Research Article

Fatty acid profile, secondary compounds and antioxidant activities in the fresh forage, hay and silage of sainfoin (*Onobrychis viciifolia*) and sulla (*Hedysarum coronarium*)

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Abstract

Background: Sainfoin (*Onobrychis viciifolia*) and sulla (*Hedysarum coronarium*) are forage legumes usually preserved to optimize their utilization as feedstuffs. However, the method of preservation modifies the chemical composition differently in both legume species. Secondary compounds (such as proanthocyanidins, fatty acids, carotenoids and tocopherols) present in forages affect the quality of animal products. Therefore, the effect of preservation on the contents of secondary compounds should be investigated. Accoridngly, samples of sainfoin and sulla were directly freeze-dried (fresh), dried at ambient temperature (hay) and vacuum-packaged for 82 days (silage).

Results: In both legumes, the total fatty acid and C18:3 *n*-3 contents decreased and C16:0 increased with preservation ($P < 0.001$), with a greater effect for the hays than for the silages. For both legumes, the lutein, neoxanthin and violaxanthin contents decreased to a greater extent in the silages than in the hays ($P < 0.001$). Both hays exhibited the lowest β-carotene concentrations ($P < 0.001$). The α-tocopherol contents decreased in hays, but not in silages, compared to the fresh forages of both legumes ($P < 0.001$). The antioxidant activities were lower in the silages than in the hays and fresh forages ($P < 0.001$) and were very strongly related to the contents of polyphenols and proanthocyanidins ($P < 0.001$).

Conclusion: Haymaking affected the fatty acid, carotenoid and α-tocopherol contents to a greater extent but had a lesser effect on the antioxidant activities than silage-making, which were very strongly related to their contents of polyphenols and proanthocyanidins.
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Keywords: carotenoids; tocopherols; proanthocyanidins; condensed tannins; ABTS; DPPH

INTRODUCTION

Sainfoin (*Onobrychis viciifolia*) and sulla (*Hedysarum coronarium*), perennial forage legumes that are grown extensively in Mediterranean areas, have positive agronomic, environmental, and nutraceutical attributes.[1] In particular, legumes exhibit drought tolerance in areas with low rainfall and light-free draining soil and regenerate the soil due to their capacity to fix nitrogen (N).[2] Sainfoin and sulla exhibit high productive capacities (e.g. 15 and 25 tonnes dry matter ha^{-1}, respectively) with a peak in maximal production in the first spring cutting,[3] which makes it advisable to preserve these forages as hay or silage to optimize their use for animal feeding. Fresh sainfoin and sulla have high nutritional value[4] as a result of their high contents of crude protein and C18:3 *n*-3,[5] the most useful fatty acid (FA) enhancing meat and milk quality[6] and secondary compounds with antioxidant properties [polyphenols, carotenoids, tocopherols and proanthocyanidins

(PAC)[7,8]], which may improve product quality parameters such the shelf life[9] and the antioxidant status[10] because they are partially transferred and deposited in animal products (milk and

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meat). A recent study reported that silage-making decreased the contents of polyphenols and extractable PAC (EPAC), although there were increases in protein-bound (PB-PAC) and fibre-bound PAC (FB-PAC) to different extents in sainfoin and sulla, whereas haymaking decreased only polyphenols and increased PB-PAC in sulla.[7] However, there is scarce information on the FA and secondary compounds of these preserved legumes, especially for sulla. In a meta-analysis of several forage species, Glasser et al.[6] reported that haymaking caused a slight decrease in total FA content as a result of an important decrease in C18:3 and an increase in C16:0, whereas total FA was similar and C18:3 decreased by 5% in wilted silages. Regarding the carotenoids, haymaking and silage-making decreased their contents depending on the process conditions and on the species.[11] The present study aimed to investigate the hypothesis that haymaking decreases levels of FA, carotenoids, tocopherols and antioxidant (AO) activities in both legumes (probably differently) to a greater extent than ensiling because of the exposure to light and oxygen of hay. Accordingly, the present study investigated (i) the effects of the preservation methods (e.g. hay and silage *versus* fresh forage) of sainfoin and sulla on the FA profiles, the carotenoid and tocopherol contents and AO activities and (ii) the relationships among AO activities and the secondary compounds (polyphenols, PAC, carotenoids and tocopherols).

