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Evaluation of the Nutritive Value and the Fatty Acid, Phenol, Tannin and Terpenoid Contents of Nine Pastures in an Alpine District during the Summer Season

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Abstract: A study was conducted over the summer of 2014 on nine Alpine pastures in the Chisone and Susa Valleys (NW Italy). The aim was to characterize the variation in the chemical composition, gross energy, in vitro true digestibility (IVTD), in vitro neutral detergent fiber digestibility (NDFD), fatty acids (FA), total phenols, total and condensed tannin contents, and terpenoid profile. The dry matter, ash, crude protein, acid detergent fiber, lignin, and gross energy contents of the pastures were found to differ. All the pastures had good IVTD (706–829 g/kg DM) and NDFD (487–694 g/kg NDF) values. The most abundant FAs in all the pastures were α-linolenic (354–519 g/kg of the total FAs), linoleic (75–110 g/kg of the total FAs), and palmitic acid (64–89 g/kg of the total FAs) and they differed significantly among pastures. No significant differences were found in the total phenols, or in the total and condensed tannin contents among pastures. Fifty-eight terpenoids were detected and 4-cyclopentene-1, 3-dione, β-caryophyllene, and eucalyptol were the most abundant. The terpenoids differed both qualitatively and quantitatively among pastures. The results highlight the importance of the great biodiversity of pastures, which provide a balanced distribution of fundamental nutritional elements and bioactive compounds in grasslands.

Keywords: chemical composition; digestibility; nutritive value; grassland; bioactive compound

1. Introduction

Knowledge of the botanical composition of pastures, in terms of the nutritional quality, the fatty acid (FA) and tannin contents and the terpenoid profile, is a key element in ensuring good coverage of the nutritional needs of livestock and correct classification of the pastures [1].

Several studies have shown that forage species, as well as maturity and environmental conditions, may determine considerable variations in the FA content of pastures [1–3] and forages [4–6]. Alpine pastures have been reported to be rich in phenols, and tannins in particular, and these compounds have been studied because of their potential role in the reduction of ruminant methane production through a modification of ruminal fermentation [7,8]. The interest in forage tannins for ruminants is also related

to their capacity to reduce the loss of native plant FAs, such as α-linolenic acid (ALA), during rumen digestion by means of the partial inhibition of ruminal biohydrogenation [9], as observed in both in vitro and in vivo studies [10,11].

Terpenoids are important constituents of the essential oils of plants and are emitted, by plants of different botanical species, as semiochemicals [12]. Moreover, they are more abundant in dicotyledons, such as the *Apiaceae* and *Asteraceae* plant families, but are present in lower amounts in some others, like *Fabaceae*. Monocotyledons are usually poor in these compounds, although, in some cases, they can emit terpenoids as a defense against insects [13].

From a physiological point of view, terpenoids are produced at different levels during the phenological stage of plants, and their production is influenced to a great extent by environmental factors [14]. Moreover, a potential impact of terpenes on milk and dairy products, especially when derived from diverse wild pastures has been shown [15]. A much greater diversity of the terpenes of milk obtained from animals grazing on pasture than of the milk derived from animals reared in confined systems has in fact been suggested [15]. These authors reported that several studies had found that different monoterpenes and sesquiterpenes were able to fully discriminate milk from highland, lowland, pasture, and indoor feeding.

In comparison to indoor feeding substances, mountain pastures are usually rich in plant families characterized by high levels of terpenoids. These strongly scented molecules are transferred directly to the milk fat, and they offer unique characteristics to many protected designations of origin cheeses produced during the grazing season all over the Alps. For this reason, their terpenoid profiles have been proposed as biomarkers to trace mountain cheeses [16]. Mountain pastures are therefore essential for the local economy and land conservation and are of paramount importance for rural sustainable development [17].

To provide valuable support for the natural environment and economic revitalization of local Alpine valley communities, the aim of this research was to characterize the nutritional quality and bioactive compounds of nine Alpine summer pastures sampled from the end of June to early July in the Chisone and Susa Valleys (NW Italy). Fresh grass derived from these grasslands is the prevalent forage resource of the dairy cows in these valleys. These cows are used to produce a typical cheese named "Plaisentif," which is produced during the violet (*Viola tricolor*) flowering period, this being a mandatory requirement for the product specifications of this typical Alpine cheese.

2. Materials and Methods

2.1. Pasture Sites, Sampling, and Phyto-Pastoral Analysis

A phyto-pastoral survey was conducted in nine summer pastures located at different altitudes from 1620–2070 m a.s.l. (Table 1), as described by Peiretti et al. [18]. Six of these pastures are located in the Chisone Valley and they have been identified herein with the following abbreviations: A1, A2, A3, A6, A7, and A8, while the other three pastures, A4, A5, and A9, are in the Susa Valley.

Table 1. Sampling date and geolocation of the investigated pastures (A1–A9) during the 2014 summer season.

	A1	A2	A3	A4	A5	A6	A7	A8	A9
Sampling date	03/07/2014	30/06/2014	25/06/2014	03/07/2014	25/06/2014	25/06/2014	30/06/2014	30/06/2014	03/07/2014
Latitude	45°2′51″	45°3′50″	44°57′5″	44°57′24″	44°57′21″	44°58′55″	44°59′12″	45°3′43″	44°54′59″
Longitude	7°7′21″	7°2′2″	6°57′20″	6°48′33″	6°50′50″	6°55′43″	6°54′36″	7°2′52″	6°53′42″
Altitude	2040	1870	1807	1620	2070	1785	1867	1900	1870

These pastures are semi-natural grasslands, without any kind of fertilization, with the exception of pasture A4, which was seeded with resistant plants (monocotyledons and *Leguminosae*) and is used as a ski slope in winter. They were traditionally grazed under rotational grazing systems. None of the

pastures were ever grazed before the sampling day and, in agreement with the owners of the pastures, were always grazed the day after the sampling.

