JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

pubs.acs.org/JAFC Article

Low Concentrations of Protein- and Fiber-Bound Proanthocyanidins in Sainfoin (*Onobrychis viciifolia*) Are Stable across Accessions, Growth Stages, and Drought Conditions

Carsten S. Malisch,* Lasse Lewandowski, Juha-Pekka Salminen, Friedhelm Taube, and Andreas Lüscher

Cite This: *J. Agric. Food Chem.* 2020, 68, 7369−7377 Read Online

ACCESS | Metrics & More | Article Recommendations

ABSTRACT: Proanthocyanidins (PAs) in forages may be present in either soluble (S-PA) or non-extractable (NE-PA) form. Despite potential benefits of the NE-PA in ruminant nutrition, few studies have analyzed NE-PA in forages. This study examined the impact of a range of conditions on S-PA as well as protein- and fiber-bound PA (the NE-PA fractions) in sainfoin (*Onobrychis viciifolia*). Thus, five sainfoin accessions in either generative or vegetative stage were subjected to drought for 18 weeks and sampled repeatedly for PA analysis. Drought-stressed plants increased S-PAs on average by 59% across all accessions yet only in the vegetative stage. In contrast, NE-PA concentrations were generally lower (on average 15% of the total PAs) and unaffected by drought. Thus, for sainfoin, the low and stable concentration of NE-PAs across accessions, growth stages, and drought conditions should have a low, predictable impact on the future sainfoin analyses and feeding studies.

KEYWORDS: *Onobrychis viciifolia, non-extractable proanthocyanidins (NE-PA), condensed tannins, water stress, HCl−butanol assay*

INTRODUCTION

Grasslands and particularly grasslands containing legumes, herbs, or both have been considered a promising solution to increase the sustainability of ruminant-based or mixed production systems by providing high biomass and energy yields as well as a range of other ecosystem services at moderate nutrient inputs.[1] Recently, it has been discovered that this potential is exacerbated by plant-specialized metabolites, which occur naturally in many dicotyledonous species.[1−3] Of these, proanthocyanidins (PAs, syn. condensed tannins) have been at the focus of research as a result of multiple positive effects on both animal health and environmental effects of ruminant husbandry.[3,4] For example, forages in the feed ration containing PAs have been shown to improve animal health by their anthelmintic effects[5−7] and the reduction of pasture bloat.[8,9] In terms of product quality, PAs have the potential to improve fatty acid composition by increasing ω-3 fatty acid content in milk and meat through inhibition of ruminal biohydrogenation.[10,11] In addition, PAs can contribute to improving the greenhouse gas balance of ruminant-based production systems by directly inhibiting methanogenic bacteria to reduce methane emissions[12,13] and shifting nitrogen excretions from urine to feces to reduce the ammonia and nitrous oxide emission potential of manure.[7,14]

PAs are oligomeric or polymeric plant-specialized metabolites synthesized from flavan-3-ol units via the flavonoid pathway. The chemical structures of the PAs are highly heterogeneous and have been shown to vary in their bioactivity.[13] They are grouped according to structural characteristics to ensure comparable properties. The main structural properties with reported relevance for the bioactive effect include the mean degree of polymerization (mDP) and the procyanidin (PC)/prodelphinidin (PD) ratio, both of which reportedly affect the protein precipitation capacity of the PAs, the functional property of tannins presumably responsible for many positive effects of PAs.[15,16]

In previous determinations of PA concentrations, the analysis was often limited mainly to the acetone/water-soluble PA fraction. In addition to the fraction that is soluble in organic solvents, there is also a non-extractable PA (NE-PA) fraction.[17] The NE-PA fraction consists of the protein-bound PAs (Pb-PAs) and the fiber-bound PAs (Fb-PAs). However, because most studies to date focused on the S-PA fraction, very little is known about NE-PA in general and specifically about Pb-PA and Fb-PA, despite their potential effects in ruminant nutrition.

While information is limited about the mode of action and the bioactive potential of Pb-PA and Fb-PA in ruminants, their effects are limited to the post-ruminal digestion. This is because most PA−protein complexes are assumed to be stable between pH 3.5 and 7.0, as it occurs in the rumen.[18] Between abomasum and ileum, however, the pH shifts to 2, resulting in a dissociation, at least in part, of the protein−PA complexes. Hence, the overall concentration of dissolved PA can increase in the abomasum by solubilizing previously bound NE-PA, in addition to the initial S-PA fraction of the forage.[19] The PAs in

Received: March 6, 2020
Revised: June 18, 2020
Accepted: June 23, 2020
Published: June 23, 2020

https://dx.doi.org/10.1021/acs.jafc.0c01540
J. Agric. Food Chem. 2020, 68, 7369−7377

the abomasum are, however, not the exact sum of S-PA and NE-PA as a result of (a) forages not being entirely digested, with an approximate digestibility of often around 60%, and (b) a partial biotransformation or depolymerization of PAs occurring in the digestive tract.[20] Nevertheless, a higher anthelmintic effect was detected in the abomasum compared to the rumen as a result of higher PA concentrations as a result of dissociated NE-PA complexes.[20,21] As a result of a higher digestibility of protein compared to fiber, a higher dissociation rate and, hence, PA concentration increments in the abomasum compared to the rumen can be expected from Pb-PAs compared to Fb-PAs. In addition, this would suggest a higher bioactivity of young and leafy plants compared to old plants with high stem shares, because the latter contains lower protein and higher fiber concentrations. However, estimations of the effectiveness of Pb-PA and Fb-PA fractions remain difficult. This is for the following reasons: (i) the above-mentioned effect of the digestibility of the plant species on the dissociation of additional NE-PA and the reduction in PAs as a result of their partial degradability, with the degradation rate depending upon the PA structure;[21,22] (ii) there may be a large variability in the NE-PA concentration both across and within plant species as well as across plant development and growth stage;[23] and (iii) the NE-PA share of the total PA in the forage can be increased by forage conservation, because it has been shown that previously soluble PAs bind to protein during ensiling in sainfoin and other legumes, thus significantly increasing Pb-PA in silage compared to fresh material.[23,24] Consequently, more analyses where not only S-PA but also Pb-PA and Fb-PA are determined are needed to unravel this complexity and understand how choice of forage and forage conservation affect their bioactive effects.

In addition to these technical factors that can affect the PA concentration and composition postharvest, three main sources of variability have been established that currently affect PA concentrations prior to harvest even within any species: the variability across genotypes (accessions and cultivars),[25,26] the variability as a result of the growth stage,[27−29] and the variability as a result of environmental conditions, particularly as a result of drought.[28,30] Drought stress has the additional advantage that, unlike herbivore stress (particularly from insects that feed on individual leaves), it triggers a systemic rather than local response in the plant, thus aiding the representativeness of the subsample.[31] This is because, if only single leaves are affected by herbivory, local responses can only increase the PA concentrations in the affected or adjacent leaves, whereas systemic responses increase the concentration more homogeneously across the entire plant. As a result, this study aimed to identify whether these three main sources of known variability of S-PAs in plants would also affect the concentration of Pb-PA and Fb-PA. To do this, sainfoin was used as a model plant as a result of its comparatively high PA concentrations and palatability.[6,32,33] Additionally, sainfoin has competitive yields[6,34] and has illustrated potential to increase live-weight gains in ruminants.[4] Accordingly, this study aims at answering the following research questions: (1) How large are the protein- and fiber-bound PA fractions in sainfoin compared to the soluble PAs across a range of accessions? (2) How do these fractions change in response to drought, and is this response consistent across the growth stage (vegetative or generative) of the plants? (3) Is the response to drought and growth stage consistent across the accessions?