MATERIALS AND METHODS

The growing conditions, management of the forages and preservation conditions are detailed in Rufino-Moya et al.[7] Briefly, sainfoin (*Onobrychis viciifolia* cv Reznos) and sulla (*Hedysarum coronarium* cv Carmen) were sown each in three plots in autumn at seeding rates of 100 and 20 kg ha^{-1}, respectively, at CITA Research Institute at Zaragoza (41°42′N, 0°49′W, altitude 216 m a.s.l., annual mean temperature of 15 ± 7.3 °C and annual average rainfall of 296 ± 78 mm, Spain). During spring, irrigation was applied every 15–21 days. Sainfoin and sulla were harvested at the late and early bloom stages, respectively. Samples obtained randomly in each plot ($n = 10$ per plot) were mixed and divided into samples for fresh forage, hay and silage. The samples destined to haymaking were extended in elevated 10-cm 'mosquito' nets. For 16 days, they were sun-dried, except during unfavourable climatic conditions (rain or strong wind) when they were stored indoors. The samples that were destined to ensiling were wilted for 1 day protected from wind and rain, chopped (3–5 cm) and vacuum-ensiled in polyamide:polyethylene (30:90) plastic bags (120 μm) for 82 days at room temperature and were protected from light (final sainfoin pH 4.2 ± 0.01 and sulla pH 5.2 ± 0.11). The number of samples, chemical compositions, polyphenol contents and PACs of the fresh forages, hays and silages of sainfoin and sulla are reported in Table 1. The contents of polyphenols and fractions of PAC were determined as described in Rufino-Moya et al.[7] in accordance with the methods of Terrill et al.,[12] Grabber et al.[13] and Wolfe et al.[14]

Chemical analysis

Samples of the forages were immediately frozen and stored at −80 °C until freeze-drying just after collection for fresh forages, after 16 days of drying for hays and immediately after pH measurement for silages. After freeze-drying, the samples were ground through a 0.2-mm screen (Rotary Mill, ZM200; Retsch, Haan, Germany) and stored in total darkness at −80 °C until further analyses of FA, secondary compounds and AO activities. All

analyses were run in triplicate. The samples that were used to determine the secondary compounds and AO activities were protected from direct light.

The FA profile was determined by using gas chromatography with a flame ionization detector. The FA of the freeze-dried forages (500 mg) were extracted[15] as FA methyl esthers (FAME) using C19:0 as an internal standard and determined in a Bruker Scion 436-GC (Bruker, Billerica, MA, USA) gas chromatograph equipped with a CP-8400 autosampler and an SP-2560 capillary column (100 m × 0.25 mm × 0.2 μm) (Supelco, Saint Louis, MO, USA). The FAME identifications were based on their retention times that were compared with those of the standard FAME mixtures GLC-532, GLC-401, GLC-643 and GLC-642 (Nu-Chek Prep, Elysian, MN, USA) and quantification was performed as described in ISO 12966-4:2015.[16] The individual FA were grouped into total saturated FAs (SFAs), monounsaturated FAs (MUFAs) and polyunsaturated FAs (PUFAs).The carotenoids and tocopherols in the feedstuffs were extracted following the procedure described in Blanco et al.[17] The chromatographic procedure was that employed by Chauveau-Duriot et al.[18] and used an ACQUITY UPLC H-Class liquid chromatograph (Waters, Milford, MA, USA) equipped with a silica-based bonded phase column (Acquity UPLC HSS T3, 1.8 μm × 2.1 mm × 150 mm column; Waters); absorbance detector (Acquity UPLC Photodiode Array PDA eλ Detector; Waters) and fluorescence detector (2475 Multi λ Fluorescence Detector; Waters). Carotenoids were detected by the absorbances at 450 nm and tocopherols by the fluorescence emissions at $\lambda_{exc} = 295$ and $\lambda_{emi} = 330$ nm and were identified and quantified according to Blanco et al.[17]

The AO activities were determined with the total radical-scavenging activity [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)] and the free radical scavenging activity [2,2-diphenyl-1-picrylhydrazyl (DPPH) assay] and the ferric reducing antioxidant power (FRAP). First, AO activity extracts were obtained following the procedure described by Roncero-Ramos et al.[19] Freeze-dried samples (100 mg) were mixed with 5 mL of methanol: ultrapure water 50:50 (v:v), adjusted to pH 2 with HCl in an ultrasound bath (10 min at room temperature), followed by agitation in rotatubes for 50 min. Subsequently, the samples were centrifuged (15 min at $3600 \times g$ at 4 °C). The supernatant was collected, and the extraction was repeated using 5 mL of an acetone:ultrapure water 70:30 (v:v) solution. The methanolic and aqueous acetone extracts were then combined, and the volume was made up to 10 mL. Finally, the AO activity extracts were diluted 1:20 for the assays, except for 1:10 for those of the samples of sainfoin silage, fresh sulla and sulla hay for the DPPH assay and 1:5 for sulla silage for the three assays. Twelve replicates of each AO activity extract were obtained.

