< 0·0001		< 0·0001		< 0·0001		< 0·0001		< 0·0001		< 0·0001		< 0·0001	

R1 and R2 refer to two replicate samples of the same accession collected from different plots. s.e.m. = standard error of the means.

information for comparing their nutritional values. Total VFA and individual VFA concentrations were affected (decreased or increased) with +PEG treatment (Table 5). However, a significant increase ($P < 0{\cdot}0001$) upon +PEG treatment was observed only for propionic and valeric acids. Also, no accession × PEG interaction effect on GP (Table 3) and CH_4 production was found (Table 4). In contrast, Azuhnwi *et al.* (2011) reported a significant increase in GP after adding PEG to sainfoin accessions. The difference could also be due to different plant maturity at harvest (Koupai-abyazani *et al.*, 1993; Theodoridou *et al.*, 2011), which could affect the plant CT and nutritional compositions.

The accessions investigated in the present study differed greatly in CT structures in terms of tannin polymer size (mDP values) and flavanol composition (Table 2). These sorts of differences can affect CT-binding properties (Frazier *et al.*, 2010) and thus digestion and nutritional value of forages (Mueller-Harvey, 2006). However, the mechanisms by which tannins reduce ruminal degradation of different dietary components have not yet been established. Sainfoin contains a heterogeneous mix of different tannin structures, which probably affects their interactions with plant components. It may also yield complexes that vary in the extent of their ruminal protein protection. For instance, a higher PD:PC ratio reflects more hydrogen bonding sites, which may enhance the affinity of CT (Aerts *et al.*, 1999) and thus slow down ruminal protein and fibre degradation. In agreement with this, the PCA analysis (Figure 1) showed a strong positive correlation between PD:PC and fibre, but the correlation with protein was weak; this reflects the relative impacts of fibre and CP on the fermentation products in the presence of this sainfoin CT.

Principal component analysis for investigating multiple trait relationships

As differences in CT contents did not reveal clear positive or negative effects on GP (Table 3), CH_4 (Table 4) or VFA production (Table 5), we subjected the data to principal component analysis (PCA) in order to examine the relationships among multiple traits (CT composition, nutritional content, CH_4, GP and VFA). Principal component analysis showed correlations among forage composition and CT structural features with CH_4 production in some accessions (Figure 1). Even though individual constituents covered a wide range (Table 2), the relationships between CH_4 production and forage constituents were not conclusive. In some accessions, CH_4 production was negatively related to nutritional components such as NDF and ADF (Figure 1). The PCA loading plot showed that in some sainfoin accessions producing CH_4, production is negatively associated with variables that relate to forage quality (such as fibre) and to flavanol composition (e.g. PD:PC ratio). On the other hand, according to Taweel *et al.* (2005), fermentation of non-structural carbohydrates in forage diets generates more propionic acid. However, total water-soluble carbohydrate tended to show a weak relationship with both propionic acid and CH_4 production in some sainfoin accessions. This result agrees with the elevated water-soluble carbohydrate concentrations in perennial ryegrass cultivars, which were also not strongly related to *in vitro* total gas production, CH_4 production and organic matter digestibility (Lovett *et al.*, 2004).

The present screening study suggested that the accessions collected across the world and evaluated under similar conditions exhibited substantial variation in terms of ruminal *in vitro* CH_4 production.

However, sainfoin CT contents did not affect CH_4 production, but other multiple factors appear also involved. Principal component analysis was able to cluster accessions into several groups according to their overall similarity which might require further investigations. The grouping explained by the PD:PC ratio appears to contain an important source of variation that is negatively related to CH_4 production, and, therefore, sainfoin accessions numbered 1012R2, 1017R2, 1026R2, 1043R2, 1103R2, 1123R2 and 1264R2 deserve further examination. We conclude that during evaluation of different sainfoin accessions for their rumen *in vitro* CH_4 production focus should not only give to CT contents and macronutrients, but should also take into account the CT structures.

Conflict of interest

The authors have no conflict of interest.

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