MATERIALS AND METHODS

Experimental Setup. The experimental setup has already been described before.[25,28] Briefly, the experiment was established near Zürich [latitude 47° 44′ N, longitude 8° 53′ E, 482 m above sea level (asl)] on calcic cambisol soil. The soil had a pH of about 7.1 and a depth of at least 0.75 m. The experiment was performed with five different sainfoin accessions (first experimental factor "accession"). Four registered varieties ('Perly', 'Taja', 'Esparsette', and 'Visnovsky') as well as the Turkish accession 'CPI 63750'. The plants were sown in late May, with no treatments being applied in the establishment year. In June of the first experimental year (i.e., 13 months after sowing), half of the plants were subjected to drought stress for 17 weeks (from June 12 to October 17) using stationary rainout shelters, while the others grew under rainfed conditions (second experimental factor "drought"). The rainout shelters were 23 m long, 4 m wide, and 2.4 m high. The covers were selected to have a high light transmission of 90% (UV-B, 70%). The effect of the shelters on soil water potential was measured in half of the drought and control plots using MPS-2 sensors (METER Group, Pullman, WA, U.S.A.) at 20 cm soil depth. After rainout shelters were erected, initially no effect on the soil water potential (SWP) was detectable in the first 3 weeks at a depth of 20 cm. From week 14 onward, the drought treatments reached SWPs in excess of −1.5 MPa, which is commonly considered the permanent wilting point (Figure 1). In the rainfed control, we irrigated using simulated rainfall events with an equivalent of 20 mm, whenever the SWP dropped below −0.2 MPa.

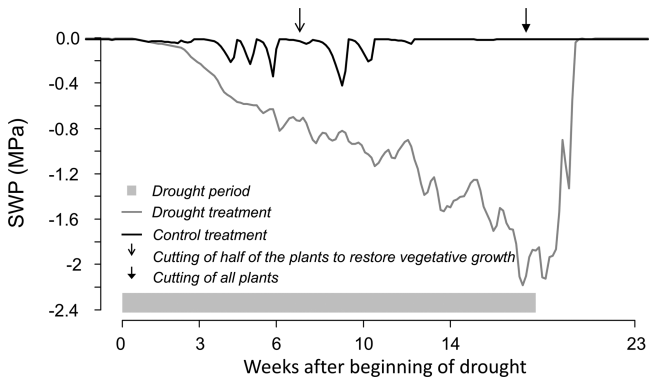

Figure 1. Development of the SWP in megapascals over time at a depth of 20 cm. At the cut in week 7 after the beginning of the drought period, only half of the plants were harvested to reset their growth stage back to vegetative (see Table 1), while the other plants continued with the generative growth stage. At the final cut at the end of the drought period (week 17), all plants were cut.

Additionally, half of the plants of both drought treatments (rainfed control and drought stress) were cut 7 weeks after the start of the drought period at the onset of flowering to restore vegetative growth. The other half of the plants remained uncut to allow for continuation of generative growth (third experimental factor "growth stage"; Table 1).

The experiment was carried out as a split-plot design. The four main plot treatment combinations, derived from the combinations of the two factors growth stage and drought, were repeated twice each, resulting in eight main plots, separated by large boundaries to prevent effects as a result of lateral water flows and shading of the cut plants by the uncut plants. The five accessions were nested as the subfactor and were randomly distributed in each of the eight main plots, resulting in 40 subplots. From each accession, three individual plants were sampled per subplot, resulting in a total of 120 plants.

Sampling and Sample Processing. Sampling took place at five different sampling events: 3, 6, 10, 14, and 23 weeks after the onset of drought stress. Five leaves of comparable age were removed from the middle of the respective stems. All leaves of an individual plant were pooled. After removal, the leaves were frozen and stored within 1 h at

https://dx.doi.org/10.1021/acs.jafc.0c01540
J. Agric. Food Chem. 2020, 68, 7369−7377

Table 1. Growth Stage of *Onobrychis viciifolia* Plants over the Experimental Period as Affected by an Additional Cut of Half of the Plants at Week 7 (+Cut), while the Other Half Was Not Cut (−Cut)[a]

sampling event	weeks after beginning of drought	growth stage (cut)	growth stage (+cut)
1	3	vegetative	vegetative
2	6	gen (flowering)	gen (flowering)
3	10	gen (immature seeds)	vegetative
4	14	gen (ripe seeds)	vegetative
5	23	vegetative	vegetative

[a]This additional cut set back the plants to vegetative growth. At the end of the drought period (week 17), all of the plants were cut and, thereafter, grew vegetatively. gen = generative.

−70 °C until they were processed and analyzed. Subsequently, the plant samples were freeze-dried using a Plant Sublimator 3 × 4 × 5 (ZIRBUS technology GmbH, Bad Grund, Germany). The plant material was then ground with a MM 400 ball mill (Retsch Technology GmbH, Hann, Germany) in 25 mL tungsten carbide containers with four tungsten carbide balls with a diameter of 7 mm.

The soluble (S-PA), protein-bound (Pb-PA), and fiber-bound (Fb-PA) PA fractions were determined using the HCl−butanol assay according to Terrill et al.[35] Although both Makkar et al.[36] and Grabber et al.[37] identified plant species where the method according to Terrill et al. can underestimate NE-PA concentrations, the distinct advantage by Terrill et al. is that it allows for the separation between the Pb-PA and Fb-PA fractions rather than just analyzing the entire NE-PA fraction together. We deem this a distinct advantage, particularly because we hypothesized (a) a difference in potential bioactivity between Pb-PA and Fb-PA as a result of their differences in digestibility and, hence, dissociation in the digestive tract and (b) differences in the composition of Pb-PA and Fb-PA as a result of the growth stage, with the vegetative plants having been expected to be richer in Pb-PA and lower in Fb-PA than the generative plants. This was anticipated as a result of the continuous increase of fiber and decrease in protein with ongoing plant maturation. Still, a comparison between the improved method by Grabber et al. using acetone as a co-solvent and the original method from Terrill et al. was performed and yielded comparable results, with slightly higher NE-PA concentrations being found according to Terrill et al. ($y = 0.85x + 0.05$; $R^2 = 0.94$).

Briefly, the extraction procedures for the different fractions were as follows: for the soluble PAs, 20 ± 0.5 mg of plant material from each sample was extracted overnight with 1.4 mL of an 80:20 acetone/water solution. After extraction, the solution was centrifuged at 9000 rpm for 10 min and the supernatant with the soluble PAs was collected. The extraction was performed twice, and the organic solvent was evaporated using an Eppendorf concentrator plus (Eppendorf AG, Hamburg, Germany) to obtain the PAs in aqueous solution. The aqueous extracts were freeze-dried using a Beta 1-8 LDplus freeze dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) and then stored at −20 °C. Before S-PA was analyzed, the freeze-dried extracts were dissolved in 1 mL of distilled water and filtered through a 0.2 μm polytetrafluoroethylene (PTFE) filter.

