University of Reading

Large variability of proanthocyanidin content and composition in sainfoin (onobrychis viciifolia)

Article

Accepted Version

It is advisable to refer to the publisher's version if you intend to cite from the work.

To link to this article DOI: http://dx.doi.org/10.1021/acs.jafc.5b04946

Publisher: American Chemical Society

Article

Large variability of proanthocyanidin content and composition in sainfoin (Onobrychis viciifolia)

Carsten Stefan Malisch, Andreas Lüscher, Nicolas Baert, Marica Therese Engström, Bruno Studer, Christos Fryganas, Daniel Suter, Irene Mueller-Harvey, and Juha-Pekka Salminen

Just Accepted

Large variability of proanthocyanidin content and composition in sainfoin (*Onobrychis viciifolia*)

Carsten S. Malisch[†,#], Andreas Lüscher[*,†], Nicolas Baert[§], Marica T. Engström[§], Bruno Studer[#], Christos Fryganas[‡], Daniel Suter[†], Irene Mueller-Harvey[‡], Juha-Pekka Salminen[§]

† Forage Production and Grassland Systems, Institute for Sustainability Sciences ISS, Agroscope, Reckenholzstrasse 191, CH-8046 Zurich

Forage Crop Genetics, Institute of Agricultural Sciences, ETH Zurich, CH-8092 Zurich

§ Laboratory of Organic Chemistry and Chemical Biology, Department of Chemistry, University of Turku, FI-20500 Turku

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

1

Abstract

2   Proanthocyanidins (PAs) in sainfoin (*Onobrychis viciifolia* Scop.) are of interest to ameliorate

3   the sustainability of livestock production. However, sainfoin forage yield and PA

4   concentrations, as well as their composition, require optimization. Individual plants of 27

5   sainfoin accessions from four continents were analyzed with LC-ESI-QqQ-MS/MS for PA

6   concentrations and simple phenolic compounds. Large variability existed in PA concentrations

7   (23.0-47.5 mg g^{-1} leaf dry matter (DM)), share of prodelphinidins (79-96%), and mean degree

8   of polymerization (11-14) among, but also within, accessions. PAs were mainly located in

9   leaves (26.8 mg g^{-1} DM), while stems had less PAs (7.8 mg g^{-1} DM). Overall, high yielding

10   plants had lower PA leaf concentrations ($R^2 = 0.16$, $P <0.001$) and fewer leaves ($R^2 = 0.66$,

11   $P <0.001$). However, the results show that these two trade-offs between yield and bioactive PAs

12   can be overcome.

Keywords

14   Condensed tannins, LC-ESI-QqQ-MS/MS, Yield, Breeding, Polyphenols

Introduction

16   Sainfoin (*Onobrychis viciifolia* Scop.) is a traditional forage legume that was an important

17   forage source as early as the mid-16th century.[1] While it was a very important forage crop for a

18   long time, it lost importance in the second half of the 20th century, due to other forages

19   exhibiting higher yields and better tolerance towards frequent cutting.[2] Nowadays, as livestock

20   farming has attracted public debate, due to, among other reasons: (i) contributions to climate

21   change, both directly (greenhouse gas emissions) and indirectly (deforestation), and (ii) sources

22   of nutrient pollution for water bodies, resulting both from feed and livestock production,

23   sainfoin has benefited from a renewed interest (www.legumeplus.eu).[3] Because of various

24   beneficial properties, mostly linked to the presence of proanthocyanidins (PAs) (syn. condensed

25   tannins) and the ability to fix nitrogen directly from the air (reviewed by Lüscher *et al*, 2014

26   and Wang *et al*, 2015),[4,5] sainfoin is potentially considered a partial solution. The beneficial

27   properties include an ability to grow on marginal soils without mineral nitrogen fertilizer, thus

28   reducing environmental pollution and competition with food production; lower nitrous oxide

29   emissions due to a shift of the nitrogen excretion pathway from urine to a more stable form in

30   feces; and there are also some indications that PAs may have the potential to reduce methane

31   emissions. Additional benefits include lower burdens of parasitic gastrointestinal nematodes

32   and bloat prevention, thereby increasing animal health and welfare.[6,7] Furthermore, sainfoin has

33   a good forage quality with higher crude protein and total sugar contents than birdsfoot trefoil

34   (*Lotus corniculatus*) and chicory (*Cichorium intybus*).[8]

35   Individual PA compounds in a plant can be constructed of either procyanidin (PC) or

36   prodelphinidin (PD) sub-units, but it is quite common to find oligomeric (2-10 sub-units) and

37   polymeric PAs (>10 sub-units) that contain both PC and PD units. In addition, individual plants

38   synthesize a mixture of tens to hundreds of different oligomers and polymers. Among these

39   types of PC/PD mixtures of oligo- and polymers, PD-rich PAs have generally shown higher

40 anti-parasitic activity than PC-rich PAs,[9,10] and PAs rich in polymers have been shown to

41 improve anti-parasitic effects and the potential for reducing methane emission.[11] This may be

42 related to the fact that PAs with higher molecular weights (i.e. polymer size) are better able to

43 interact with macromolecules,[12,13] although this may not be the sole factor. However, higher

44 PC share on the other hand, seems to enhance protein-protection, which is important for

45 ruminant nutrition and also for sustainable livestock production. The exact reasons for this

46 remain unclear, but PDs are expected to have a higher protein binding affinity than PCs based

47 on their ability to form more hydrogen bonds. This would make tannin-protein complexes

48 consisting of PD-rich tannins more difficult to dissociate in the digestive tract and thus lead to

49 higher fecal nitrogen losses in ruminants.[7] Therefore, farmers will need sainfoin varieties with

50 both a PA composition that is optimized for animal health and for nutrition, and with sufficient

51 concentrations of these optimized PAs.[9,10] Interestingly, anthelmintic properties may also be

52 enhanced by monomeric flavonoids,[14,15] which are very common in many plants. As recent

53 findings have determined that the average composition of PAs is, at least in part, heritable,[16,17]

54 the major remaining obstacle is the variability in PA concentration, which is required for

55 optimization. However, until recently, screening of large numbers of individual plants

56 simultaneously for PA concentrations and their composition was not feasible, which prohibited

57 detailed characterization of this variability. Hence, with the exception of one accession, the

58 differences in PA concentrations among individual plants have never been established.[18] The

59 previous limitations have since been overcome by a new method for UPLC-MS/MS analysis of

60 extractable PAs, which allows high-throughput rates for the measurement of PA composition,

61 such as the mean degree of polymerization (mDP) and PC/PD ratios, allowing for large

62 screenings of individual plants for their PA concentration and composition.[19]

63 As PA-based bioactivity of sainfoin is of interest for increasing the sustainability of livestock

64 production systems, we evaluated the existing variability of PA properties (PA concentration

4

65   and composition) and of agronomic properties (yield and leaf share) in sainfoin. Special

66   emphasis was placed (i) on variability in PA properties at different levels, such as among

67   accessions, among individual plants within accessions and among plant organs within

68   individual plants and (ii) on the correlation between the PA properties of a plant and its yield.

69   Our findings will be of substantial value for the optimization of sainfoin as a bioactive forage,

70   by establishing that the required variability in PA concentration and composition is available

71   and that the concomitant improvement of yields and PA properties in sainfoin is possible.

