JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Characterization of Two *Agrostis*–*Festuca* Alpine Pastures and Their Influence on Cheese Composition

Milena Povolo,[†] Valeria Pelizzola,[†] Luigi Passolungo,[†] Elisa Biazzi,[‡] Aldo Tava,[‡] and Giovanna Contarini*[,†]

[†]Consiglio per la Ricerca e la Sperimentazione in Agricoltura, Centro di Ricerca per le Produzioni Foraggere e Lattiero-Casearie, Via A. Lombardo 11, 26900 Lodi, Italy

[‡]Consiglio per la Ricerca e la Sperimentazione in Agricoltura, Centro di Ricerca per le Produzioni Foraggere e Lattiero-Casearie, Viale Piacenza 69, 26900 Lodi, Italy

ABSTRACT: Recently, there has been a renewed interest in mountain farming, and several studies have been carried out on milk and cheese obtained in the unique environmental conditions of the Alps, a 1300 km mountain chain, located in the north of Italy. In this paper, the influence, on some cheese constituents, of two very similar mountain grasslands, both dominated by *Festuca*–*Agrostis*, was investigated. The two pastures were located in the same area in the southeastern Italian alpine region and differed in sunshine orientation and exposure. Milk obtained from cows grazing on these pastures was used to produce a semi-hard traditional cheese. The differences observed between the cheeses of the two areas for both some hydrocarbons (1-phytene and 2-phytene) and *trans*-fatty acids can be explained by a different rumen environment created by the botanical composition of the two pastures. The multidisciplinary approach can be considered a successful strategy, suitable for studying markers of authenticity.

KEYWORDS: *Hydrocarbons, fatty acids, biodiversity, pasture, cheese*

INTRODUCTION

Over the past decade, there has been a renewed interest in alpine farming probably stimulated, among other reasons, by the increased request for high-quality animal products obtained from extensive farming systems. This type of farming management can be found in the unique environmental conditions of the Alps, a 1300 km mountain chain, located in the north of Italy, bordering France, Switzerland, Germany, Austria, and Slovenia. In these areas, pasture grazing can be practiced from June to September and represents an important resource that could be more fully exploited, because it has markedly positive direct and indirect effects on several traits of animal products.

Several studies have shown that milk and cheese, obtained from grazing cows, have particular characteristics that are often related to health-giving properties for humans. In particular, a considerable increase of the content of ω-3 and conjugated linoleic fatty acids (FAs) in milk and cheese is known to be the main effect of the (high) grass-based feeding strategy.[1−3] These positive characteristics have been demonstrated to be enhanced by alpine pasture feeding, located at different altitudes.[4−8]

Transhumance in the Alps or seasonal migration between valley and high pastures is a traditional practice that has shaped much of the landscape in the Alps, because without it, most areas below 2000 m would be forests. While tourism and industry contribute much to today's alpine economy, seasonal migration is still practiced by local farmers who move the cattle to higher pastures during the summer season. Altitude, topography, clime, bedrock type, and grazing intensity underlie the high biodiversity that characterizes the meadows of the alpine pastures, particularly from a botanical point of view. The influence of this biodiversity on the composition of milk and cheese produced in alpine pastures has been investigated by several researchers, aiming to detect a link between the grazing region and the dairy product. Lourenço et al.[9] reviewed literature on the effects of the changes induced by leguminous and biodiverse forages on milk and tissue FA composition. They concluded that the chemical composition of pastures together with the secondary plant metabolites play a significant role in the rumen FA metabolism.

Collomb et al.[10] detected significant correlations between the occurrence of plant families and species and the concentrations of FAs of milk fat produced in regions at different altitudes.

Other authors[11−14] demonstrated, in alpine-produced milk and cheese, that both terpene and FA changes were linked to the botanical changes of the mountain pastures. Moreover, other techniques, such as nuclear magnetic resonance (NMR) and isotope ratio mass spectrometry (IRMS), were used in combination with chemometric methods for determining the geographical origin of dairy products.[15,16]

More recently, in a previous research,[17] by applying a particular experimental design that minimized the other possible sources of variations, we detected significant differences in non-volatile hydrocarbons and FAs of cheese originating from two different alpine pastures.