To extract the Pb-PA fraction, the remaining solid pellet was treated with 600 μL of a sodium dodecyl sulfate (SDS)/mercaptoethanol buffer (10 g L^{-1} SDS and 50 g L^{-1} 2-mercaptoethanol dissolved in 10 mM Tris/chloride adjusted to pH 8). The mixture was then heated to 100 °C while being shaken at 450 rpm in a ThermoMixer F2.0 (Eppendorf AG, Hamburg, Germany). Subsequently, the samples were cooled on ice for 10 min and centrifuged at 9000 rpm for 10 min. The supernatant containing the Pb-PAs was collected, and the extraction was repeated. The two supernatant extracts were combined, and the residue was retained to provide samples for the Fb-PA determination. To measure the PA concentration in the different extracts, the colorimetric HCl−butanol assay was used.[35] In a triplicate repeat, 160 μL of extract of the S-PA and Pb-PA fractions was added to 960 μL of a HCl−butanol (5:95, v/v) solution. To the pellet containing the fiber-bound PAs, 120 μL of a SDS buffer (10 g L^{-1} SDS dissolved in 10 mM Tris/chloride adjusted to pH 8) and 1200 μL of the HCl−butanol solution were added. The samples were then heated to 90 °C for 90 min in the Thermomix at 0 rpm and then cooled to room temperature on ice. Three replicates of the PA standard of known concentration (for details, see below) and a HCl−butanol reagent blank were included in each run as well. The standard was used to determine the efficiency of each run, and the HCl−butanol reagent blank determined the absorption zero value. The absorption was determined at 550 nm in a Libra S22 spectrophotometer (Biochrom, Ltd., Cambridge, U.K.).

Calibration Curve. To create a representative standard, 50 sainfoin samples from the experiment were selected randomly and subsampled to accumulate 10 g of biomass for a PA extraction. For the standard, the soluble PAs were extracted from the plant material using three extraction steps as described above, resulting in a total of 1 L of an 80:20 acetone/water solution. After extraction, acetone was evaporated using a rotary evaporator (Büchi Labortechnik, Flawil, Switzerland). The remaining aqueous phase was subsequently freeze-dried using a Beta 1-8 LDplus freeze dryer, and the resulting powder was run through 100 g of Sephadex LH-20 (Sigma-Aldrich Chemie GmbH, Munich, Germany), packed in water in a 48 × 300 mm column (Kimble Chase, Vineland, NJ, U.S.A.) and eluted with water, followed by acetone/water solutions in increasing concentrations (1000 mL of H_2O, 500 mL of 20:80 Ac/H_2O, 500 mL of 40:60 Ac/H_2O, 500 mL of 60:40 Ac/H_2O, and 500 mL of 80:20 Ac/H_2O) with a Masterflex L/S peristaltic pump (Cole-Parmer LLC, Vernon Hills, IL, U.S.A.) at 5 mL min^{-1}. The fractions were concentrated in the rotary evaporator and then freeze-dried. PAs were found in the 60:40 and 80:20 acetone/water fractions, and hence, these fractions were pooled. Subsequently. the purity of the standard material was determined by ultra-performance liquid chromatography−tandem mass spectrometry (UPLC−MS/MS). From the purified PAs, a dilution series was prepared containing values from 0.0625 to 1.25 mg mL^{-1}, with absorption values showing an excellent fit ($R^2 = 0.998$; $p < 0.001$).

Statistical Analysis. The effect of the treatments on the dependent variables y (soluble PA concentration, protein-bound PA concentration, and fiber-bound PA concentration) was determined using a linear mixed regression model. For each specific sampling event, the model was

$$y_{ikl} = \alpha \times \text{growth stage} + \beta \times \text{drought} + \gamma \times \text{accession} + \lambda_1 \times \text{plot}_k + \lambda_2 \times \text{subplot}_l + e_i \quad (1)$$

where y is the estimated effect of the factors growth stage, drought, and accession as well as their interactions and y_{ikl} is the effect of a single plant i in plot k and subplot l. The effect of the independent variables cut, drought, and accession is represented by the fixed parameters α, β, and γ. The random parameters λ_1 and λ_2 estimate the variance within a plot and subplot, respectively. A normal distribution is assumed for the error e, and the mean as well as the variance σ^2 is 0.

To determine the effect of the treatments over the entire duration of the experiment, eq 1 was extended to the following equation:

$$y_{jklm} = \alpha \times \text{growth stage} + \beta \times \text{drought} + \gamma \times \text{accession} + \delta \times \text{sampling} + \lambda_1 \times \text{plot}_k + \lambda_2 \times \text{subplot}_l + \lambda_3 \times \text{plant} + e_j \quad (2)$$

The additional parameter δ represents the effect of the sampling event on y, and y_{jklm} is the effect of a plant m in plot k and subplot l at sampling j. The variance of the plants over different sampling events is estimated by the random coefficient λ_3. All analyses were performed

https://dx.doi.org/10.1021/acs.jafc.0c01540
J. Agric. Food Chem. 2020, 68, 7369−7377

Table 2. Concentration of Soluble (S-PA), Protein-Bound (Pb-PA), and Fiber-Bound (Fb-PA) PAs and Total PAs for Each Accession Averaged over All Sampling Events[a]

	[PA] (mg g^{-1} of DM)			
accession	S-PA	Pb-PA	Fb-PA	total
CPI 63750	15.0 (1.2) b	1.6 (0.2) a	0.6 (0.04) a	17.2 (1.3) b
Esparsette	15.2 (1.2) b	1.6 (0.1) a	0.6 (0.05) a	17.4 (1.2) b
Perly	11.3 (1.2) a	1.6 (0.2) a	0.7 (0.04) a	13.6 (1.1) a
Taja	15.4 (1.2) b	1.8 (0.1) a	0.6 (0.03) a	17.8 (1.1) b
Visnovsky	12.3 (1.3) ab	1.8 (0.1) a	0.5 (0.03) a	14.7 (1.3) ab

[a]Values in parentheses show the standard error (SE). Different letters indicate differences among the accessions ($p < 0.05$).

with the software R,[38] and the calculation of the model was performed with the package "nlme".

RESULTS

Unless otherwise stated, only sampling events at 10 and 14 weeks were considered in the comparison of treatments to show the effects of drought stress and the growth stage on PA concentrations. These sampling events were selected because only those can compare different growth stages (as a result of the additional cut, see Table 1). Drought effects on biomass were published previously.[28]

What Is the Composition of S-PA, Pb-PA, and Fb-PA in Sainfoin? The average concentrations of S-PA, Pb-PA, and Fb-PA in sainfoin across all treatments and sampling events were 13.8 mg g^{-1} of dry matter (DM), 1.7 mg g^{-1} of DM, and 0.6 mg g^{-1} of DM, respectively (Table 2). Thus, overall, the S-PA fraction accounted for 86% of the total PAs. The concentration of S-PA differed among accessions. Averaged over all sampling events and treatments, 11.3 mg g^{-1} of DM Perly had lower ($p < 0.01$) S-PA concentrations than Taja, Esparsette, and CPI 63750 (with their average being 15.2 mg g^{-1} of DM). However, no significant differences were observed in the concentration of Pb-PA or Fb-PA among accessions. In the plants from the rainfed control that continued their growth into the generative stage, all PA concentrations increased evenly between weeks 3 and 14 and the share of S-PA remained between 82 and 83% for the entire growth period of sainfoin, while the Pb-PA and Fb-PA fraction remained continuously at 13% and between 4 and 5%, respectively. This was a result of linear increments in the S-PA concentration from 11.1 to 13.2 mg g^{-1} of DM, while the Pb-PA and Fb-PA concentrations increased slightly from 1.7 to 2.1 mg g^{-1} of DM and from 0.5 to 0.7 mg g^{-1} of DM, respectively (Figure 2).

