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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

48   bound phenolics.[14,15] Therefore, the present study evaluated whether pre-treatment

49   with NaOH would enable compositional analysis of proanthocyanidins in ensiled

50   sainfoin by thiolytic degradation with benzyl mercaptan.

51

52   **MATERIALS AND METHODS**

53   **Chemicals.** Hydrochloric acid (concentrated, 36%), acetone (analytical reagent

54   grade), dichloromethane (HPLC grade) and acetonitrile (HPLC grade) were

55   purchased from ThermoFisher Scientific Ltd (Loughborough, U.K.); (±)-taxifolin

56   (98%) from Apin Chemicals (Abingdon, U.K.); benzyl mercaptan (98%), catechin (**3**),

57   epicatechin (4), gallocatechin (**1**), epigallocatechin (**2**), quercetin (95%), kaempferol

58   (98%), gallic acid and 3,4-dihydroxybenzoic acid from Sigma-Aldrich (Poole, U.K.);

59   rutin (98%) from Lancaster Synthesis (Lancaster, UK) and formononetin (98%) from

60   Acros Organics (Loughborough, UK). Deionized water was obtained from a Milli-Q

61   System (Millipore, Watford, U.K.). Quercetin, kaempferol and formononetin were

62   dissolved in methanol/water (1:1, v/v) using six concentrations (n = 2) and peak

63   areas were used for the calibration curve.

64

65   **Plant Materials.** Sainfoin (*Onobrychis viciifolia* 'Perly') was sown at the Swiss

66   Federal Research Station ALP (Posieux, altitude: 650 m) in April 2012 and harvested

67   at the early flowering stage on 9 July 2012. A bulked sample (5 kg) was freeze-dried

68   (= fresh/freeze-dried sample) and had a dry matter (DM) content of 169.4 g/kg fresh

69   material and an organic matter (OM) content of 923.2 g/kg DM. The samples were

70   wilted for 24 h in the field in a swath to minimize leaf loss and then chopped (1-2 cm)

71   with a Mex GT chaff cutter (Poettinger, Grieskirchen, Austria). The wilted samples

72   (DM content was 377.9 g/kg fresh wet material; OM content was 913.2 g/kg DM)

4

73 were ensiled without additives in 30 L barrels. The barrels were filled, compressed

74 by hand and stored at room temperature in a dark room for 86 d. The room was

75 neither cooled nor heated in the barn facilities (without insulation). The room

76 temperature was recorded every week and ranged from 23.0 °C (August) to 17.7 °C

77 (October). On average it was 20.4 °C. On opening the silo after 86 days of ensiling,

78 the pH was 4.51 ± 0.03, the concentrations of short chain volatile fatty acids were

79 6.72 ± 0.94 (mg/kg DM) for acetate, 56.9 ± 5.96 (mg/kg DM) for lactate and 1.02 ±

80 0.06 (mg/kg DM) for butyrate. The silage was mixed by hand in a tray, subsamples

81 were taken, freeze–dried, ground to pass a <1 mm screen sieve with an impeller

82 SM1 cutting mill (Retsch, Haan, Germany) and stored at room temperature. The

83 silage DM content was 370.7 g/kg, and the OM content was 911.3 g/kg DM. Three

84 other sainfoin silage samples (SF1 to SF3, field replicates) were prepared in INRA

85 Theix (France) according to Copani *et al.*[9]

86

87 **Preparation of a Purified Proanthocyanidin Fraction and Acetone-Water**

88 **Extracts.** A purified proanthocyanidin fraction was isolated for use as a

89 proanthocyanidin standard in the acetone-butanol-HCl assay[16] and also for the

90 proanthocyanidin degradation test with NaOH. Sainfoin 'Cotswold Common' was

91 harvested on 24 July 2007 from Hartley Farm near Seven Springs (Cheltenham,

92 Gloucestershire, U.K.), manually separated from weeds, and freeze-dried. It was

93 ground using a impeller SM1 cutting mill (Retsch, Haan, Germany) to pass <8 mm

94 and then ground again to <1 mm. The ground sainfoin (20 g) was extracted with

95 acetone/water (7:3, v/v; 250 mL, 1 h). Acetone was removed under vacuum and the

96 aqueous phase was loaded on a Sephadex LH-20 column. The column was rinsed,

97 firstly with water, and then with acetone/water (3:7), and the proanthocyanidin

98   fraction was eluted with acetone/water (1:1, v/v). Thiolysis[10] coupled to HPLC-MS

99   revealed that this fraction had a very high proanthocyanidin content (98 g/100 g

100   fraction) with an mean degree of polymerization value of 9.7, a

101   procyanidin/prodelphinidin ratio of 44.1/55.9 and a *cis/trans* flavan-3-ol ratio of

102   81.9/18.1.