The aim of this work, unlike the previous research,[17] was the investigation of the influence, on some cheese constituents, of two very similar mountain grasslands, both dominated by *Festuca*–*Agrostis*. The two pastures were located in the same area but differed in sunshine orientation and exposure, and

Received: November 8, 2012
Revised: December 19, 2012
Accepted: December 21, 2012
Published: December 21, 2012

these factors influenced more the specific contribution of each plant species than their botanical diversity.

MATERIALS AND METHODS

Experiment Organization. The experimental trial, designed according to Povolo et al.,[17] was conducted in a pasture of the southeastern Italian alpine region [approximately 1647 m above mean sea level (AMSL); latitude, 45°57′; longitude, 11°24′], named Malga Dosso di Sotto. The identification of the experimental paddocks, on the basis of the botanical characteristics, was made in the spring, before the cattle went up to the alpine region. The total surface area of the paddocks was 5.42 ha, and the slope varied from 15° to 20°.

The experimental trial (Figure 1) was begun in late July. Two groups of brown alpine cows were put to graze, with each group in an

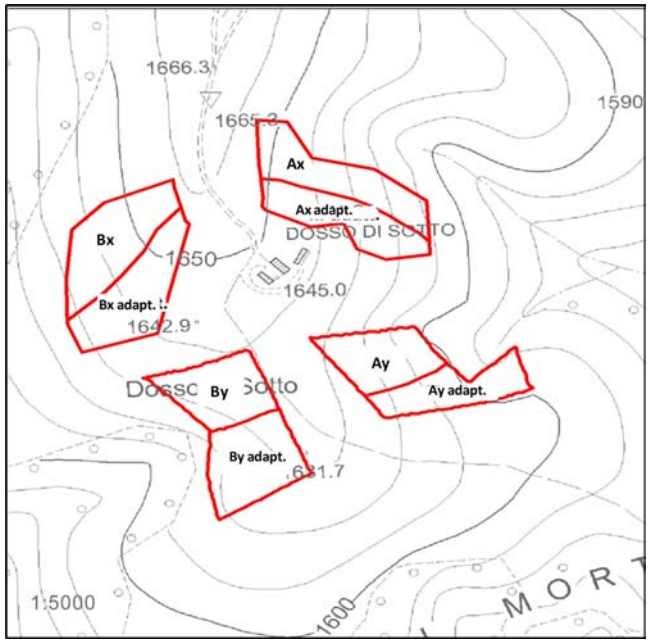

Figure 1. Map of the experimental paddocks.

adaptation paddock (Ax adaptation and Bx adaptation). The two groups (10 animals each) were homogeneous for age, stage of lactation, and general physiological conditions. After 6 days of adaptation, the groups were moved to the corresponding experimental paddocks Ax and Bx, where they grazed for 6 days and received about 2.5 kg cow^{-1} day^{-1} of concentrate. During that period, cows were milked twice a day, directly in the paddock, with a mobile milking machine, and milk was converted into a typical regional cheese, named Asiago. The mean milk daily production was 20.8 ± 2.2 kg cow^{-1} day^{-1}.

At the end of the first 6 day experimental period, the group of cows that had grazed in the paddock Ax was moved on the adaptation paddock By (By adaptation) and vice versa for the second group of cows (Bx to Ay adaptation). The experiment was then repeated as in the first stage, including the cheese production.

Pasture Sampling and Characterization. The pasture sampling (A and B) was carried out 3 times (different days), 1 week before the entrance of the animals into the trial paddocks (Ax, Ay, Bx, and By), and each sample (100 g) was collected in triplicate. Additional pasture samples were taken before and after the entrance of cows to determine the ratio between consumed and available forage, according to Smit et al.[18]

The detailed characterization of the vegetation of the different paddocks was carried out, according to Daget and Poissonet.[19] The botanical composition of the grasslands was expressed as a percentage of specific contribution (C_s) of each species with respect to the number of the total species identified. The Shannon index (SH),[20] which gives a measure of the botanical biodiversity of the pastures, and the pastoral value (PV), which measures the nutritional value of a pasture from its abundance, quality, and herbivore preference,[21] were calculated.

