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Impact of variation in structure of condensed tannins from sainfoin (*Onobrychis viciifolia*) on *in vitro* ruminal methane production and fermentation characteristics

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Condensed tannins and in vitro methane production

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Summary

Our study investigated the effects of condensed tannins (CT) on rumen *in vitro* methane (CH_4) production and fermentation characteristics by incubating lucerne in buffered rumen fluid in combination with different CT extracts at 0 (control), 40, 80 and 120 g CT/kg DM. Condensed tannins were extracted from 4 sainfoin accessions: Rees "A", CPI63763, Cotswold Common and CPI63767. Gas production (GP) was measured using a fully automated GP apparatus with CH_4 measured at distinct time points. Condensed tannins differed substantially in terms of polymer size and varied from 13 (Rees "A") to 73 (CPI63767) mean degree of polymerization, but had relatively similar characteristics in terms of CT content, procyanidin:prodelphinidin (PC:PD) and *cis:trans* ratios. Compared to control, addition of CT from CPI63767 and CPI63763 at 80 and 120 g CT/kg DM reduced CH_4 by 43 and 65%, and by 23 and 57%, respectively, after 24 h incubation. Similarly, CT from Rees "A" and Cotswold Common reduced CH_4 by 26 and 46%, and by 28 and 46%, respectively. Addition of increasing level of CT linearly reduced the maximum rates of GP and CH_4 production, and the estimated *in vitro* organic matter digestibility. There was a negative linear and quadratic ($p < 0.01$) relation between CT concentration and total volatile fatty acid (VFA) production. Inclusion of 80 and 120 g CT/kg DM reduced ($p < 0.001$) branched-chain VFA production and acetate:propionate ratio and was lowest for CPI63767. A decrease in proteolytic activity as indirectly shown by a change in VFA composition favoring a shift towards propionate and reduction in branched-chain VFA production varied with type of CT, and was highest for CPI63767. In conclusion, these results suggest that tannin polymer size is an important factor affecting *in vitro* CH_4 production which may be linked to the CT interaction with dietary substrate or microbial cells.

Keywords: sainfoin, condensed tannin, polymer size, methane, *in vitro*

Introduction

Plants produce a vast array of different tannin types and concentrations (Khanbabaee and Van Ree, 2001; Mueller-Harvey, 2006; Huemmer and Schreier, 2008). For a long time tannins have been considered as an anti-nutritional factor in animal nutrition (Mueller-Harvey, 2006). Whether tannins exert positive or negative effects appears to depend on the type and level of tannins in the plants (Barry and McNabb, 1999; Min et al., 2003), the amount ingested and the animal species involved (Frutos et al., 2004; Mueller-Harvey, 2006). It is of note, however, that only a few studies investigated their bioactivities and included a full tannin analysis. Most of these studies compared the procyanidin-rich *Lotus corniculatus* (birdsfoot trefoil) and the prodelphinidin-rich *Lotus pedunculatus* (big trefoil) tannin types (Molan et al., 2001; Min et al., 2003; Tavendale et al., 2005). However, condensed tannins (CT) vary considerably even within a single plant species (Koupai-Abyazani et al., 1993; Marais et al., 2000; Stringano et al., 2012). Studies are needed to test how this variation affects their biological activity in relation to ruminant nutrition, nitrogen and methane (CH_4) emission.

Tannins of various origins have been shown to inhibit ruminal CH_4 production either when fed to ruminants as tannin-containing forages (Woodward et al., 2001; Puchala et al., 2005) and as tannin extracts tested *in vitro* (Tavendale et al., 2005; Pellikaan et al., 2011b; Hassanat and Benchaar, 2013) or fed *in vivo* (Beauchemin et al., 2007; Animut et al., 2008; Bhatta et al., 2013). To date, this anti-methanogenic activity appears to be variable and could not be explained by simply grouping the tannins into hydrolysable or condensed tannin types (Bhatta et al., 2009; Pellikaan et al., 2011b), suggesting that specific chemical structural properties are responsible for their anti-methanogenic activity. However, to our knowledge, limited studies are available focusing on chemical structural composition of CT to elucidate which chemical property is most responsible in reducing ruminal CH_4 production.

77 The objective of this study was to investigate the structural variation of semi-purified
78 condensed tannin extracts which had been obtained from 4 sainfoin accessions on rumen *in*
79 *vitro* CH_4 production and fermentation characteristics. We hypothesize that the mean degree
80 of polymerization (i.e. polymer size) of the tannin molecule is the most important property
81 determining its activity to inhibit *in vitro* CH_4 production.

82 **Material and methods**

83 **Plant samples for preparation of condensed tannin extracts**

84 Four sainfoin accessions were selected from the EU 'HealthyHay' sainfoin germplasm
85 collection based on their distinct differences in terms of CT structure (Stringano et al., 2012).
86 The 4 accessions were: accession number 1165 (Rees "A"), 1123 (CPI63763), 1262
87 (Cotswold Common) and 1127 (CPI63767). The plants were grown at the National Institute
88 of Agricultural Botany (NIAB; Cambridge, UK). Growing conditions and source of seeds
89 were described previously (Carbonero et al., 2011). Sainfoin accessions were harvested when
90 about 50% of stems showed open flowers on the lowest half of the flower stem. Plant
91 material was packed in special bags (Nalgene low density polyethylene bags; 22.9 × 45.7
92 cm), stored at -20 °C, freeze-dried and then ground to pass an 8-mm sieve using an impeller
93 mill (Retsch GmbH, SM1, Haan, Germany), and subsequently ground to pass a 1-mm sieve
94 (Retsch GmbH, ZM 100, Haan, Germany).

95 **Extraction of condensed tannins**

96 The CT extracts were prepared as described by Stringano et al. (2011). Briefly, 25 g of
97 ground (1 mm) sainfoin sample was extracted once with acetone/water (200 ml; 7:3, v/v)
98 containing ascorbic acid (1 g/liter) for 40 min. Chlorophyll was removed from the
99 acetone/water solution by extracting twice with dichloromethane (200 ml). Acetone was then
100 removed on a rotary evaporator and the aqueous phase was concentrated in vacuum (< 40

101 °C), and subsequently freeze-dried to yield CT extracts. The extracts were stored at -20 °C
102 until use.

103 **Analysis of condensed tannin extracts**

104 Condensed tannin extracts were analyzed for CT content and structural properties by thiolysis
105 as described by Gea et al. (2011). Briefly, freeze-dried extract (4 mg) was weighed into a
106 glass tube and 1.0 ml methanol was added, followed by 50 µl of acidified methanol (3.3 ml
107 concentrated HCl in 100 ml methanol) and 100 µl benzylmercaptan in methanol (5:95, v/v).
108 The reaction mixture was stirred at 40 °C for 30 minutes. The reaction was stopped by
109 cooling in an ice-water bath. Water (250 µl) and then dihydroquercetin in methanol (50 µl;
110 0.047 mg/ml) as the internal standard was added. Samples were then analyzed by high
111 performance liquid chromatography. This provided information on monomeric flavanol
112 composition (Figure 1) and allowed calculation of the mean degree of polymerization (mDP),
113 procyanidin:prodelphinidin (PC:PD) and *cis:trans* flavanol ratios (Gea et al., 2011; Eq. 1, 2
114 and 3).