Materials and Methods

Chemicals and Reagents

74   Technical grade acetone for extraction was purchased from VWR (Haasrode, Belgium). Formic

75   acid (HCOOH) and LC-MS Chromasolv acetonitrile for the UHPLC-ESI-QqQ-MS were

76   obtained from Sigma-Aldrich (Seelze, Germany) and catechin for the catechin stock solution

77   was acquired from Sigma-Aldrich (St. Louis, MO, USA). Kaempferol-3-*O*-rutinoside,

78   myricitrin, caffeoylquinic acid, arbutin, quercetin-*O*-rutinoside for the calibration curves, as

79   well as kaempferol-7-*O*-glucoside, kaempferol-7-*O*-neohesperoside, kaempferol-3-*O*-

80   glucoside, hyperoside and quercetin glucopyranoside for the stock solutions were obtained from

81   Extrasynthese (Genay, France). Digalloylglucose (98% purity, as determined by UPLC-DAD)

82   for the calibration of galloylated compounds was purified by J.-P. Salminen (University of

83   Turku, Turku, Finland) from a *Betula pubescens* leaf extract by a combination of Sephadex LH-

84   20 gel chromatography and semipreparative HPLC. Water was purified with a Millipore

85   Synergy water purification system from Merck KGaA (Darmstadt, Germany). Sephadex LH-

86   20 was obtained from GE Healthcare (GE Healthcare, Uppsala, Sweden).

87 **Site description**

88 Plant material was harvested from a field experiment in Rümlang (47°44'N 8°53'E,

89 482 m a.s.l), near Zurich, Switzerland. The soil is a calcic cambisol of at least 0.75 m depth and

90 ranges from loam to clay loam. The volume of the soil skeleton was measured at 5-10% and

91 the pH was 7.1. Except where otherwise noted, all data were generated under the following

92 environmental conditions: cumulative precipitation from the sowing of the experiment (June,

93 1st) to the harvest of the samples (September, 24th) was 559 mm (annual cumulative

94 precipitation: 1165 mm) at the Agroscope research station, which is 1 km from the field site

95 (shortest, direct route). The average temperature over the same time frame was 17.7 °C (average

96 annual temperature: 9.8 °C).

97 **Experimental design**

98 In a common garden experiment, seeds of 27 sainfoin accessions (Table S1) were sown in 8

99 replicates in a randomized complete block design. We aimed to cover a large range of

100 variability, and thus the accessions were selected according to their status of cultivation,

101 covering a range from wild accessions to fully registered cultivars, as well as according to their

102 geographic origin, covering 12 different countries from four different continents. Each

103 accession was sown in a row with 0.5 m distance between accessions, and out of germinated

104 seedlings, healthy individuals that were 0.25 m apart from each other (within rows) were

105 selected for further examination, and all others were removed. Each row consisted of 13 plants,

106 from which 9 were experimental plants: the two plants on each end were eliminated to decrease

107 margin effects. The design, thus, added up to a total of 1944 experimental plants (27 accessions

108 x 8 replicates (i.e. blocks) x 9 individuals), with a subset of the 9 individuals per block and

109 accession being used for the various analyses described below.

6

110   **Sampling**

111   For the chemical analysis, from each of the 27 accessions, at least 12 plants were selected

112   according to size (large, medium and small size, from a subset of 4 blocks), to determine the

113   possible trade-off between plant size and PA concentration, and to cover the whole range of

114   variability within and among the accessions. Altogether, polyphenol extracts from a subset of

115   364 individual plants from all accessions was analyzed by UPLC-ESI-QqQ-MS/MS. Per plant,

116   five whole leaves (leaflets including petiole and rachis) of a comparative developmental stage

117   (intermediate age) were taken from each plant. Additionally, young (freshly unfolded) and old

118   leaves (Figure S1), as well as stems, were harvested simultaneously from a subset of seven

119   accessions to study the variability of PA among plant organs. Sampling was conducted in the

120   morning of a cool, cloudy autumn day, to prevent evapotranspiration and biological

121   degradation. Immediately after sampling, samples were cooled on dry ice and within an hour

122   stored in a -70 °C freezer. Prior to grinding and extraction, samples were taken out of the

123   freezer, immediately dipped in liquid nitrogen and loaded into a precooled Freeze Drying Plant

124   Sublimator 3x4x5 (ZIRBUS technology GmbH, Bad Grund, Germany) for lyophilization.

125   For agronomical measurements, all 1944 experimental plants, including the subset used for

126   chemical analysis, were oven dried at 40 °C, with high air throughput rates, to constant weight.

127   In addition to dry matter, the leaf ratio (leaf dry mass to plant total dry mass) was determined

128   for 545 plants, which included 102 large and medium plants used for chemical analysis.

129   **Extraction and sample preparation**

130   After freeze drying, chemical samples were ground using a MM 400 ball mill (Retsch

131   Technology GmbH, Haan, Germany) in 25 mL tungsten carbide containers with four tungsten

132   carbide balls (7 mm diameter). Plant material (20 mg) was weighed into 2 mL Eppendorf tubes

133   and stored at -20 °C. For extraction, 1.4 mL of acetone/ H_2O (80:20, v/v) was added into the

7

134   Eppendorf tubes, which were then shaken for 15 minutes, and the plant/solvent mixture was

135   allowed to macerate in a fridge overnight to enhance the extraction efficiency of especially

136   large PAs. [20, 21] The tubes were then shaken in a planar shaker for 3 hours before centrifuging

137   at 12,000 g and decanting the solvent. The solvent samples were concentrated for

138   approximately 2 hours in an Eppendorf concentrator plus (Eppendorf AG, Hamburg, Germany)

139   to remove the acetone, while the plant residues were extracted with a new 1.4 mL of acetone/

140   H_2O (80:20, v/v) for an additional 3 hours. The two extracts were then combined and

141   concentrated into the water phase. Subsequently, the extracts were frozen and freeze dried with

142   a Christ Alpha 2-4 (B. Braun Biotech International, Melsungen, Germany) overnight and stored

143   at -20 °C. Prior to injection in the UPLC-MS/MS, samples were dissolved in 1 mL of ultrapure

144   water, shaken for 10 minutes, filtered with 0.2 μm PTFE syringe filters (VWR International,

145   Radnor, PA, USA) and diluted 4-fold with ultrapure water.

146   **Treatment of extracts with Sephadex LH-20**

147   For identification of the main monomeric phenolic compounds, equal plant subsamples of 200

148   mg were taken from 50 randomly selected samples, to obtain a 10 g pooled sample. The pooled

149   sample was then extracted according to the protocol above, but solvent quantities were adjusted

150   according to the increased plant biomass. Of the freeze-dried crude extract, 2 g were dissolved

151   in a small quantity of ultrapure water and loaded onto a Sephadex LH-20 column, which had

152   been equilibrated with 100% water. Solvents were pumped with a flow rate of 5 mL/min with

153   the following gradient: water fraction (100% H_2O, 1000 mL), MeOH:H_2O (50:50, v/v;

154   500 mL), acetone:H_2O (20:80, v/v; 500 mL), acetone:H_2O (40:60, v/v; 500 mL), acetone:H_2O

155   (60:40, v/v; 500 mL) and acetone:H_2O (80:20, v/v; 500 mL). The fractions were collected

156   separately, organic solvents removed with a rotary evaporator, and the remaining aqueous

157   extracts frozen and lyophilized.

