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Sodium Hydroxide Enhances Extractability and Analysis of Proanthocyanidins in Ensiled Sainfoin (*Onobrychis viciifolia*)

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ABSTRACT: Little information exists on the effects of ensiling on condensed tannins or proanthocyanidins. The acetone−butanol−HCl assay is suitable for measuring proanthocyanidin contents in a wide range of samples, silages included, but provides limited information on proanthocyanidin composition, which is of interest for deciphering the relationships between tannins and their bioactivities in terms of animal nutrition or health. Degradation with benzyl mercaptan (thiolysis) provides information on proanthocyanidin composition but proanthocyanidins in several sainfoin silages have proved resistant to thiolysis. We now report that a pre-treatment step with sodium hydroxide prior to thiolysis was needed to enable their analysis. This alkaline treatment increased their extractability from ensiled sainfoin and facilitated especially the release of larger proanthocyanidins. Ensiling reduced assayable proanthocyanidins by 29% but the composition of the remaining proanthocyanidins in silage resembled those of the fresh plants.

KEYWORDS: *silage, thiolysis, unextractable tannins, alkaline pre-treatment, reaction products*

INTRODUCTION

Sainfoin (*Onobrychis viciifolia*) is a perennial forage legume that grows in parts of Europe, the U.S. and Canada.[1] Ruminant animals generate safer forms of environmental nitrogen emissions on sainfoin diets,[2] can safely graze it as it is non-bloating[1] and suffer lower intestinal parasite burdens.[3,4] These health and environmental benefits have been attributed to its proanthocyanidins (Figure 1).[5] Sainfoin is suitable for processing into silage, which provides energy and protein during periods of feed shortages.[6] It has, however, also been shown that preservation, such as ensiling, lowers proanthocyanidin extractability in different forage legumes.[5,7]

Currently, there is no information on the effects of ensiling on proanthocyanidin composition. Few methods exist for analyzing tannins in fermented samples and the HCl-butanol method is currently the most widely used method for silages[6,8,9] but provides only limited information on proanthocyanidin structures. In contrast, the milder acid-catalyzed degradation of proanthocyanidins with benzyl mercaptan (*i.e.* thiolysis) yields quantitative data on the composition of flavan-3-ols in extension and terminal units and enables calculation of the mean degree of polymerization of the proanthocyanidins.[10] Surprisingly, however, although proanthocyanidins can be quantitated by the HCl-butanol assay in silages,[11,12] thiolysis[10] with benzyl mercaptan at 40 ˚C did not detect any proanthocyanidins in several silages.

There is some evidence that the decrease of extractable proanthocyanidins in sainfoin and other plants after ensiling is caused by the binding of proanthocyanidins to protein and fiber.[5,7,11] Recently, White *et al*.[13] reported that alkaline hydrolysis with NaOH improved the extraction of A-type procyanidins from cranberry pomace and NaOH is also commonly used to facilitate the extraction of various esterified or

bound phenolics.[14,15] Therefore, the present study evaluated whether pre-treatment with NaOH would enable compositional analysis of proanthocyanidins in ensiled sainfoin by thiolytic degradation with benzyl mercaptan.

MATERIALS AND METHODS

Chemicals. Hydrochloric acid (concentrated, 36%), acetone (analytical reagent grade), dichloromethane (HPLC grade) and acetonitrile (HPLC grade) were purchased from ThermoFisher Scientific Ltd (Loughborough, U.K.); (±)-taxifolin (98%) from Apin Chemicals (Abingdon, U.K.); benzyl mercaptan (98%), catechin (**3**), epicatechin (4), gallocatechin (**1**), epigallocatechin (**2**), quercetin (95%), kaempferol (98%), gallic acid and 3,4-dihydroxybenzoic acid from Sigma-Aldrich (Poole, U.K.); rutin (98%) from Lancaster Synthesis (Lancaster, UK) and formononetin (98%) from Acros Organics (Loughborough, UK). Deionized water was obtained from a Milli-Q System (Millipore, Watford, U.K.). Quercetin, kaempferol and formononetin were dissolved in methanol/water (1:1, v/v) using six concentrations (n = 2) and peak areas were used for the calibration curve.

Plant Materials. Sainfoin (*Onobrychis viciifolia* 'Perly') was sown at the Swiss Federal Research Station ALP (Posieux, altitude: 650 m) in April 2012 and harvested at the early flowering stage on 9 July 2012. A bulked sample (5 kg) was freeze-dried (= fresh/freeze-dried sample) and had a dry matter (DM) content of 169.4 g/kg fresh material and an organic matter (OM) content of 923.2 g/kg DM. The samples were wilted for 24 h in the field in a swath to minimize leaf loss and then chopped (1-2 cm) with a Mex GT chaff cutter (Poettinger, Grieskirchen, Austria). The wilted samples (DM content was 377.9 g/kg fresh wet material; OM content was 913.2 g/kg DM)

were ensiled without additives in 30 L barrels. The barrels were filled, compressed by hand and stored at room temperature in a dark room for 86 d. The room was neither cooled nor heated in the barn facilities (without insulation). The room temperature was recorded every week and ranged from 23.0 °C (August) to 17.7 °C (October). On average it was 20.4 °C. On opening the silo after 86 days of ensiling, the pH was 4.51 ± 0.03, the concentrations of short chain volatile fatty acids were 6.72 ± 0.94 (mg/kg DM) for acetate, 56.9 ± 5.96 (mg/kg DM) for lactate and 1.02 ± 0.06 (mg/kg DM) for butyrate. The silage was mixed by hand in a tray, subsamples were taken, freeze–dried, ground to pass a <1 mm screen sieve with an impeller SM1 cutting mill (Retsch, Haan, Germany) and stored at room temperature. The silage DM content was 370.7 g/kg, and the OM content was 911.3 g/kg DM. Three other sainfoin silage samples (SF1 to SF3, field replicates) were prepared in INRA Theix (France) according to Copani *et al*.[9]

Preparation of a Purified Proanthocyanidin Fraction and Acetone-Water Extracts. A purified proanthocyanidin fraction was isolated for use as a proanthocyanidin standard in the acetone-butanol-HCl assay[16] and also for the proanthocyanidin degradation test with NaOH. Sainfoin ‘Cotswold Common’ was harvested on 24 July 2007 from Hartley Farm near Seven Springs (Cheltenham, Gloucestershire, U.K.), manually separated from weeds, and freeze-dried. It was ground using a impeller SM1 cutting mill (Retsch, Haan, Germany) to pass <8 mm and then ground again to <1 mm. The ground sainfoin (20 g) was extracted with acetone/water (7:3, v/v; 250 mL, 1 h). Acetone was removed under vacuum and the aqueous phase was loaded on a Sephadex LH-20 column. The column was rinsed, firstly with water, and then with acetone/water (3:7), and the proanthocyanidin

fraction was eluted with acetone/water (1:1, v/v). Thiolysis[10] coupled to HPLC-MS revealed that this fraction had a very high proanthocyanidin content (98 g/100 g fraction) with an mean degree of polymerization value of 9.7, a procyanidin/prodelphinidin ratio of 44.1/55.9 and a *cis/trans* flavan-3-ol ratio of 81.9/18.1.