103   In addition, separate extracts were prepared from the fresh/freeze-dried and ensiled

104   sainfoin samples (5 g) from Posieux with acetone/water (7:3, v/v; 63 mL, 1 h) as

105   previously described.[10] The residues remaining after this acetone-water extraction

106   were also kept for proanthocyanidin analysis (= solvent-extracted residue).

107

108   **Thiolysis of the Purified Proanthocyanidin Fraction, Acetone/Water Extracts**

109   **and Solvent-Extracted Residues.** The purified proanthocyanidin fraction (8 mg),

110   acetone-water extracts and plant residues that remained after the acetone-water

111   extraction (8 mg) were placed into a 100 mm x 16 mm screw cap glass tube (Fisher

112   Scientific, Loughborough, U.K.) with a stirring magnet (10 x 5 x 5 mm). Methanol (1.5

113   mL) was added followed by methanol acidified with concentrated HCl (3.3%; 500 μL)

114   and benzyl mercaptan (50 μL). Tubes were capped and placed into a water bath at

115   40 °C for 1 h under vigorous stirring. [10] The reaction was stopped by placing the tube

116   in an ice bath for 5 min. Distilled water (2.5 mL) and the internal standard, taxifolin in

117   methanol (500 μL; 0.1 mg/mL), were added and thoroughly mixed. The mixture was

118   transferred into a vial (800 μL), closed with a crimp top and analyzed by HPLC-MS

119   within 24 h.

120

121   **Sodium Hydroxide Pre-Treatment of Plant Samples and Solvent-Extracted**

122   **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

173 proanthocyanidin fraction described above was used as standard for the calibration

174 curve.

175

176 **Statistical Analysis.** The proanthocyanidin parameters (content, mean degree of

177 polymerization, % prodelphinidin, % *cis-flavanols*) were subjected to a two-way

178 analysis of variance (ANOVA) performed with repeated measures analysis to test the

179 effect of NaOH concentration and reaction time. All analyses were determined by

180 Systat 9 (SPSS Ltd.). Differences between means were determined using the

181 protected LSD ($\alpha = 0.01$).

182

183 **RESULTS AND DISCUSSION**

184 **Optimization of Alkaline Pre-treatment for Ensiled Sainfoin.** Several NaOH

185 concentrations, temperatures and reaction times were tested initially. These trials

186 showed that low NaOH concentrations (0.05 and 0.1 M) did not improve

187 proanthocyanidin detection after thiolysis and reactions at 60 $^\circ$C induced

188 considerable proanthocyanidin degradation (data not shown). These NaOH studies

189 were repeated several times over the course of three months and generated similar

190 results. All of these initial studies were conducted under nitrogen and produced

191 rather large standard deviations that were successfully reduced once the NaOH

192 reaction was performed under argon. Subsequent experiments, therefore, explored

193 0.5, 1, 2, 3 and 4 M NaOH concentrations and 5, 15, 30, 60 min reaction times at 40

194 $^\circ$C under argon. Time ($P < 0.001$) and NaOH concentration ($P < 0.001$ to 0.008) had

195 a significant effect on the thiolysis results in terms of proanthocyanidin content, mean

196 degree of polymerization, percentages of prodelphinidins and *cis* flavan-3-ols within

197 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

222   polymerization, which suggested either proanthocyanidin depolymerization or

223   degradation as observed previously.[13]

224

225   **Effect of NaOH on Purified Tannins.** Next, the effects of the optimized NaOH

226   reaction conditions were tested on a purified sainfoin proanthocyanidin fraction in

227   order to identify marker compounds that might be indicative of any

228   proanthocyanidin−derived degradation products from the NaOH reaction when

229   applied directly to silage samples. HPLC chromatograms before and after NaOH pre-

230   treatment are shown in Figure 2. Flavan-3-ol terminal units (peaks 1 to 4) and

231   extension units (peaks 6 to 11) are still visible after 15 min (Figure 2B) but start to

232   disappear after 60 min (Figure 2C). These chromatograms revealed a rapid loss of