8

158   **UPLC-MS/MS analysis**

159   The UPLC-MS/MS analysis was conducted according to Engström *et al.*[19] on the Acquity

160   UPLC system (Waters Corp., Milford, MA, USA), interfaced to a Xevo TQ triple-quadrupole

161   mass spectrometer with electrospray ionization (ESI) (Waters Corp., Milford, MA, USA). In

162   brief, the UPLC system was equipped with an autosampler, a binary solvent manager, a 100

163   mm × 2.1 mm i.d., 1.7 μm, an Acquity UPLC BEH Phenyl column (Waters Corp., Wexford,

164   Ireland), and a diode array detector. The flow rate was set to 0.5 mL/min, and the mobile phase

165   consisted of two solvents: acetonitrile (A) and 0.1% aqueous formic acid (B) with the following

166   gradient profile: 0−0.5 min, 0.1% A in B (isocratic); 0.5−5.0 min, 0.1−30% A in B (linear

167   gradient); 5.0−6.0 min, 30−35% A in B (linear gradient); 6.0−9.5 min, column wash and

168   stabilization. Data collection of both UV and MS occurred continuously from 0 to 6 min.

169   Negative ESI mode was used, with ESI conditions as follows: capillary voltage, 2.4 kV;

170   desolvation temperature, 650 °C; source temperature, 150 °C; desolvation and cone gas (N_2),

171   1000 and 100 L/h, respectively; and argon as collision gas.

172   **Standards and method performance**

173   Before each run, a flavonoid mix stock solution containing 4 μg mL^{-1} each of kaempferol-7-*O*-

174   glucoside,   kaempferol-7-*O*-neohesperoside,   kaempferol-3-*O*-glucoside,   quercetin-3-*O*-

175   galactoside and quercetin-3-*O*-glucoside, in a mixture of acetonitrile / 0.1% aqueous formic

176   acid (1:4 v/v) was injected twice to assess the performance of the system (stability of the UPLC

177   retention times, and *m/z* values of the MS detector). Furthermore, a catechin stock solution,

178   containing 1 μg mL^{-1} of catechin in a mixture of acetonitrile / 0.1% aqueous formic acid (1:4

179   v/v) was injected five times every ten samples, to account for possible changes in the

180   quantitative performance of the MS/MS system for polyphenols throughout the 110 minutes

181   that was required for each analysis set of 10 samples. Quantitative results were corrected for

9

182   possible fluctuations in the system's quantitative performance within each analysis set, as well

183   as among different sets. Replicate analyses (the same sample injected 10 times) were tested for

184   quantitative results, and the relative standard deviation (RSD) was $\pm$ 2.8% (range $\pm$ 0.8 - 6.5%,

185   Table 1) for all compounds that were analyzed quantitatively.

Calibration curves

187   In addition to the quantitative measurements of PA concentration and the determination of its

188   composition (PC/PD ratio, mDP, and largest mean degree of polymerization maxmDP), we

189   performed a qualitative analysis of 24 individual phenolic compounds, six of which were also

190   quantified and thus required calibration curves for quantification (Table 1). Identification of

191   compounds is described in the supporting information. Dilution series from stock solutions of

192   40 μg mL^{-1} were prepared for calibration of kaempferol-3-*O*-rutinoside, myricitrin, chlorogenic

193   acid, and rutin and were diluted with H_2O. The dilution range was from 40 μg mL^{-1} to 0.3125

194   μg mL^{-1}. The arbutin dilution series from a stock solution of 200 μg mL^{-1} diluted in H_2O ranged

195   from 200 μg mL^{-1} to 20 μg mL^{-1}, and the dilution series of digalloylglucose was prepared from

196   a stock solution of 10 μg mL^{-1} and ranged from 10 μg mL^{-1} to 0.375 μg mL^{-1} and diluted with

197   H_2O. Calibration curves for PC and PD concentrations were produced as described in

198   Engstroem *et al.* (2014) from purified PA stock solutions (1.0 μg mL^{-1}) of a PC-rich sample

199   (*Salix caprea* leaves: 95% pure, as determined by thiolysis) and a PD-rich sample (*Trifolium*

200   *repens* flowers: 98% pure, as determined by thiolysis),[19] respectively, by diluting with

201   acetonitrile/H_2O (20:80, v/v). The dilution range was from 1.0 μg mL^{-1} to 0.01 μg mL^{-1}.

202   Calibration curves were used to determine the linear range for quantification. The mDP was

203   determined according to Engström *et al.* (2014)[19] by calculating the ratio of terminal and

204   extension units for both PCs and PDs (eqn S1). As larger PAs tend to elute later, the maxmDP

205   was calculated by utilizing the same method as for mDP, but only integrated the terminal units

206   and extension units from a retention time window from 3.70 to 5.50 minutes, which enabled a

10

207   strong enough signal for the terminal and extension units of the larger PAs. Thus, the maxmDP

208   is not the largest polymer size found in the analyzed sample per se, but it shows reliably the

209   mean degree of polymerization for the largest PA polymers that elute in that given retention

210   time window (Figure S2). The maxmDP could also have been calculated from a later retention

211   time window, but this approach would generate less reliable data from samples with low

212   amounts of such PAs.

Statistical Analysis

214   The primary response variables analyzed were plant weight, leaf ratio, PA concentration, mDP,

215   and the share of prodelphinidins. Because accessions were selected for high variability both in

216   their geographic origin and their cultivation status (i.e. cultivars, wild accessions), both criteria

217   were initially tested for their impact on the response variables. It turned out that the geographic

218   origin of an accession was of minor importance in explaining variation in the response

219   variables. Therefore, regarding the following analyses, accessions were grouped only for their

220   cultivation status without consideration of the origin. The 27 accessions were assigned to one

221   of three groups of cultivation status. Cultivar/cultivated (hereafter referred to as "cultivars")

222   were accessions that have been cultured substantially and were, in most cases, even registered

223   as cultivars. Ecotype/landrace (referred to as "landraces") were "adapted to a specific region or

224   location, such as a farm", i.e. a very small scale, with landrace adaptation being driven by

225   human intervention and ecotype adaptation driven by natural selection pressures.[22]

226   Wild/unknown (referred to as "wild") were either wild accessions or accessions where the

227   cultivation status was not well established, which hints at a very low level of cultivation.

228   The effects of the cultivation status and the accession on the response variables were analyzed

229   with linear mixed regression.[23] With y being one of the response variables (plant weight, etc.,

230   see above), the model was:

231 $y_{ijkm} = \alpha*status_j + b*accession_k + g*block_m + e_i$ eqn 1

232 with y_{ijkm} being the response of the ith plant of cultivation status j and accession k in block m.

233 The fixed parameter α estimates the mean response of the cultivation status j. To consider the

234 variation of accessions within their status, b was modeled as a random parameter with $b \sim N(0,$

235 $\sigma_b^2)$. Likewise, block was modeled as a random parameter with $g \sim N(0, \sigma_g^2)$. The error e_i was

236 assumed to be normally distributed with zero mean and variance σ^2. The model contrasts were

237 used to infer differences among groups of the cultivation status.