Are Pb-PA and Fb-PA Concentrations Affected by Drought or the Growth Stage? In the first 6 weeks, when the water deficit in the sheltered plots was still comparably small (Figure 1), drought stress was limited and, thus, had no effect on the concentration of S-PA, Pb-PA, or Fb-PAs (Figure 2). After 10 weeks, the vegetative plants stage showed a 47% higher S-PA concentration under drought stress than under control conditions ($p < 0.05$), while after 14 weeks, this difference was 73% ($p < 0.01$). Contrary to that, generative plants showed no significant differences in the S-PA concentration. Hence, the response of S-PA to drought is dependent upon the growth stage (growth stage × drought interaction; $p < 0.01$; Table 3). As a result, the S-PA concentration of drought-stressed plants in their vegetative stage was 44% higher than that of generative plants ($p < 0.05$; Table 4).

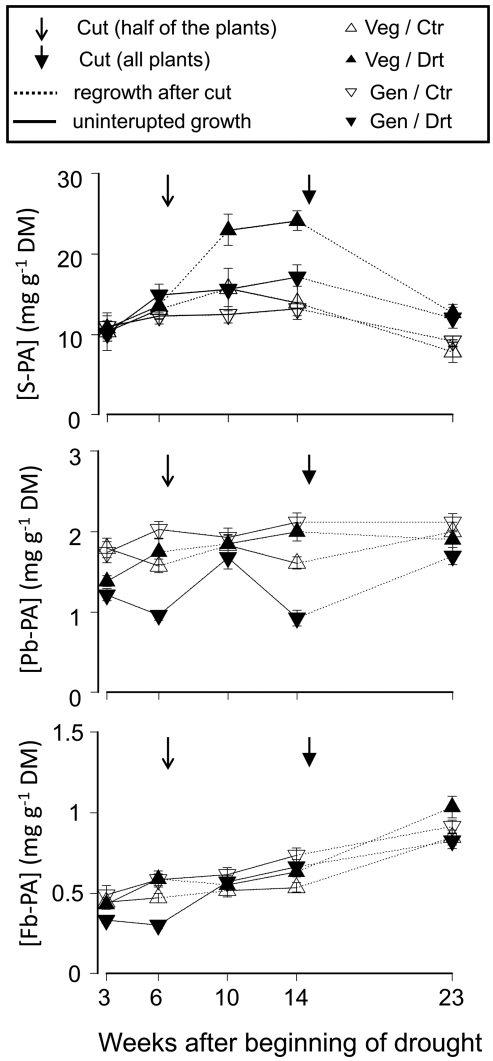

Figure 2. Change in concentration of soluble (S-PA), protein-bound (Pb-PA), and fiber-bound (Fb-PA) PAs during the experimental period, as affected by drought (Drt) compared to the rainfed control (Ctr) and growth stage (Veg, vegetative; Gen, generative). Growth stages differed at weeks 10 and 14. For details on growth stages, see Table 1. The error bars indicate the standard error of the mean.

With regard to the Pb-PA and Fb-PA concentrations, there was no effect of drought (drought, ns; Figure 3), irrespective of the growth stage of the plants (cut × drought, ns; Table 3). Thus, the absolute differences in concentration across treatments were small compared to those of the S-PA, and the Pb-PA concentration varied from 1.3 mg g^{-1} of DM in the generative drought-stressed plants to 2.1 mg g^{-1} of DM in the

https://dx.doi.org/10.1021/acs.jafc.0c01540
J. Agric. Food Chem. 2020, 68, 7369−7377

Table 3. Analysis of Variance (ANOVA) Table of the Linear Mixed Model To Determine Treatment Effects of Soluble (S-PA), Protein-Bound (Pb-PA), and Fiber-Bound (Fb-PA) PAs at Weeks 10 and 14 (the Two Samplings When Growth Stages Differed; Table 1)[a]

treatment	F value	p value
S-PA (mg g^{-1} of DM)		
$acc_{4,16}$	2.99	<0.05
$sampling_{1,94}$	1.80	0.18
$G{\times}D_{3,4}$	22.40	<0.01
$acc/sampling_{4,94}$	2.17	0.08
$acc/G{\times}D_{12,16}$	1.79	0.14
$sampling/G{\times}D_{3,94}$	2.30	0.08
$acc/sampling/G{\times}D_{12,94}$	0.66	0.79
Pb-PA (mg g^{-1} of DM)		
$acc_{4,16}$	1.70	0.2
$sampling_{1,94}$	4.91	<0.05
$G{\times}D_{3,4}$	3.45	0.13
$acc/sampling_{4,94}$	0.80	0.53
$acc/G{\times}D_{12,16}$	1.04	0.46
$sampling/G{\times}D_{3,94}$	8.10	<0.001
$acc/sampling/G{\times}D_{12,94}$	1.10	0.37
Fb-PA (mg g^{-1} of DM)		
$acc_{4,16}$	2.46	0.08
$sampling_{1,94}$	9.15	<0.01
$G{\times}D_{3,4}$	0.66	0.62
$acc/sampling_{4,94}$	0.18	0.95
$acc/G{\times}D_{12,16}$	3.24	<0.05
$sampling/G{\times}D_{3,94}$	0.32	0.81
$acc/sampling/G{\times}D_{12,94}$	2.14	<0.05

[a]Abbreviations are acc, accession; sampling, sampling event (weeks 10 and 14); and G×D, treatment combinations of factors growth stage and drought. Subscripts indicate numerator and denominator degrees of freedom.

generative rainfed plants (Table 4). Likewise, the Fb-PA concentration was generally between 0.5 and 0.7 mg g^{-1} of DM, irrespective of the treatment.

Do Pb-PA and Fb-PA Fractions and Their Response to Drought Differ across Sainfoin Accessions? While the previous results have been pooled across all accessions, the subsequent analyses will illustrate to which degree these responses are similar across accessions and, hence, can be considered representative for sainfoin.

The response of both the S-PA and Pb-PA concentrations to the treatments did not differ across the five sainfoin accessions (accession × cut × drought, ns; Table 3). Contrary to that, the change in the Fb-PA concentration as a result of the treatments was different across the sainfoin accessions (accession × cut × drought; $p < 0.05$). However, despite these differences, the absolute values of the Fb-PA concentration across accessions and treatments remained small and ranged from 0.5 to 0.8 mg g^{-1} of DM (Figure 3).

In comparison of generative and vegetative plants, accessions only differed significantly in their S-PA concentration in vegetative plants ($p < 0.05$) and their Fb-PA concentration in generative plants ($p < 0.01$; Figure 3). There was no difference in the response to drought across accessions for any PA fraction in either growth stage (Figure 3).

DISCUSSION

S-PA Constitutes the Largest Fraction of the Total PAs in Sainfoin. The values for S-PAs measured with the HCl−butanol method correlated with previously determined results of the same samples using UPLC−MS/MS ($p < 0.001$; $R^2 = 0.74$).[28] Also, the proportions of the S-PA, Pb-PA, and Fb-PA fractions within the total PA were in accordance with recently determined values of Girard et al., who had found S-PA shares of 75−79% of the total PAs and Pb-PA shares of 15−18% and Fb-PA shares of 6−7% for the variety Perly.[23] Also, the measured total PA concentrations of 14−18 mg g^{-1} of DM and the order in concentration across accessions were similar to leaf PA concentrations and PD shares of the accessions Perly, Visnovsky, Taja, and CPI 63750 previously determined by thiolysis and the HCl−butanol assay.[26,39−41]

Generally, the Pb-PA and Fb-PA shares of the total PAs in sainfoin appear rather low compared to other species. In the birdsfoot trefoil (*Lotus corniculatus*) variety 'Polom', Pb-PAs shares of 41% and fiber-bound PAs of 12% were reported.[23] High shares of NE-PAs were also identified in plant species with relevance for human nutrition. Accordingly, the saskatoon berry (*Amelanhcier alnifolia*) had a NE-PA share of 37%, and red and green grapes (*Vitis vinifera* L.) even contained 44 and 63% NE-PAs, respectively.[42] However, these species were exceptions, and the other 10 tested species in that study exhibited shares of NE-PAs between 4 and 18%; thus, the observed NE-PA concentrations of sainfoin can be considered a typical range.