In addition, separate extracts were prepared from the fresh/freeze-dried and ensiled sainfoin samples (5 g) from Posieux with acetone/water (7:3, v/v; 63 mL, 1 h) as previously described.[10] The residues remaining after this acetone-water extraction were also kept for proanthocyanidin analysis (= solvent-extracted residue).

Thiolysis of the Purified Proanthocyanidin Fraction, Acetone/Water Extracts and Solvent-Extracted Residues. The purified proanthocyanidin fraction (8 mg), acetone-water extracts and plant residues that remained after the acetone-water extraction (8 mg) were placed into a 100 mm x 16 mm screw cap glass tube (Fisher Scientific, Loughborough, U.K.) with a stirring magnet (10 x 5 x 5 mm). Methanol (1.5 mL) was added followed by methanol acidified with concentrated HCl (3.3%; 500 µL) and benzyl mercaptan (50 µL). Tubes were capped and placed into a water bath at 40 ˚C for 1 h under vigorous stirring. [10] The reaction was stopped by placing the tube in an ice bath for 5 min. Distilled water (2.5 mL) and the internal standard, taxifolin in methanol (500 µL; 0.1 mg/mL), were added and thoroughly mixed. The mixture was transferred into a vial (800 µL), closed with a crimp top and analyzed by HPLC-MS within 24 h.

Sodium Hydroxide Pre-Treatment of Plant Samples and Solvent-Extracted Residues. Fresh/freeze-dried sainfoin, ensiled sainfoin or acetone-water extracted

plant residues (150 mg) were placed into a screw cap tube containing a stirring magnet. Air was replaced with argon before adding aqueous NaOH (0.5, 1, 2, 3, 4 M; 2 mL). The tube was flushed once more with argon just before capping, placed into a water bath at 40 ˚C and stirred for 5, 15, 30, 60 min at 1500 rpm. The tube was cooled for 2 min in an ice bath, the solution was neutralized with 4 M HCl, stirred, distilled water (1 mL) was added and the tube was left stirring to cool for another 2 min. The sample was centrifuged at 3000 rpm for 1 min, frozen and freeze-dried before thiolysis.

***In Planta* Thiolysis.** The original untreated and NaOH treated samples (150 mg), which were the freeze-dried sainfoin sample from Posieux, the ensiled sainfoin samples from Posieux and Theix and the acetone-water solvent extracted residues from the Posieux samples, were reacted with the thiolysis reagent (2 mL methanol, 1 mL of 3.3% HCl in methanol, and 100 µL benzyl mercaptan). The tubes were capped immediately and placed in a water bath at 40 ˚C for 1 h under vigorous stirring. The reaction was stopped by placing the tubes in an ice bath for 5 min. The samples were centrifuged at 3000 rpm for 3 min, and supernatants (1 mL) were transferred to clean screw cap glass tubes. Distilled water (4 mL) was added to the supernatants with the internal standard, taxifolin in methanol (500 µL; 0.1 mg/mL). The tubes were capped, shaken, and analyzed by HPLC-MS within 24 h.

HPLC-MS Analysis. Flavan-3-ols and their benzyl mercaptan-adducts were identified by HPLC-MS analysis on an Agilent 1100 Series HPLC system and an API-ES Hewlett Packard 1100 MSD detector (Agilent Technologies, Waldbronn, Germany). Samples (20 µL) were injected into the HPLC at room temperature and

148 the column used was a 250 mm x 4.6 mm i.d., 3 µm, ACE C_{18} column with a 10 mm
149 x 4.6 mm i.d. guard cartridge of the same material (Hichrom Ltd, Theale, U.K.). The
150 HPLC system consisted of a G1379A degasser, G1312A binary pump, a G1313A
151 ALS autoinjector and a G1314A VWD UV detector. Data were acquired with
152 ChemStation software (version A 10.01 Rev. B.01.03). The flow rate was 0.75
153 mL/min using 1% acetic acid in water (solvent A) and HPLC-grade acetonitrile
154 (solvent B). The following gradient was employed: 0-35 min, 0-36% B; 35-40 min,
155 36-50% B; 40-45 min, 50-100% B; 45-55 min, 100-0% B; 55-60 min, 0% B. Eluting
156 compounds were recorded at 280 nm. Mass spectra were recorded in the negative
157 ionization scan mode from *m/z* 100−1000 using the following conditions: capillary
158 voltage, -3000 V; nebulizer gas pressure, 35 psi; drying gas, 12 mL/min; and dry
159 heater temperature, 350 ˚C. Flavan-3-ol terminal and extension units were
160 quantitated relative to taxifolin.[10] This provided information on proanthocyanidin
161 content and composition in terms of flavan-3-ol terminal and extension units. It also
162 allowed calculation of the mean degree of polymerization, the percentage of
163 procyanidin and prodelphinidin tannins and *cis*- and *trans* flavan-3-ols.[10]
164
165 **Proanthocyanidin Analysis by the Acetone–Butanol–HCl Assay.** The
166 acetone−butanol−HCl reagent[16] was also used to analyze freeze-dried fresh and
167 ensiled materials. Samples (5 mg) were placed in glass screw cap tubes with the
168 acetone−butanol−HCl reagent (10 mL) and a small magnetic stirrer. Tubes were
169 heated at 70 ˚C for 2.5 hours, cooled to room temperature and centrifuged for 1 min
170 at 3000 rpm. Absorbance was recorded at 555 nm in a CE 2040-2000 series
171 UV/visible spectrophotometer (Cecil, London, U.K.). The acetone−butanol−HCl
172 reagent was used as a blank and all samples were run in triplicate. The purified

proanthocyanidin fraction described above was used as standard for the calibration curve.

Statistical Analysis. The proanthocyanidin parameters (content, mean degree of polymerization, % prodelphinidin, % *cis-flavanols*) were subjected to a two-way analysis of variance (ANOVA) performed with repeated measures analysis to test the effect of NaOH concentration and reaction time. All analyses were determined by Systat 9 (SPSS Ltd.). Differences between means were determined using the protected LSD (α = 0.01).