238 Variability within accessions was evaluated by analysis of the population standard deviations

239 of the 27 accessions. To this aim, a modified version of eqn 1 was used:

240 $y_{ik} = \beta*accession_k + g*block_m + e_{ki}$ eqn 2

241 Here, the fixed parameter β estimates the mean response of accession k, with the random

242 variable block, as defined above. The variance parameter is $Var(e_{ki}) = \sigma^2\delta_k^2$, with δ being a ratio

243 that represents k variances, one for each accession. Inference of the average variances of the

244 three groups j of the cultivation status were derived from a model similar to eqn 2, but using

245 only three variance estimates, i.e. with $Var(e_{ji}) = \sigma^2\delta_j^2$. To achieve normality and

246 homoscedasticity of the error variance, the plant weight was log transformed in eqs 1 & 2; and

247 the PD share was logit transformed, because its values were restricted between zero and one.[23]

248 P-values of all correlations were calculated utilizing the Pearson's product moment correlation

249 coefficient, while checking the data for outliers. All analyses were performed using the

250 statistical software R,[24] with Figure 2 being generated using the R-package 'multcompView'.[25]

Results and Discussion

Variability among accessions

Over all 27 accessions, the concentration of PAs in leaves varied by a factor of 2, with the accession average ranging from 23.0 mg g^{-1} DM to 47.5 mg g^{-1} DM (Figure 1). The other PA properties varied less: the accession means of PD share in leaves ranged from 79% to 96% of the PAs and mDP ranged from 11 to 14. The maxmDP ranged from 23 to 32 flavan-3-ol units (results not shown, as maxmDP was strongly related to mDP (R^2: 0.75, $P <0.001$)). The largest variability occurred in the forage yield, where the smallest accession had, on average, 0.2 g DM plant^{-1}, whereas the largest accession yielded an average 20.3 g DM plant^{-1} (Figure 1). The accessions had a highly significant impact on all four parameters described above ($P <0.001$).

The cultivation status of the accessions contributed significantly to the overall variability among accessions described above. With their group mean of 32.7 mg g^{-1} DM, wild accessions had higher ($P <0.001$) leaf PA concentrations than cultivars (26.5 mg g^{-1} DM) and landraces (25.6 mg g^{-1} DM) (Figure 1). In contrast, group means for yield were clearly higher ($P <0.001$) for cultivars (9.7 g DM plant^{-1}) and landraces (8.9 g DM plant^{-1}) than for wild accessions (3.0 g DM plant^{-1}). These effects of cultivation status may be explained by the fact that until now, breeding efforts have been aimed at improving the agronomic performance of sainfoin, as is stated for the breeding of the cultivars Nova and Melrose, bred in Canada.[26] Improving yields is a major aim of breeding for all crops and has also been successful during recent decades for forage crops.[27] In addition, visual scoring of yield is fast and easy to conduct with a limited amount of labor. This explains why PAs were not a breeding target in the past. However, a recently developed, novel technique has made it possible now to scan large numbers of plants for PAs and thus to exploit the beneficial impacts PAs have on animal health and the environment.[19]

275   While low PA concentrations in cultivars may be insufficient for the bioactivity of sainfoin, we

276   also found that forage yield was of substantial importance for producing high amounts of PAs

277   per plant. This is because the difference between the forage yields in cultivars as compared to

278   those in wild accessions was, on average, 76%, whereas the respective difference in PA

279   concentration was, on average, only 20% (Figure 1). Consequently, when calculating the

280   amount of PAs per plant (eqn S2), as determined by the biomass and PA concentration exhibited

281   in Figure 1, cultivars achieved on average 332 mg plant^{-1} (median: 298 mg plant^{-1}), which was

282   higher compared to wild accessions (P <0.01) with 263 mg plant^{-1} (median: 177 mg plant^{-1}).

283   Landraces were not significantly different from either cultivars or wild accessions with 256 mg

284   plant^{-1} (median: 234 mg plant^{-1}).

285   **Variability within accessions**

286   The variability among individual plants within accessions was huge. For example, the standard

287   deviation of PA concentration in leaves of WKT10 was 10.6 mg g^{-1} DM (Figure 1), meaning

288   that approximately 1/3 of the plants had PA leaf concentrations of above 58.1 mg g^{-1} DM (mean

289   PA of WKT10 + 1 standard deviation) or below 36.9 mg g^{-1} DM (mean - 1 standard deviation).

290   This range in PA within accessions was comparable to the range among the accession means:

291   the greatest mean PA value of all 27 accessions was 47.5 mg PA g^{-1} DM in WKT10, while the

292   smallest PA value was 23.0 mg g^{-1} DM in Wiedlisbach. The largest variability within accession

293   was found with respect to plant yields. There, the standard deviation for CPI 63820 identified

294   one third of the plants to be either above 21.6 g DM plant^{-1} or below 0.6 g DM plant^{-1}, compared

295   to the range in accession means of 20.3 g DM plant^{-1} to 0.3 g DM plant^{-1}. Figure 1 further shows

296   that the variabilities within the accessions (standard deviation) for mDP and PD share were

297   slightly smaller than that of PA leaf concentration.

14

298   The cultivation status had a distinct effect on the variability exhibited within accessions: with

299   a group mean standard deviation of 7.5 mg g^{-1} DM for PA leaf concentration, wild accessions

300   had, on average, a greater (P <0.001) standard deviation than cultivars (5.9 mg g^{-1} DM) and

301   landraces (5.3 mg g^{-1} DM). For plant weight, the average standard deviation in wild accessions

302   (2.9 g DM plant^{-1}) was also larger (P <0.001) than in both cultivars (2.0 g DM plant^{-1}) and

303   landraces (1.9 g DM plant^{-1}). This may be explained, at least for registered cultivars, by the fact

304   that uniformity is one of the criteria that cultivars have to fulfill for registration, according to

305   paragraph 6 of Council Directive 2002/53/EC of 13 June 2002 from the European Union.[28]

306   Still, the observed variability is invaluable for the optimization of sainfoin.

307   **Variability within the plant**

308   The mean leaf concentration of PAs (averaged over all three age classes of leaves) was

309   26.8 mg g^{-1} DM, and, thus was almost 3.5 times higher (P <0.001) than the stem concentration

310   of PAs, which was only 7.8 mg g^{-1} DM (Figure 2). This is consistent with the literature, although

311   the extent of the difference was more pronounced in our findings.[29,30] The composition of the

312   PAs in sainfoin leaves might also be better suited for anthelmintic effects and methane

313   suppression: leaves had longer (P <0.001) polymers (mDP: 13, maxmDP: 28) than stems (mDP:

314   5, maxmDP: 10), and a higher (P <0.001) share of PDs (89%) than stems (60%). Furthermore,

315   quercetin-*O*-rutinoside (rutin) and kaempferol-*O*-rutinoside (nicotiflorin) were also

316   significantly higher (P <0.001) in leaves than in stems. Leaves had, on average, concentrations

317   of 5.9 mg g^{-1} DM rutin and 0.7 mg g^{-1} DM nicotiflorin, compared to stems with 1.4 mg g^{-1} DM

318   and 0.0 mg g^{-1} DM, respectively (Figure 2). Rutin has been shown to exhibit antioxidant and

319   anti-inflammatory properties.[31] Additionally, both rutin and nicotiflorin are also expected to

320   enhance the anthelmintic of PAs. A study by Barrau (2005)[14] found that in high concentrations,

321   rutin resulted in 25% and nicotiflorin in 30% reduction of the larval migration of *Haemonchus*

322   *contortus*, as compared to the negative control treatment. The study did not, however, quantify

323   whether this effect was additive with the effect of PAs, or whether an interaction between PAs

324   and the flavonoids occurred. The large differences in PAs found in stems and leaves

325   demonstrate that the leaf share of a plant may be as, or even more, important for PA

326   concentration and PA composition of the whole forage plant than the concentration and

327   composition of PA in the leaves alone.

328   The three leaf age classes differed significantly in their PA properties, although the differences

329   were clearly smaller than those observed between leaves and stems (Figure 2).