Cheese Manufacture and Sampling. Asiago is a semi-cooked and semi-hard traditional cheese. For its production, the milk of the afternoon milking was mixed with the milk of the morning milking and was heated at about 35 °C. Coagulation was achieved in about 20 min after the addition of the rennet. The curd was then broken into small parts (the size of a grain of rice) and cooked in two stages at two temperatures: 40 and 47 °C. The curd was extracted and placed in molds for forming. The cheese was then salted by spreading salt over the surface. The last step was the aging process, which lasted 90 days and took place, within the area of origin, in warehouses where the storage temperature and relative humidity varied between 10 and 15 °C and 80−85%, respectively. The average weight of the cheese mold was 12 kg.

A total of 24 Asiago cheeses were produced during the trial, 12 from treatment A (A1−A12) and 12 from treatment B (B1−B12). A slice of about 250 g was taken from each cheese and stored at −20 °C until the analyses.

Reagents. High-purity standards of squalane (99%), 3-methyl cyclohexanone (99%), squalene (≥98%), *n*-alkanes from C_6 to C_{34} (99%), and methyl esters (purities ranging from 99.5 to 99.8%) of FAs and phytanic acid were supplied from Sigma-Aldrich (Milan, Italy). Phytane (99%) was purchased from Ultra Scientific (Bologna, Italy). All of the compounds detected in the evaluation of the essential oil were purchased from Sigma-Aldrich, except those identified only by their retention indices (RIs) (Table 3).

n-Hexane used in the whole extraction procedure of the hydrocarbon fraction was a Suprasolv solvent (Merck, Darmstadt, Germany). All of the other reagents were of analytical grade and purchased from Sigma-Aldrich.

Chemical Composition. Dry matter (DM), protein, fat, and ash contents of cheese samples and crude protein (CP), ash, neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) of forage samples were determined according to Association of Official Analytical Chemists (AOAC) methods.[22] The DM content was determined by drying the samples in a forced draft oven at 60 °C, to a constant weight, and was expressed as g 100 g^{-1}. Ash, CP, NDF, ADF, and ADL were expressed as g 100 g^{-1} of DM. Each sample was analyzed in triplicate.

Pasture Essential Oil Composition. Plant material (100 g) and 0.34 mg of 3-methylcyclohexanone, added as an internal standard, were steam-distilled with odor-free water in a Clevenger-type apparatus for 1 h. The distillate was saturated with NaCl, extracted with freshly distilled diethyl ether (3 × 100 mL), dried over anhydrous Na_2SO_4, and concentrated in a rotary evaporator to give a pale-yellow oil. The obtained volatile oils were diluted in diethyl ether and analyzed by gas chromatography−mass spectrometry (GC−MS) and gas chromatography with a flame ionization detector (GC−FID), applying the analytical condition described by Tava et al.[23] The identification of the oil components was performed by their RIs, authentic reference compounds, peak matching library search, as well as published mass spectra.[24,25] RIs were calculated using a *n*-alkane series (C_6−C_{34}) under the same GC conditions as for the samples. The individual components were expressed as mg kg^{-1} of fresh forage. Each sample was analyzed in duplicate.

Cheese Fat Extraction. Frozen cheese samples were thawed slowly at refrigerator temperature (4 °C) and finely grated. An amount of 20 g of grated cheese was weighed in a 60 mL screw-cap glass tube and warmed in a water bath at 60 °C for 20−30 min. Furthermore, the fat fraction was separated by centrifugation as reported by Povolo et al.[26]

Hydrocarbon Fraction Analysis. The hydrocarbon fraction of cheese and pasture samples was analyzed by GC−MS as described by Povolo et al.[17] The identification of the compounds was made using the National Institute of Standards and Technology (NIST) library,[24] the MS literature data, the injection of authentic standards, when available, and the comparison of the RIs with published data. Phytyl esters were previously identified.[26] The quantification of the compounds was performed by relating the peak abundance to that of

squalane (internal standard), and the amount was expressed as mg kg^{-1} of fat and mg kg^{-1} of DM for cheese and forage, respectively. Each sample was analyzed in duplicate.