233   proanthocyanidins (from 100 g to less than 20 g/100 g fraction) and a change in the

234   mean degree of polymerization from 9.7 to less than 6 within 5 min. The most

235   noticeable effect was the appearance of a 'hump', which is likely to stem from

236   oxidized or polymerized proanthocyanidins and suggested that many more reaction

237   products were formed over time during NaOH treatment.[17]

238   Several of the degradation products (peaks a to g, Figure 2) were tentatively

239   assigned based on their *m/z*-values and literature reports: peak a with an *m/z* value

240   of 169.2 (RT = 13.96 min) could stem from gallic acid and peak c with an *m/z* of

241   153.2 (RT = 19.57 min) from 3,4-dihydroxybenzoic acid as these are typical products

242   from base-catalyzed/degradation reaction of the B-rings of prodelphinidins and

243   procyanidins, respectively.[18] Other plausible proanthocyanidin oxidation/degradation

244   products are peaks b and d with *m/z* values of 303.3 (RT = 14.31 min) and 319.2 (RT

245   = 24.05 min), respectively, which might be α-ketoretro-chalcones derived from

246   base-catalyzed opening of the C-ring of catechin (**3**) or epicatechin (**4**) moieties in

247 procyanidins and gallocatechin (**1**) or epigallocatechin (**2**) moieties in prodelphinidins

248 followed by the cleavage of the interflavanyl bond under base-catalysis.[18,19] Peak e

249 (*m/z* of 309.2; RT 31.87 min) could have come from an epigallocatechin (**2**) oxidation

250 product as reported after H_2O_2 treatment;[20] peak g (*m/z* of 427.3; RT = 47.75 min)

251 from theacitrinin A;[19] and peak f (*m/z* of 310.2; RT = 45.29 min) could be the

252 rearranged benzylsulfanyl indan derivative of catechinic acid.[18]

253

254 **NaOH Pre-Treatment for *in Planta* Analysis of Proanthocyanidins in Ensiled**

255 **Sainfoin.** Although NaOH generated several degradation products from the pure

256 proanthocyanidins (Figures 2B and C), none of these products nor the polymeric

257 hump were seen when ensiled samples were treated with NaOH (Figure 3). In

258 contrast to the NaOH-treated pure proanthocyanidin fraction, all flavan-3-ol terminal

259 and extension units were clearly detectable in the treated silage (peaks labelled 2 to

260 4 and 7 to 11; Figure 3B).

261 Without the NaOH treatment, direct thiolysis of the plant materials yielded 3.1 and

262 0.4 g proanthocyanidins/100 g dry weight for fresh and ensiled samples, respectively

263 (Table 1). In comparison, the acetone−butanol−HCl assay gave much higher values

264 of 4.7 and 3.9 g/100 g dry weight for these samples. However, when the ensiled

265 sample was first treated with NaOH and then thiolyzed, proanthocyanidin content

266 increased from 0.4 g to 2.2 g/100 g dry weight in the silage. It can also be seen that

267 the sum of acetone/water extractable (0.4 g/100 g dry weight) plus residual (1.9

268 g/100 g dry weight) proanthocyanidins was comparable to the directly determined

269 result in the silage (2.3 g *vs* 2.2 g/100 g dry weight).

270 NaOH facilitated the release of prodelphinidins (69.9% with NaOH *vs* 50.1% without

271 NaOH) and of larger proanthocyanidin polymers (mean degree of polymerization-

272 values of 9.1 *vs* 4.4) from the silage and a similar trend can be seen in the NaOH-

273 treated plant residue. Table 2 shows that these changes stemmed from a 10-fold

274 increase in the concentration of epigallocatechin (**2**) extension units (1.2 *vs* 12.7

275 mg/g) and a 2- to 4-fold increase in all other flavan-3-ol concentrations. Taken

276 together, the results demonstrate that NaOH facilitated especially the release of

277 larger prodelphinidins from the residue, and that ensiling profoundly reduced

278 proanthocyanidin extractability. This NaOH/thiolysis reaction was then also applied

279 to other sainfoin silages, which had proanthocyanidins that had proved completely

280 resistant to thiolysis, and the results are shown in Table 3.

281 Ensiling caused proanthocyanidin contents to fall by 29% from 3.1 g in the fresh to

282 2.2 g/100 g dry weight in the ensiled sample (Table 1), whereas a loss of 17% from

283 4.7 g to 3.9 g/100 g dry weight was found with the acetone-butanol-HCl reagent.