330   Proanthocyanidins changed from an average concentration of 38.8 mg g^{-1} DM in young leaves,

331   to 20.9 mg g^{-1} DM in old leaves ($P <0.001$) (Figure 2). The mDP increased ($P <0.001$) from 12

332   to 14, between young and old leaves, while the PD share changed ($P <0.05$) from young (90%)

333   to old (88%) leaves. Rutin decreased ($P <0.01$) from 6.9 mg g^{-1} DM to 5.3 mg g^{-1} DM, and

334   nicotiflorin increased ($P <0.001$) from 0.75 mg g^{-1} DM to 0.99 mg g^{-1} DM with aging of leaves.

335   It has to be considered, however, that such concentration changes do not imply anything about

336   the rates of PA synthesis. A graphical vector analysis (GVA), as proposed by Koricheva

337   (1999),[32] revealed that at first (young to intermediate aged leaves) the PAs showed

338   predominantly a dilution effect, which indicates that the leaf biomass growth rates were higher

339   than the PA synthesis rates. This reduced the concentration despite the fact that the amount of

340   PA produced per leaf continued to increase. From intermediate-aged to old leaves, there was a

341   shift towards a reduced synthesis rate of PAs (Figure 3), in which case even the PA amount

342   decreased between intermediate and old leaves, although biomass of the leaves increased in the

343   same time period. This indicates that either PAs were metabolized, or became insoluble and

344   were, thus, not measured by our method, which only detects soluble PAs. This could, for

345   example, happen when tannins are embedded into cell walls, as previously recorded for

346   ellagitannins (syn. hydrolysable tannins).[33] However, we did a follow up analysis on the

347   extraction residues of 30 leaf samples (young, intermediate, and old leaves of 10 plants), which

348　　were tested for insoluble PAs with the modified HCl-butanol analysis.[34] These analyses did not

349　　find an increase in insoluble PAs with the aging of leaves (results not shown). Nevertheless, the

350　　observed reduction in PA amounts agrees with another study by Lees,[35] in which PAs were

351　　located with light and electron microscopy in leaves of different developmental stages. These

352　　authors found that although PAs were very abundant in young leaves, they seemed to disappear

353　　with the aging of the leaf until the cells were almost entirely devoid of PAs.

354　　**Trade-offs: the growth rate hypothesis**

355　　To optimize sainfoin as a bioactive forage, it does not only need amelioration of PA properties

356　　(concentration and composition) but also an improved biomass yield. Yield increase is crucial

357　　in two respects: to produce higher amounts of PAs (at a given concentration of PA in that

358　　biomass) and to make sainfoin more competitive compared to high yielding non-PA forage

359　　species. However, there are potential trade-offs that may restrict the concomitant increase of

360　　sainfoin yield and PA properties. One potential trade-off between yield and PA properties is

361　　based on the growth rate hypothesis (GRH) (also known as: resource availability

362　　hypothesis).[36,37] The GRH is based on the fact that plants only have limited resources, and each

363　　resource can only be invested in either growth or defense mechanisms, such as plant secondary

364　　metabolites (e.g. PAs). Over all plants, such a trade-off was significant (Figure 4A, $R^2 = 0.16$,

365　　$P <0.001$). However, plant yield only explained 16% of the observed variability in PA leaf

366　　concentration and this effect was negligible above 5 g DM plant^{-1} ($R^2 = 0.01$, NS), as seen from

367　　the regression line. In addition, within each yield group (e.g. plants of 10-20 g DM), the range

368　　in PA concentrations was huge, and a threefold difference in PA concentrations is possible. In

369　　conclusion, in the yield range that is of interest for plant breeding (large plants), this trade-off

370　　is negligible and a large variability in PA leaf concentration is available that will allow

371　　improvement of PA leaf concentrations and yields.

372   Besides a concomitant increase in PA leaf concentration and yield as discussed above,

373   ameliorating PA composition (at a given concentration of PA) and yield in parallel is a second

374   strategy. The composition of PAs are considered to be at least as important as PA concentration

375   for the bioactivity of PA-containing feeds and their beneficial or antinutritional activity to

376   animal health.[7,11] Our results on PA composition demonstrate neither a negative relationship

377   (trade-off) between plant yield and mDP (R^2: 0.01, NS) nor PD share (R^2: 0.02, NS) (see

378   supporting information). Accordingly, enhancing the PAs by improving their composition

379   seems possible and should not be hampered by a parallel increase of the forage yield.

380   **Trade-offs: Reduced leaf share with increased plant size**

381   Another potential trade-off between yield and PA properties is based on the leaf share of the

382   plant. Given the on average 3.5 times higher PA concentration in leaves as compared to stems

383   (Figure 2) the leaf share of the plant becomes an important factor in determining PA

384   concentration of the entire forage[38,39] and studies generally found reduced leaf share in bigger

385   plants.[40] In fact, Figure 4B shows such a negative correlation (trade-off) between yield and leaf

386   share ($R^2 = 0.66$, $P < 0.001$). However, when observing only plants larger than 20 g DM, leaf

387   share appears quite stable at about 43%, as can be seen in the regression line (Figure 4B) and

388   no significant effect on leaf share occurred anymore with higher plant weight. In addition, the

389   variability in leaf share was quite large and exhibited a 2-fold variation for three out of four

390   plant size classes (vertical lines, Figure 4B) above 20 g DM. This indicates that yields could be

391   extensively increased without compromising the leaf share, and that there seems to be enough

392   variability to increase the leaf share at any given plant size. Finally, the possible increments in

393   yield without further reductions in leaf share for plant weights above 20 g DM are also of

394   importance, as Borreani *et al.* identified the leaves to be richer in crude protein and lower in

395   Neutral Detergent Fiber (NDF) than the stems (on average 227.7 and 83.3 g kg^{-1} DM crude

396   protein and 240.0 and 527.7 g kg^{-1} DM NDF for leaves and stems, respectively).[41] While

18

397　　Azuhnwi *et al.* in a comparison of 15 different sainfoin accessions found significant differences

398　　in both crude protein and NDF between these accessions, the range of 156-182 g kg^{-1} DM for

399　　crude protein and 378-417 g kg^{-1} DM for NDF, was much lower than the differences between

400　　leaves and stems.[42]

401　　In conclusion, we found that a large variability exists for breeding a sainfoin ideotype with high

402　　yields and large amounts of PAs with every possible combination of PD share and mDP, which

403　　will be identified to be ideal to obtain high bioactivity. This is of particular importance, as

404　　bioactivity is currently the main driver for the cultivation of sainfoin, yet cultivation becomes

405　　only attractive once the forage yield is competitive with other forage legumes. Our results

406　　suggest three independent strategies to increase bioactivity in the entire forage, which could

407　　have an additive effect, if applied together: (1) to ameliorate the composition of PAs, (2) to

408　　increase the overall PA concentration in sainfoin organs, and (3) to increase the share of leaves,

409　　which are the organs with the highest PA concentration. These findings further strengthen the

410　　opportunities offered by sainfoin to ameliorate the sustainability of livestock production.

Abbreviations used

PAs, Proanthocyanidins; DM, Dry matter; PD, Prodelphinidins; PC, Procyanidins; mDP, Mean

Degree of Polymerization; GRH, Growth rate hypothesis; ESI, Electrospray ionization; RSD,

Relative standard deviation; maxmDP, largest mDP; GVA, Graphical vector analysis; NDF,

Neutral Detergent Fiber

Author information

Corresponding Author

* (A.L.) Phone: +41 58 468 7273. E-mail: andreas.luescher@agroscope.admin.ch

Funding

Notes

The authors declare no competing financial interest.