The method to determine ABTS was based on that of Jimenez-Escrig et al.[20] with slight modifications. A 7 mM ABTS solution was prepared and mixed at a 1:1 ratio (v:v) with a 2.45 mmol L^{-1} solution of $K_2S_2O_8$, shaken and allowed to react overnight (approximately 16 h). Subsequently, a solution of ABTS$^{-\cdot}$ in absolute ethanol was prepared (absorbance value of 0.700 ± 0.02 at 730 nm). Then, 20 μL of the diluted extracts and 280 μL of ABTS$^{-\cdot}$ solution were mixed for 30 min at room temperature. Finally, absorbance at 730 nm was measured using an EPOCH microplate spectrophotometer (BioTek, Winooski, VT, USA). The method for determining DPPH was based on Morales and Jiménez-Pérez,[21] with some modifications. Briefly, 74 mg L^{-1} DPPH solution (absorbance value of 1.8 at 520 nm) was freshly prepared. Then, 50 μL of the diluted extracts and 250 μL of DPPH solution were allowed to

Table 1. Chemical composition, polyphenols and proanthocyanidins (PAC) of the fresh forage, hay and silage of sainfoin and sulla

Item	Fresh	Hay	Silage	SE	*P*-value
Sainfoin					
N	5	5	5		
Dry matter (DM) (g kg^{-1})	233 c	899 a	329 b	4.7	< 0.001
Ash (DM) (g kg^{-1})	82	80	86	2.1	0.52
Crude protein (DM) (g kg^{-1})	194 a	169 b	169 b	3.2	0.01
Neutral detergent fibre (DM) (g kg^{-1})	392 b	470 a	456 a	8.1	0.004
Acid detergent fibre (DM) (g kg^{-1})	258 b	340 a	332 a	6.8	0.001
Lignin (DM) (g kg^{-1})	69 c	94 b	108 a	0.9	< 0.001
Polyphenols (DM) (eq-g tannic acid kg^{-1})	51 a	47 a	34 b	1.4	0.001
PAC (DM) (eq-g PAC kg^{-1})					
Total PAC	37 a	40 a	33 b	0.9	0.02
Extractable PAC	31 a	32 a	8 b	0.7	< 0.001
Protein-bound PAC	4.8 b	6.5 b	17.2 a	0.37	< 0.001
Fibre-bound PAC	1.8 c	2.3 b	7.7 a	0.07	< 0.001
Sulla					
n	4	5	5		
Dry matter (DM) (g kg^{-1})	142 c	932 a	171 b	3.4	< 0.001
Ash (DM) (g kg^{-1})	130 c	144 b	176 a	1.0	< 0.001
Crude protein (DM) (g kg^{-1})	218 a	198 b	193 b	1.6	< 0.001
Neutral detergent fibre (DM) (g kg^{-1})	349 c	409 b	483 a	2.4	< 0.001
Acid detergent fibre (DM) (g kg^{-1})	251 c	309 b	368 a	1.5	< 0.001
Lignin (DM) (g kg^{-1})	54 c	90 b	123 a	1.1	< 0.001
Polyphenols (DM) (eq-g tannic acid kg^{-1})	35 a	28 b	20 c	0.6	< 0.001
PAC (DM) (eq-g PAC kg^{-1})					
Total PAC	30 a	32 a	23 b	0.5	< 0.001
Extractable PAC	20.7 a	20.1 a	2 b	0.35	< 0.001
Protein-bound PAC	6.8 b	9 a	7.5 b	0.20	0.003
Fibre-bound PAC	2.2 c	3.3 b	13.5 a	0.18	< 0.001

react for 60 min at room temperature. Finally, absorbance at 520 nm was measured using the spectrophotometer. The method for determining FRAP was carried out according to Benzie and Strain,[22] with slight modifications. The FRAP solution was freshly prepared and kept at 37 °C; for the determinations, the solution was mixed in a 1:1:10 ratio (v:v:v) with a solution of 10 mmol L^{-1} 2,4,6-Tris(2-pyridyl)-*s*-triazine and 40 m$_M$ HCl, and a solution of 20 mmol L^{-1} FeCl$_3$·H$_2$O and a solution of 0.3 mol L^{-1} acetate buffer pH 3.6 was adjusted with HCl. Then, 20 μL of the diluted extracts and 280 μL of FRAP solution were allowed to react for 30 min at 37 °C. Finally, absorbance at 595 nm was measured using the spectrophotometer. Calibration was performed with Trolox (> 97% purity; Sigma-Aldrich, Sant Louis, MO, USA) standards (concentrations: 0.01 to 0.1 mg mL^{-1}, which were diluted in 50% [methanol:ultrapure water 50:50 (v:v) and adjusted to pH 2 with HCl] and a 50% [acetone:ultrapure water 70:30 (v:v) solution], which were subjected to the same process as the diluted extracts.