115

$$\mathrm{mDP} = \frac{\text{amount of extension and terminal flavanol units (mol)}}{\text{amount of terminal flavanol units (mol)}} \quad (1)$$

116

$$\mathrm{PC{:}PD} = \frac{\text{percentage of C} + \text{EC units}}{\text{percentage of GC} + \text{EGC units}} \quad (2)$$

117

$$\mathrm{cis{:}\,trans} = \frac{\text{percentage of EC} + \text{EGC units}}{\text{percentage of C} + \text{GC units}} \quad (3)$$

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

119 **Substrate and condensed tannin preparations**

120 Effects of CT on *in vitro* CH_4 production and fermentation kinetics were examined using the
121 tannin-free lucerne (*Medicago sativa*) as a substrate. Lucerne was harvested at 50% flowering
122 stage, freeze-dried and ground to pass a 1-mm sieve (Retsch GmbH, ZM 100, Haan, Germany).
123 The chemical composition of lucerne was: OM = 800.0 g/kg DM; CP = 188.0 g/kg DM; NDF
124 = 279.5 g/kg DM and ADF = 211.2 g/kg DM. Condensed tannins were prepared at 3 effective
125 concentrations: 40, 80 and 120 g CT/kg of substrate DM. The extracts used in the present study
126 differed in their CT contents and range from 5 to 11 g CT/100 g extract (Table 1). Therefore,
127 the amount of CT extract required to achieve these 3 effective CT concentrations was weighed
128 separately into Eppendorf vials and dissolved in 2 ml of Millipore water (Milli-Q Academic,
129 Amsterdam, The Netherlands) and added to the fermentation bottles at the onset of *in vitro*
130 incubation. Condensed tannin extracts were dissolved in water to ensure its proper
131 homogenization with the substrate.

132 **Rumen *in vitro* gas and methane production measurements**

133 Cumulative gas production (GP) was measured using a fully automated time related GP
134 apparatus (Cone et al., 1996) and CH_4 production at distinct time points as described by
135 Pellikaan et al. (2011a). Approximately 250 mg of substrate was weighed into 250 ml
136 fermentation bottles (Schott, Germany). Bottles were then randomly distributed within each
137 GP unit, such that bottles with each substrate-CT extract treatment combination including the
138 blanks were incubated in each GP unit. Rumen fluid was obtained from 3 ruminally fistulated
139 lactating Holstein-Friesian dairy cows. Donor cows were fed a grass and maize silage mixture
140 in the morning and afternoon and 7-8 kg/d of concentrate according to their milk production.
141 The handling of the animals was approved by the institutional animal care and use committee
142 of Wageningen University (Wageningen, The Netherlands) and in accordance with the Dutch
143 legislation on the use of experimental animals.

144 Rumen fluid was collected prior to the morning feeding by suction method using a solid
145 perforated plastic tube (85 cm long and 2.5 cm in diameter). Rumen fluid once collected was
146 transferred into pre–warmed and carbon dioxide (CO_2) flushed thermos flasks, transported
147 quickly to the laboratory, pooled and filtered through 2 layers of cheesecloth into a flask
148 flushed with CO_2. Filtered rumen fluid was mixed with the buffered mineral solution at 1:2
149 ratios (v/v) as described by Cone et al. (1996) with constant stirring and continuous flushing
150 with CO_2, while maintained in a water bath set to 39 ^{o}C. Then, 30 ml buffered rumen fluid
151 mixture was subsequently dispensed in the fermentation bottle prewarmed to 39 ^{o}C. Finally,
152 CT solution was immediately added into the fermentation bottle and incubated in a water bath
153 maintained at 39 ^{o}C and shaking at 40–50 movements per minute. Control bottles containing
154 substrate and buffered rumen fluid (i.e. without CT) were injected with 2 ml of Millipore
155 water.
156 The study was designed as a randomized complete block design with incubation run
157 considered as a block. Each treatment and control were incubated in duplicate within a run
158 and replicated in 2 runs on different days. Two bottles in each run were included as a blank
159 (containing only buffered rumen fluid) and GP for each bottle was corrected for the blank
160 values. The amount of gas, CH_4 and VFA produced were adjusted to the total amount of
161 organic matter (OM) incubated and expressed per gram of incubated OM (substrate OM plus
162 extra OM supplied with CT extracts).
163 Methane concentration in the headspace of the fermentation bottle was measured by gas
164 chromatography (GC; GC8000Top, CE Instruments, Milan, Italy). To allow gas sampling
165 from the headspace, the fermentation bottles were fitted with a side port sealed with a screw
166 cap that is fitted with an air-tight septum (GRACE, XLB-11 Septa 7/16, Breda, The
167 Netherlands) as illustrated by Pellikaan et al. (2011a). At distinct time points of incubation (0,
168 2, 4, 6, 8, 10, 12, 24, 26, 28, 30, 32, 48, 50, 52, 54 and 72 h), 10 µl aliquots of the bottles

169 headspace gas were sampled through this opening using a gas tight syringe (Gastight® # 1701
170 Hamilton 1701N, 10 µl Syringe, Point Style 5, Bonaduz, Switzerland) and analyzed for CH_4
171 concentration using GC. The GC was fitted with a flame ionization detector and stainless
172 steel column (6 m long, 0.53 mm i.d., 25 µm film thicknesses) packed with PoraPack Q 50-
173 80 mesh (GRACE, Breda, The Netherlands). The temperatures of the injector, column, and
174 detector were maintained at 150 ^{o}C, 60 ^{o}C and 150 °C, respectively. The carrier gas was
175 nitrogen, and the pressure for nitrogen, hydrogen and air was set at 100, 50 and 100 kPa,
176 respectively. The CH_4 concentration was calculated by external calibration, using a certified
177 gas mixture containing known composition of CH_4 (Linde Gas Benelux, Schiedam, The
178 Netherlands). Peak areas were determined by automatic integration system software (Chrom–
179 Card data system Version 2.4, 2006, Rodano Milan, Italy) for GC.
180 Cumulative CH_4 production was calculated following the procedure described by Pellikaan et
181 al. (2011b; Eq. 4) by taking the sum of the increased amount of CH_4 in the bottle headspace
182 between 2 successive valve openings and the amount of CH_4 vented from the bottle.

$$M = \sum_{i=1}^{n} \{V_{HS}(C_{i+1} - C_i) + G_{i+1}C_{i+1}\} \quad (4)$$

183 where M, cummulative CH_4 production (ml/g of incubated OM); V_{HS}, the bottle headspace
184 volume (ml); C_i and C_{i+1}, CH_4 concentration in the bottle headspace gas at i and i+1 valve
185 openings, respectively; G_{i+1}, the amount of gas (ml) vented at i+1 valve opening; and n, total
186 number of valve openings.