FA Composition. The cheese FA determination and identification were performed by applying the same procedure as in the study by Povolo et al.[17] In addition, the identification of phytanic acid was carried out by both GC−MS analysis and a comparison to the retention time of the authentic standard (Sigma-Aldrich). The result of each FA was expressed as a percentage of the total area of the peaks eluted, excluding solvent. Saturated fatty acid (SFA), monounsaturated fatty acid (MUFA), polyunsaturated fatty acid (PUFA), branched fatty acid, and *trans*-fatty acid (TFA) were then calculated by summing the percentage of the molecules belonging to each chemical class. Each sample was analyzed in duplicate.

Statistical Analysis. The effect of the different pastures was calculated by the analysis of variance (ANOVA) using XLSTAT 7.5 package (Addinsoft, France).

RESULTS AND DISCUSSION

Characterization of the Vegetation Types. At the end of the experiment, the percentage of DM consumed by the cattle was about 62% in treatment A and 65% in treatment B.

Table 1 shows the results of the botanical characterization of pastures A and B. Both vegetation types belonged to the *Agrostis*−*Festuca* grassland class, with these species being the most represented species in the two areas. Nevertheless, the botanical composition showed interesting differences. First, from the quantitative point of view, pasture B was characterized by a higher (42.6% in B versus 26.3% in A), although not statistically significant, content of all of the species belonging to the gramineae family (Poaceae), and this was the main reason for its higher PV. Otherwise, pasture A showed a significantly higher content of leguminosae (Fabaceae) (15.3% in A versus 7.7% in B), together with the presence of a higher number of different species. The latter influenced the SH, which is a measure of the species diversity calculated from not only the number of species but also their relative abundances (4.9 in A versus 3.9 in B).

Among the other family/species, only the Asteraceae, with the high contribution of *Achillea* gr. *millefolium*, and *Plantago media*, provided significant differences.

The chemical composition and quality indices of the forages (A and B) were 31.1 ± 2.0 and 28.6 ± 1.8 for DM, 6.5 ± 0.2 and 6.2 ± 0.2 for ash, 11.2 ± 0.8 and 10.4 ± 0.7 for CP, 61.2 ± 1.1 and 62.9 ± 2.0 for NDF, 37.5 ± 2.5 and 39.2 ± 1.6 for ADF, and 6.3 ± 0.7 and 6.3 ± 0.1 for ADL, respectively. No statistically significant differences were observed between the values, and both forages showed good protein content and provided a high value of NDF.

Table 2 reports the essential oil composition of the two vegetation types. Essential oils are complex mixtures of volatile compounds mainly derived from three biosynthetic pathways: the mevalonate pathway leading to terpenes, the shikimic acid pathway leading to phenylpropanoids, and short linear chain compounds, such as C_6 alcohols, aldehydes, and esters, originating from the breakdown of saturated and unsaturated FAs.[27,28]

A total of 27 compounds belonging to the chemical classes of aldehydes, alcohols, mono- and sesquiterpenes, phenyl propanoic derivatives, and esters were detected and identified in both vegetation types. A wide range of concentrations was observed, and limonene (27.8 in A versus 29.0 in B), carvone (26.8 in A versus 18.9 in B), and germacrene D (13.6 in A versus 19.7 in B) were the most abundant compounds.

Table 1. Mean Values and Standard Error of the Mean (SEM) of the Specific Contribution (C_s, %) of the Different Botanical Species, Single and Grouped by Family, Detected in Pastures A and B