The botanical composition of the pastures was determined according to the linear analysis method proposed by Daget and Poissonet [19] following the procedure described by Peiretti et al. [18]. The vegetation of the investigated areas was surveyed along 20 m transects laid out on representative and homogeneous meadows. A metric ribbon was used to trace two transects and an iron rod was inserted into the turf at 50 cm intervals (40 insertions along each transect). The plants in contact with the iron rod were recorded at each insertion.

The chemical analysis sampling was performed using the hand-plucking technique [20]: two separate samples (2 kg each) of herbage were collected in ten points per field above and below the metric ribbon in the transect area, respectively. Plants were cut to a 1–2 cm stubble height, with edging shears, in the morning after evaporation of the dew and were never collected on rainy days. All the samples were immediately refrigerated until the arrival at the laboratory.

The vegetation species were identified by means of the Pignatti dichotomous key [21], and complete results of the phyto-pastoral analysis were reported by Peiretti et al. [18], together with the number of times a plant species was present in a given survey (Species Frequency, SF), the ratio between the SF of a considered species and the sum of the SF of all the species that were present (Species Contribution, SC).

2.2. Chemical Analysis

An aliquot of 200 g was used for each pasture sample, according to the Association of Official Analytical Chemists method [22] to determine the dry matter (DM) content (#925.40) in duplicate. Another aliquot of 200 g was immediately refrigerated, freeze-dried, and then brought to room temperature, ground in a Cyclotec mill (Tecator, Herndon, VA, USA) to pass through a 1-mm sieve and stored for qualitative analyses. The freeze-dried samples were analyzed by means of the AOAC methods for total N (#984.13) and ash (#923.03) [22]. Neutral detergent fiber (NDFom), and acid detergent fiber (ADFom) were determined as described by Van Soest et al. [23] and expressed exclusive of residual ash, while lignin was determined by solubilization of cellulose with sulfuric acid, as described by Robertson and Van Soest [24], using an Ankom200 Fiber Analyzer (Ankom Technology Corp., Fairport, NY, USA). The NDF of pasture samples was analyzed without sodium sulfite or α-amylase. An adiabatic calorimeter bomb (IKA C7000, Staufen, Germany) was used to determine the gross energy (GE) content, while the lipid content was quantified according to the Hara and Radin method [25]. In vitro true digestibility (IVTD) was determined using an Ankom-Daisy incubator (Ankom Technology Corp.) and the in vitro neutral detergent fiber digestibility (NDFD) concentration was subsequently determined using a fiber analyzer (Ankom Technology Corp., Fairport, NY, USA), as previously reported by Peiretti et al. [18]. All the determinations were performed in duplicate.

2.3. Fatty Acid Analysis

The FA analysis was performed on a freeze-dried pasture sample (2 g) according to the method described by Revello Chion et al. [26]. The FA methyl esters (FAME) in hexane were injected into a gas chromatograph (Dani Instruments S.P.A. GC 1000 DPC; Cologno Monzese, Italy) equipped with a flame ionization detector (FID) and a PTV injection port. The separation of the FAME was performed with a Supelcowax-10 fused silica capillary column (60 m, 0.32 mm (i.d.), 0.25 lm). The peak area was measured using a Dani DDS 1000 Data Station. Each peak was identified according to pure methyl ester standards (Supelco, Bellefonte, PA, USA) and the data were expressed as relative values. The FA composition was expressed as g/100 g of FA.

2.4. Phenolic Fraction Determination

The following phenolic fractions: total phenols (TP), total tannins (TT), and condensed tannins (CT) were determined in the samples. Sample determinations of TP and TT were carried out on the basis of the method described by Makkar et al. [27]. In this way, TP and TT were assessed by means of

a Folin–Ciocalteu reactive, using the ability of polyvinylpolypyrrolidone to bind tannins and therefore to separate non-tannin phenols from tannin phenols. Both the TP and TT values were expressed as mg/kg of DM. The condensed tannins (CT) were analyzed by means of the butanol–HCl–iron method, as described by Porter et al. [28]. CT values were given as leucocyanidin equivalents.

2.5. Terpenoid Analysis

Terpenoid analysis was carried out on a freeze-dried pasture sample (200 mg), extracted without the use of solvents, according to the method described by De Noni and Battelli [29], by means of dynamic headspace extraction (Dani Instrument, Cologno Monzese, Italy). Briefly, the extraction conditions were: 5 min equilibrium at 65 °C, purging with 500 ml helium (high-grade purity), adsorption at 40 °C on a Tenax-TA trap (270 mg), desorption at 280 °C for 3 min. The obtained data were expressed as arbitrary units, as the peak area of the Total Ion Chromatogram $\times 10^{-6}$.

2.6. Statistical Analysis

The variability in the nutritive value, FA, total phenols, and tannin contents of the pastures was analyzed, to establish their statistical significance, by means of an analysis of variance (ANOVA), using the Statistical Package for Social Science [30] to test the effect of pasture. When the values of F were significant ($p < 0.05$), the Ryan-Einot-Gabriel-Welsch range test was used to detect any differences in the means [31]. To examine the relationships between the flora characteristics and the terpenoid profile of the pastures Principal Component Analysis was used [32].