Statistical analysis

The statistical analyses were performed using SAS, version 9.1 (SAS Institute Inc., Cary, NC, USA). The analyses were performed separately for sainfoin and sulla because they were collected in different phenological stages. The FA profiles, secondary compounds and antioxidant activities were analysed with a general linear model that used the preservation method as a fixed effect. The least square means and their associated SEs and differences between means were obtained using Tukey

correction. The relationships between the AO activities and polyphenols, PAC, carotenoids and tocopherols were analysed by linear correlation using the CORR procedure in SAS. Stepwise multiple regression analyses were performed using the AO activity parameters as dependent variables and the secondary compounds as independent variables. *P* < 0.05 was considered to be statistically significant.

RESULTS

Sainfoin

Most of the individual FAs were more affected by haymaking than by ensiling (*P* < 0.05) (Table 2). Sainfoin hay contained higher proportions of all SFAs than fresh forage and silage (*P* < 0.05), except for C12:0, which was similar in the hay and the silage (*P* > 0.05). Silage had higher proportions than fresh forage of C14:0, C15:0 and C16:0 (*P* < 0.05). Consequently, the total SFAs were greatest in the hay forage, intermediate in silage and lowest in fresh forage (*P* < 0.001). Regarding individual MUFAs, the silage had greater C16:1 9c than hay and fresh forage, and higher C18:1 11c levels than the fresh forage (*P* < 0.05), with no differences in total MUFAs (*P* > 0.05). The fresh forage contained the greatest PUFAs, the silage contained intermediate levels, and the hay contained the lowest PUFAs (*P* < 0.001). Haymaking decreased C18:2 *n*-6 compared to fresh forage, whereas no effect was seen during ensiling (*P* < 0.001), and C18:3 *n*-3 decreased during both haymaking and ensiling respect to fresh forage, with the largest decrease in haymaking

Table 2. Effect of the preservation method on the fatty acids (FA), carotenoids, tocopherols and antioxidant (AO) activity of sainfoin

Item	Fresh	Hay	Silage	SEM	P
n	5	5	5		
FA, g FA kg^{-1} total FA					
C12:0	7.7 b	10.7 a	9.5 ab	0.33	0.02
C14:0	6.1 c	10 a	7.5 b	0.10	< 0.001
C15:0	1.2 b	2.6 a	1.5 b	0.07	< 0.001
C16:0	252 c	308 a	274 b	2.03	< 0.001
C16:1-9c	9.8 b	9.8 b	11.5 a	0.25	0.02
C18:0	126 b	165 a	137 b	2.91	0.001
C18:1-9c	20	24	22	1.24	0.46
C18:1-11c	3.1 b	4.3 ab	4.8 a	0.16	0.01
C18:2 n-6	127 a	107 b	132 a	1.50	< 0.001
C18:3 n-3	446 a	358 c	401 b	4.25	< 0.001
Saturated (SFA)	393 c	497 a	429 b	4.42	< 0.001
Monounsaturated (MUFA)	33	39	39	1.31	0.27
Polyunsaturated (PUFA)	574 a	465 c	532 b	4.76	< 0.001
Total FA (DM))g FA kg^{-1})	287 a	186 c	233 b	3.75	< 0.001
Carotenoids (DM) (mg kg^{-1})					
Neoxanthin	34 a	15 b	0 c	0.2	< 0.001
Violaxanthin	15 a	6 b	0 c	0.8	< 0.001
Lutein	174 a	104 b	66 c	0.6	< 0.001
Zeaxanthin	16 b	11 c	21 a	3.0	< 0.001
All-E-β-carotene	101 a	41 c	53 b	0.1	< 0.001
9z-β-carotene	10 b	4 c	14 a	0.4	< 0.001
13z-β-carotene	5 b	1.5 c	11.9 a	0.54	< 0.001
Tocopherols (DM) (mg kg^{-1})					
α-tocopherol	122 a	61 b	118 a	4.3	< 0.001
γ-tocopherol	9 b	11 a	10 ab	0.3	0.05
AO activity (DM) (eq-mmol trolox kg^{-1})					
Total radical-scavenging activity (ABTS)	360 a	335 a	246 b	10.9	0.003
Free radical scavenging activity (DPPH)	278 a	264 a	139 b	4.0	< 0.001
Ferric reducing antioxidant power (FRAP)	310 a	289 a	189 b	7.0	< 0.001