Nevertheless, it should be noted that, while the low concentration of Pb-PAs and Fb-PAs in sainfoin appears promising to estimate bioactivity based on the S-PA concentration alone, caution needs to be taken when assuming anthelmintic or antimethanogenic bioactivity from concentration alone, because the structure has been found to differ between S-PA and NE-PA. The structure of NE-PA has generally been found to have larger polymers and, in the case of Visnovsky, also higher PD shares.[43] These results correspond with the findings that, on average, the PD share and mDP were higher in the abomasum, where the NE-PA

Table 4. Concentration of Soluble (S-PA), Protein-Bound (Pb-PA), and Fiber-Bound (Fb-PA) PAs and Total PAs Dependent upon Treatments Averaged over Sampling Events at Weeks 10 and 14 (When Growth Stages Differed; Table 1)[a]

	[PA] (mg g^{-1} of DM)			
treatment	S-PA	Pb-PA	Fb-PA	total
Ctr/Gen	13.1 (0.1) a	2.1 (0.3) b	0.7 (0.01) a	15.9 (0.2) a
Ctr/Veg	15.2 (1.3) a	1.8 (0.4) ab	0.5 (0.1) a	17.5 (0.8) a
Drt/Gen	16.7 (1.5) a	1.3 (0.1) a	0.6 (0.03) a	18.7 (1.6) a
Drt/Veg	24.1 (0.8) b	2.0 (0.0) ab	0.6 (0.03) a	26.7 (0.9) b

[a]Treatments: with (Drt) or without (Ctr) rain exclusion combined with generative (Gen) or vegetative (Veg) growth. Different letters indicate differences among the accessions ($p < 0.05$).

https://dx.doi.org/10.1021/acs.jafc.0c01540
J. Agric. Food Chem. 2020, 68, 7369−7377

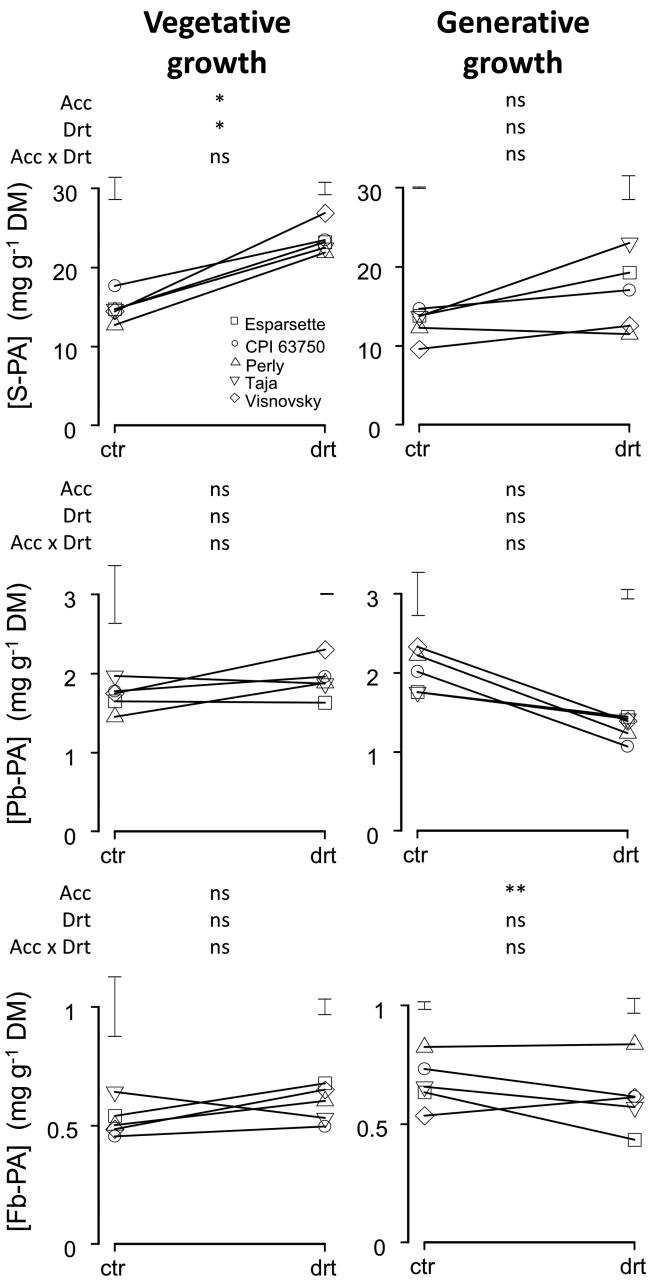

Figure 3. Effect of the drought stress, growth stage, and accession on the concentration of soluble (S-PA), protein-bound (Pb-PA), and fiber-bound (Fb-PA). Treatments: drought stress (Drt) or rainfed control (Ctr) and accession (Acc). Values are the means of the two sampling events at weeks 10 and 14 after the beginning of the drought. Error bars are standard errors of the mean from all accessions. Levels of significance of the tested factors (LMM): (ns) $p \geq 0.1$, (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$.

undergos dissociation compared to the rumen of cattle fed with sainfoin.[20] Because larger ellagitannin polymers derived from rosebay willowherb (*Epilobium angustifolium*) were shown to have higher antimethanogenic effects,[44] NE-PA might have relatively more potent PAs compared to S-PA as a result of the structure. Nevertheless, with regard to sainfoin, with the few existing studies on the impact of structural characteristics on the bioactivity (predominantly anthelmintic bioactivity assessments) on sainfoin to date, the effect of changes in mDP had either comparably small effects on the anthelmintic properties of sainfoin[45] or the effects were inconclusive across different nematode species.[46] Still, because these studies did not use purified PA oligomers and polymers, no direct link between the mDP in the PAs of sainfoin and their bioactivity exists to date.

Drought Only Affected Concentration of S-PA but Not Pb-PA and Fb-PA. While the observed drought effect on S-PAs was in accordance with a previous study,[30] the effect of drought on Pb-PAs and Fb-PAs has not been evaluated for sainfoin before. Drought has been shown to reduce neutral and acid detergent fiber (NDF and ADF) as well as lignin in several forage species.[47] This is an indirect effect from the impeded plant growth and development under drought conditions, resulting in younger plants at identical harvest dates. Consequently, both drought and growth stage were anticipated to effect plant size and, hence, protein/fiber ratios. Thus, for this experiment, control treatments were also anticipated to have higher Fb-PA concentrations as a result of faster maturation. However, in sainfoin, drought had not affected CP, NDF, or ADF concentrations across the whole plant in previous experiments,[48] and hence, the general assumption that drought would have affected NE-PA as a result of increments in the Fb-PA content does not seem to hold true for sainfoin. Contrary to that, previous studies with northern red oak (*Quercus rubra*) had documented that, under warm climates, the total NE-PA concentration in green leaves under drought was significantly higher than in ambient or wet conditions.[49] The share of NE-PA, on the other hand, was lower in ambient conditions than in either dry or wet conditions. A study with red maple (*Acer rubrum*) observed that the concentration of NE-PA was actually decreased under drought, when the temperature was not changed as well.[50] They did, however, observe that independent of comparably small concentration changes, the reactivity of PAs from drought-stressed trees was increased disproportionally large. Consequently, the changes in the concentration (or the absence of such) might not suffice to conclude anthelmintic or antimethanogenic properties of the PAs.