RESULTS AND DISCUSSION

Optimization of Alkaline Pre-treatment for Ensiled Sainfoin. Several NaOH concentrations, temperatures and reaction times were tested initially. These trials showed that low NaOH concentrations (0.05 and 0.1 M) did not improve proanthocyanidin detection after thiolysis and reactions at 60 ˚C induced considerable proanthocyanidin degradation (data not shown). These NaOH studies were repeated several times over the course of three months and generated similar results. All of these initial studies were conducted under nitrogen and produced rather large standard deviations that were successfully reduced once the NaOH reaction was performed under argon. Subsequent experiments, therefore, explored 0.5, 1, 2, 3 and 4 M NaOH concentrations and 5, 15, 30, 60 min reaction times at 40 ˚C under argon. Time ($P < 0.001$) and NaOH concentration ($P < 0.001$ to 0.008) had a significant effect on the thiolysis results in terms of proanthocyanidin content, mean degree of polymerization, percentages of prodelphinidins and *cis* flavan-3-ols within proanthocyanidins. The mean degree of polymerization was also significantly (P<

198 0.001) affected by a time x NaOH concentration interaction. Overall, the length of the
199 reaction time had the greatest effect on all parameters followed by NaOH
200 concentration.
201 The highest proanthocyanidin content (2.17 g/100 g dry weight; cv = 4.3%) was
202 obtained with 1 M NaOH and a 15 min reaction time. The mean degree of
203 polymerization was relatively stable at 0.5, 1 and 2 M NaOH; and a value of 9.1, with
204 the 1 M NaOH and 15 min pre-treatment, was comparable to 8.2 in the fresh sainfoin
205 (Table 1). The percentage of prodelphinidins remained stable between 15 and 60
206 min with 1 and 2 M NaOH, and the percentage of *trans* flavan-3-ols increased only
207 slightly over 60 min.
208 A closer look at the flavan-3-ol composition revealed that more terminal catechin (**3**)
209 and especially epigallocatechin (**2**) units were detectable with the lowest NaOH
210 concentrations (0.5 and 1 M) and 15 and 30 min reaction times (Table 2). The
211 epicatechin (**4**) concentration from the 1 M NaOH reaction was low, but remained
212 constant over time and produced consistently small errors in contrast to the 0.5 and
213 2 M NaOH treatments. The highest extension unit concentrations of gallocatechin
214 (**1**), epigallocatechin (**2**) and epicatechin (**4**) were obtained with 1 M NaOH and 5 or
215 15 min reaction times. However, the differences were not significant and standard
216 errors were generally smaller at 15 min compared to 5 or 60 min. Thus, we chose
217 the 1 M NaOH and 15 min pre-treatment because this gave the highest
218 proanthocyanidin content based on a maximal release of epigallocatechin (**2**),
219 epicatechin (**4**) and gallocatechin (**1**) units, the most stable mean degree of
220 polymerization and consistently low standard errors. Longer hydrolysis times and
221 especially higher NaOH concentrations (2 to 4 M) led to lower mean degrees of

polymerization, which suggested either proanthocyanidin depolymerization or degradation as observed previously.[13]

Effect of NaOH on Purified Tannins. Next, the effects of the optimized NaOH reaction conditions were tested on a purified sainfoin proanthocyanidin fraction in order to identify marker compounds that might be indicative of any proanthocyanidin−derived degradation products from the NaOH reaction when applied directly to silage samples. HPLC chromatograms before and after NaOH pre-treatment are shown in Figure 2. Flavan-3-ol terminal units (peaks 1 to 4) and extension units (peaks 6 to 11) are still visible after 15 min (Figure 2B) but start to disappear after 60 min (Figure 2C). These chromatograms revealed a rapid loss of proanthocyanidins (from 100 g to less than 20 g/100 g fraction) and a change in the mean degree of polymerization from 9.7 to less than 6 within 5 min. The most noticeable effect was the appearance of a 'hump', which is likely to stem from oxidized or polymerized proanthocyanidins and suggested that many more reaction products were formed over time during NaOH treatment.[17]

Several of the degradation products (peaks a to g, Figure 2) were tentatively assigned based on their *m/z*-values and literature reports: peak a with an *m/z* value of 169.2 (RT = 13.96 min) could stem from gallic acid and peak c with an *m/z* of 153.2 (RT = 19.57 min) from 3,4-dihydroxybenzoic acid as these are typical products from base-catalyzed/degradation reaction of the B-rings of prodelphinidins and procyanidins, respectively.[18] Other plausible proanthocyanidin oxidation/degradation products are peaks b and d with *m/z* values of 303.3 (RT = 14.31 min) and 319.2 (RT = 24.05 min), respectively, which might be α-ketoretro-chalcones derived from base-catalyzed opening of the C-ring of catechin (**3**) or epicatechin (**4**) moieties in

procyanidins and gallocatechin (**1**) or epigallocatechin (**2**) moieties in prodelphinidins followed by the cleavage of the interflavanyl bond under base-catalysis.[18,19] Peak e (*m/z* of 309.2; RT 31.87 min) could have come from an epigallocatechin (**2**) oxidation product as reported after H_2O_2 treatment;[20] peak g (*m/z* of 427.3; RT = 47.75 min) from theacitrinin A;[19] and peak f (*m/z* of 310.2; RT = 45.29 min) could be the rearranged benzylsulfanyl indan derivative of catechinic acid.[18]

NaOH Pre-Treatment for *in Planta* Analysis of Proanthocyanidins in Ensiled Sainfoin. Although NaOH generated several degradation products from the pure proanthocyanidins (Figures 2B and C), none of these products nor the polymeric hump were seen when ensiled samples were treated with NaOH (Figure 3). In contrast to the NaOH-treated pure proanthocyanidin fraction, all flavan-3-ol terminal and extension units were clearly detectable in the treated silage (peaks labelled 2 to 4 and 7 to 11; Figure 3B).

Without the NaOH treatment, direct thiolysis of the plant materials yielded 3.1 and 0.4 g proanthocyanidins/100 g dry weight for fresh and ensiled samples, respectively (Table 1). In comparison, the acetone−butanol−HCl assay gave much higher values of 4.7 and 3.9 g/100 g dry weight for these samples. However, when the ensiled sample was first treated with NaOH and then thiolyzed, proanthocyanidin content increased from 0.4 g to 2.2 g/100 g dry weight in the silage. It can also be seen that the sum of acetone/water extractable (0.4 g/100 g dry weight) plus residual (1.9 g/100 g dry weight) proanthocyanidins was comparable to the directly determined result in the silage (2.3 g *vs* 2.2 g/100 g dry weight).