3. Results

3.1. Botanical Composition of the Pastures

The main forage groups present in the sampled pastures are reported in percentage in Figure 1. The A1 pasture in the Chisone Valley was dominated by *Gramineae* and *Leguminosae* (36% and 20% of SC, respectively), A2 by *Gramineae* and *Leguminosae* (40% and 14% of SC, respectively), A3 by *Gramineae* and *Leguminosae* (both 23% of SC), A6 by *Gramineae* and *Umbelliferae* (20% and 19% of SC, respectively), A7 by *Asteraceae* and *Gramineae* (28% and 25% of SC, respectively) and A8 by *Gramineae* and *Asteraceae* (34% and 14% of SC, respectively). In the Susa Valley pastures, A4 was dominated by *Leguminosae* and *Gramineae* (25% and 21% of SC, respectively), A5 by *Gramineae* and *Asteraceae* (26% and 20% of SC, respectively) and A9 by *Leguminosae* and *Gramineae* (18% and 17% of SC, respectively).

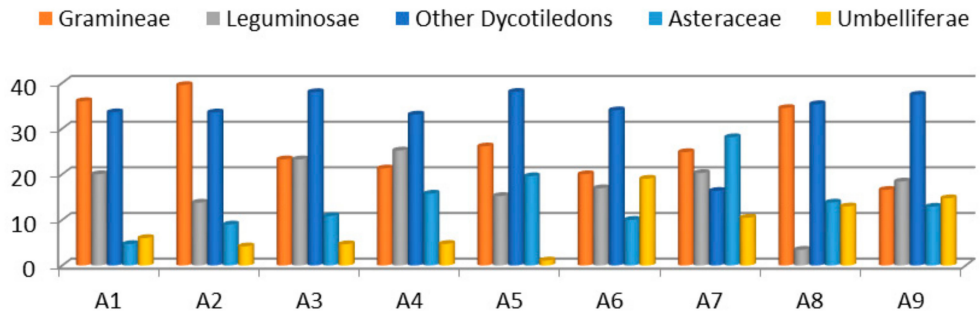

Figure 1. Main forage groups (as the mean percentages of the total number of collected plants) present in the pastures (A1–A9).

Apart from the above-quoted predominant families, there was also a considerable presence of other dicotyledons in some pastures, and in particular: *Polygonaceae* and *Orobanchaceae* in A2 (13% and 7% of SC, respectively), *Rubiaceae* and *Lamiaceae* in A4 (16 and 9% of SC, respectively), *Violaceae* in A6 (9% of SC), *Dipsacaceae* in A9 (8% of SC), and *Ranunculaceae* in A8 (11% of SC).

3.2. Chemical Composition and In Vitro Digestibility

The chemical composition and in vitro digestibility of the pastures are reported in Table 2. No differences were observed in the lipid and NDFom contents. The mean values were 228 g/kg fresh matter for dry matter (DM), 81 g/kg DM for ash, 138 g/kg DM for crude protein (CP), 343 g/kg DM for ADFom, 102 g/kg DM for lignin and 17.7 MJ/kg DM for GE contents in the pastures differed significantly ($p < 0.05$).

Table 2. Chemical composition (g/kg DM basis), gross energy (GE), in vitro true digestibility (IVTD), in vitro neutral detergent fiber digestibility (NDFD) of the pastures (A1–A9).

	A1	A2	A3	A4	A5	A6	A7	A8	A9	S.E.M.
DM (g/kg)	218.8^{ab}	190.3^{a}	229.2^{b}	276.8^{c}	207.4^{ab}	195.3^{ab}	225.8^{ab}	231.8^{b}	273.7^{c}	7.2
Ash	77.8^{abc}	75.0^{abc}	64.7^{ab}	63.4^{a}	82.1^{abc}	106.6^{d}	94.7^{cd}	88.0^{bcd}	74.9^{abc}	3.3
Crude protein	158.0^{d}	150.2^{bcd}	157.7^{d}	116.1^{a}	153.0^{cd}	130.4^{abc}	127.4^{ab}	126.4^{ab}	122.0^{ab}	3.5
Lipid	23.7	19.1	15.5	17.8	18.0	14.0	15.2	14.8	17.5	1.1
NDFom	562.6	587.6	534.6	575.1	509.2	515.8	522.9	551.0	523.7	8.8
ADFom	333.5^{a}	333.4^{a}	329.7^{a}	403.2^{b}	325.4^{a}	349.0^{a}	338.1^{a}	337.5^{a}	339.5^{a}	6.0
Lignin	89.7^{ab}	109.3^{ab}	117.3^{b}	98.7^{ab}	108.4^{ab}	98.9^{ab}	79.2^{a}	103.4^{ab}	110.4^{b}	3.0
GE (MJ/kg DM)	17.5^{a}	17.8^{a}	18.5^{b}	17.6^{a}	17.6^{a}	17.6^{a}	17.5^{a}	17.6^{a}	17.8^{a}	0.1
IVTD (g/kg DM)	829.3	790.3	811.8	705.7	829.1	819.8	803.4	816.5	759.9	11.1
NDFD (g/kg NDF)	694.4	643.5	648.7	487.0	664.0	650.1	621.3	669.0	540.5	19.3

^{a,b,c,d} Within a row, values with different letters differ ($p < 0.05$); S.E.M., Standard Error Mean.

No differences were observed in the IVTD and NDFD digestibilities. All the pastures are highly digestible, in fact, IVTD ranged between 705 and 829 g/kg DM, while NDFD ranged between 487 and 669 g/kg NDF.

3.3. Fatty Acid Profile

Regarding the FA content, the most abundant FAs in all the pastures were ALA, linoleic acid (LA) and palmitic acid (PA) and they significantly differed among pastures ($p < 0.05$). Some minor FAs (stearic, oleic, and γ-linolenic acid) overall accounted for 45 to 73 g/kg of the total FAs, and they did not differ significantly among pastures (Table 3).