As mentioned above, protein and fiber shares are affected by plant maturation, and hence, the lack of effect from the growth stage across all PA fractions was surprising. With regard to the S-PAs, previous studies have repeatedly observed higher concentrations with increasing plant maturity.[27,41,51] However, this was a result of whole-plant analyses, where the concentration increment was mainly driven by the development of the plant reproductive organs, which are high in PAs.[27] When leaves are only looked at, Guglielmelli et al.[52] found PA concentrations to decrease with the increasing growth stage of sainfoin. This was attributed to a dilution effect, because the PA synthesis has been observed to be the maintain activity throughout plant growth, yet the fast leaf expansion and biomass accumulation resulted in a reduced PA concentration per dry matter. Because in this study only leaves were sampled, the reduced concentration in total PA and S-PA in leaves of the generative compared to vegetative plants, independent of drought or rainfed conditions, is in accordance with this. However, we anticipated simultaneous increments in particularly Fb-PA, because over the course of ontological plant development, cell wall proportions increase, accompanied by a decrease in proteins and other cell contents.[53] In earlier experiments with sainfoin, the average crude protein (CP) concentrations across the entire plant were 21% before flowering and 18% during full flower, while crude fiber

https://dx.doi.org/10.1021/acs.jafc.0c01540
J. Agric. Food Chem. 2020, 68, 7369−7377

concentrations were 17% before flowering and 23% during flowering.[54] Because seed formation had already occurred at sampling events 3 and 4 in this study, an even larger increment in fiber concentrations and, hence, Fb-PAs was expected in generative plants, with a simultaneous decrease in the proportion of S-PAs and Pb-PAs. The reason that we did not observe this is likely due to the fact that Fb-PA concentrations were generally so low that the relative small increments in crude fiber that have been previously documented did not suffice to change the fractions of PAs that are bound to fiber.

Response of PA to Drought Is Generally Uniform across Accessions. A large variability in the S-PA concentration both within and across different sainfoin accessions had been identified several times in previous research.[25,26] As a result of this, the observed bioactivity has also varied substantially with, for example, liveweight changes in lambs feeding sainfoin either decreasing[10] or increasing[4] compared to lucerne as a PA-free control. Hence, changes in the PA composition across accessions would complicate the predictability of the bioactivity further, particularly because the Pb-PA and Fb-PA fractions are rarely ever analyzed, even within a single environment. Differences in the PA composition across cultivars have been identified, for example, for birdsfoot trefoil cultivars 'Polom' and 'Bull', with the Pb-PA fractions being either 38 or 13%, respectively, yet generally very few studies have analyzed differences in the PA composition across cultivars of the same species. Additionally, to our knowledge, no study thus far has determined these differences in accessions across a range of environments and/or plant growth stages. Hence, these findings will be useful for the planning of future experiments and will help to assess the importance of the S-PA, Pb-PA, and Fb-PA fractions in sainfoin.

Generally, the results of this study show that the proportions of non-extractable PAs in sainfoin were much lower compared to soluble PAs. Therefore, the additional release of bound PAs in the lower digestive tract of ruminants from feeding sainfoin is also likely to be correspondingly lower. This increases the likelihood that the analysis of S-PA is most important for fresh sainfoin. This will, however, be different for silage samples, because the ensiling process can transform S-PA into the NE-PA form.[3,23,24] Furthermore, the fact that protein- and fiber-bound PAs did not differ between accessions and were also unaffected by drought can be considered promising. Consequently, studies analyzing soluble PAs of any sainfoin cultivar from any environmental condition are likely to be representative for the bioactivity of sainfoin. However, care needs to be taken because previous studies have frequently shown that the concentration does not suffice to conclude bioactivity, and hence, feeding trials will be required to establish the bioactive effects of non-extractable PAs in sainfoin.

AUTHOR INFORMATION

Corresponding Author

Carsten S. Malisch − *Grass and Forage Science/Organic Agriculture, Institute of Crop Science and Plant Breeding, Christian-Albrechts-Universität zu Kiel, 24118 Kiel, Germany;* orcid.org/0000-0002-2905-8885; Email: c.malisch@posteo.de

Authors

Lasse Lewandowski − *Grass and Forage Science/Organic Agriculture, Institute of Crop Science and Plant Breeding, Christian-Albrechts-Universität zu Kiel, 24118 Kiel, Germany*

Juha-Pekka Salminen − *Natural Chemistry Research Group, Department of Chemistry, University of Turku, 20500 Turku, Finland;* orcid.org/0000-0002-2912-7094

Friedhelm Taube − *Grass and Forage Science/Organic Agriculture, Institute of Crop Science and Plant Breeding, Christian-Albrechts-Universität zu Kiel, 24118 Kiel, Germany*

Andreas Lüscher − *Forage Production and Grassland Systems, Agroscope, 8046 Zürich, Switzerland*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jafc.0c01540

Funding

These investigations were supported by the European Commission (PITN-GA-2011-289377, "LegumePlus" Project). The authors are very grateful to the United States Department of Agriculture (USDA) Agricultural Research Service (ARS)−Germplasm Resources Information Network (GRIN) germplasm database for the provision of the seeds of the sainfoin accessions.

Notes

The authors declare no competing financial interest.

REFERENCES

(1) 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 Forage Sci.* **2014**, *69*, 206−228.

(2) Poutaraud, A.; Michelot-Antalik, A.; Plantureux, S. Grasslands: A source of secondary metabolites for livestock health. *J. Agric. Food Chem.* **2017**, *65*, 6535−6553.

(3) Mueller-Harvey, I.; Bee, G.; Dohme-Meier, F.; Hoste, H.; Karonen, M.; Kölliker, R.; Lüscher, A.; Niderkorn, V.; Pellikaan, W. F.; Salminen, J.-P.; Skøt, L.; Smith, L. M. J.; Thamsborg, S. M.; Totterdell, P.; Wilkinson, I.; Williams, A. R.; Azuhnwi, B. N.; Baert, N.; Brinkhaus, A. G.; Copani, G.; Desrues, O.; Drake, C.; Engström, M.; Fryganas, C.; Girard, M.; Huyen, N. T.; Kempf, K.; Malisch, C.; Mora-Ortiz, M.; Quijada, J.; Ramsay, A.; Ropiak, H. M.; Waghorn, G. C. Benefits of condensed tannins in forage legumes fed to ruminants: Importance of structure, concentration, and diet composition. *Crop Sci.* **2019**, *59*, 861−885.

(4) Waghorn, G. Beneficial and detrimental effects of dietary condensed tannins for sustainable sheep and goat production-Progress and challenges. *Anim. Feed Sci. Technol.* **2008**, *147*, 116−139.

(5) Klongsiriwet, C.; Quijada, J.; Williams, A. R.; Mueller-Harvey, I.; Williamson, E. M.; Hoste, H. Synergistic inhibition of *Haemonchus contortus* exsheathment by flavonoid monomers and condensed tannins. *Int. J. Parasitol.: Drugs Drug Resist.* **2015**, *5*, 127−134.