187 **Curve fitting and calculations**

188 Cumulative gas and CH_4 production curves were fitted iteratively with a triphasic and
189 monophasic Michaelis-Menten equation (Groot et al., 1996; Eq. 5), respectively, using the non-
190 linear least squares regression procedure in SAS (SAS, 2010).

$$\mathrm{OMCV} = \sum_{i=1\ or\ 3}^{n} \frac{\mathrm{A}_i}{1 + (\mathrm{B}_i/\mathrm{t})^{\mathrm{C}_i}} \quad (5)$$

where OMCV, gas or CH_4 production (ml/g of incubated OM); A, the asymptotic gas production (ml/g of incubated OM); B, time at which half of the asymptotic gas or CH_4 production has been reached ($t_{1/2}$, h); C, the sharpness of the switching characteristics of the profile; and t, the time (h).

The maximum rate of gas or CH_4 production (Rmax, ml/h) was calculated as described by Bauer et al. (2001; Eq. 6).

$$\mathrm{Rmax} = \frac{\mathrm{A} \times \mathrm{B}^{\mathrm{C}} \times \mathrm{C} \times \mathrm{TRmax}^{(-\mathrm{C}-1)}}{(1 + \mathrm{B}^{\mathrm{C}} \times \mathrm{TRmax}^{-\mathrm{C}})^2} \quad (6)$$

where A, asymptote gas or CH_4 production (ml/g of incubated OM); B, time of incubation at which half of the asymptote gas or CH_4 has been formed ($t_{1/2}$, h); C, the sharpness of the switching characteristic of the profile.

***In vitro* organic matter digestibility**

The *in vitro* organic matter digestibility (IVOMD) was estimated according to the equation given by Menke and Steingass (1988; Eq. 7) based on 24 h gas production and nutrient composition of the substrate.

$$\mathrm{IVOMD}\ (\%) = 14.88 + 0.8893 \times \mathrm{GP} + 0.0448 \times \mathrm{CP} + 0.0651 \times \mathrm{A} \quad (7)$$

where GP, 24 h net gas production (ml/200 mg DM); CP, crude protein (%) and A, ash (%) contents of the substrate.

Analytical procedures

Substrate sample was freeze dried, ground using a Wiley mill through a 1-mm sieve and analyzed for DM (ISO 6496, 1999), ash (ISO 5984, 2002) and N (ISO 5983, 2005). Crude protein content was calculated as: CP = 6.25 × N. Neutral detergent fiber and ADF were

analyzed using an ANKOM2000 Fiber Analyzer (ANKOM Technology Corporation, NY, USA).

The VFA sample (750 µl) from each bottle after 72 h incubation was acidified with equal volume of orthophosphoric acid solution (1:1, v/v) and stored at -20 ^{o}C pending for further analysis. The VFA concentration was analyzed by GC as described by Taweel et al. (2005). The VFA concentration in the medium was corrected for the VFA concentration of blank (i.e. rumen fluid plus buffer) and expressed as mM/g of incubated OM.

Statistical analysis

All duplicate bottles per treatment within run were averaged prior to statistical analysis. Fermentation bottle was considered as an experimental unit. Data were subjected to analysis of variance based on a complete randomized design within a 4 × 4 factorial arrangement of treatments using the GLM procedure in SAS (SAS, 2010). For each CT type, the effects of CT concentration on gas, CH_4, VFA and kinetic parameters were analysed for orthogonal polynomial contrasts. The model included treatment (CT level) as a fixed effect and block (run) as a random effect. Least square means for control and treatments are reported. Treatment effects were declared significant at $p \leq 0.05$ and tendency at $0.05 < p \leq 0.10$.

Results

Chemical composition of lucerne and chemical characteristics of condensed tannins

The analyzed organic matter (OM) content of lucerne was somewhat lower than the expected. Since GP, CH_4 production and kinetic parameters were expressed per unit of OM for all treatments, the lower OM content of the lucerne does not affect differences between treatments. There was a large variation in CT average polymer sizes (mDP) among the 4 CT types but much less variation in content, PC:PD and *cis:trans* ratios (Table 1). The mDP values varied from 13 to 73 (Rees "A" vs. CPI63767) and the CT contents from 5 to 11 g

CT/100 g extract (CPI63767 vs. Rees "A"). The PC:PD ratios ranged from 23:77 to 29:71 (CPI63763 vs. Rees "A" and Cotswold Common) and *cis:trans* ratios from 68:32 to 79:21 (CPI63763 vs. Cotswold Common).

Effect of condensed tannins on total gas and methane production

Cumulative gas (ml/g of incubated OM) was reduced by the type and level of CT compared with the control (Table 2). A linear ($p < 0.001$) reduction was observed with increasing CT level for all CT type after 72 h. On average less gas was produced by inclusion of CT from CPI63767 and followed by Cotswold Common, Rees "A" and CPI63763 CT at 12, 24 and 72 h. Condensed tannins from CPI63767 when added at ≥ 80 g CT/kg of substrate DM consistently gave the lowest GP at 12, 24 and 72 h compared with the control and the other CT types. All types of CT when added at 80 and 120 g CT/kg of substrate DM have linearly decreased GP.

The effect of CT on CH_4 production is presented in Table 3. Condensed tannins from CPI63767 were the most effective in reducing CH_4 production followed by CT from CPI63763, Rees "A", and Cotswold Common. Addition of CT at 40 g CT/kg of substrate DM, except CPI63767, did not affect CH_4 production. Inclusion of CT from CPI63767 at 80 and 120 g CT/kg of substrate DM reduced ($p < 0.001$) CH_4 by 43 and 65% compared with the control after 24 h incubation. Similarly, CT from CPI63763 reduced ($p < 0.001$) CH_4 by 23 and 57% after 24 h, whilst Rees "A" and Cotswold Common reduced CH_4 by about 26 and 46%, and 28 and 46%, respectively. Inclusion of CT at 120 g CT/kg reduced CH_4 production by 28 % (Rees "A" and Cotswold Common) and 63% (CPI63767) compared with the control after 72 h of incubation. Methane production expressed per unit IVOMD was 33.6 ml/g of OM degraded for control and 28.6, 20.4 and 12.5 ml/g of OM degraded for 40, 80 and 120 g CT/kg of substrate DM; resulting in a 15, 39 and 63% reduction for the respective CT levels (Table 3).