Acknowledgements

20

Supporting information:

Supporting information contains: List of all examined accessions with their status of cultivation, origin country and ploidy, a visual comparison of different leaf ages, the calculation for the mean degree of Polymerization (mDP) and the largest mean degree of polymerization (maxmDP), the formula for the calculation for amount of proanthocyanidins, a description of the identification of phenolic compounds and the correlation between plant weight and the PD share and between plant weight and the maxmDP. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1)   Chorley, P., Early Evidence of Sainfoin Cultivation around Paris. *Agricultural History Review* **1981,** *29*, 118-124.

(2)   Boller, B.; Schubiger, F.X.; Koelliker, R., Red Clover; In *Fodder Crops and Amenity Grasses.* 1st Edition. Boller, B., Posselt, U. K., Veronesi, F., Eds.; Springer: New York City, New York, **2010**; Vol 5, 439-456.

(3)   Kingston-Smith, A.H.; Edwards, J.E.; Huws, S.A.; Kim, E.J.; Abberton, M., Plant-based strategies towards minimising 'livestock's long shadow'. *Proceedings of the Nutrition Society* **2010,** *69*, 613-620.

(4)   Lüscher, A.; Mueller-Harvey, I.; Soussana, J.F.; Rees, R.M.; Peyraud, J.L., Potential of legume-based grassland–livestock systems in Europe: a review. *Grass and Forage Science* **2014,** *69*, 206-228.

(5)   Wang, Y.; McAllister, T.A.; Acharya, S., Condensed Tannins in Sainfoin: Composition, Concentration, and Effects on Nutritive and Feeding Value of Sainfoin Forage. *Crop Science* **2015,** *55*, 13-22.

(6)   Aufrere, J.; Dudilieu, M.; Andueza, D.; Poncet, C.; Baumont, R., Mixing sainfoin and lucerne to improve the feed value of legumes fed to sheep by the effect of condensed tannins. *Animal* **2013,** *7*, 82-92.

(7)   Mueller-Harvey, I., Unravelling the conundrum of tannins in animal nutrition and health. *Journal of the Science of Food and Agriculture* **2006,** *86*, 2010-2037.

(8)   Scharenberg, A.; Arrigo, Y.; Gutzwiller, A.; Soliva, C.R.; Wyss, U.; Kreuzer, M.; Dohme, F., Palatability in sheep and in vitro nutritional value of dried and ensiled sainfoin (*Onobrychis viciifolia*) birdsfoot trefoil (*Lotus corniculatus*), and chicory (*Cichorium intybus*). *Archives of Animal Nutrition* **2007,** *61*, 481-496.

(9)   Kommuru, D.S.; Barker, T.; Desai, S.; Burke, J.M.; Ramsay, A.; Mueller-Harvey, I.; Miller, J.E.; Mosjidis, J.A.; Kamisetti, N.; Terrill, T.H., Use of pelleted sericea lespedeza (*Lespedeza cuneata*) for natural control of coccidia and gastrointestinal nematodes in weaned goats. *Veterinary Parasitology* **2014,** *204*, 191-198.

(10)   Mechineni, A.; Kommuru, D.S.; Gujja, S.; Mosjidis, J.A.; Miller, J.E.; Burke, J.M.; Ramsay, A.; Mueller-Harvey, I.; Kannan, G.; Lee, J.H., et al., Effect of fall-grazed sericea lespedeza (*Lespedeza cuneata*) on gastrointestinal nematode infections of growing goats. *Veterinary Parasitology* **2014,** *204*, 221-228.

(11)   Hatew, B.; Stringano, E.; Mueller-Harvey, I.; Hendriks, W.H.; Carbonero, C.H.; Smith, L.M.J.; Pellikaan, W.F., Impact of variation in structure of condensed tannins from sainfoin (*Onobrychis viciifolia*) on *in vitro* ruminal methane production and fermentation characteristics. *Journal of Animal*

21

474   *Physiology and Animal Nutrition*. [Online early access]. doi: 10.1111/jpn.12336. Published Online: May
475   8th, 2015. (accessed: September 30th, 2015).
476   (12)   Huang, X.D.; Liang, J.B.; Tan, H.Y.; Yahya, R.; Long, R.; Ho, Y.W., Protein-binding affinity
477   of *leucaena* condensed tannins of differing molecular weights. *Journal of Agricultural and Food*
478   *Chemistry* **2011,** *59*, 10677-10682.
479   (13)   Zeller, W.E.; Sullivan, M.L.; Mueller-Harvey, I.; Grabber, J.H.; Ramsay, A.; Drake, C.; Brown,
480   R.H., Protein Precipitation Behavior of Condensed Tannins from *Lotus pedunculatus* and *Trifolium*
481   *repens* with Different Mean Degrees of Polymerization. *Journal of Agricultural and Food Chemistry*
482   **2015,** *63*, 1160-1168.
483   (14)   Barrau, E.; Fabre, N.; Fouraste, I.; Hoste, H., Effect of bioactive compounds from sainfoin
484   (*Onobrychis viciifolia* Scop.) on the in vitro larval migration of *Haemonchus contortus*: role of tannins
485   and flavonol glycosides. *Parasitology* **2005,** *131*, 531-538.
486   (15)   Klongsiriwet, C.; Quijada, J.; Williams, A.R.; Mueller-Harvey, I.; Williamson, E.M.; Hoste, H.,
487   Synergistic inhibition of *Haemonchus contortus* exsheathment by flavonoid monomers and condensed
488   tannins. *International Journal for Parasitology: Drugs and Drug Resistance* **2015,** *5*, 127-134.
489   (16)   Lattanzio, V., Bioactive polyphenols: Their role in quality and storability of fruit and vegetables.
490   *Journal of Applied Botany* **2003,** *77*, 128-146.
491   (17)   Scioneaux, A.; Schmidt, M.; Moore, M.; Lindroth, R.; Wooley, S.; Hagerman, A., Qualitative
492   variation in proanthocyanidin composition of populus species and hybrids: genetics is the key. *Journal*
493   *of Chemical Ecology* **2011,** *37*, 57-70.
494   (18)   Regos, I.; Urbanella, A.; Treutter, D., Identification and quantification of phenolic compounds
495   from the forage legume sainfoin (*Onobrychis viciifolia*). *Journal of Agricultural and Food Chemistry*
496   **2009,** *57*, 5843-5852.
497   (19)   Engström, M.T.; Pälijärvi, M.; Fryganas, C.; Grabber, J.H.; Mueller-Harvey, I.; Salminen, J.-P.,
498   Rapid qualitative and quantitative analyses of proanthocyanidin oligomers and polymers by UPLC-
499   MS/MS. *Journal of Agricultural and Food Chemistry* **2014,** *62*, 3390-3399.
500   (20)   Salminen, J.-P., Effects of sample drying and storage, and choice of extraction solvent and
501   analysis method on the yield of birch leaf hydrolyzable tannins. *Journal of Chemical Ecology* **2003,** *29*,
502   1289-1305.
503   (21)   Baert, N.; Karonen, M.; Salminen, J.-P., Isolation, characterisation and quantification of the
504   main oligomeric macrocyclic ellagitannins in *Epilobium angustifolium* by ultra-high performance
505   chromatography with diode array detection and electrospray tandem mass spectrometry. *Journal of*
506   *Chromatography A.*, [Online early access]. doi:10.1016/j.chroma.2015.09.050. Published Online:
507   September 21st, 2015. http://www.sciencedirect.com/science/article/pii/S0021967315013564 (accessed
508   September 30th, 2015).
509   (22)   Boller, B.; Greene, S., Genetic Resources. In *Fodder Crops and Amenity Grasses.* 1st Edition.
510   Boller, B., Posselt, U. K., Veronesi, F., Eds.; Springer: New York City, New York, **2010**; Vol. 5, 13-38.
511   (23)   Pinheiro, J.; Bates, D.; DebRoy, S.; Sarkar, D.; R Core Team. *nlme: linear and nonlinear mixed*
512   *effects models*, R package version 3.1-117; **2014.**
513   (24)   R Core Team *R: A language and environment for statistical computing*, Vienna, Austria, **2014**.
514   (25)   Figueroa-Pérez, M.G.; Rocha-Guzmán, N.E.; Pérez-Ramírez, I.F.; Mercado-Silva, E.; Reynoso-
515   Camacho, R., Metabolite profile, antioxidant capacity, and inhibition of digestive enzymes in infusions
516   of peppermint (*Mentha piperita*) grown under drought stress. *Journal of Agricultural and Food*
517   *Chemistry* **2014,** *62*, 12027-12033.
518   (26)   Goplen, B.P.; Richards, K.W.; Moyer, J.R., Sainfoin for western Canada. 3rd Edition.
519   Agriculture Canada: Ottawa, Canada, **1991**; 1-22.
520   (27)   Walter, A.; Studer, B.; Kölliker, R., Advanced phenotyping offers opportunities for improved
521   breeding of forage and turf species. *Annals of Botany* **2012**, *110*, 1271-1279.
522   (28)   Council of the European Union, Council Directive 2002/53/EC of 13 June 2002 on the common
523   catalogue of varieties of agricultural plant species. In *Official Journal* **2002**; Vol. L *193*, 0001 - 0011.
524   (29)   Theodoridou, K.; Aufrere, J.; Andueza, D.; Le Morvan, A.; Picard, F.; Stringano, E.; Pourrat,
525   J.; Mueller-Harvey, I.; Baumont, R., Effect of plant development during first and second growth cycle
526   on chemical composition, condensed tannins and nutritive value of three sainfoin (*Onobrychis viciifolia*)
527   varieties and lucerne. *Grass and Forage Science* **2011,** *66*, 402-414.