		C_s (%)			
family	species	A	B	SEM	*p*
Poaceae		26.3	42.6	3.8	0.164
	Agrostis tenuis Sibth.	10.7	18.9	1.7	0.133
	Festuca gr. *rubra*	5.1	9.4	4.1	0.659
	Phleum alpinum	1.4	4.4	1.0	0.255
	Poa pratensis	1.7	9.0	1.1	0.087
	Nardus stricta	2.3	1.0	0.5	0.615
	Koeleria pyramidata	2.7		0.4	
	Poa alpina	2.4		0.4	
Rosaceae		15.2	8.6	1.9	0.219
	Alchemilla vulgaris	8.6	8.6	1.5	0.982
	Potentilla crantzii	6.6		1.6	
Fabaceae		15.3	7.7	0.6	0.028
	Trifolium pratense	6.4	3.8	1.0	0.315
	Trifolium repens	3.6	4.0	0.9	0.852
	Lotus corniculatus	3.5		0.3	
	Anthyllis vulneraria	1.8		0.4	
Asteraceae		7.5	14.5	0.3	0.018
	Achillea gr. *millefolium*	2.6	9.6	0.5	0.017
	Cirsium acaule	1.5		0.9	
	Taraxacum officinale		4.9	1.3	
	Carlina acaulis	1.1		0.1	
	Carduus nutans	2.3		0.8	
Plantaginaceae		5.1	3.0	0.4	
	Plantago media	5.1	1.3	0.4	0.037
	Veronica chamaedrys		1.7	0.2	
Apiaceae	*Carum carvi*	3.0	3.2	0.8	0.937
Ranuncolaceae	*Ranunculus acris*	3.0	6.5	1.7	0.427
Rubiaceae		2.4	1.4	0.2	0.156
	Cruciata laevipes		1.4	0.1	
	Galium gr. *mollugo*	2.4		0.4	
Caryophyllaceae		1.7	4.3	1.2	0.392
	Stellaria graminea	1.7	3.1	1.2	0.615
	Cerastium holosteoides		1.1	0.1	
Cypearaceae	*Carex pallescens*	2.9		0.2	
Scrophulariaceae		2.6		1.4	
	Rhinanthus alectorolophus	1.6		1.5	
	Euphrasia rotskoviana	1.0		0.2	
Hypericaceae	*Hypericum maculatum*	1.3		0.6	
Lamiaceae	*Thymus* gr. *serpyllum*	1.1		0.6	
Polygonaceae	*Polygonum viviparum*	1.2		1.1	
others		11.3	8.2		
SH		4.9	3.9		0.002
PV		25.7	34.3		0.044

Among the compounds detected, only seven showed differences statistically significant: α-pinene, β-pinene, 1,8-cineol, camphor, borneol, bornylacetate, and germacrene D. All of these compounds were higher in pasture B than in pasture A. When these results were compared to those of the specific contribution of each species to the pasture (Table 1), it was observed that the only species showing a difference statistically significant with higher values in pasture B than in pasture A was *A. millefolium*. This species is an herbaceous perennial plant belonging to the Asteraceae family and widespread in most temperate regions. Because of pharmacological properties of its

dx.doi.org/10.1021/jf304756w | J. Agric. Food Chem. 2013, 61, 447−455

Table 2. Mean Values (mg kg^{-1} of Fresh Sample) and SEM of the Essential Oil Composition of the Two Vegetation Types (A and B)

compound	identification method[a]	vegetation type A	vegetation type B	SEM	p
hexanal	MSa/MSr/PI	0.4	0.5	0.10	0.609
cis-3-hexenol	MSa/MSr/PI	4.2	3.4	0.47	0.448
α-pinene	MSa/MSr/PI	1.1	2.4	0.29	0.048
benzaldehyde	MSa/MSr/PI	1.4	1.2	0.48	0.778
sabinene	MSr/PI	2.5	1.4	0.34	0.132
β-pinene	MSa/MSr/PI	1.2	3.4	0.24	0.001
oct-1-en-3-ol	MSa/MSr/PI	2.6	2.9	0.25	0.634
limonene	MSa/MSr/PI	27.8	29.0	6.18	0.920
1,8-cineol	MSa/MSr/PI	1.0	2.9	0.30	0.009
phenylacetaldehyde	MSa/MSr/PI	1.1	1.0	0.20	0.910
linalool	MSa/MSr/PI	1.6	0.7	0.14	0.065
nonanal	MSa/MSr/PI	0.9	0.9	0.07	0.870
2-phenylethanol	MSa/MSr/PI	0.7	0.5	0.13	0.467
camphor	MSa/MSr/PI	1.0	3.1	0.44	0.040
borneol	MSa/MSr/PI	1.3	5.9	0.56	0.002
α-terpineol	MSa/MSr/PI	0.7	0.8	0.15	0.841
carvone	MSa/MSr/PI	26.8	18.9	7.61	0.723
bornylacetate	MSa/MSr/PI	0.2	0.7	0.07	0.008
p-vinylguaiacol	MSr/PI	0.1	0.1	0.03	0.421
eugenol	MSa/MSr/PI	0.2	0.2	0.04	0.865
α-copaene	MSr/PI	0.2	0.4	0.03	0.068
β-elemene	MSr/PI	0.1	0.1	0.03	0.898
trans-cariophyllene	MSa/MSr/PI	1.9	2.4	0.19	0.189
coumarin	MSa/MSr/PI	0.8	0.6	0.34	0.814
germacrene D	MSr/PI	13.6	19.7	1.09	0.019
bicyclogermacrene	MSr/PI	1.1	1.6	0.25	0.361
cariophyllene oxide	MSa/MSr/PI	0.7	0.6	0.26	0.906