NaOH facilitated the release of prodelphinidins (69.9% with NaOH *vs* 50.1% without NaOH) and of larger proanthocyanidin polymers (mean degree of polymerization-

values of 9.1 *vs* 4.4) from the silage and a similar trend can be seen in the NaOH-treated plant residue. Table 2 shows that these changes stemmed from a 10-fold increase in the concentration of epigallocatechin (**2**) extension units (1.2 *vs* 12.7 mg/g) and a 2- to 4-fold increase in all other flavan-3-ol concentrations. Taken together, the results demonstrate that NaOH facilitated especially the release of larger prodelphinidins from the residue, and that ensiling profoundly reduced proanthocyanidin extractability. This NaOH/thiolysis reaction was then also applied to other sainfoin silages, which had proanthocyanidins that had proved completely resistant to thiolysis, and the results are shown in Table 3.

Ensiling caused proanthocyanidin contents to fall by 29% from 3.1 g in the fresh to 2.2 g/100 g dry weight in the ensiled sample (Table 1), whereas a loss of 17% from 4.7 g to 3.9 g/100 g dry weight was found with the acetone-butanol-HCl reagent. These results are in line with other studies that reported lower proanthocyanidin contents after ensiling.[5] However, there is also some evidence that ensiling can produce variable results, as others[7] found no change in proanthocyanidin content when birdsfoot trefoil or sulla were ensiled; although it is worth pointing out that these authors had used an HCl-butanol method, which yields lower total proanthocyanidin contents than the acetone-HCl-butanol method used here.[16] Ensiling appears to affect mainly extractable proanthocyanidins, which accounted for 81% in the fresh but for only 18% in the ensiled samples. This implies that ensiling substantially increased the proportion of residual or bound proanthocyanidins (Table 1) and is in accord with literature data.[7,8,21] It would appear that NaOH affected the measured proanthocyanidin content by releasing bound proanthocyanidins from the residue (Tables 1 and 3).

However, ensiling appears to have caused hardly any changes in the composition of the assayable proanthocyanidins (Table 2). In agreement with the literature, epigallocatechin (**2**) and epicatechin (**4**) extension units accounted for the majority of flavan-3-ols in sainfoin proanthocyanidins[22,23] and residues contained a higher percentage of prodelphinidins than extracts (Table 1).[10]

Whilst purified proanthocyanidins were readily degraded by NaOH (Figure 2) and several reaction products (peaks a to g) were detected, there was some evidence that proanthocyanidins in the fresh sample were also degraded by NaOH (Table 1): measured proanthocyanidin contents changed from 3.1 g to 2.4 g/100 g (P = 0.05), mean degrees of polymerization from 8.2 to 5.3 (P = 0.01) and the percentages of prodelphinidins from 69% to 73% (P = 0.05), but inspection of the HPLC-MS chromatograms showed no or only trace amounts of any of the proanthocyanidin degradation products that had been observed with the pure proanthocyanidin fraction. In contrast, we could find no evidence that NaOH caused depolymerization of ensiled proanthocyanidins, as proanthocyanidins in fresh (by thiolysis) and ensiled sainfoin (by NaOH-thiolysis) had similar mean degrees of polymerization (whole plants: 8.2 *vs* 9.1; residues: 8.1 *vs* 8.3) and prodelphinidin percentages (whole plants: 69.1 *vs* 69.9%; residues: 72.0 *vs* 72.9%).

It would appear that in the absence of a NaOH pre-treatment, benzyl mercaptan reacted mainly with the extractable proanthocyanidins, as the quantities were the same, i.e. 0.4 g/100 g for the ensiled plant and acetone/water extract (Table 1). Benzyl mercaptan also seemed to react preferentially with procyanidins rather than prodelphinidins in all samples, as shown by the higher procyanidin percentages (31% in fresh and 50% in ensiled plants), which were almost identical for the whole plants and the extracts (Table 1). This might be due to the fact that procyanidins

were more soluble in acetone/water than prodelphinidins in these samples and seems to suggest that proanthocyanidins need to be 'free' in order to react with benzyl mercaptan. An alternative explanation for these procyanidin-prodelphinidin differences could be that interflavanyl links were more difficult to break with benzyl mercaptan in larger than smaller proanthocyanidin polymers as larger polymers in sainfoin tend to be prodelphinidins.[10]

Effect of NaOH on Other Polyphenolic Compounds in Ensiled Sainfoin. Several flavonoids[22,24] and isoflavones[25] were also detected in the silage and tentatively assigned based on their *m/z* values (Figures 1 and 3). They were quantitated using authentic standards (provided there were no co-eluting impurities) and this showed that NaOH treatment reduced the concentrations of some of these compounds by up to 44% (Figure 3B). The concentration without and with the NaOH treatment were as follows: rutin (4.5 *vs* 4.3 mg/g dry weight), afzelin (1.4 *vs* 1.1 mg/g dry weight), quercetin (1.8 *vs* 1.1 mg/g dry weight), kaempferol (0.9 *vs* 0.5 mg/g dry weight), isorhamnetin (0.3 *vs* 0.2 mg/g dry weight), formononetin (0.10 *vs* 0.06 mg/g dry weight) and afromosin (0.06 *vs* 0.04 mg/g dry weight).

On the Nature and Reactivity of Residual Proanthocyanidins in Silage. Relatively little is known about the reactions of proanthocyanidins with other plant constituents in processed plant samples. The method of Terrill *et al*.[8] distinguishes between extractable and protein-bound proanthocyanidins using solvents designed to dissociate hydrogen bonds and hydrophobic interactions. However, Hagerman[26] reported that covalent bonds can also be formed between proanthocyanidins and amino acids such as L-lysine and L-cysteine under neutral to alkaline conditions. The

346 reaction between proanthocyanidins and the amino group in L-lysine gives rise to *N*-
347 quinoyls or Schiff's bases and the sulfhydryl group of L-cysteine can generate a
348 covalent thioether linkage. Other studies reported oxidative coupling between
349 catechin (**3**) and L-lysine[27] and also between thiols in cysteine, glutathione, 3-
350 mercaptohexan-1-ol and the A− or B−rings of epigallocatechin gallate (**2**) or between
351 thiols in peptides and rosmarinic acid.[28,29] All of these reactions can take place under
352 slightly acidic conditions, *i.e.* at a pH of 4 to 6, and could, therefore, occur during
353 ensiling.[30] Covalent linkages may prevent reaction with benzyl mercaptan as
354 reported recently for proanthocyanidin-glycosides, which were, however, detected
355 with butanol-HCl;[31] this could account for the larger proanthocyanidin loss measured
356 by thiolysis (29%) than by acetone-HCl-butanol (17%).
357 Covalent links could also have been generated via proanthocyanidin oxidation by
358 oxidases, which are released upon cell death and remain active during the initial
359 stages of ensiling.[32,33] Thus, any intermolecular oxidative reactions formed between
360 proanthocyanidins and other cellular components are likely to generate covalent
361 cross linkages that may resist thiolysis. In addition, flavan-3-ols reacting with the
362 carbonyl group in aldehydes could also generate thiolysis-resistant bonds and such
363 flavan-3-ol−aldehyde adducts were reported in wine.[34] Aldehydes are present in
364 legume silages and result from the degradation of amino acids, organic acids and
365 fatty acids.[35]
366 Sodium hydroxide (0.1 to 10 M NaOH under nitrogen for 30 to 60 min)[36] is widely
367 used for releasing phenolics from cell wall carbohydrates that are linked via ester or
368 ether bonds.[14,36,37] Ensiling may have given rise to enzymatic esterification and ester
369 bonds are the most likely bonds to be hydrolyzed by this short, 15 min, 1 M NaOH
370 treatment at 40 °C, although hydrogen bonds in tannin-protein or tannin-