Within a parameter, means with different superscript differ at $P < 0.05$.
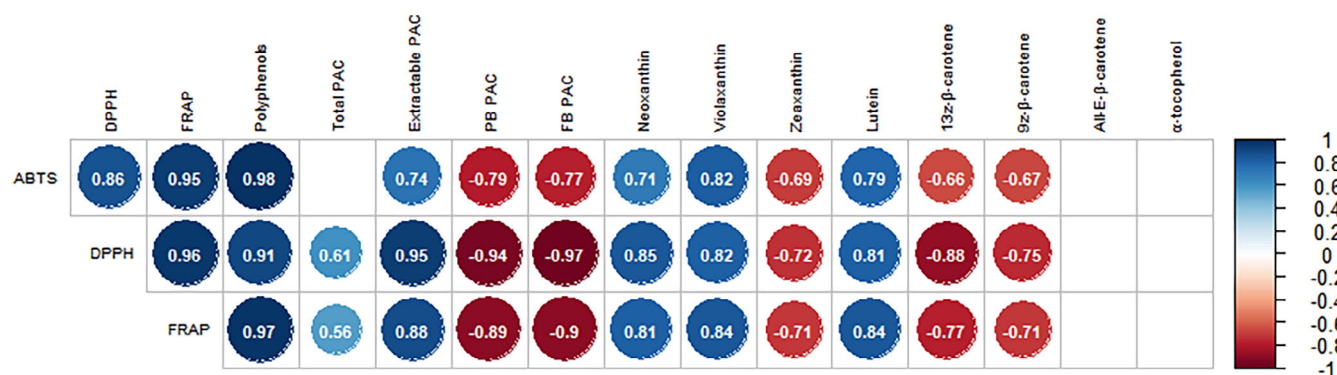
Figure 1. Significant Pearson correlations between antioxidant activity parameters [total radical-scavenging activity (ABTS), free radical scavenging activity (DPPH), ferric reducing antioxidant power (FRAP)] and polyphenols, proanthocyanidins (PAC), carotenoids and tocopherols in sainfoin.

($P < 0.001$). The total FA levels decreased by 35% for haymaking and by 19% for ensiling ($P < 0.001$).

The preservation method had an effect on all detected xanthophylls, carotenes and tocopherols ($P < 0.001$) (Table 2). Preservation decreased neoxanthin, violaxanthin, lutein and all-E-beta-carotene ($P < 0.001$), with a greater extent in the silage than in the hay. Haymaking decreased zeaxanthin, 9z- and

13z-β-carotene contents, whereas ensiling increased their contents ($P < 0.05$). Regarding tocopherols, haymaking decreased α-tocopherol and increased γ-tocopherol content ($P < 0.05$) whereas ensiling had no effect. Ensiling reduced the AO activities between 32% and 50% ($P < 0.05$) but haymaking did not affect the AO activities in comparison with fresh forage ($P > 0.05$). The AO activities were positively and strongly correlated with the

Table 3. Multiple regression equations for total radical-scavenging activity (ABTS), free radical scavenging activity (DPPH) and ferric reducing anti-oxidant power (FRAP) using the contents of polyphenols and proanthocyanidins (PAC) (mg kg^{-1}) in sainfoin

Dependent variable	Independent variable	a[a]	b[b]	SB[c]	Partial R[b]	Adjusted R[b]	MSE[d]
ABTS	Polyphenols	−2.1	7.2	0.38	0.966	0.963	152
DPPH	Polyphenols	123	2.72	0.42	0.045	0.985	67.2
	Total PAC		1.16	0.6	0.004		
	Fibre-bound PAC		−15	1.45	0.939		
FRAP	Polyphenols	5.2	4.98	0.45	0.944	0.978	80.9
	Extractable PAC		1.63	0.34	0.037		

[a] Intercept.
[b] Regression coefficients.
[c] Standard error of *b*.
[d] Mean square error of the model.