22

528 (30)   Stringano, E.; Carbonero, C.H.; Smith, L.M.J.; Brown, R.H.; Mueller-Harvey, I.,
529 Proanthocyanidin diversity in the EU 'HealthyHay' sainfoin (Onobrychis viciifolia) germplasm
530 collection. *Phytochemistry* **2012,** *77*, 197-208.
531 (31)   Lee, C.-C.; Shen, S.-R.; Lai, Y.-J.; Wu, S.-C., Rutin and quercetin, bioactive compounds from
532 tartary buckwheat, prevent liver inflammatory injury. *Food & Function* **2013,** *4*, 794-802.
533 (32)   Koricheva, J., Interpreting phenotypic variation in plant allelochemistry: problems with the use
534 of concentrations. *Oecologia* **1999,** *119*, 467-473.
535 (33)   Salminen, J.-P.; Ossipov, V.; Pihlaja, K., Distribution of hydrolysable tannins in the foliage of
536 Finnish birch species. In *Zeitschrift für Naturforschung C*, **2002**; *57*, 248-256.
537 (34)   Grabber, J.H.; Zeller, W.E.; Mueller-Harvey, I., Acetone enhances the direct analysis of
538 procyanidin- and prodelphinidin-based condensed tannins in lotus species by the butanol-HCl-iron
539 assay. *Journal of Agricultural and Food Chemistry* **2013,** *61*, 2669-2678.
540 (35)   Lees, G.L.; Gruber, M.Y.; Suttill, N.H., Condensed tannins in sainfoin .2. occurrence and
541 changes during leaf development. *Canadian Journal of Botany-Revue Canadienne De Botanique* **1995,**
542 *73*, 1540-1547.
543 (36)   Coley, P.D.; Bryant, J.P.; Chapin, F.S., Resource availability and plant antiherbivore defense.
544 *Science* **1985,** *230*, 895-899.
545 (37)   Nancy Stamp, Out of the quagmire of plant defense hypotheses. *The Quarterly Review of*
546 *Biology* **2003,** *78*, 23-55.
547 (38)   Häring, D.A.; Scharenberg, A.; Heckendorn, F.; Dohme, F.; Luscher, A.; Maurer, V.; Suter, D.;
548 Hertzberg, H., Tanniferous forage plants: agronomic performance, palatability and efficacy against
549 parasitic nematodes in sheep. *Renewable Agriculture and Food Systems* **2008,** *23*, 19-29.
550 (39)   Haring, D.A.; Suter, D.; Amrhein, N.; Lushcer, A., Biomass allocation is an important
551 determinant of the tannin concentration in growing plants. *Annals of Botany* **2007,** *99*, 111-120.
552 (40)   Lemaire, G.; Gastal, F., N uptake and distribution in plant canopies. In *Diagnosis of the Nitrogen*
553 *Status in Crops*; Lemaire, G., Ed. Springer Berlin Heidelberg: **1997**; 3-43.
554 (41)   Borreani, G.; Peiretti, P.G.; Tabacco, E., Evolution of yield and quality of sainfoin (*Onobrychis*
555 *viciifolia* Scop.) in the spring growth cycle. *Agronomie* **2003,** *23*, 193-201.
556 (42)   Azuhnwi, B.N.; Boller, B.; Martens, M.; Dohme-Meier, F.; Ampuero, S.; Gunter, S.; Kreuzer,
557 M.; Hess, H.D., Morphology, tannin concentration and forage value of 15 Swiss accessions of sainfoin
558 (*Onobrychis viciifolia* Scop.) as influenced by harvest time and cultivation site. *Grass and Forage*
559 *Science* **2011,** *66*, 474-487.
560 (43)   Veitch, N.C.; Regos, I.; Kite, G.C.; Treutter, D., Acylated flavonol glycosides from the forage
561 legume, *Onobrychis viciifolia* (sainfoin). *Phytochemistry* **2011,** *72*, 423-429.
562 (44)   Marais, J.P.J.; Mueller-Harvey, I.; Brandt, E.V.; Ferreira, D., Polyphenols, condensed tannins,
563 and other natural products in *Onobrychis viciifolia* (Sainfoin). *Journal of Agricultural and Food*
564 *Chemistry* **2000,** *48*, 3440-3447.
565 (45)   Lu, Y.R.; Sun, Y.; Foo, L.Y.; McNabb, W.C.; Molan, A.L., Phenolic glycosides of forage
566 legume *Onobrychis viciifolia*. *Phytochemistry* **2000,** *55*, 67-75.

567 **Figure captions**

568 **Figure 1: A) Proanthocyanidin concentration [PA] in leaves, B) mean degree of**

569 **polymerization (mDP), C) share of prodelphinidins (PD), and D) the plant weight of**

570 **sainfoin accessions, arranged in order of their status of cultivation and - within status -**

571 **increasing plant weight.**

572 Displayed are predicted means and population standard deviations of each accession based on

573 regression analysis (eqs 1 & 2). Different letters among group values indicate significant

574 differences at $P \leq 0.05$: upper case for group means (eqn 1), lower case for standard deviations

575 (eqn 2). Note the log-scale in panels C) and D). Accession NA/RCAT028437 is abbreviated as

576 RCAT and Cholderton-Hampshire Common as Hampshire.

577 **Figure 2: A) Proanthocyanidin concentration [PA], B) mean degree of polymerization**

578 **(mDP), C) maximum degree of polymerization (maxmDP), D) share of prodelphinidins**

579 **(PD), E) quercetin-*O*-rutinoside concentration, and F) nicotiflorin concentration in leaves**

580 **of different ages and stems.**

581 Boxes display medians (bold line), the first and third quartile (lower and upper line of box), and

582 whiskers extending to the most extreme data point, which is less than 1.5 times of the

583 interquartile range. Different letters among medians indicate significant differences at $P \leq 0.05$.