carbohydrate complexes might also be disrupted. Interestingly, Grabber *et al.*[37] successfully incorporated epicatechin (**4**) into lignin with peroxidase and ester-linked components were subsequently analyzed after hydrolysis with NaOH.

In conclusion, this is the first report of an analytical method capable of characterizing proanthocyanidin contents and composition in an ensiled animal feed. A 15 min pre-treatment at 40 °C with 1 M NaOH under argon was required to release bound proanthocyanidins and enabled their subsequent analysis by thiolytic degradation. The composition of assayable proanthocyanidins resembled that of the original proanthocyanidins in the fresh plant, but 29% of the original proanthocyanidins could not be detected by this NaOH-thiolysis treatment and 17% by the acetone-butanol-HCl reagent. This suggests that fermentation had caused considerable proanthocyanidin losses or structural changes. Ensiling also had a major effect on the extractability of proanthocyanidins and most remained in the silage residue after the acetone/water extraction, *i.e.* 86%. It would be interesting to explore whether the presence of such unextractable proanthocyanidins may be responsible for the enhanced anthelmintic (deworming) activities, which have been observed when feeding ensiled proanthocyanidin-containing samples.[38,39] We venture to hypothesize that these bound proanthocyanidins may act post-ruminally in the form of 'slow-release' compounds against parasitic nematodes in the small intestine, although this will require further research.

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NOTE

The authors declare no competing financial interest.

AUTHORS CONTRIBUTION

AR and IMH designed the study. AGB and MG produced sainfoin silage samples from Posieux (Switzerland) under the supervision of FDM and GB. GC produced silages samples from Theix (France) under the supervision of VN. AR carried out the study and analyzed the data. AR co-wrote the manuscript with IMH. CD contributed to analysis of proanthocyanidins. All authors critically read and approved the final manuscript.

Supporting Information

Analysis of variance used to assess effects of reaction times and NaOH concentrations on the proanthocyanidin parameters (Table S1). Thiolytic degradation of proanthocyanidins with benzyl mercaptan (Figure S1). Changes in proanthocyanidin contents and mean degrees of polymerization with different NaOH concentrations over a 60 minute time period (Figure S2). Changes in molar percentages of prodelphinidins and *trans* flavan-3-ols with different NaOH concentrations over a 60 minute time period (Figure S3). Changes in the contents of terminal flavan-3-ol units with different NaOH concentrations over a 60 minute time period (Figure S4). Changes in the contents of extension flavan-3-ol units with different NaOH concentrations over a 60 minute time period (Figure S5). Tentative assignments of several reaction products after treating a pure sainfoin proanthocyanidin fraction with NaOH and benzylmercaptan (Figure S6). This material is available free of charge via the Internet at http://pubs.acs.org.

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FIGURE CAPTIONS

Figure 1. Flavan-3-ol monomeric subunits of proanthocyanidins and other phenolics detected in sainfoin silage (dashed numbers, 1' to 10', refer to peak numbers in Figure 3).

Figure 2. HPLC chromatograms after thiolysis of a pure sainfoin proanthocyanidin fraction. A. without NaOH pre-treatment, B. after 15 min, and C. after 60 min of NaOH pre-treatment. **1**, gallocatechin; **2**, epigallocatechin; **3**, catechin; **4**, epicatechin; **5**; internal standard (taxifolin); **6**, 3,4-*trans*–gallocatechin-benzyl mercaptan; **7**, 3,4-*cis*–gallocatechin-benzyl mercaptan; **8**, epigallocatechin-benzyl mercaptan; **9**, 3,4-*trans*–catechin-benzyl mercaptan; **10,** 3,4-*cis*–catechin-benzyl mercaptan; **11**, epicatechin-benzyl mercaptan; *****, unidentified peaks.

Figure 3. HPLC chromatograms after *in situ* thiolysis of proanthocyanidins in ensiled sainfoin without (A) and with (B) NaOH pre-treatment (1 M NaOH, 40 °C, 15 min). **2**, epigallocatechin; **3**, catechin; **4**, epicatechin; **5**, internal standard (taxifolin); **7**, 3,4-*cis*–gallocatechin-benzyl mercaptan; **8**, epigallocatechin-benzyl mercaptan; **9**, 3,4-*trans*–catechin-benzyl mercaptan; **10**, 3,4-*cis*–catechin-benzyl mercaptan; **11**, epicatechin-benzyl mercaptan; ***,** unidentified compound; **1′**, coumaric acid glycoside; **2′**, rutin; **3′**, coumaric acid; **4′**, afzelin; **5′**, isorhamnetin-rutinoside; **6′**, quercetin; **7′**, kaempferol; **8′**, isorhamnetin; **9′**, formononetin; **10′**, afromosin.

Table 1. Proanthocyanidin Contents and Compositions of Fresh/Freeze-Dried and Ensiled Sainfoin after Thiolysis in the Absence and Presence of a NaOH (1M, 40 °C, 15 min) Pretreatment (SD in parenthesis, n = 3).