Table 3. Fatty acid (g/kg of total FA), total phenols (g/kg DM), total tannin (g/kg DM), and condensed tannin (mg leucocyanidin equivalent/g DM) of the pastures (A1–A9).

	A1	A2	A3	A4	A5	A6	A7	A8	A9	S.E.M.
Palmitic acid	63.9^{a}	65.9^{ab}	88.8^{b}	88.9^{b}	85.3^{ab}	86.2^{ab}	81.9^{ab}	87.6^{b}	79.8^{ab}	2.5
Stearic acid	16.4	16.3	26.4	23.3	21.2	24.6	23.3	26.3	33.0	1.5
Oleic acid	11.2	14.6	18.4	17.6	13.4	10.7	11.9	16.1	19.5	1.0
Linoleic acid	104.0^{bcd}	104.1^{cd}	88.5^{ab}	88.9^{abc}	104.4^{cd}	124.7^{e}	105.0^{cd}	74.9^{a}	110.2^{de}	8.4
γ-Linolenic acid	23.8	21.5	11.5	11.8	22.3	9.8	12.7	10.7	20.9	1.6
α-Linolenic acid	354.3^{a}	395.7^{ab}	519.1^{c}	503.2^{bc}	470.5^{abc}	438.3^{abc}	453.2^{abc}	518.7^{bc}	420.2^{abc}	14.3
Total phenols	36.3	44.3	61.2	49.2	53.5	52.7	37.0	42.5	52.0	2.6
Total tannins	29.4	34.8	49.9	39.2	43.5	43.4	24.6	34.4	41.4	2.6
Condensed tannins	82.6	243.1	224.3	179.7	71.6	157.1	23.9	71.6	114.6	22.7

^{a,b,c,d,e} Within a row, values with different letters differ ($p < 0.05$); S.E.M., Standard Error Mean.

3.4. Total Phenols, and Total and Condensed Tannins

No significant differences were found in the total phenols, or in the total and condensed tannin contents among pastures (Table 3). The TP and TT values ranged from 36 to 61 g/kg DM and from 25 to 50 g/kg DM, respectively. The values of the CT content ranged from 24 to 243 mg leucocyanidin equivalent/kg DM, respectively.

3.5. Terpenoid Profile

A total of 58 volatiles, mainly terpenes, were found in the collected pasture samples (Table 4). 4-cyclopentene-1,3-dione, β-caryophyllene, and eucalyptol were the most abundant terpenes, and they were detected in different concentrations in the pasture samples. Other volatiles (δ-3-carene, allo-ocimene, γ-curcumene, copaene) were only present in a few locations. Therefore, a great difference in the composition of the volatiles was observed among the pastures.

Table 4. Terpenoid content range (the min and max data are expressed as arbitrary units of the Total Ion Chromatogram peak areas × 10^{-6}) of the pastures (A1–A9).