584 **Figure 3**: **Graphical vector analysis for leaves of sainfoin (*Onobrychis viciifolia*),**

585 **comparing the concentration and amount of proanthocyanidins (PA).**

586 Arrows follow aging of leaves from young to intermediate aged leaves, and from there to old

587 leaves. Grey dotted lines are isolines for leaf biomass; arrows crossing the lines indicate

588 changes in biomass. Synthesis rate changes are defined by angle relative to biomass isolines

589 and can be identified with the help of the black arrows on the top right. Error bars indicate

590 standard error.

24

591   **Figure 4: A) Proanthocyanidin concentration [PA] in leaves, and B) leaf share of**

592   **individual plants both compared to plant yield.**

593   Data are for the first harvests of the years 2013 and 2014. The equation for the exponential

594   trendline and its regression analysis are denoted at the bottom right.

595

Tables

Table 1: Chromatographic, UV, and mass spectral characteristics of individual phenolic compounds identified in the sainfoin extract [1].

Comp.	R_T	[M-H]⁻	MS²	Fragments of daughter ion	λ_{max}	Tentative ID	MRM	RSD
1	1.18	271	108		222, 282	Arbutin[a,b]		6.5
2	2.01	331	169		222, 272	1-O-Monogalloylglucose[a]	331>169	2.9
3	2.44	353	191, 179, 135	108	219, 323	Caffeoylquinic acid[b]	353>191	3.5
4	2.87	353	191	171, 155, 137, 115, 108	244, 324	Chlorogenic acid[a,b]	353>191	2.5
5	2.92	325	163, 119	119	ND	Coumaric acid glucoside[b,e]		
6	2.92	625	463, 301, 299	301	255, 352	Quercetindihexoside	625>300	
7	3.32	337	191, 173, 163, 119	173, 127, 111	230, 310	Coumaroylquinic acid[b,e]	337>191	
8	3.65	337	191, 173, 163, 127, 119	173, 137, 127, 111	234, 304	Coumaroylquinic acid[b,e]	337>191	
9	3.68	325	163, 119	117, 101	219, 279	Coumaric acid glucoside[b,e]		4.2
10	2.93	771	609, 462, 301	300, 272, 194	255, 352	Quercetin-3-O-rutinoside-7-O-β-D-glucoside[e]		3.9
11	3.56	755	301		255, 352	Quercetin-3-O-rhamnosylrutinoside[b,d,e]		1.4
12	3.79	739	285, 284		265, 347	Kaempferol-3-O-rhamnosylrutinoside[b,e]		1.4
13	3.89	609	301, 300, 271, 255		255, 352	Quercetin-3-O-rutinoside[a,b,e]	609>300	2.2
14	4.20	593	285		265, 346	Kaempferol-3-O-rutinoside[a,b,e]	593>285	0.8
15	4.24	505	301		254, 351	Quercetinacetylhexoside		
16	4.28	623	315, 300		254, 352	Isorhamnetin-3-O-rutinoside[b]		
17	4.62	489	285, 284, 255, 227		265, 335	Kaempferolacetylhexoside		
18	3.55	625	316, 271		258, 355	Myricetin-3-O-rutinoside[b,e]		2.0
19	4.41	947	623, 609, 301, 179	300	252, 334	Quercetinferuloyltriglycoside		

27

20	3.94	463	317, 316, 287, 271, 179, 151		228, 349	Myricetin-3-O-rhamnoside[a,b]	463>316
21	4.02	463	301, 300, 271, 255, 179, 151		255, 353	Quercetin-3-O-glucoside[a,b]	463>300
22	4.33	447	285, 255, 227		265, 348	Kaempferol-3-O-glucoside[a,b]	447>284
23	5.10	961	<u>755</u>, 301, 300, 179	300	243, 335	Quercetin 3-O-(4′′′-O-E-sinapoyl)-α-rhamnopyranosyl-(1′′′ → 2′′) [α-rhamnopyranosyl-(1′′ ′′ → 6′′)]-β-glucopyranoside[c]	
24	5.12	931	<u>755</u>, 301, 300	300	243, 334	Quercetin 3-O-(4′′′-O-E-feruloyl)-α-rhamnopyranosyl-(1′′′ → 2′′)[α-rhamnopyranosyl-(1′′ ′′ → 6′′)]-β-glucopyranoside[c]	

[1] RSD is the relative standard deviation of a replicate quantitative analysis of all the main compounds included in the quantifications. Oligomeric and polymeric proanthocyanidins are not shown in the table since they do not elute as sharp chromatographic peaks. They were identified and quantified by MS/MS according to Engström et al. (2014)

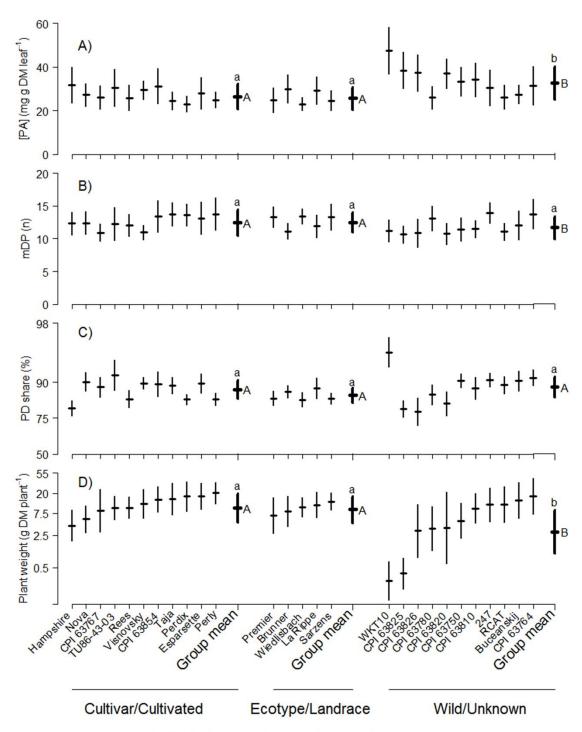[a] identification compared to a reference standard

[b] as previously identified by Regos et al. (2009) [18]

[c] as previously identified by Veitch et al. (2011) [43]

[d] as previously identified by Marais et al. (2000) [44]

[e] as previously identified by Lu et al. (2000) [45]

Figures

Figure 1

Figure 2

Figure 3

A)

B)

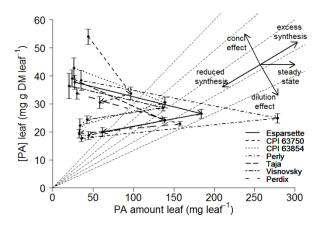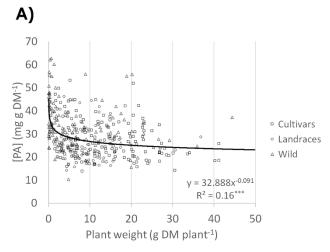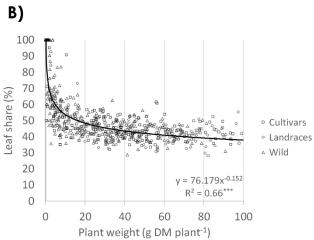**Figure 4**

Graphic for table of contents

Agronomic and chemical variability in sainfoin

Yield   Leafiness   UPLC-chromatogram