	PA (g/100 g DW)	**mDP**	**PC** %	**PD** %	***cis*** %	***trans*** %
Fresh/freeze-dried sainfoin (Posieux)						
Plant	3.1 (0.2)	8.2 (0.0)	30.9 (0.1)	69.1 (0.1)	82.3 (0.1)	17.3 (0.1)
Plant + NaOH	2.4 (0.1)	5.3 (0.1)	27.3 (0.5)	72.7 (0.5)	83.2 (0.1)	16.8 (0.1)
Acetone/water extract	2.5 (0.1)	11.4 (0.1)	30.3 (0.6)	69.7 (0.6)	82.2 (0.4)	17.8 (0.4)
Residue	0.6 (0.1)	8.1 (0.1)	28.0 (0.1)	72.0 (0.1)	87.5 (0.0)	12.5 (0.0)
Residue + NaOH	0.7 (0.1)	6.6 (0.3)	30.3 (0.5)	69.7 (0.5)	85.7 (0.3)	14.3 (0.3)
Ensiled sainfoin (Posieux)						
Plant	0.4 (0.2)	4.4 (0.4)	49.9 (6.2)	50.1 (6.2)	82.6 (0.5)	17.5 (0.5)
Plant + NaOH	2.2 (0.1)	9.1 (0.2)	30.1 (0.4)	69.9 (0.4)	83.0 (0.8)	17.0 (0.8)
Acetone/water extract	0.4 (0.1)	11.5 (1.5)	49.5 (5.0)	50.5 (2.8)	87.0 (5.5)	13.0 (5.5)
Residue	0.6 (0.1)	6.2 (0.7)	27.4 (0.7)	72.6 (0.7)	87.3 (0.7)	12.7 (0.7)
Residue + NaOH	1.9 (0.1)	8.3 (0.1)	27.3 (0.8)	72.9 (0.8)	79.4 (0.6)	20.5 (0.6)

cis: molar percentage of epicatechin plus epigallocatechin subunits; DW: dry weight; mDP: mean degree of polymerization; PA: proanthocyanidins; PC: procyanidins (molar percentage of catechin plus epicatechin subunits); PD: prodelphinidins (molar percentage of gallocatechin plus epigallocatechin subunits); SD: standard deviation; *trans*: molar percentage of catechin plus gallocatechin subunits.

Table 2. Concentrations of Flavan-3-ol Terminal and Extension Units (mg flavan-3-ol/g DW) and Relative Molar Percentages (%) in Fresh/Freeze-Dried and Ensiled Sainfoin after Thiolysis in the Absence and Presence of a NaOH (1M, 40 °C, 15 min) Pretreatment (SD in parentheses, n = 3).

	Terminal units						**Extension units**							
	EGC		**C**		**EC**		**GC**		**EGC**		**C**		**EC**	
	mg/g	**%**	**mg/g**	**%**	**mg/g**	**%**	**mg/g**	**%**	**mg/g**	**%**	**mg/g**	**%**	**mg/g**	**%**
Fresh sainfoin														
Plant	2.1 (0.1)	6.7 (0.1)	0.7 (0.1)	2.1 (0.0)	1.0 (0.1)	3.3 (0.1)	3.6 (0.2)	11.7 (0.1)	15.7 (0.7)	50.7 (0.1)	1.1 (0.1)	3.5 (0.0)	6.8 (0.3)	21.9 (0.1)
Plant+NaOH	3.3 (0.1)	13.9 (0.1)	0.4 (0.1)	1.7 (0.1)	0.8 (0.1)	3.4 (0.1)	2.6 (0.1)	11.0 (0.2)	11.5 (0.4)	47.8 (0.2)	1.0 (0.2)	4.1 (0.1)	4.3 (0.1)	18.1 (0.2)
AW extract	1.0 (0.1)	4.1 (0.1)	0.6 (0.1)	2.3 (0.1)	0.6 (0.1)	2.5 (0.1)	3.1 (1.2)	12.4 (0.1)	13.3 (0.1)	53.3 (0.5)	0.8 (1.5)	3.1 (0.4)	5.6 (0.3)	22.5 (0.3)
Residue	0.5 (0.1)	7.6 (0.1)	0.1 (0.1)	1.9 (0.0)	0.2 (0.1)	2.9 (0.1)	0.5 (0.1)	8.0 (0.0)	3.4 (0.1)	56.4 (0.1)	0.2 (0.1)	2.6 (0.0)	1.2 (0.1)	20.6 (0.1)
Residue+NaOH	0.7 (0.1)	10.0 (0.4)	0.2 (0.1)	2.2 (0.2)	0.2 (0.1)	3.0 (0.2)	0.3 (0.1)	4.4 (1.1)	3.9 (0.1)	55.3 (3.3)	0.3 (0.1)	4.9 (0.8)	1.4 (0.2)	20.1 (0.8)
Ensiled sainfoin														
Plant	0.4 (0.3)	10.2 (4.1)	0.1 (0.1)	3.5 (0.4)	0.4 (0.1)	9.4 (2.2)	0.4 (0.2)	8.8 (0.6)	1.2 (0.5)	31.0 (1.6)	0.2 (0.1)	5.1 (0.4)	1.3 (0.3)	31.9 (3.9)
Plant+NaOH	1.3 (0.7)	6.1 (0.3)	0.4 (0.1)	2.0 (0.1)	0.7 (0.1)	3.0 (0.1)	1.4 (0.1)	6.4 (0.1)	12.7 (0.1)	57.5 (0.1)	0.5 (0.1)	4.0 (0.0)	4.6 (0.1)	21.0 (0.3)
AW extract	0.1 (0.1)	1.0 (0.4)	0.2 (0.1)	4.0 (0.4)	0.2 (0.1)	4.0 (0.4)	0.2 (0.1)	4.1 (5.8)	1.8 (0.0)	45.4 (3.0)	0.2 (0.3)	4.9 (1.2)	1.5 (0.7)	36.7 (3.5)
Residue	0.7 (0.1)	12.1 (1.7)	0.1 (0.1)	2.0 (0.1)	0.1 (0.1)	2.3 (0.3)	0.5 (0.1)	8.3 (0.5)	3.1 (0.1)	52.2 (2.8)	0.1 (0.1)	2.3 (0.2)	1.2 (0.2)	20.7 (0.1)
Residue+NaOH	1.5	8.1	0.4	1.9	0.4	2.1	2.6	13.9	9.7	50.9	0.9	4.9	3.5	18.3

	(0.1)	(0.1)		(0.0)	(0.1)		(0.1)	(0.1)		(0.1)	(0.1)		(0.6)		(0.1)	(0.7)		(0.1)	(0.1)

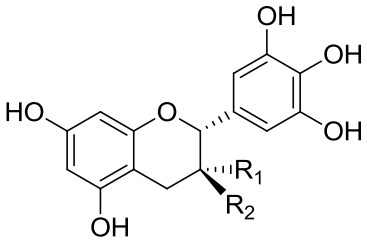
AW: acetone-water; C: catechin; DW: dry weight of plant material; EC: epicatechin; EGC: epigallocatechin; GC: gallocatechin; SD: standard deviation.

Table 3. Analysis of Thiolysis-Resistant Sainfoin Silages from Theix by the Acetone-Butanol-HCl (ABH) and the Thiolysis Assays in the Absence and Presence of the NaOH Pretreatment (SD in parentheses, n = 3).