284 These results are in line with other studies that reported lower proanthocyanidin

285 contents after ensiling.[5] However, there is also some evidence that ensiling can

286 produce variable results, as others[7] found no change in proanthocyanidin content

287 when birdsfoot trefoil or sulla were ensiled; although it is worth pointing out that

288 these authors had used an HCl-butanol method, which yields lower total

289 proanthocyanidin contents than the acetone-HCl-butanol method used here.[16]

290 Ensiling appears to affect mainly extractable proanthocyanidins, which accounted for

291 81% in the fresh but for only 18% in the ensiled samples. This implies that ensiling

292 substantially increased the proportion of residual or bound proanthocyanidins (Table

293 1) and is in accord with literature data.[7,8,21] It would appear that NaOH affected the

294 measured proanthocyanidin content by releasing bound proanthocyanidins from the

295 residue (Tables 1 and 3).

296   However, ensiling appears to have caused hardly any changes in the composition of

297   the assayable proanthocyanidins (Table 2). In agreement with the literature,

298   epigallocatechin (**2**) and epicatechin (**4**) extension units accounted for the majority of

299   flavan-3-ols in sainfoin proanthocyanidins[22,23] and residues contained a higher

300   percentage of prodelphinidins than extracts (Table 1).[10]

301   Whilst purified proanthocyanidins were readily degraded by NaOH (Figure 2) and

302   several reaction products (peaks a to g) were detected, there was some evidence

303   that proanthocyanidins in the fresh sample were also degraded by NaOH (Table 1):

304   measured proanthocyanidin contents changed from 3.1 g to 2.4 g/100 g ($P = 0.05$),

305   mean degrees of polymerization from 8.2 to 5.3 ($P = 0.01$) and the percentages of

306   prodelphinidins from 69% to 73% ($P = 0.05$), but inspection of the HPLC-MS

307   chromatograms showed no or only trace amounts of any of the proanthocyanidin

308   degradation products that had been observed with the pure proanthocyanidin

309   fraction. In contrast, we could find no evidence that NaOH caused depolymerization

310   of ensiled proanthocyanidins, as proanthocyanidins in fresh (by thiolysis) and ensiled

311   sainfoin (by NaOH-thiolysis) had similar mean degrees of polymerization (whole

312   plants: 8.2 vs 9.1; residues: 8.1 vs 8.3) and prodelphinidin percentages (whole

313   plants: 69.1 vs 69.9%; residues: 72.0 vs 72.9%).

314   It would appear that in the absence of a NaOH pre-treatment, benzyl mercaptan

315   reacted mainly with the extractable proanthocyanidins, as the quantities were the

316   same, i.e. 0.4 g/100 g for the ensiled plant and acetone/water extract (Table 1).

317   Benzyl mercaptan also seemed to react preferentially with procyanidins rather than

318   prodelphinidins in all samples, as shown by the higher procyanidin percentages

319   (31% in fresh and 50% in ensiled plants), which were almost identical for the whole

320   plants and the extracts (Table 1). This might be due to the fact that procyanidins

321 were more soluble in acetone/water than prodelphinidins in these samples and

322 seems to suggest that proanthocyanidins need to be 'free' in order to react with

323 benzyl mercaptan. An alternative explanation for these procyanidin-prodelphinidin

324 differences could be that interflavanyl links were more difficult to break with benzyl

325 mercaptan in larger than smaller proanthocyanidin polymers as larger polymers in

326 sainfoin tend to be prodelphinidins.[10]

327

328 **Effect of NaOH on Other Polyphenolic Compounds in Ensiled Sainfoin.** Several

329 flavonoids[22,24] and isoflavones[25] were also detected in the silage and tentatively

330 assigned based on their m/z values (Figures 1 and 3). They were quantitated using

331 authentic standards (provided there were no co-eluting impurities) and this showed

332 that NaOH treatment reduced the concentrations of some of these compounds by up

333 to 44% (Figure 3B). The concentration without and with the NaOH treatment were as

334 follows: rutin (4.5 *vs* 4.3 mg/g dry weight), afzelin (1.4 *vs* 1.1 mg/g dry weight),

335 quercetin (1.8 *vs* 1.1 mg/g dry weight), kaempferol (0.9 *vs* 0.5 mg/g dry weight),

336 isorhamnetin (0.3 *vs* 0.2 mg/g dry weight), formononetin (0.10 *vs* 0.06 mg/g dry

337 weight) and afromosin (0.06 *vs* 0.04 mg/g dry weight).