(6) Häring, D. A.; Scharenberg, A.; Heckendorn, F.; Dohme, F.; Luscher, A.; Maurer, V.; Suter, D.; Hertzberg, H. Tanniferous forage plants: Agronomic performance, palatability and efficacy against parasitic nematodes in sheep. *Renew. Agric. Food Syst.* **2008**, *23*, 19−29.

(7) Azuhnwi, B. N.; Hertzberg, H.; Arrigo, Y.; Gutzwiller, A.; Hess, H. D.; Mueller-Harvey, I.; Torgerson, P. R.; Kreuzer, M.; Dohme-Meier, F. Investigation of sainfoin (*Onobrychis viciifolia*) cultivar differences on nitrogen balance and fecal egg count in artificially infected lambs. *J. Anim. Sci.* **2013**, *91*, 2343−2354.

(8) Wang, Y.; Berg, B. P.; Barbieri, L. R.; Veira, D. M.; McAllister, T. A. Comparison of alfalfa and mixed alfalfa-sainfoin pastures for grazing cattle: Effects on incidence of bloat, ruminal fermentation, and feed intake. *Can. J. Anim. Sci.* **2006**, *86*, 383−392.

(9) Min, B. R.; Pinchak, W. E.; Hernandez, K.; Hernandez, C.; Hume, M. E.; Valencia, E.; Fulford, J. D. Effects of plant tannin

supplementation on animal responses and in vivo ruminal bacterial populations associated with bloat in heifers grazing wheat forage. *Prof. Anim. Sci.* **2012**, *28*, 464−472.
(10) Girard, M.; Dohme-Meier, F.; Silacci, P.; Ampuero Kragten, S.; Kreuzer, M.; Bee, G. Forage legumes rich in condensed tannins may increase n-3 fatty acid levels and sensory quality of lamb meat. *J. Sci. Food Agric.* **2016**, *96*, 1923−1933.
(11) Toral, P. G.; Hervás, G.; Missaoui, H.; Andrés, S.; Giráldez, F. J.; Jellali, S.; Frutos, P. Effects of a tannin-rich legume (*Onobrychis viciifolia*) on in vitro ruminal biohydrogenation and fermentation. *Span. J. Agric. Res.* **2016**, *14*, No. e0602.
(12) Tavendale, M. H.; Meagher, L. P.; Pacheco, D.; Walker, N.; Attwood, G. T.; Sivakumaran, S. Methane production from in vitro rumen incubations with *Lotus pedunculatus* and *Medicago sativa*, and effects of extractable condensed tannin fractions on methanogenesis. *Anim. Feed Sci. Technol.* **2005**, *123*−*124*, 403−419.
(13) 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. *J. Anim. Physiol. Anim. Nutr.* **2016**, *100*, 348−360.
(14) Grosse Brinkhaus, A.; Wyss, U.; Arrigo, Y.; Girard, M.; Bee, G.; Zeitz, J. O.; Kreuzer, M.; Dohme-Meier, F. In vitro ruminal fermentation characteristics and utilisable CP supply of sainfoin and birdsfoot trefoil silages and their mixtures with other legumes. *Animal* **2017**, *11*, 580−590.
(15) Lorenz, M. M.; Alkhafadji, L.; Stringano, E.; Nilsson, S.; Mueller-Harvey, I.; Udén, P. Relationship between condensed tannin structures and their ability to precipitate feed proteins in the rumen. *J. Sci. Food Agric.* **2014**, *94*, 963−968.
(16) Jonker, A.; Yu, P. The occurrence, biosynthesis, and molecular structure of proanthocyanidins and their effects on legume forage protein precipitation, digestion and absorption in the ruminant digestive tract. *Int. J. Mol. Sci.* **2017**, *18*, 1105.
(17) Pérez-Jiménez, J.; Torres, J. L. Analysis of nonextractable phenolic compounds in foods: The current state of the art. *J. Agric. Food Chem.* **2011**, *59*, 12713−12724.
(18) Jones, W. T.; Mangan, J. L. Complexes of condensed tannins of sainfoin (*Onobrychis viciifolia* Scop) with fraction 1 leaf protein and with submaxillary mucoprotein, and their reversal by polyethylene glycol and pH. *J. Sci. Food Agric.* **1977**, *28*, 126−136.
(19) Kariuki, I. W.; Norton, B. W. The digestion of dietary protein bound by condensed tannins in the gastro-intestinal tract of sheep. *Anim. Feed Sci. Technol.* **2008**, *142*, 197−209.
(20) Desrues, O.; Mueller-Harvey, I.; Pellikaan, W.; Enemark, H. L.; Thamsborg, S. M. Condensed tannins in the gastrointestinal tract of cattle after sainfoin (*Onobrychis viciifolia*) intake and their possible relationship with anthelmintic effects. *J. Agric. Food Chem.* **2017**, *65*, 1420−1427.
(21) Quijada, J.; Drake, C.; Gaudin, E.; El-Korso, R.; Hoste, H.; Mueller-Harvey, I. Condensed tannin changes along the digestive tract in lambs fed with sainfoin pellets or hazelnut skins. *J. Agric. Food Chem.* **2018**, *66*, 2136−2142.
(22) Kronberg, S. L.; Zeller, W. E.; Waghorn, G. C.; Grabber, J. H.; Terrill, T. H.; Liebig, M. A. Effects of feeding *Lespedeza cuneata* pellets with *Medicago sativa* hay to sheep: Nutritional impact, characterization and degradation of condensed tannin during digestion. *Anim. Feed Sci. Technol.* **2018**, *245*, 41−47.
(23) Girard, M.; Dohme-Meier, F.; Kragten, S.; Große-Brinkhaus, A.; Arrigo, Y.; Wyss, U.; Bee, G. Modification of the proportion of extractable and bound condensed tannins in birdsfoot trefoil (*Lotus corniculatus*) and sainfoin (*Onobrychis viciifolia*) during wilting, ensiling and pelleting processes. *Biotechnol. Anim. Husb.* **2018**, *34*, 1−19.
(24) Huang, Q. Q.; Jin, L.; Xu, Z.; Acharya, S.; McAllister, T. A.; Hu, T. M.; Iwaasa, A.; Schellenberg, M.; Peng, K.; Wang, Y. Effects of conservation method on condensed tannin content, ruminal degradation, and in vitro intestinal digestion of purple prairie clover (*Dalea purpurea* Vent.). *Can. J. Anim. Sci.* **2016**, *96*, 524−531.
(25) Malisch, C. S.; Lüscher, A.; Baert, N.; Engström, M. T.; Studer, B.; Fryganas, C.; Suter, D.; Mueller-Harvey, I.; Salminen, J.-P. Large variability of proanthocyanidin content and composition in sainfoin (*Onobrychis viciifolia*). *J. Agric. Food Chem.* **2015**, *63*, 10234−10242.
(26) Stringano, E.; Carbonero, C. H.; Smith, L. M. J.; Brown, R. H.; Mueller-Harvey, I. Proanthocyanidin diversity in the EU 'HealthyHay' sainfoin (*Onobrychis viciifolia*) germplasm collection. *Phytochemistry* **2012**, *77*, 197−208.
(27) Theodoridou, K.; Aufrere, J.; Andueza, D.; Le Morvan, A.; Picard, F.; Stringano, E.; Pourrat, J.; Mueller-Harvey, I.; Baumont, R. Effect of plant development during first and second growth cycle on chemical composition, condensed tannins and nutritive value of three sainfoin (*Onobrychis viciifolia*) varieties and lucerne. *Grass Forage Sci.* **2011**, *66*, 402−414.
(28) Malisch, C. S.; Salminen, J.-P.; Kölliker, R.; Engström, M. T.; Suter, D.; Studer, B.; Lüscher, A. Drought effects on proanthocyanidins in sainfoin (*Onobrychis viciifolia* Scop.) are dependent on the plant's ontogenetic stage. *J. Agric. Food Chem.* **2016**, *64*, 9307−9316.
(29) Häring, D. A.; Suter, D.; Amrhein, N.; Lüscher, A. Biomass allocation is an important determinant of the tannin concentration in growing plants. *Ann. Bot.* **2007**, *99*, 111−120.
(30) Selmar, D.; Kleinwächter, M. Stress enhances the synthesis of secondary plant products: The impact of stress-related over-reduction on the accumulation of natural products. *Plant Cell Physiol.* **2013**, *54*, 817−26.
(31) Häring, D. A.; Huber, M. J.; Suter, D.; Edwards, P. J.; Lüscher, A. Plant enemy-derived elicitors increase the foliar tannin concentration of *Onobrychis viciifolia* without a trade-off to growth. *Ann. Bot.* **2008**, *102*, 979−987.
(32) 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*). *Arch. Anim. Nutr.* **2007**, *61*, 481−496.
(33) Khalilvandi-Behroozyar, H.; Dehghan-Banadaky, M.; Rezayazdi, K. Palatability, in situ and in vitro nutritive value of dried sainfoin (*Onobrychis viciifolia*). *J. Agric. Sci.* **2010**, *148*, 723−733.
(34) Malisch, C. S.; Suter, D.; Studer, B.; Lüscher, A. Multifunctional benefits of sainfoin mixtures: Effects of partner species, sowing density and cutting regime. *Grass Forage Sci.* **2017**, *72*, 794−805.
(35) Terrill, T. H.; Rowan, A. M.; Douglas, G. B.; Barry, T. N. Determination of extractable and bound condensed tannin concentrations in forage plants, protein concentrate meals and cereal grains. *J. Sci. Food Agric.* **1992**, *58*, 321−329.
(36) Makkar, H. P. S.; Gamble, G.; Becker, K. Limitation of the butanol−hydrochloric acid−iron assay for bound condensed tannins. *Food Chem.* **1999**, *66*, 129−133.
(37) Grabber, J. H.; Zeller, W. E.; Mueller-Harvey, I. Acetone Enhances the Direct Analysis of Procyanidin- and Prodelphinidin-Based Condensed Tannins in Lotus Species by the Butanol-HCl-Iron Assay. *J. Agric. Food Chem.* **2013**, *61*, 2669−2678.
(38) R Core Team. *R: A Language and Environment for Statistical Computing*; R Foundation for Statistical Computing: Vienna, Austria, 2020.
(39) Azuhnwi, B. N.; Boller, B.; Dohme-Meier, F.; Hess, H. D.; Kreuzer, M.; Stringano, E.; Mueller-Harvey, I. Exploring variation in proanthocyanidin composition and content of sainfoin (*Onobrychis viciifolia*). *J. Sci. Food Agric.* **2013**, *93*, 2102−2109.
(40) Hatew, B.; Hayot Carbonero, C.; Stringano, E.; Sales, L. F.; Smith, L. M. J.; Mueller-Harvey, I.; Hendriks, W. H.; Pellikaan, W. F. Diversity of condensed tannin structures affects rumen in vitro methane production in sainfoin (*Onobrychis viciifolia*) accessions. *Grass Forage Sci.* **2015**, *70*, 474−490.
(41) Aufrere, J.; Dudilieu, M.; Poncet, C. In vivo and in situ measurements of the digestive characteristics of sainfoin in comparison with lucerne fed to sheep as fresh forages at two growth stages and as hay. *Animal* **2008**, *2*, 1331−1339.