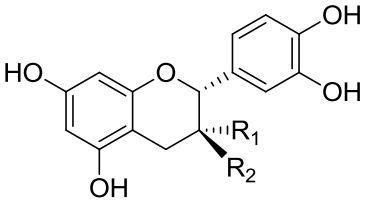
	PA (ABH) g/100 g DW	**PA** (thiolysis) g/100 g DW	**mDP**	**PC** %	**PD** %	***cis*** %	***trans*** %	**Terminal units** (%)			**Extension units** (%)		
								C	**EC**	**EGC**	**C**	**EC**	**EGC**
SF1 (INRA Theix)													
Plant	2.6 (0.1)	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Plant+NaOH		1.2 (0.1)	2.2 (0.1)	27.3 (0.1)	72.7 (0.1)	96.5 (0.1)	3.5 (0.1)	1.6 (0.1)	3.4 (0.1)	40.8 (1.5)	2.0 (0.1)	20.4 (0.1)	31.9 (1.5)
SF2 (INRA Theix)													
Plant	2.8 (0.1)	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Plant+NaOH		1.1 (0.1)	2.7 (0.1)	28.0 (0.2)	72.0 (0.2)	96.2 (0.0)	3.8 (0.0)	1.8 (0.1)	3.3 (0.1)	32.2 (1.7)	2.1 (0.1)	20.9 (0.1)	39.9 (1.9)
SF3 (INRA Theix)													
Plant	2.5 (0.1)	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Plant+NaOH		0.5 (0.1)	4.3 (0.9)	42.8 (0.2)	57.3 (0.2)	93.6 (0.3)	6.4 (0.3)	2.8 (0.3)	5.9 (0.7)	15.5 (4.3)	3.5 (0.1)	30.5 (0.8)	41.8 (4.5)

%: relative molar percentages; C: catechin; *cis:* epicatechin plus epigallocatechin subunits; DW: dry weight; EC: epicatechin; EGC: epigallocatechin; mDP: mean degree of polymerization; nd: none detected; PA: proanthocyanidins; PC: procyanidins (catechin plus

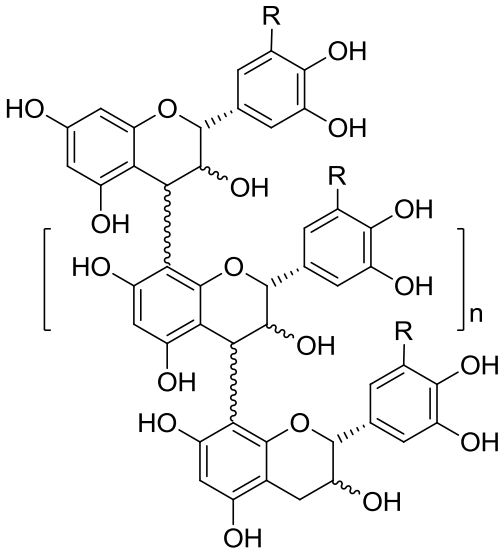
epicatechin subunits); PD: prodelphinidins (gallocatechin plus epigallocatechin subunits); SD: standard deviation; *trans*: catechin plus gallocatechin subunits.



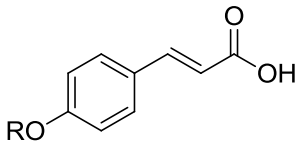
1, R_1 = H, R_2 = OH, Gallocatechin (a *trans*-flavan-3-ol)
2, R_1 = OH, R_2 = H, Epigallocatechin (a *cis*-flavan-3-ol)



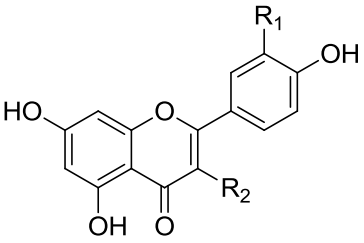
3, R_1 = H, R_2 = OH, Catechin (a *trans*-flavan-3-ol)
4, R_1 = OH, R_2 = H, Epicatechin (a *cis*-flavan-3-ol)



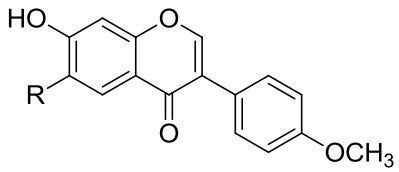
Example of proanthocyanidins:
R = H, procyanidins
R = OH, prodelphinidins



1', R = hexoside, Coumaroyl-*O*-glycoside
3', R = H, Coumaric acid



2', R_1 = OH, R_2 = *O*-rutinoside, Rutin
4', R_1 = H, R_2 = *O*-rhamnoside, Afzelin
5', R_1 = OCH_3, R_2 = *O*-rutinoside, Isorhamnetin-rutinoside
6', R_1 = OH, R_2 = OH, Quercetin
7', R_1 = H, R_2 = OH, Kaempferol
8', R_1 = OCH_3, R_2 = OH, Isorhamnetin