	Compound	Chemical Abstract Service Number	Retention Index	Pasture																	
				A1		A2		A3		A4		A5		A6		A7		A8		A9	
				min	max	min	max	min	max	min	max	min	max	min	max	min	max	min	max	min	max
1	α-pinene	80-56-8	*1023*	11.68	62.69	9.78	16.42	19.78	43.31	0.73	1.22	7.15	30.51	31.83	36.61	47.84	53.41	1.50	4.96	11.80	59.07
2	camphene	79-92-5	*1065*	2.11	12.20	1.40	4.68	4.62	6.29	0.13	0.20	2.24	2.54	2.16	2.81	2.67	19.97	0.20	0.85	1.44	5.32
3	L-β-pinene	18172-67-3	*1105*	3.82	5.85	1.47	1.76	2.83	7.84	1.29	1.77	7.64	10.54	1.96	2.19	6.66	8.76	0.27	0.55	0.39	3.40
4	β-pinene	127-91-3	*1113*	11.66	15.08	8.26	11.02	13.60	41.18	3.26	5.60	23.93	34.14	4.77	6.83	23.57	41.29	0.34	1.46	1.92	24.94
5	unidentified		*1125*	-	-	0.65	3.40	1.66	6.86	-	-	-	6.29	-	-	-	-	-	-	-	-
6	δ-3-carene	13466-78-9	*1125*	-	-	-	-	-	-	-	-	-	1.41	8.37	22.36	-	-	-	-	5.26	8.17
7	sabinene	3387-41-5	*1142*	7.61	10.09	-	-	-	-	-	-	-	-	-	-	10.55	14.87	-	-	-	-
8	β-myrcene	123-35-3	*1166*	32.75	89.77	-	-	-	23.72	1.25	2.15	-	1.10	15.79	36.34	7.16	16.81	-	-	-	5.84
9	α-terpinene	99-86-5	*1185*	-	5.24	1.46	2.46	-	7.67	-	-	1.40	5.58	8.75	20.01	13.53	15.37	-	-	7.06	10.74
10	limonene	5989-27-5	*1204*	12.79	41.70	9.54	69.15	9.10	24.29	1.31	3.24	7.63	10.62	26.45	31.16	31.42	37.96	-	1.13	7.96	10.28
11	eucalyptol	470-82-6	*1217*	14.95	15.35	12.15	35.07	44.98	96.58	2.91	4.01	12.47	25.88	12.50	38.89	149.37	244.43	2.13	5.71	28.75	47.22
12	β-ocymene	3779-61-1	*1237*	4.23	16.92	-	0.60	1.23	1.65	-	-	0.53	0.59	2.85	4.91	3.01	4.87	-	0.16	0.58	2.23
13	γ-terpinene	99-85-4	*1252*	13.02	15.83	3.98	5.32	5.77	23.11	1.57	1.62	8.38	8.80	27.02	44.46	27.55	31.98	0.90	3.24	13.90	50.86
14	*o*-cymene	527-84-4	*1278*	8.63	11.69	1.53	3.44	3.48	30.25	0.29	0.34	2.25	20.50	13.34	14.23	32.29	44.32	1.01	1.17	19.29	91.13
15	terpinolene	586-62-9	*1290*	20.01	21.42	3.33	4.35	-	11.12	-	1.85	1.79	2.50	22.57	27.89	4.90	13.61	1.15	3.11	2.01	2.75
16	*allo*-ocimene	3016-19-1	*1377*	5.48	43.02	0.25	0.29	0.68	1.13	0.21	0.25	0.42	0.68	6.65	13.22	3.53	5.70	-	0.34	0.65	1.62
17	matsutakeol	3391-86-4	*1453*	33.93	33.81	22.57	29.28	29.02	35.29	18.85	18.96	26.71	33.77	14.66	22.77	27.24	42.86	12.88	44.92	19.39	32.08
18	t-chrysanthenol	38043-83-3	*1491*	1.19	1.44	0.00	0.60	0.71	0.94	0.37	0.51	0.68	0.70	3.70	11.38	11.77	25.66	-	0.52	1.06	5.80
19	α-copaene	1000360-33-0	*1511*	5.19	21.05	2.38	4.98	5.91	8.83	1.89	5.79	2.00	8.94	7.74	11.61	13.72	26.81	0.36	2.52	5.25	6.35
20	(t.t)3.5-octadien-2-one	30086-02-3	*1531*	4.41	8.39	2.71	3.69	2.32	2.45	1.25	1.65	1.22	1.37	2.08	4.84	4.80	5.17	1.15	3.33	1.66	1.71
21	t-chrisanthenyl acetate	50764-55-1	*1543*	5.33	13.01	0.79	2.75	10.90	12.97	2.04	2.23	1.94	2.01	7.82	8.71	3.34	35.89	1.63	4.19	-	-
22	2-bornanone	464-49-3	*1546*	-	-	-	15.39	-	-	-	-	-	-	-	-	-	147.47	-	1.66	17.71	23.85
23	linalyl acetate	115-95-7	*1550*	4.51	6.58	0.47	2.33	-	3.69	-	0.35	1.62	2.46	1.47	2.93	10.25	72.83	-	0.44	4.06	9.00
24	calarene	17334-55-3	*1558*	2.07	9.92	1.08	2.44	2.26	9.05	-	-	0.30	1.42	2.16	6.50	6.92	11.66	-	0.82	1.76	2.36
25	4-cyclopentene-1.3-dione	930-60-9	*1604*	80.59	98.18	44.94	60.05	55.14	237.28	10.20	10.26	1.03	1.77	3.48	25.74	69.11	138.91	53.70	210.23	56.52	104.18
26	β-elemene	33880-83-0	*1611*	-	14.53	-	11.69	-	-	-	1.71	-	-	3.90	8.31	-	-	-	-	-	-
27	isoledene	95910-36-4	*1617*	6.10	14.20	3.62	6.13	-	16.09	0.81	3.88	-	-	6.38	9.37	19.79	22.50	0.67	7.48	5.40	7.92
28	β-caryophyllene	87-44-5	*1623*	60.02	162.31	7.39	14.12	22.45	29.32	18.19	26.57	18.51	21.09	33.38	45.29	57.46	98.28	1.62	11.40	46.77	77.63
29	*cis*-calamenene	72937-55-4	*1657*	5.33	22.74	3.40	6.92	8.92	16.59	1.47	6.34	1.83	7.48	16.37	15.80	22.53	31.77	0.53	4.47	6.72	9.14
30	unidentified		*1662*	6.72	26.76	5.71	10.87	12.54	23.45	2.05	10.37	3.02	10.46	18.55	24.52	34.92	46.62	0.73	6.07	10.54	13.90
31	β-farnesene	28973-97-9	*1669*	12.45	24.63	1.34	1.41	3.60	4.91	12.49	12.98	1.54	1.90	-	5.98	27.50	47.94	0.20	5.35	32.06	36.47
32	β-sesquiphellandrene	555-10-2	*1684*	4.64	20.59	5.91	12.77	-	-	1.59	7.15	-	-	-	-	-	-	-	-	7.86	10.13
33	α-elemene	5951-67-7	*1696*	18.88	103.70	-	-	16.03	30.29	3.86	13.94	3.23	11.67	21.70	37.82	-	-	0.62	6.94	16.05	22.03
34	unidentified		*1696*	-	-	7.54	14.47	-	-	-	-	-	0.96	-	-	45.66	64.43	-	-	-	-
35	γ-curcumene	28976-68-3	*1702*	2.00	13.31	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
36	δ-elemene	20307-84-0	*1706*	-	-	-	23.62	-	-	-	-	-	13.18	-	-	-	-	1.02	6.65	-	-
37	γ-muurolene	30021-74-0	*1706*	6.84	26.92	-	11.86	15.25	28.36	2.70	10.80	3.46	13.18	17.64	24.45	45.18	63.38	-	-	12.15	15.18
38	10-epi-β-acoradiene	28477-64-7	*1710*	-	5.78	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
39	viridiflorene	21747-46-6	*1713*	3.99	12.95	3.56	5.52	3.16	7.44	0.54	2.25	0.75	2.85	3.70	4.93	-	8.79	-	1.95	4.53	5.03