https://dx.doi.org/10.1021/acs.jafc.0c01540
J. Agric. Food Chem. 2020, 68, 7369−7377

(42) Hellström, J. K.; Mattila, P. H. HPLC determination of extractable and unextractable proanthocyanidins in plant materials. *J. Agric. Food Chem.* **2008**, *56*, 7617−7624.
(43) Gea, A.; Stringano, E.; Brown, R. H.; Mueller-Harvey, I. In situ analysis and structural elucidation of sainfoin (*Onobrychis viciifolia*) tannins for high-throughput germplasm screening. *J. Agric. Food Chem.* **2011**, *59*, 495−503.
(44) Baert, N.; Pellikaan, W. F.; Karonen, M.; Salminen, J.-P. A study of the structure-activity relationship of oligomeric ellagitannins on ruminal fermentation in vitro. *J. Dairy Sci.* **2016**, *99*, 8041−8052.
(45) Williams, A. R.; Fryganas, C.; Ramsay, A.; Mueller-Harvey, I.; Thamsborg, S. M. Direct anthelmintic effects of condensed tannins from diverse plant sources against *Ascaris suum*. *PLoS One* **2014**, *9*, No. e97053.
(46) Quijada, J.; Fryganas, C.; Ropiak, H. M.; Ramsay, A.; Mueller-Harvey, I.; Hoste, H. Anthelmintic activities against *Haemonchus contortus* or *Trichostrongylus colubriformis* from small ruminants are influenced by structural features of condensed tannins. *J. Agric. Food Chem.* **2015**, *63*, 6346−6354.
(47) Peterson, P. R.; Sheaffer, C. C.; Hall, M. H. Drought effects on perennial forage legume yield and quality. *Agron. J.* **1992**, *84*, 774−779.
(48) Küchenmeister, K.; Küchenmeister, F.; Kayser, M.; Wrage-Mönnig, N.; Isselstein, J. Influence of drought stress on nutritive value of perennial forage legumes. *Int. J. Plant Prod.* **2013**, *7*, 693−710.
(49) Top, S. M.; Preston, C. M.; Dukes, J. S.; Tharayil, N. Climate influences the content and chemical composition of foliar tannins in green and senesced tissues of *Quercus rubra*. *Front. Plant Sci.* **2017**, *8*, 423.
(50) Tharayil, N.; Suseela, V.; Triebwasser, D. J.; Preston, C. M.; Gerard, P. D.; Dukes, J. S. Changes in the structural composition and reactivity of *Acer rubrum* leaf litter tannins exposed to warming and altered precipitation: Climatic stress-induced tannins are more reactive. *New Phytol.* **2011**, *191*, 132−45.
(51) Li, Y.; Iwaasa, A. D.; Wang, Y.; Jin, L.; Han, G.; Zhao, M. Condensed tannins concentration of selected prairie legume forages as affected by phenological stages during two consecutive growth seasons in western Canada. *Can. J. Plant Sci.* **2014**, *94*, 817−826.
(52) Guglielmelli, A.; Calabro, S.; Primi, R.; Carone, F.; Cutrignelli, M. I.; Tudisco, R.; Piccolo, G.; Ronchi, B.; Danieli, P. P. In vitro fermentation patterns and methane production of sainfoin (*Onobrychis viciifolia* Scop.) hay with different condensed tannin contents. *Grass Forage Sci.* **2011**, *66*, 488−500.
(53) Osbourn, D. F. The feeding value of grass and grass products. In *Grass, Its Production and Utilization*; Holmes, W., Ed.; Blackwell Scientific Publications: Oxford, U.K., 1980; pp 70−124.
(54) Baker, C. J. L.; Heimberg, M.; Alderman, G.; Eden, A. Studies of the composition of sainfoin. *J. Agric. Sci.* **1952**, *42*, 382−394.

https://dx.doi.org/10.1021/acs.jafc.0c01540
J. Agric. Food Chem. 2020, 68, 7369−7377