9', R = H, Formononetin
10', R = OCH_3, Afromosin

Figure 1

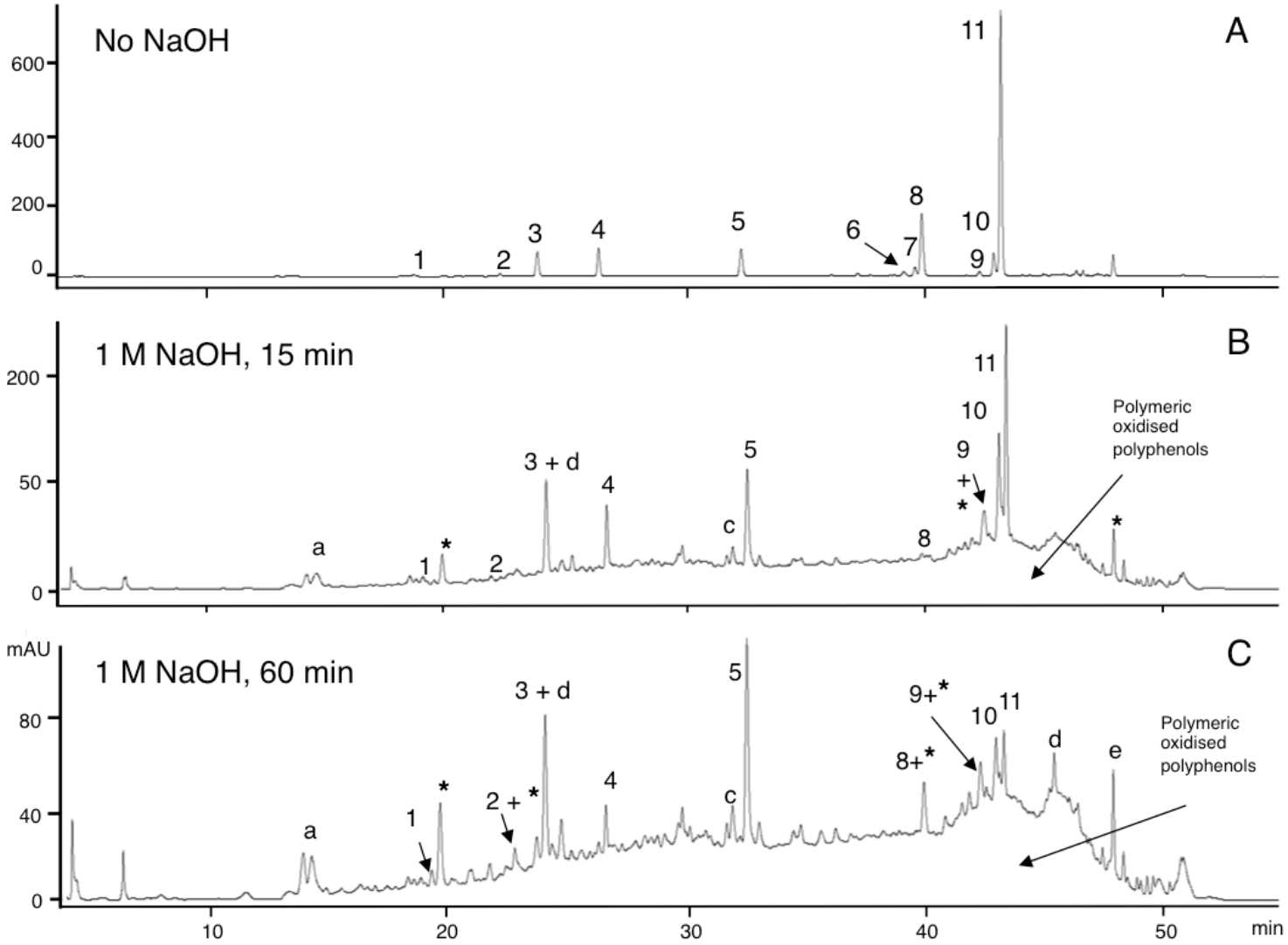

No NaOH
A
600
400
200
0
1
2
3
4
5
6
7
8
9
10
11
1 M NaOH, 15 min
B
200
50
0
a
1
*
2
3 + d
4
c
5
8
9
+
*
10
11
Polymeric
oxidised
polyphenols
*
mAU
1 M NaOH, 60 min
C
80
40
0
a
1
*
2 +
*
3 + d
4
c
5
8+*
9+*
10
11
d
e
Polymeric
oxidised
polyphenols
10
20
30
40
50
min

Figure 2

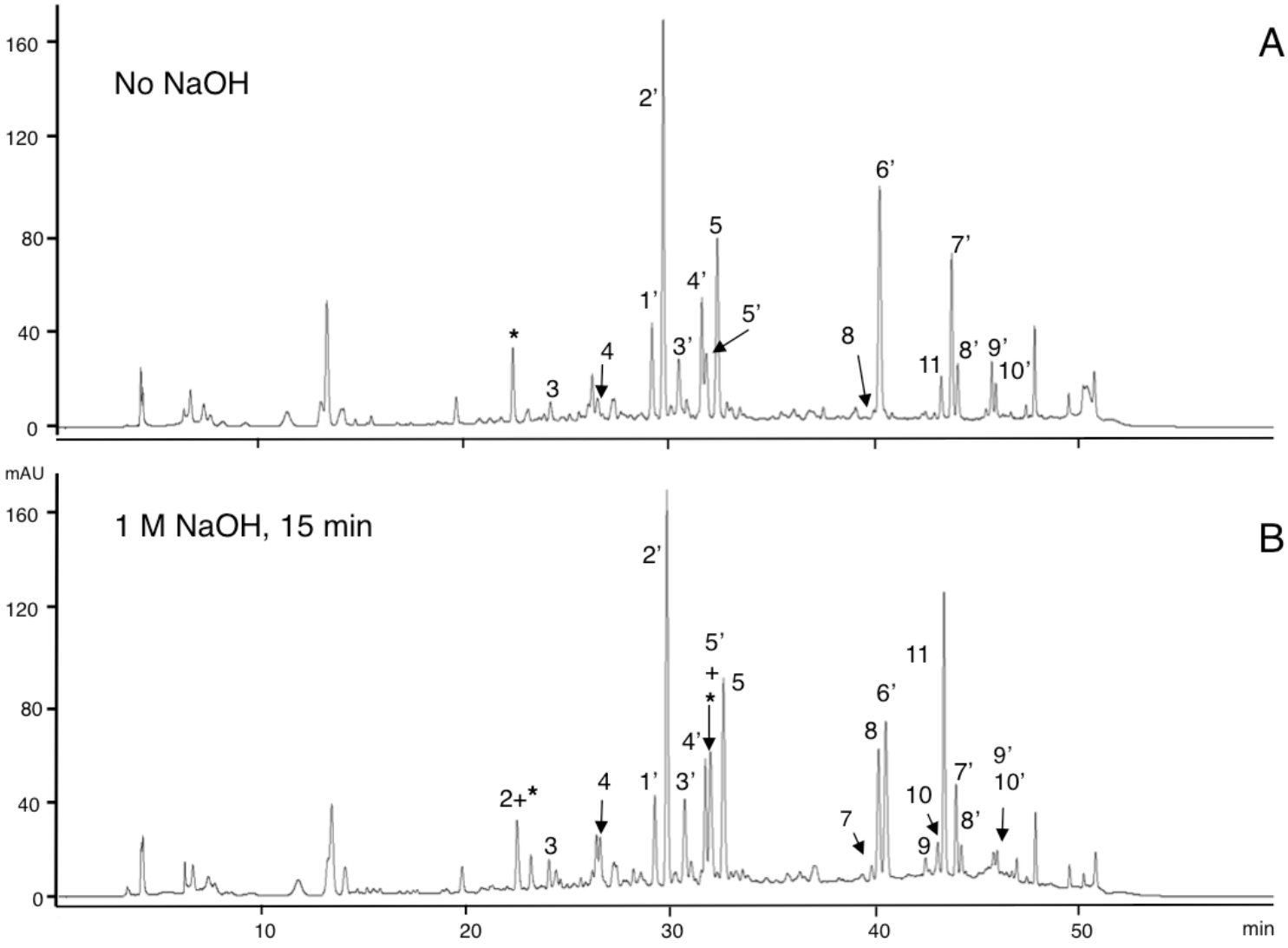

A
No NaOH
B
1 M NaOH, 15 min
mAU
min
0
40
80
120
160
10
20
30
40
50
2'
6'
5
7'
4'
1'
5'
3'
4
8
11
8'
9'
10'
3
*
2+*
5'
+
*
7
9
10

Figure 3

Table of Contents Graphic