Table 4. Effect of the preservation method on the fatty acids (FA), carotenoids, tocopherols and antioxidant (AO) activity of sulla

	Fresh	Hay	Silage	SEM	*P*-value
n	4	5	5		
FA (g FA kg^{-1} total FA)					
C12:0	7.5 c	13.3 a	10.2 b	0.32	< 0.001
C14:0	5.4 c	8.8 a	6.6 b	0.13	< 0.001
C15:0	1.6 c	3 a	2 b	0.05	< 0.001
C16:0	256 b	304 a	291 a	3.1	< 0.001
C16:1-9c	6.7 b	6.7 b	8.8 a	0.19	< 0.001
C18:0	138 b	175 a	147 b	2.2	< 0.001
C18:1-9c	26 b	34 a	25 b	0.2	< 0.001
C18:1-11c	3.2 b	3.8 ab	4.6 a	0.12	0.003
C18:2 *n*-6	94 a	83 b	86 b	0.9	0.002
C18:3 *n*-3	462 a	368 c	419 b	4.4	< 0.001
Saturated (SFA)	409 c	504 a	457 b	5.1	< 0.001
Monounsaturated (MUFA)	36 c	45 a	38 b	0.3	< 0.001
Polyunsaturated (PUFA)	556 a	451 c	504 b	5.2	< 0.001
Total FA (DM) (g FA kg^{-1})	265 a	206 c	234 b	3.4	< 0.001
Carotenoids (DM) (mg kg^{-1})					
Neoxanthin	34 a	19 b	0 c	0.4	< 0.001
Violaxanthin	21 a	7 b	0 c	0.3	< 0.001
Lutein	134 a	111 b	84 c	3.2	< 0.001
Zeaxanthin	5 b	7 b	22 a	0.4	< 0.001
All-*E*-*β*-carotene	72 a	32 b	65 a	1.7	< 0.001
9*z*-*β*-carotene	8.7 b	2.2 c	13.9 a	0.27	< 0.001
13*z*-*β*-carotene	2.2 a	0.7 b	2.3 a	0.14	< 0.001
Tocopherols (DM) (mg kg^{-1})					
α-tocopherol	63 a	13 b	70 a	2.2	< 0.001
γ-tocopherol	4 ab	2.4 b	5 a	0.25	0.005
AO activity (DM) (eq-mmol trolox kg^{-1})					
Total radical-scavenging activity (ABTS)	228 a	197 a	113 b	5.1	< 0.001
Free radical scavenging activity (DPPH)	160 a	134 b	48 c	2.6	< 0.001
Ferric reducing antioxidant power (FRAP)	218 a	175 b	61 c	5.0	< 0.001

Within a parameter, means with different supercript differ at *P* < 0.05.

polyphenols and EPAC (*P* < 0.001), moderately related to total PAC (TPAC) and xanthophylls (except for zeaxanthin) (*P* < 0.001) (Fig. 1). The AO activities presented negative correlations with FB-PAC, PB-PAC, zeaxanthin, and 9*z*- and 13*z*-*β*-carotenes (*P* < 0.05 to 0.001) (Fig. 1). Regarding the multiple regression equation for AO activities, only polyphenols and PAC were admitted in the models (Table 3). For ABTS, polyphenols content was the only independent variable admitted in the model. For DPPH, FB-PAC was the variable that accounted for most of the variability, followed by polyphenols and TPAC. For FRAP, polyphenols content was the first variable in the model for FRAP followed by EPAC (which had a minor contribution).
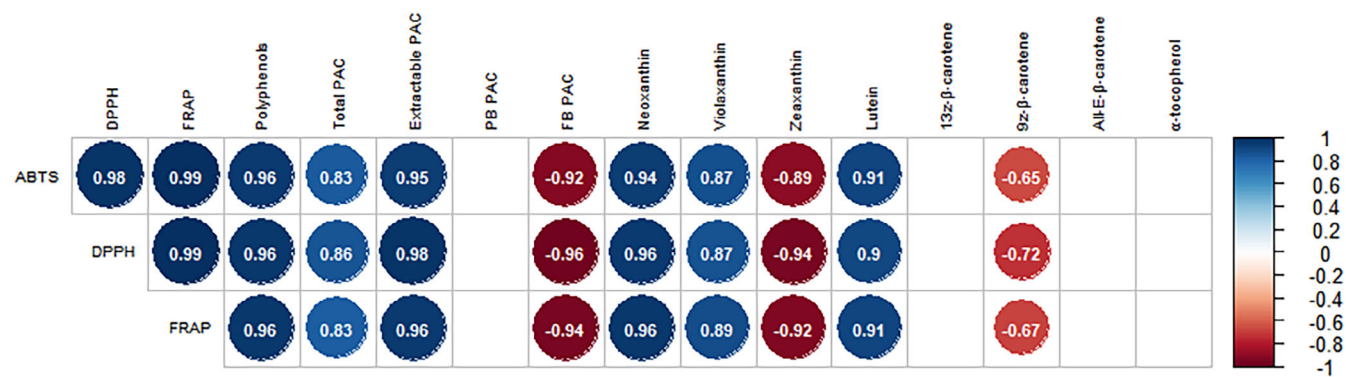
Figure 2. Significant pearson correlations between antioxidant activity parameters [total radical-scavenging activity (ABTS), free radical scavenging activity (DPPH), ferric reducing antioxidant power (FRAP)] and polyphenols, proanthocyanidins (PAC), carotenoids and tocopherols in sulla.