338

339 **On the Nature and Reactivity of Residual Proanthocyanidins in Silage.**

340 Relatively little is known about the reactions of proanthocyanidins with other plant

341 constituents in processed plant samples. The method of Terrill *et al.*[8] distinguishes

342 between extractable and protein-bound proanthocyanidins using solvents designed

343 to dissociate hydrogen bonds and hydrophobic interactions. However, Hagerman[26]

344 reported that covalent bonds can also be formed between proanthocyanidins and

345 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-

371 carbohydrate complexes might also be disrupted. Interestingly, Grabber *et al.*[37]

372 successfully incorporated epicatechin (**4**) into lignin with peroxidase and ester-linked

373 components were subsequently analyzed after hydrolysis with NaOH.

374 In conclusion, this is the first report of an analytical method capable of characterizing

375 proanthocyanidin contents and composition in an ensiled animal feed. A 15 min pre-

376 treatment at 40 °C with 1 M NaOH under argon was required to release bound

377 proanthocyanidins and enabled their subsequent analysis by thiolytic degradation.

378 The composition of assayable proanthocyanidins resembled that of the original

379 proanthocyanidins in the fresh plant, but 29% of the original proanthocyanidins could

380 not be detected by this NaOH-thiolysis treatment and 17% by the acetone-butanol-

381 HCl reagent. This suggests that fermentation had caused considerable

382 proanthocyanidin losses or structural changes. Ensiling also had a major effect on

383 the extractability of proanthocyanidins and most remained in the silage residue after

384 the acetone/water extraction, *i.e.* 86%. It would be interesting to explore whether the

385 presence of such unextractable proanthocyanidins may be responsible for the

386 enhanced anthelmintic (deworming) activities, which have been observed when

387 feeding ensiled proanthocyanidin-containing samples.[38,39] We venture to hypothesize

388 that these bound proanthocyanidins may act post-ruminally in the form of 'slow-

389 release' compounds against parasitic nematodes in the small intestine, although this

390 will require further research.

391

395

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 $^{\circ}$C, 15 min) Pretreatment (SD in parenthesis, n = 3).

	PA	mDP	PC	PD	cis	trans
	(g/100 g DW)		%	%	%	%
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)	(0.1)	(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
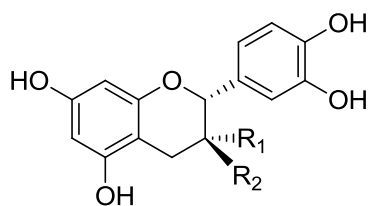
epicatechin subunits); PD: prodelphinidins (gallocatechin plus epigallocatechin subunits); SD: standard deviation; *trans*: catechin plus gallocatechin subunits.
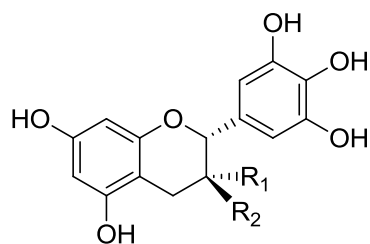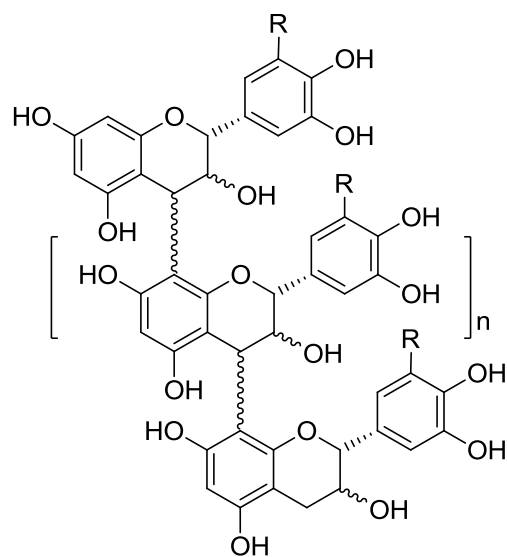
1, R$_1$ = H, R$_2$ = OH, Gallocatechin (a *trans*-flavan-3-ol)
2, R$_1$ = OH, R$_2$ = H, Epigallocatechin (a *cis*-flavan-3-ol)