Table 4. *Cont.*

	Compound	Chemical Abstract Service Number	Retention Index	Pasture																	
				A1		A2		A3		A4		A5		A6		A7		A8		A9	
				min	max	min	max	min	max	min	max	min	max	min	max	min	max	min	max	min	max
40	borneol	507-70-0	*1714*	-	18.58	-	0.79	1.05	2.10	-	-	0.19	-	-	4.08	3.21	65.53	-	-	-	-
41	epizonarene	41702-63-0	*1723*	6.34	28.50	4.55	8.76	12.76	25.96	2.84	13.32	3.01	12.44	20.77	24.57	36.68	46.20	-	4.26	11.97	14.94
42	α-muurolene	10208-80-7	*1731*	7.07	30.15	4.92	9.62	11.64	29.61	1.43	7.55	2.15	8.96	13.57	18.82	43.54	51.02	-	4.48	8.06	11.28
43	eremophilene	10219-75-7	*1736*	12.53	141.01	3.66	12.97	5.93	6.50	1.27	7.26	1.11	6.91	13.50	21.90	14.04	15.99	-	3.13	6.34	6.90
44	carvone	99-49-0	*1743*	2.98	5.13	1.07	21.47	2.16	2.86	0.13	12.75	-	0.53	2.08	3.76	12.19	120.46	-	1.53	2.06	13.90
45	δ-cadinene	483-76-1	*1754*	10.35	50.07	8.42	14.36	18.75	32.61	3.65	16.39	4.26	18.20	27.17	34.13	-	47.20	-	7.91	15.40	19.96
46	β-cadinene	523-47-7	*1755*	-	-	-	-	-	-	-	-	-	-	-	-	-	68.78	-	-	-	-
47	germacrene	28387-44-2	*1759*	-	-	-	-	10.91	20.96	-	-	-	2.52	-	-	-	-	-	4.63	-	-
48	γ-cadinene	39029-41-9	*1760*	11.31	29.55	4.55	8.02	-	-	2.22	8.95	-	10.57	13.29	18.57	27.57	40.72	-	-	9.43	12.44
49	copaene	3856-25-5	*1772*	-	25.00	-	-	-	-	-	-	1.41	5.33	-	-	-	-	-	-	-	-
50	cadinadiene	16728-99-7	*1774*	-	4.22	2.30	2.97	4.44	9.22	-	4.02	-	5.33	-	12.05	9.85	17.87	-	-	-	-
51	selina-3.7(11)-diene	6813-21-4	*1775*	-	-	-	2.10	-	-	-	-	-	-	-	17.41	3.95	10.40	-	-	-	-
52	α-cadinene	24406-05-1	*1780*	2.01	9.02	-	3.21	4.16	7.95	0.66	3.31	0.91	3.99	6.20	8.38	10.25	15.99	-	1.89	2.98	3.88
53	calamenene	483-77-2	*1808*	3.06	10.40	2.73	4.30	6.74	10.03	2.09	6.77	2.61	6.63	8.88	9.42	11.83	14.11	1.11	2.57	5.72	6.79
54	α-patchoulene	560-32-7	*1835*	1.46	8.40	-	0.58	0.70	1.49	0.27	0.54	0.15	1.36	3.56	10.90	2.85	3.75	0.10	0.30	0.96	1.00
55	α calacorene	21391-99-1	*1869*	1.29	6.44	0.85	1.76	2.40	4.22	0.78	2.29	0.66	2.30	3.01	4.99	5.11	6.26	0.19	0.94	3.98	5.20
56	thymol	89-83-8	*2043*	0.32	0.58	-	0.10	0.58	16.39	-	0.31	0.16	0.45	0.59	0.63	0.60	0.70	-	-	0.48	72.28
57	carvacrol	499-75-2	*2070*	0.15	0.20	-	0.21	-	0.54	-	0.12	-	-	-	-	0.86	1.51	0.19	0.21	0.32	3.02
58	cadalene	483-78-3	*2102*	1.33	1.34	0.57	0.73	0.74	1.16	0.18	0.65	0.27	0.55	0.64	1.18	1.64	2.09	0.80	1.30	0.72	0.87

Regarding the total terpenoids, a huge difference was detected among the pastures, with location A7 being eight-fold richer than the A4 pasture (Figure 2). The relative composition of the volatile organic compounds also differed. Apart from the most abundant terpenoids cited above, the A1 pasture was mainly characterized by eremophilene, α-elemene, β-myrcene, and copaene, and γ-curcumene and 10-epi-β-acoradiene were present only in this location.