Table 5. Multiple regression equations for total radical-scavenging activity (ABTS), free radical scavenging activity (DPPH) and ferric reducing antioxidant power (FRAP) using the contents of polyphenols, proanthocyanidins (PAC) and carotenoids (mg kg^{-1}) in sulla

Dependent variable	Independent variable	a[a]	b[b]	SB[c]	Partial R[b]	Adjusted R[b]	MSE[d]
ABTS	Polyphenols	9.5	4.9	1.26	0.923	0.951	133
	Extractable PAC		2.45	0.81	0.04		
DPPH	Extractable PAC	−6.24	2.81	0.36	0.955	0.991	21.9
	Polyphenols		2.46	0.69	0.035		
	Neoxanthin		720	307	0.004		
FRAP	Polyphenols	41.7	3.72	1.43	0.930	0.98	99.9
	Extractable PAC		5.34	0.96	0.045		
	PB-PAC		−8.82	3.38	0.012		

[a] Intercept.
[b] Regression coefficients.
[c] Standard error of b.
[d] Mean square error of the model.

Sulla

Preservation had an effect on all individual and total FAs, secondary compounds and AO activities in sulla ($P < 0.001$) (Table 4). In general, individual and total SFAs and MUFAs increased, whereas the PUFAs decreased with both preservation methods, and haymaking exhibited a greater effect than ensiling ($P < 0.001$). Among carotenoids, ensiling and haymaking decreased neoxanthin, violaxanthin and lutein, with ensiling exhibiting the greatest effect ($P < 0.001$). Haymaking also decreased β-carotenes and α-tocopherol ($P < 0.001$). Ensiling increased zeaxanthin and 9z-β-carotene contents ($P < 0.001$) but had no effect on the other carotenes and tocopherols ($P > 0.05$). Regarding the AO activities, haymaking decreased DPPH and FRAP values by 16% and 20%, respectively ($P < 0.05$) whereas ensiling decreased ABTS, DPPH and FRAP values by 50%, 70% and 72%, respectively ($P < 0.001$). The AO activities were positively and very strongly correlated with the polyphenols, EPAC, TPAC, neoxanthin, lutein and violaxantin ($P < 0.001$) (Fig. 2) and negatively correlated with FB-PAC, zeaxantin and 9-z-β-carotene ($P < 0.05$ to 0.001). The multiple regression equations for AO activities included polyphenols, PPAC and neoxanthin (Table 5). For ABTS, polyphenols content accounted for 92% of the variability followed by EPAC, which only improved the accuracy 0.4%. Extractable PAC content was the first variable in the model for DPPH, followed by polyphenols and neoxanthin, which had minor contributions. For FRAP model, the inclusion of polyphenols accounted for 93% of the variability, followed by EPAC and PB-PAC.

DISCUSSION

To the best of our knowledge, the FA profiles, carotenoid and tocopherol contents, and antioxidant activities of hay, silage and fresh forage have not been compared before in sainfoin and sulla. The FA proportions of fresh sainfoin, hay and silage are in line with those reported by Lobón et al.,[9] Toral et al.[23] and Girard et al.,[24] respectively. Previous work has reported data for the carotenoids and tocopherols in fresh sainfoin,[25,26] but not for silage or hay. Cabiddu et al.[27] reported the FA profiles for fresh sulla at different stages and Priolo et al.[28] reported that fresh sulla before flowering contained the most abundant C18:3 n-3, C16:0 and C18:2 n-6 contents, but no data on the FA, carotenoids, tocopherols and antioxidant activity of preserved sulla are available.