More than 50% of total CH_4 was produced in the first 12 h of incubation for the control treatment, which was considerably more than when substrate was incubated with CT. The proportions of CH_4 in total GP (v/v) showed a linear reduction ($p < 0.001$) for CPI63767 (21.9% to 12.1%) and CPI63763 (21.9% to 14.7%), and a linear and quadratic effect for Cotswold Common and Rees "A" after 72 h incubation (Figure 2). A higher proportion of CH_4 for Rees "A" (22.8%) and Cotswold Common (24.3%) were measured when CT was added at 40 g CT/kg compared with control (21.9%) after 72 h of incubation (Table 3). The same trend was observed after 24 h of incubation. Depending on the type of CT, CH_4 produced as a proportion of total gas during 24 h incubation varied from 18.6% (control) to 13.2% (CPI63767), 15.5% (CPI63763), 15.5% (Rees "A") and 16.0% (Cotswold Common) when CT was added at 80 g CT/kg substrate DM.

Table 4 presents a set of substrate fermentation kinetic parameters. Asymptotic GP for the first phase (A1; the first 3 h of incubation, which corresponds to fermentation of the soluble and rapidly fermentable fraction; Groot et al., 1996) decreased linearly ($p < 0.01$) with increasing level of CT for all CT type. The asymptotic GP for the second phase (A2; 3-20 h of incubation, which corresponds to fermentation of the non-soluble fraction) only showed a linear ($p < 0.01$) decrease for Cotswold Common and CPI63767. The asymptotic GP for the third phase (A3; 20-72 h of incubation, corresponds to microbial turn over) was both linearly ($p < 0.01$) and quadratically ($p < 0.01$) affected by CT from CPI63767. The rate of GP (Rmax1) decreased linearly ($p < 0.05$) with increasing level of CT, but was unaffected for CPI63763. The half-time of asymptotic GP in the first phase (B1) was longer for CT from Cotswold Common compared to control. In general, all CT types gave a longer B1 when added at 80 and 120 g CT/kg of substrate DM compared with the control. Similarly, increasing CT level from CPI63767 caused a higher reduction ($p < 0.01$) of both Rmax1 and asymptotic CH_4 production. For all CT types, the B1 of the asymptotic CH_4 production was

affected in a linear and quadratic manner ($p < 0.001$). The asymptotic CH_4 production was also affected both linearly and quadratically, but only for CPI63767. On average, half of the asymptotic CH_4 production was reached after 18.6 h (CPI63767), 18.8 h (CPI63763), 37.3 h (Rees "A") and 25.6 h for Cotswold Common as compared with 8.9 h for the control.

Effects of condensed tannins on volatile fatty acids

Condensed tannins had linear and quadratic effects on total and individual VFA production (Table 5). There was a negative linear and quadratic relation between CT level and VFA production for CPI63767, and linear effect for Cotswold Common. The proportion of propionate linearly increased at the expense of acetate, and butyrate decreased for all CT types and levels of inclusion compared to the control. The highest increase in propionate production was observed for CT from CPI63767 added at 120 g CT/kg of substrate DM. A similar increase in propionate was observed when CT was added at 80 g CT/kg of substrate DM. The ratio of acetate: propionate was the lowest for the same CT when added at 80 and 120 g CT/kg of substrate DM. The reduction in acetate:propionate ratio was 20% and 40% when CT was added at 80 and 120 g CT/kg of substrate DM, respectively, compared with only 3% reduction when CT was added at 40 g CT/kg of substrate DM. On average the decline (% relative to control) in acetate:propionate ratio was 29% (CPI63767), 22% (CPI63763), 17% (Rees "A") and 16% for Cotswold Common.

Discussion

Chemical property of condensed tannins

Condensed tannins (CT) from sainfoin (*Onobrychis viciifolia*) are rich in prodelphinidins and have a considerable spread of molecular weights, PC:PD ratios and tannin contents (Marais et al., 2000; Regos et al., 2009; Stringano et al., 2012). The 'HealthyHay' sainfoin germplasm collection provided a unique opportunity to obtain contrasting CT in terms of molecular

309 weights but otherwise relatively similar characteristics in CT content, PC:PD and *cis:trans*
310 ratios (Table 1). Non-tannin components in acetone/water extracts consist of soluble
311 carbohydrates (mainly sucrose), monomeric flavonoids and their glycosides and phenolic
312 acids (Marais et al., 2000; Regos et al., 2009). These semi-purified CT extracts were used in
313 the current study in order to assess the effects of different sainfoin CT types on rumen *in vitro*
314 CH_4 production and fermentation characteristics. Previous approaches have tested
315 commercially available tannins, which were either water or alcohol extracts, and found that
316 the potential to reduce CH_4 production, or affect rumen fermentation and protein degradation
317 varied with type and level of tannins (Getachew et al., 2008; Hassanat and Benchaar, 2013).
318 Similarly, other studies, in which acetone/water extracts were added to *in vitro* systems,
319 reported the effects of tannins on CH_4 production and their role in animal feed vary
320 depending on the type and concentrations of tannins (Pellikaan et al., 2011b; Sivakumaran et
321 al., 2006; Beauchemin et al., 2007).
322 The CT used in the present study consisted mainly of prodelphinidins and a wide polymer
323 size (mDP values) ranging from 13 to 73 (Table 1). However, the literature contains
324 surprisingly contradictory information on the effect of tannin polymer size on anti-
325 methanogenic properties. Tavendale et al. (2005) found that CT polymers with a mDP value
326 of 12.5 (DP range from 4 – 13) completely inhibited methane production, but CT oligomers
327 with mDP values of 4.5 to 6.6 (DP range from 2 – 7) had no inhibitory effect. In contrast,
328 Field et al. (1989) found that autoxidized oligomers in particular had anti-methanogenic
329 effects, whilst their autoxidized high molecular weight polymers showed no inhibiting
330 effects. These seeming contradictions could arise from the fact that the autoxidized polymers
331 from Field et al. (1989) are unlike the naturally occurring plant polymers studied by
332 Tavendale et al. (2005). Another explanation could be that condensed tannins are highly
333 specific in their anti-methanogenic properties just as reported previously for hydrolysable

334 tannins (Pellikaan et al., 2011b) and for condensed tannins (Hassanat and Benchaar, 2013). It
335 is also important to note that mDP values describe the 'average CT' in a mixture of different
336 tannin molecules and thus the CT distribution profiles will include tannins of different
337 molecular weights (Stringano et al., 2011).