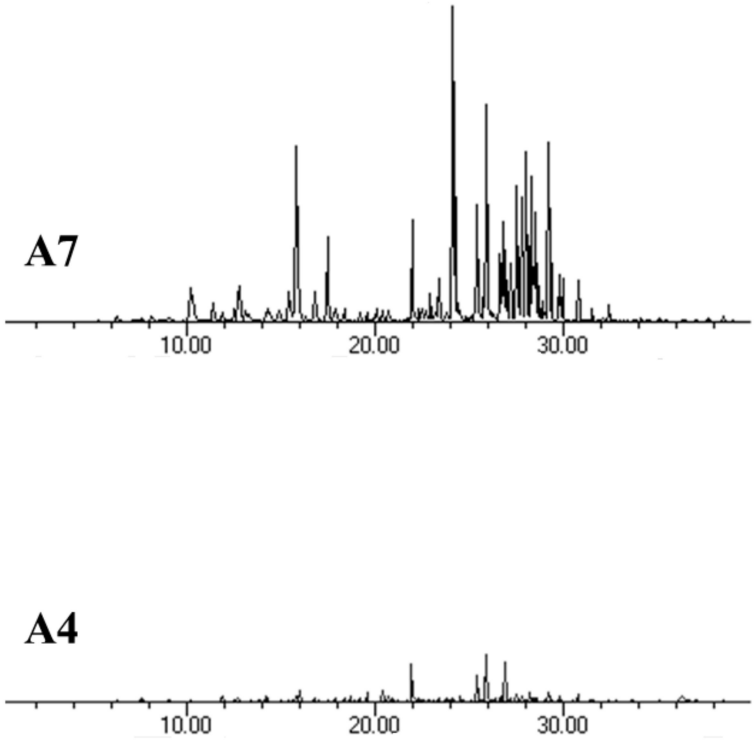

Figure 2. Example of chromatograms of the terpenoid analysis performed according to the method described by De Noni and Battelli [29]. The letters (A7 and A4) refer to the richest and poorest pastures, respectively.

With regard to the other locations, the A2 pasture was mainly characterized by limonene and δ-elemene, the A3 pasture was characterized by eucalyptol and germacrene, the A4 pasture, which showed the lowest level of total terpenoids, was characterized by β-farnesene, the A5 pasture was characterized by β-pinene and eucalyptol and the A6 pasture was characterized by δ-3-carene, 3,7(11)-selinadiene, γ-terpinene, and α-pinene. Moreover, almost all the individual terpenoids in the A7 pasture showed the highest concentrations, and it is therefore not surprising that the A7 pasture was the richest location in total volatiles: among these volatiles, the most relevant were eucalyptol, 2-bornanone, carvone, γ-muurolene, and α-pinene.

However, the A8 pasture was as poor as the A4 pasture in total terpenoids and did not show any particular compound, other than the previously cited ones that were present in all the samples. Finally, the A9 pasture was characterized by *o*-cymene and thymol.

The huge variability in the terpenoid levels was also shown by means of a Principal Component Analysis, which was applied to all the data (Figure 3). The richest pastures (A7 and A1) are visible on the left side of the plot, which is shown in Figure 3, while the poorest (A4 and A8) can be observed on the right side. Interestingly, the two samplings performed in the same pasture showed very different terpenoid profiles.

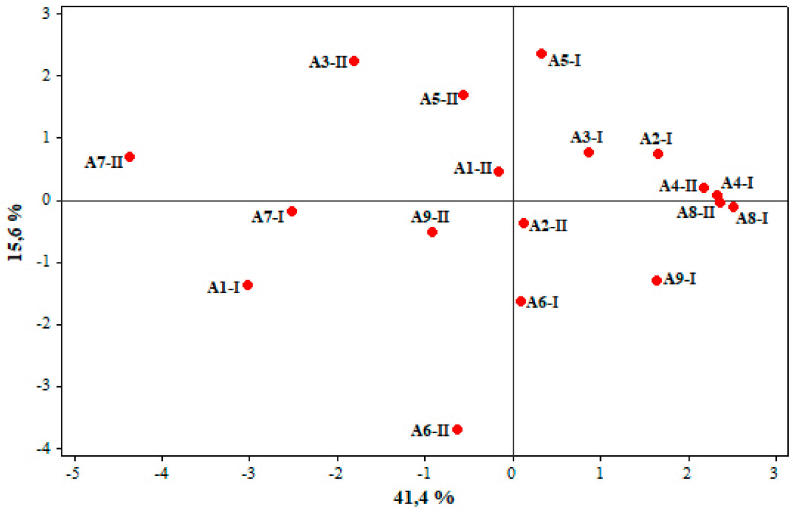

Figure 3. Principal Component Analysis applied to the volatiles identified in the first (I) and second (II) replicates of each pasture (A1–A9).

4. Discussion

The results of present study could be useful for dairy farmers in the studied area, and more in general in the Alpine district, to characterize the nutritional quality and bioactive compounds present in the pastures and to correlate them to those of high-quality dairy products, to highlight possible markers capable of linking the product to the production area, and to discriminate these mountain products from those derived from intensive dairy farming.

Overall, the chemical data were in agreement with the average values of all the species reported by Bovolenta et al. [33], even though the here examined pastures are generally more fibrous, as well as less proteic and energetic.

With regards to the fiber fractions and CP content of our study, the NDFom content in the different sampled pastures was similar to those previously reported in a study carried out by Peiretti et al. [1] in the same alpine environment. The ADFom content only differed from the highest recorded content (403 g/kg DM) in the A4 pasture, which was also the lowest in altitude and resulted to be the pasture with the lowest CP content (116 g/kg DM). A similar trend between the ADFom and CP contents, which revealed an effect of altitudinal zone, was also observed by Roukos et al. [34], who performed a nutritional quality study on herbage botanical component samples taken from three altitudinal zones (lower, middle, and upper) of a mountainside grassland in North-West Greece. These authors found a CP content that ranged from 111 to 163 g/kg DM, corresponding to an ADFom content ranging from 362 to 277 g/kg DM in the lower and upper altitudinal zones, respectively. Different frequencies of *Leguminosae* at different altitudes can influence the ADFom and CP contents of a pasture [1], and this points out that the altitudinal zone has an important effect on the nutritive value of grasses, legumes, and forbs, as previously reported by Roukos et al. [34].

The NDF digestibility was similar to the values found by Mayer et al. [35] in Alpine wood pastures. The quality and digestibility of forage from grazing lands generally decrease from spring to autumn in all altitudinal zones, as reported by Mountousis et al. [36]. However, we found herbage of good quality in the summer season.