Usually, the effects of preservation on the FA profiles are related to the losses from leaves during drying[29] and from handling before ensiling, such as chopping and crushing the forage,[30] which cause oxidative losses of the unsaturated FA present in the cut forage.[31] The reduction of total FA, C18:2 and C18:3 as a result of haymaking agrees with the results of the meta-analyses of Glasser et al.,[6] with the extent of decrease being determined mainly by adverse environmental conditions. In the present study, handling was carefully performed, and the losses from leaves were minimal based on the crude protein contents.[7] The smaller decreases in total FA and C18:3 in silage compared to hay were because the silage process protects PUFAs from the oxidative losses caused by the incidence of solar radiation and atmospheric

oxygen occurring during haymaking.[31] The more pronounced decreases in the individual PUFA concentrations and increases in individual SFAs with haymaking than with ensiling agrees with the results obtained for grass[32] and for mountain pastures.[31] However, in the present study, the 9–10% decrease of C18:3 and 12–23% decrease in total FA as a result of ensiling are greater than the effects reported in wilted silages by Glasser *et al.*[6] In this sense, Boufaïed *et al.*[33] reported that ensiling increased C16:0, C18:2, C18:3 and total FA because of a loss of some components during fermentation or a loss of soluble components in silage effluent increasing the concentration of other components. However, in the present study, there was no loss of soluble components in silage effluent because the silages were vacuum-packed and all of the content (solid + liquid) was lyophilized when opening it. The degradation of most carotenoids and tocopherols caused by haymaking was more marked than that resulting from ensiling as reported in a review of different species.[11] In hay, these compounds are easily degraded in the presence of UV irradiation and oxygen,[34,35] and these losses are higher when field sun-drying is performed under rainy weather conditions.[36] In the present study, despite the careful handling during sun-drying, hay contained lower carotene contents than the fresh forages, which confirms their high susceptibility to degradation under sun exposure. Regarding the effect of ensiling in the present study, the decreases of neoxanthin, violaxanthin, lutein and all-β-carotene agree with Nozière *et al.*,[11] but not the increases of zeaxanthin and 13z- and 9-z-β-carotene. Nozière *et al.*,[11] stated that the effect of the ensiling process on the carotenoid concentrations is affected more by high pH levels (i.e. pH > 5) than by light and oxygen. Zeaxanthin is formed from violaxathin in a reaction catalysed by violaxanthin de-epoxidase, for which the action is enhanced with pH < 5.8.[37] In the present study, both silages had pH values < 5.8; therefore, ensiling increased zeaxanthin at the expense of violaxanthin in both legumes. Similarly, the increase of 13z- and 9z-β-carotene contents due to the ensiling in both legumes, except for 13z-β-carotene in sulla, could be due to the isomerization reactions of All-*E*-β-carotene in both compounds[36] because *cis* isomerization mainly takes place due to increases of temperature,[38] which is a normal occurrence during silage fermentation. Regarding α-tocopherol, the literature contains very few studies of the effects of preservation of this vitamin. In the present study, α-tocopherol contents were reduced in hay but not in silage compared to fresh legumes. In *Sida hermaphrodita* herbage, Antoszkiewicz *et al.*[39] reported lower values of α-tocopherol and greater γ-tocopherol values in silage than in fresh forage. Lindqvist *et al.*[40] concluded that, when the ensiling process included a short wilting time and good weather conditions, it resulted in small losses of α-tocopherol.

The AO activities are a computation of the antioxidant effects of secondary compounds, among which the contents of polyphenols,[10] proanthocyanidins,[41] carotenoids[42] and tocopherols[43] stand out. In the present study, the AO activities were primarily related to the contents of polyphenols, for ABTS and FRAP assays (above 92%), and FB-PAC and EPAC, for DPPH in sainfoin and sulla, respectively, discarding any relevant impact of carotenoids and tocopherols.

CONCLUSIONS

The preservation method markedly affected the percentages of the FA and the contents of carotenoids and tocopherols in both legumes. Preservation decreased total FA with an increase in saturated FA percentages and a decrease in PUFA percentages, with greater effects on hay than on silage. The xanthophyll contents (except for zeaxanthin) were decreased more by ensiling than by haymaking. Regarding β-carotenes, haymaking decreased all the contents, whereas ensiling only decreased all-*E*-β-carotene, increasing the contents of the isomers. The contents of tocopherol were only negatively affected by haymaking. The antioxidant activities decreased more with ensiling than with haymaking and were strongly related to the total polyphenols and proanthocyanidins, but not to carotenoids, which had minor effects. Animal studies should be carried out to assess the impacts on animal performance and product quality before making further recommendations.

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