338 **Effect of CT on *in vitro* methane and VFA production**

339 The inclusion of CT from Rees "A" having the lowest polymer size (mDP = 13) compared
340 with CT from CPI63767 that has the highest polymer size (mDP = 73) at the same
341 concentration affected CH_4 production differently (Table 3). We note that both CT types have
342 relatively similar PC:PD ratios (29:71 and 25:75, respectively). The inclusion of CT from
343 these two sources at 80 g CT/kg of substrate DM produced 59.3 and 46.6 ml CH_4/g of
344 incubated OM for CT from Rees "A" vs. CPI63767 after 72 h of incubation (Table 3). The
345 same trend was observed when CT was added at 120 g CT/kg of substrate DM and incubated
346 for 6, 12, and 24 h. Similarly, inclusion of CT from CPI63767 (mDP = 73; PC:PD = 25:75)
347 compared to CT from CPI63763 (mDP = 24; PC:PD = 23:73) at the same concentration, for
348 instance, 80 g CT/kg of substrate DM reduced CH_4 production to different extent. This
349 suggests that the PC:PD ratio at least for the type of CT tested in the present study is not the
350 main responsible factor explaining the differences observed in their potential of reducing *in*
351 *vitro* CH_4 production. In contrast, Molan et al. (2001) observed that CT extracts from
352 prodelphinidin-rich big trefoil (*Lotus pedunculatus*) was more active in inhibiting the growth
353 of proteolytic rumen bacteria than procyanidin-rich birdsfoot trefoil (*Lotus corniculatus*). The
354 inconsistencies between studies could be due to difference in source of CT extracts (i.e. plant
355 of origin) that might affect their activity to bind carbohydrates and proteins (McAllister et al.,
356 2005).

357 The ratio of CH_4 to total gas is an important indicator of the potential amount of CH_4
358 produced per unit OM degraded. The proportion of CH_4 in the total gas on average declined

359 from 18.6% to 11.4%; a reduction of 39% (control vs. 120 g CT/kg of substrate DM; Table
360 3). This is agreement with Hassanat and Benchaar (2013) who reported up to 40% reduction
361 of CH_4 production compared with control when substrate was incubated with condensed
362 tannins at ≥100 g/kg with minimum detrimental effects on efficiency of ruminal
363 fermentation. Similarly, Waghorn et al. (2002) found that CH_4 production was reduced by
364 31% when sheep were fed on *Lotus pedunculatus* (DMI = 935 g/day; CT content = 5.3 g/100
365 g DM) compared with *Medicago sativa*, a tannin-free legume. In the present study, addition
366 of CT at 40 g CT/kg substrate DM from Rees "A" and Cotswold Common did not inhibit
367 CH_4 production (Table 3). However, at increasing CT levels CH_4 production was inhibited.
368 This is in consistence with a meta-analysis conducted by Jayanegara et al. (2011) who
369 showed a significant negative correlation between CT concentration and *in vitro* CH_4
370 production. Equally, McMahon et al. (1999) demonstrated that there is a linear suppression of
371 *in vitro* CH_4 production with an increasing proportion of sainfoin forage in lucerne.
372 Regardless of the CT type, substrate incubated with ≥ 80 g CT/kg substrate DM was less
373 degraded than the control as reflected by a lower *in vitro* OM digestibility (IVOMD; Table
374 2). In agreement with Kaplan (2011), who compared the *in vitro* ruminal degradability of 4
375 accessions of sainfoin hay (containing extractable CT varying from 49 to 100 g/kg DM), and
376 found that estimated IVOMD of sainfoin hay is negatively correlated with its CT content. In
377 contrast, Theodoridou et al. (2011) found that sainfoin CT extracted from whole-plant, leaves
378 and stems (CT content 13.6, 9.8 and 9.0 g/kg DM for whole-plant, leaves and stems,
379 respectively) inhibited *in vitro* CH_4 production without altering its measured OM
380 digestibility. The variations among studies could be due to: 1) in the study of Theodoridou et
381 al. (2011) the IVOMD was measured, whereas in current study and that of Kaplan (2011) it
382 was estimated from gas production and chemical composition of the substrate, or 2) lower CT
383 content in the study of Theodoridou et al. (2011).

384 Addition of 120 g CT/kg substrate DM showed an average reduction of 24% in 24 h GP and a
385 63% reduction in CH_4 expressed per unit of IVOMD. This relationship between the reduction
386 in CH_4 production and CT concentration suggests that the effects of CT may be attributed for
387 an important part to a negative effect on ruminal fiber degradation such as increased
388 formation of tannin–cellulose complexes that are resistant to enzymatic digestion, or lessened
389 substrate adhesion by fibrolytic microbes (Waghorn, 2008; McAllister et al., 2005). On the
390 other hand, the linear and quadratic effect observed on VFA, or change in VFA composition
391 (i.e. linear increase in propionate proportion and decrease in acetate:propionate ratio) with
392 higher CT level (Table 5) suggest that a direct effect of CT on rumen methanogenesis is also
393 prevailing. This effect could result from a reduction of available hydrogen, which is a
394 substrate for methanogens (Bhatta et al., 2009; Smith et al., 2005; Tavendale et al., 2005).
395 The reduction in hydrogen availability can be achieved when an alternative metabolic
396 pathway such as propionate production disposes of the hydrogen produced during *in vitro*
397 fermentation of substrate (López et al., 1999).
398 The increase in CT level except CT from Rees "A" and CPI63763 reduced total VFA
399 production linearly. Hassanat and Benchaar (2013) also reported a decrease in *in vitro* VFA
400 concentration when CT level was increased from 20 to 200 g/kg of DM. However, in the
401 current study, the addition of CT from CPI63767 resulted in less acetate, butyrate and lower
402 acetate:propionate ratio when added at 80 g CT/kg of substrate DM compared with the
403 control and other CT types. This is important in terms of CH_4 reduction, since fermentation
404 of OM to acetate and butyrate produces hydrogen, which is utilized in the rumen to produce
405 CH_4, while substrates that promote production of propionate in the rumen decreases CH_4
406 production (Tavendale et al., 2005). This strong inverse relationship between propionate and
407 CH_4 production can be predicted from knowledge of interactions among ruminal microbial
408 populations (Morgavi et al., 2010). The extent of linear and quadratic reduction in total VFA