Similar FA profiles to those reported in our study were found by Peiretti et al. [1] in five Alpine pastures during the 2013 grazing period, but these authors found a higher proportion of ALA and LA (their sum ranged from 705 to 734 g/kg of the total FAs) than our results (from 458 to 608 g/kg of the total FAs). Moreover, these authors only found significant differences for the PA and oleic acid contents between pastures, but not for LA and ALA fatty acids.

The variability of the forage FA profile in the Alpine region in Italy was also studied by Revello Chion et al. [3] between May, when grazing began, to July, when haymaking was performed. They

found five dominant FAs, and ALA, LA, and PA accounted for more than 850 g/kg of the total FAs in both experimental years (2002–2003). During 2002, no significant difference was observed in the LA or PA contents throughout the growing cycle, whereas the ALA significantly decreased from 670 to 407 g/kg of the total FAs, while there was no significant change in LA or ALA content throughout the 2003 growing cycle. An FA profile similar to those reported by Revello Chion et al. [3] was found in our study, with the exception of γ-Linolenic acid and other minor FA contents. Between major FAs, the ALA content of our pastures was similar to those found by Willems et al. [37] in three different alpine pastures, while the LA content was higher than our results.

As far as the TP content of alpine pastures, Willems et al. [37] found a TP content that ranged from 23 to 46 g/kg DM in the swards of the experimental vegetation types. In our study, no significant differences were found in the TP, TT and CT contents between pastures and most of the investigated pastures showed similar or higher TP values than those found by Willems et al. [37] for an alpine vegetation type classified as a highly biodiverse herbaceous-shrub type with a moderate forage quality and high phenolic compound content.

The TP and TT concentrations in our alpine pasture were rather high, compared with those found by Khiaosa-Ard et al. [38] in a similar environment located at an altitude of 2000 m a.s.l. in the southeast of the Swiss Alps. These authors found that TP and TT ranged from 23 to 30 g/kg DM and from 10 to 17 g/kg DM, respectively, and showed that alpine forages were richer in TP and TT than the respective lowland forages. The differences in the tannin contents between our study and those found by Khiaosa-Ard et al. [38] could be related to the different botanical compositions of the Swiss pastures, which were characterized by two main vegetation types, namely *Crepido aureae–Festucetum rubrae* and *Deschampsio cespitosae–Poetum alpinae*.

A previous phyto-pastoral analysis carried out in the same sites [18] showed that the most frequent plant species found in the A3 pasture were *Onobrychis viciaefolia* and *Trifolium pratense* among the *Leguminosae* and *Dactylis glomerata*, *Poa alpina* and *Poa violacea* among the *Gramineae*. Sainfoin (*Onobrychis viciaefolia*) and red clover (*Trifolium pratense*) are known to be good sources of CT, with either beneficial or detrimental effects on sustainable ruminant production [39] and the high CT values recorded in the A2 and A3 pastures could, therefore, be related to the presence of these plant species. Khiaosa-Ard et al. [38] determined a CT content in pastures located in the southeast of the Swiss Alps that ranged from 0.3 to 3.2 g/kg DM but did not find any general anti-bacterial effect of these alpine pastures.

Mountain pastures usually contain a rich variety of terpenoids, due to the great diversity of plant species, which are affected by geographical, agronomic and climatic factors. A rich variety of terpenoids was observed also in this paper as showed by PCA (Figure 3), where the richest and poorest pastures in volatiles are visible on the left and right side of the plot, respectively.

In a similar way, in a mountain environment different from the Alpine ones investigated in our study, a similar richness in the terpenoid profile of the plant species of a mountain pasture located in an eastern region of Northern Spain has been found by Valdivielso et al. [40]. These authors detected more than 75 different individual terpenoids and reported that the total abundance of monoterpenoids was lower than that of sesquiterpenoids in most botanical families and that the most abundant monoterpene in *Lamiaceae*, *Asteraceae*, and *Ericaceae* were α-pinene, isoeugenol, β-thujene, and linalool. Regarding sesquiterpenoids, the most abundant compounds were β-caryophyllene, α-amorphene, and α-humulene. Moreover, a higher sesquiterpene/monoterpene ratio was found for the highland forage than for the lowland grazed pasture [41]. Dicotyledon grassland plants generally contain more terpenes than monocotyledons, and the contents can vary widely, according to their botanical family [42]. As a consequence, when natural dicotyledon-rich pastures are fed to dairy cows, the terpene content in their milk and cheese is higher than that of cows fed monospecific forage and concentrates [43].

Fernandez et al. [44] identified six sesquiterpenes that fully discriminated milk produced by dairy cows fed on a highland pasture, a lowland pasture, and an indoor diet. Such a remarkable quantitative

and qualitative diversity in terpenoids of Alpine pastures should be reflected in the dairy products as a richness in flavor and taste, thus enhancing their global value [45]. These findings were also highlighted by Bozoudi et al. [46], which stated that widespread terpenes in plants could be used as a marker to characterize milk from two mountainous regions of Greece.

5. Conclusions

Overall, our research has shown that DM, ash, CP, ADF, lignin, and GE differed between pastures. All the pastures had good IVTD and NDFD values. The most abundant FAs in all the pastures were α-linolenic, linoleic, and palmitic acids and their values differed significantly. No significant differences were found in the total phenols, or in the total and condensed tannin contents between pastures. Fifty-eight terpenoids were detected, and 4-cyclopentene-1, 3-dione, β-caryophyllene, and eucalyptol were the most abundant. Finally, this research has confirmed the great botanical biodiversity and the good nutritional value of the pastures sampled in the Chisone and Susa Valleys during the summer grazing season.

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