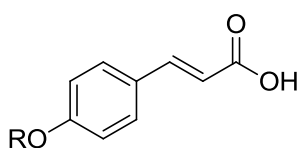**3**, R$_1$ = H, R$_2$ = OH, Catechin (a *trans*-flavan-3-ol)
4, R$_1$ = OH, R$_2$ = H, Epicatechin (a *cis*-flavan-3-ol)
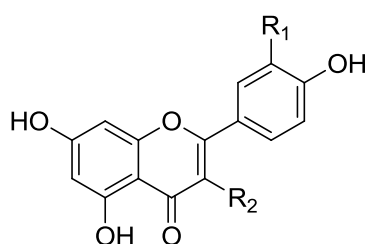
Example of proanthocyanidins:
R = H, procyanidins
R = OH, prodelphinidins

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

9', R = H, Formononetin
10', R = OCH$_3$, Afromosin

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
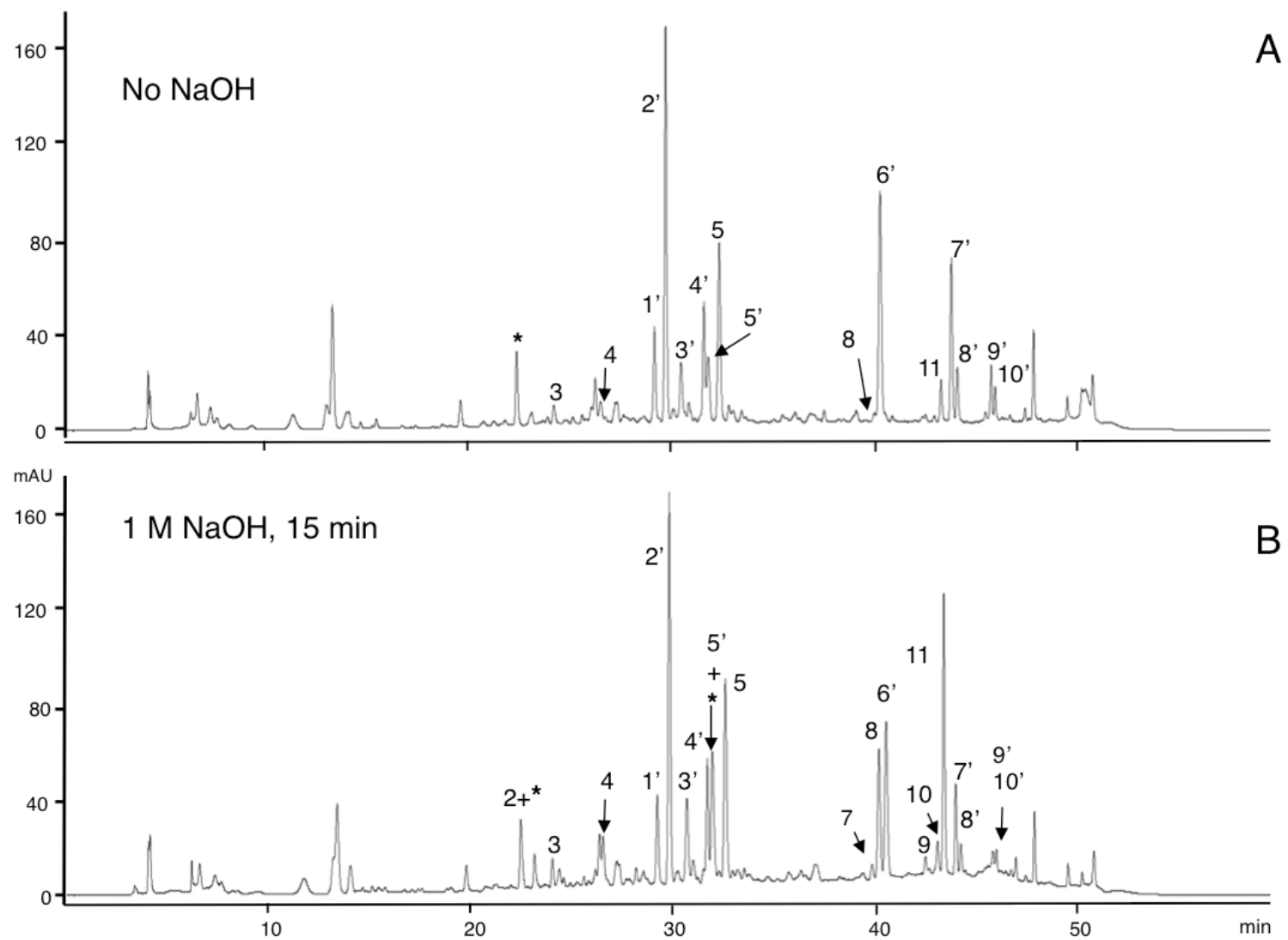**8'**, R$_1$ = OCH$_3$, R$_2$ = OH, Isorhamnetin
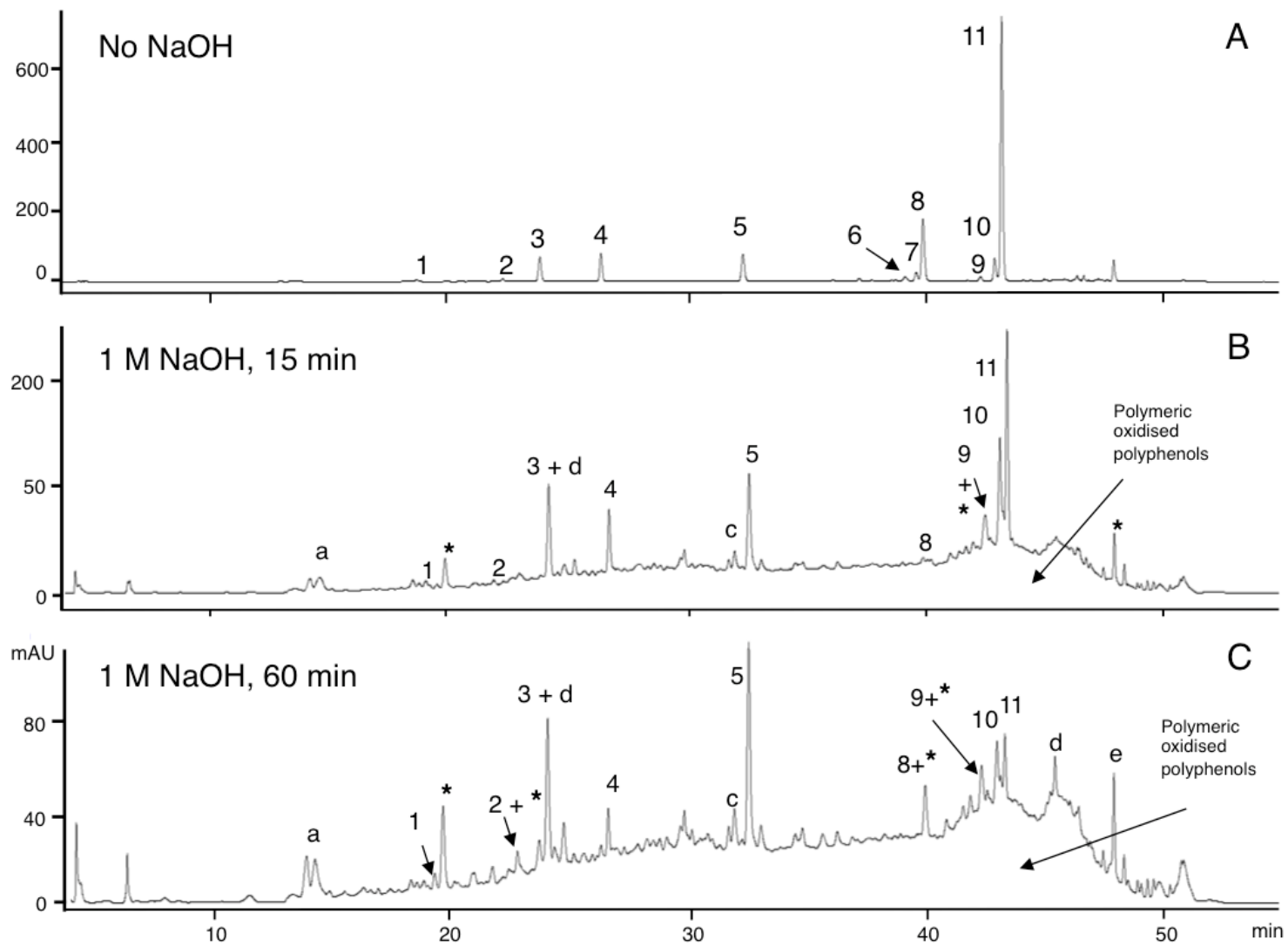
Figure 1

Figure 2

Figure 3

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