409 production, and the linear increase in propionate proportion and decrease in
410 acetate:propionate ratio for different CT types with increasing CT levels indicates an anti-
411 methanogenic effect, in which the effect depends on the type of CT. Earlier it was reported
412 the activities of CT on ruminal VFA production and composition vary depending on CT level
413 and source (Beauchemin et al., 2007; Bueno et al., 2008; Hassanat and Benchaar, 2013).
414 For better assessment of the overall environmental impact of different CT types and levels in
415 ruminant diets other greenhouse gas emissions such as N_2O will also need to be considered in
416 addition to enteric CH_4 production. This is important because CT can also affect protein
417 degradation (Martínez et al., 2006); but they can also increase the utilization of branched-
418 chain VFA (iso-acids) for microbial protein synthesis. In fact, both effects are likely, as
419 dietary CT have been reported to be associated with reduction of protein degradation in the
420 rumen (Hassanat and Benchaar, 2013; Getachew et al., 2008; Waghorn and Shelton, 1997).
421 The iso-acids VFA arise almost exlusively from the oxidative deamination of amino acids. In
422 addition, these branched-chain VFA production also decreased linearly as CT level increased
423 and becomes negative when CT were added at higher concentration (Table 5), indicating that
424 there was net uptake of these branched-chain VFA as the result of protection of proteins by
425 CT from rumen deamination. This effect was more pronounced with CT from CPI63767 and
426 added at 80 g CT/kg of substrate or higher level. In agreement with our results, Makkar et al.
427 (1988) also reported that the protein precipitation capacity of CT depends on the type of
428 tannins and the degree of polymerization. This can have a positive effect by increasing the
429 amount of rumen–escape protein as well as causing a higher flow of microbial proteins to the
430 intestine and hence improve N utilization when CT are supplied in ruminant diets.
431 Moreover, the effect observed on protein fermentation as indirectly evidenced by a change in
432 VFA composition favoring a shift towards propionate and reduction of branched-chain VFA
433 (Table 5) is consistent with a recent *in vivo* study on sainfoin of Aufrére et al. (2013), who

reported that sainfoin CT generated rumen–escape protein and enabled better utilization in the small intestine and higher N retention as the dietary proportion of sainfoin was increased.

Conclusion

Condensed tannins obtained from sainfoin accessions are promising for reducing rumen CH_4 production. There were significant differences in the anti–methanogenic activity among the CT extracts, which could be attributed to differences in tannin polymer size (mDP values). These size differences may have affected the ability of tannins to interact with dietary fiber and proteins or microbial cells. A decrease in proteolytic activity as indirectly shown by a change in VFA composition favoring a shift towards propionate and reduction in branched–chain VFA production which can be seen as a potential advantage in terms of improving N utilization by ruminants. This study generated preliminary evidence that tannin polymer size is an important factor as far as CH_4 and VFA production are concerned. Next to knowledge about the actual degree of polymerization, its proportional distributions could be of interest. A study with a wider range in CT structure (PC:PD and *cis:trans* ratios) is recommended to unambiguously assess the impact of CT structures on activity without detrimental effects on fiber degradation.

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Figure 1. Structural features of condensed tannins.

Figure 2. The effect of type and concentration of CT in the proportion of CH_4 production (expressed as percent of total gas) compared with the control after 72 h incubation (** $p < 0.01$; *** $p < 0.001$).

Table 1 Condensed tannins (CT) content, mean degree of polymerization, PC:PD and *cis:trans* ratios in aqueous acetone extracts obtained from 4 sainfoin accessions*

CT type	CT content (g/100 g extract)	mDP†	PC:PD‡	*cis:trans*§
Rees "A"	11 (2.2)	13 (2.3)	29:71 (1.4)	78:22 (4.4)
CPI63763	7 (1.9)	24 (6.7)	23:77 (2.6)	68:32 (3.9)
Cotswold Common	10 (1.5)	31 (1.6)	29:71 (0.7)	79:21 (1.7)
CPI63767	5 (0.9)	73 (3.0)	25:75 (2.0)	74:26 (2.0)

*Number in bracket indicates standard deviation.

†mDP = mean degree of polymerization (i.e., the average number of flavanol monomers per tannin polymer).

‡PC = procyanidin (i.e., CT that contain catechin and epicatechin units), PD = prodelphinidin (i.e., CT that contain gallocatechin and epigallocatechin units).

§*cis:trans* = the orientation of functional groups within a molecule.

669

Table 2 Effect of type and concentration of sainfoin CT on *in vitro* gas production (ml/g of incubated OM) and *in vitro* organic matter digestibility of lucerne

CT type	CT concentration (g/kg of substrate DM)	Time of post incubation (h)				IVOMD*
		6	12	24	72	
Rees "A"	0	216.9	257.3	290.9	309.8	62.1
	40	188.0	226.6	257.1	271.1	54.7
	80	171.4	227.9	258.6	279.6	54.9
	120	149.5	205.4	228.0	243.5	50.4
	SEM†	3.92	3.91	5.10	5.48	0.76
	Linear	<0.001	<0.001	<0.001	<0.001	<0.001
	Quadratic	0.400	0.321	0.763	0.823	0.055
CPI63763	0	216.9	257.3	290.9	309.8	62.1
	40	199.0	251.2	290.0	308.5	59.6
	80	185.9	237.0	268.1	290.5	56.4
	120	159.4	205.4	227.1	239.6	50.3
	SEM	4.24	4.34	4.52	5.99	0.67
	Linear	<0.001	<0.001	<0.001	<0.001	<0.001
	Quadratic	0.335	0.085	0.165	0.212	0.091
Cotswold Common	0	216.9	257.3	290.9	309.8	62.1
	40	186.9	237.1	268.6	284.1	56.2
	80	162.4	217.8	242.0	258.0	52.3
	120	145.1	202.3	227.6	242.0	50.2
	SEM	4.58	3.12	3.25	4.51	0.49
	Linear	<0.001	<0.001	<0.001	<0.001	<0.001
	Quadratic	0.201	0.475	0.257	0.315	0.102
CPI63767	0	216.9	257.3	290.9	309.8	62.1
	40	187.6	239.9	273.9	292.3	57.3
	80	160.7	206.1	231.9	251.6	51.1
	120	144.6	181.8	199.6	206.5	46.3
	SEM	3.50	3.69	4.20	5.76	0.62
	Linear	<0.001	<0.001	<0.001	<0.001	<0.001
	Quadratic	0.387	0.374	0.099	0.638	0.959

670 *IVOMD = *in vitro* OM digestibility (%) was determined based on 24 h gas production and
671 chemical composition of the substrate (Menke and Steingass, 1988).
672 †SEM = standard error of the means.

673

Table 3 Effect of type and concentration of sainfoin CT on *in vitro* methane production (ml/g of incubated OM), proportion of methane in total gas and methane produced per unit estimated OM degraded (ml/g of degraded OM)

CT type	CT concentration (g/kg of substrate DM)	Time of post incubation (h)				CH_4 per total gas*	CH_4 per IVOMD
		6	12	24	72		
Rees "A"	0	26.7	40.7	54.0	68.0	18.6	33.6
	40	22.1	35.6	50.9	61.8	20.0	28.1
	80	18.0	28.1	40.2	59.3	15.5	22.0
	120	12.5	19.3	28.9	49.0	12.7	14.6
	SEM†	1.25	1.25	0.66	3.99	0.46	0.39
	Linear	<0.001	<0.001	<0.001	0.012	<0.001	<0.001
	Quadratic	0.737	0.196	0.321	0.622	0.002	0.055
CPI63763	0	26.7	40.7	54.0	68.0	18.6	33.6
	40	22.6	36.4	51.2	66.8	17.6	30.5
	80	17.4	29.4	41.5	56.5	15.5	23.5
	120	10.6	16.6	23.3	35.2	10.3	11.7
	SEM	1.65	2.04	2.42	4.14	0.66	1.40
	Linear	<0.001	<0.001	<0.001	<0.001	<0.001	0.001
	Quadratic	0.437	0.070	0.073	0.442	0.199	0.104
Cotswold Common	0	26.7	40.7	54.0	68.0	18.6	33.6
	40	22.6	37.6	52.1	69.0	19.4	29.3
	80	12.9	30.9	38.8	53.3	16.1	20.3
	120	11.5	19.1	29.4	49.1	12.9	14.8
	SEM	1.59	1.67	0.76	3.35	0.41	0.32
	Linear	<0.001	<0.001	<0.001	0.002	<0.001	<0.001
	Quadratic	0.432	0.340	0.468	0.088	0.001	0.125
CPI63767	0	26.7	40.7	54.0	68.0	18.6	33.6
	40	20.1	32.9	46.3	61.0	16.9	26.5
	80	13.8	21.5	30.7	46.6	13.3	15.7
	120	9.1	13.9	18.9	24.9	9.5	8.7
	SEM	1.30	1.70	1.71	3.49	0.56	0.81
	Linear	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	Quadratic	0.493	0.962	0.261	0.074	0.086	0.924

674 *Proportion of CH_4 in total gas (%) estimated based on 24 h incubation.
675 †SEM = standard error of the means.
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Table 4 Effect of type and concentration of sainfoin CT on in vitro methane and gas production kinetics parameters of lucerne

CT type	CT concentration (g/kg of substrate DM)	Methane kinetics parameters			Gas production kinetics parameters*								
		A1	B1	Rmax1	A1	A2	A3	B1	B2	B3	Rmax1	Rmax2	Rmax3
Rees "A"	0	67.4	8.9	4.9	157.3	123.9	25.5	2.0	5.1	7.4	100.8	19.7	2.4
	40	71.1	13.9	4.0	129.5	106.2	23.6	1.6	4.1	21.9	86.2	16.4	2.0
	80	84.2	27.2	2.9	122.4	141.4	31.0	3.2	4.9	10.3	66.1	15.2	1.5
	120	87.1	70.6	1.3	98.1	98.4	18.8	1.0	5.4	11.3	50.5	13.1	1.3
	SEM†	6.61	9.77	1.00	7.76	12.41	3.18	1.40	2.05	6.39	14.36	1.33	0.27
	Linear	0.095	0.011	0.010	0.006	0.366	0.295	0.236	0.623	0.918	0.021	0.003	0.007
	Quadratic	0.890	0.113	0.536	0.833	0.060	0.042	0.025	0.925	0.032	0.973	0.633	0.812
CPI63763	0	67.4	8.9	4.9	157.3	123.9	25.5	2.0	5.1	7.4	100.8	19.7	2.4
	40	85.3	13.7	3.6	142.4	139.3	31.0	1.0	4.8	19.5	68.9	16.3	2.2
	80	67.6	14.9	3.0	133.5	136.7	30.9	1.3	4.5	16.0	73.2	14.2	1.3
	120	52.8	27.8	1.7	105.4	123.7	18.1	2.3	4.0	10.3	53.3	18.9	3.5
	SEM	5.09	3.59	0.96	6.22	2.37	3.06	0.69	0.72	2.19	15.76	1.76	0.64
	Linear	0.045	0.028	0.032	0.004	0.103	0.120	0.172	0.208	0.439	0.067	0.554	0.376
	Quadratic	0.063	0.265	0.236	0.240	0.004	0.052	0.088	0.045	0.013	0.668	0.040	0.088
Cotswold Common	0	67.4	8.9	4.9	157.3	123.9	25.5	2.0	5.1	7.4	100.8	19.7	2.4
	40	78.7	13.2	3.4	136.9	135.5	25.4	2.9	3.3	10.3	82.8	16.4	1.5
	80	53.7	17.0	2.5	96.1	110.2	21.3	3.3	3.7	11.0	63.4	13.9	1.8
	120	74.6	46.6	1.5	90.2	98.5	19.7	3.1	4.3	9.3	47.1	12.7	1.1
	SEM	4.34	2.07	1.20	7.20	4.70	6.09	1.91	2.49	6.46	17.19	1.44	0.28
	Linear	0.592	<0.001	0.001	0.002	0.005	0.391	0.715	0.921	0.502	0.036	0.003	0.010

		Quadratic	0.023	0.003	0.365	0.367	0.012	0.997	0.016	0.794	0.248	0.916	0.499	0.765
CPI63767	0		67.4	8.9	4.9	157.3	123.9	25.5	2.0	5.1	7.4	100.8	19.7	2.4
	40		75.3	13.7	3.7	140.1	140.8	30.1	2.8	4.8	15.9	78.1	14.9	2.0
	80		72.0	29.7	2.9	113.2	120.0	26.7	2.2	6.3	6.5	75.9	27.4	6.2
	120		30.7	13.1	1.2	93.5	108.5	11.1	2.3	3.8	9.9	25.6	13.2	0.9
		SEM	4.85	1.56	1.06	5.12	1.35	1.90	1.36	0.73	3.76	17.65	1.34	1.16
		Linear	0.005	0.023	<0.001	0.001	<0.001	0.004	0.353	0.283	0.923	0.015	0.250	0.859
		Quadratic	0.009	0.003	0.460	0.962	0.002	0.008	0.132	0.097	0.022	0.490	0.004	<0.001

[*]A = asymptotic gas or CH_4 production (A1, A2, A3 indicates different phases; ml/g of incubated OM); B = half-time of asymptotic gas or CH_4 production (B1, B2, B3 indicates different phases; h); Rmax = rate of maximum gas or CH_4 production ($Rmax_1$, $Rmax_2$, $Rmax_3$ indicates different phases; ml/h).

[†]SEM = standard error of the means.

Table 5 Effect of type and concentration of sainfoin CT on total VFA production and molar proportions of individual VFA

CT type	CT concentration (g/kg substrate DM)	Total VFA (mM/g OM)	Individual VFA (mol/100 mol)*					
			Ace	Pro	But	Val	BCVFA	Ace:Pro
Rees "A"	0	5.29	65.53	23.33	7.00	1.30	2.83	2.83
	40	5.37	67.10	25.35	4.75	1.23	1.63	2.65
	80	4.94	67.55	27.43	3.45	1.13	0.50	2.50
	120	5.13	63.38	33.33	2.30	1.15	-0.15	1.93
	SEM†	0.112	0.719	0.939	0.155	0.037	0.098	0.119
	Linear	0.100	0.087	<0.001	<0.001	0.006	<0.001	<0.001
	Quadratic	0.609	0.002	0.161	0.154	0.199	0.106	0.120
CPI63763	0	5.29	65.53	23.33	7.00	1.30	2.83	2.83
	40	5.34	67.63	24.68	4.30	1.78	1.65	2.75
	80	5.72	65.90	29.23	2.43	2.03	0.45	2.25
	120	5.24	60.00	37.48	1.28	2.05	-0.80	1.60
	SEM	0.134	0.644	0.690	0.076	0.042	0.072	0.080
	Linear	0.689	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	Quadratic	0.069	<0.001	0.201	0.312	<0.001	0.611	0.064
Cotswold Common	0	5.29	65.53	23.33	7.00	1.30	2.83	2.83
	40	5.17	68.53	23.73	4.90	1.15	1.70	2.90
	80	4.98	66.13	29.40	3.18	1.00	0.30	2.28
	120	4.96	63.83	33.08	2.33	0.98	-0.20	1.95
	SEM	0.093	0.674	0.833	0.124	0.028	0.075	0.118
	Linear	0.014	0.029	<0.001	<0.001	<0.001	<0.001	<0.001
	Quadratic	0.600	0.002	0.373	0.181	0.045	0.213	0.035
CPI63767	0	5.29	65.53	23.33	7.00	1.30	2.83	2.83
	40	5.56	67.80	25.73	3.70	1.45	1.35	2.65
	80	5.29	63.98	32.08	2.18	1.50	0.25	2.00
	120	4.52	56.68	42.15	1.00	1.43	-1.28	1.35
	SEM	0.117	0.528	0.685	0.123	0.028	0.081	0.097
	Linear	<0.001	<0.001	<0.001	<0.001	0.005	<0.001	<0.001
	Quadratic	0.001	<0.001	0.321	0.172	0.002	0.763	0.180

699 *Ace = acetate; Pro = propionate; But = butyrate; Val = valerate; BCVFA = branched-chain
700 VFA (iso–butyrate + iso–valerate).
701 †SEM = standard error of the means.
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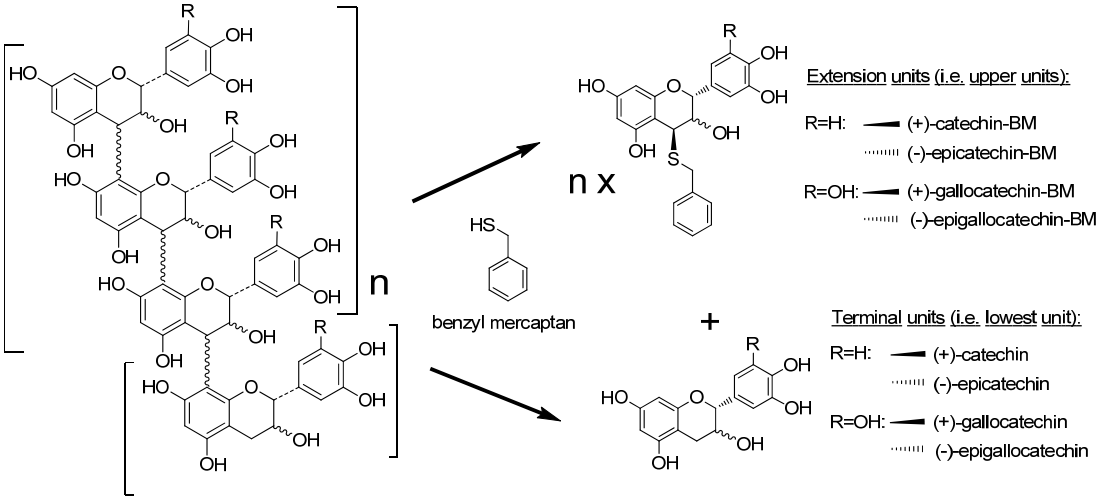
n
benzyl mercaptan
n x
+
Extension units (i.e. upper units):
R=H: (+)-catechin-BM
(-)-epicatechin-BM
R=OH: (+)-gallocatechin-BM
(-)-epigallocatechin-BM
Terminal units (i.e. lowest unit):
R=H: (+)-catechin
(-)-epicatechin
R=OH: (+)-gallocatechin
(-)-epigallocatechin

Figure 1.

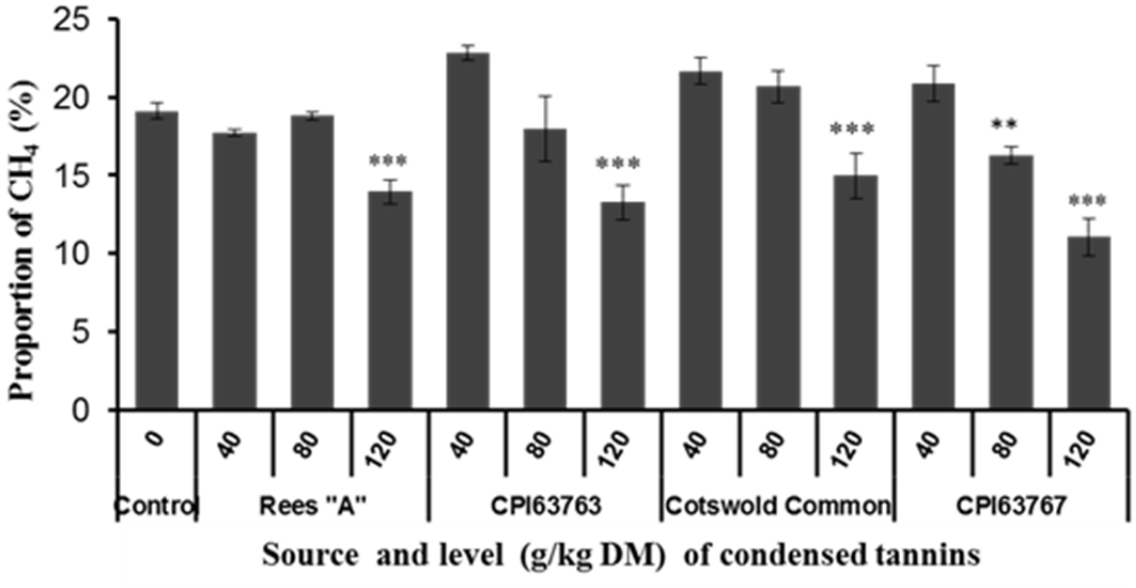
Proportion of CH_4 (%)
25
20
15
10
5
0
***
***
***
**
***
0
40
80
120
40
80
120
40
80
120
40
80
120
Contro
Rees "A"
CPI63763
Cotswold Common
CPI63767
Source and level (g/kg DM) of condensed tannins

Figure 2.