[a]Confirmation of the identification: MSa, mass spectra of authentic compounds (authentic compounds had the same RIs as the molecules detected in the samples); MSr, comparison to mass spectra reported in the literature;[25] and PI, published indexes, comparison of KI calculated with published indexes.[29]

Table 3. Mean Values (mg kg^{-1} DM) and SEM of the Main Non-volatile Hydrocarbons Detected in the Two Vegetation Types (A and B)

compound	identification method[a]	vegetation type A	vegetation type B	SEM	p
neophytadiene	MSr/PI	3.7	4.2	0.64	0.737
heneicosane (C_{21})	MSa	1.5	2.0	0.18	0.233
tricosane (C_{23})	MSa	6.9	8.6	0.96	0.398
pentacosane (C_{25})	MSa	14.5	21.9	1.99	0.115
heptacosane (C_{27})	MSa	19.9	30.8	2.93	0.113
squalene	MSa	9.2	5.6	0.63	0.029
nonacosane (C_{29})	MSa	65.3	139.9	9.21	0.007
hentriacontane (C_{31})	MSa	93.4	157.3	19.98	0.161
tritriacontane (C_{33})	MSa	95.7	89.7	21.14	0.891

[a]Confirmation of the identification: MSa, mass spectra of authentic compounds (authentic compounds had the same RIs as the molecules detected in the samples); MSr, comparison to mass spectra reported in the literature; and [44] PI, published indexes, comparison of KI calculated with published indexes.[45]

essential oil, the constituents of different species of *Achillea*, growing in different areas, were studied by several authors.[30−33] The presence of various chemotypes of *A. millefolium* occurring in nature, determined a significant variation in the chemical composition of the essential oil. Nevertheless, the different authors always found 1,8-cineol, camphor, borneol, germacrene D, and α- and β-pinene as characteristic constituents of *A. millefolium*, and these compounds were often the most concentrated compounds. This evidence led to the reasonable hypothesis that the significant higher presence of *A. millefolium* in pasture B was responsible for the differences obtained in the chemical constituents of the essential oil of this vegetation type.

The composition of non-volatile hydrocarbons (Table 3) mainly included *n*-alkanes with odd-numbered carbon atoms ranging from C_{21} to C_{33}. According to Dove and Mayes,[34] the predominant alkanes were C_{29} (65.3 in A versus 139.9 in B), C_{31} (93.4 in A versus 157.3 in B), and C_{33} (95.7 in A versus 89.7 in B). These molecules are components of the plant cuticle, a hydrophobic layer coating the epidermis of the primary plant body, which has a vital importance in protecting tissue from environmental stresses. *n*-Alkanes, as well as all other long-chain aliphatic compounds present in plant cuticular waxes, originate from a series of metabolic steps belonging to the acetyl coenzyme A biosynthetic pathway.[35]

The differences between species in their individual alkane concentrations were exploited to provide information on the composition of available and consumed herbage.[34,36,37] Under conditions in which animals were fed on complex vegetation communities, such as in this experiment, different authors[38−40] observed that it was likely that the reliability of the diet composition estimates declined because of the high number of plant species and feeding selectivity for a specific plant.

In our research, the vegetation B was characterized by a higher but not statistically significant content of Poaceae and vegetation A was characterized by a higher content of Fabaceae. The main species contributing to these results were *Agrostis tenuis* and *Festuca* gr. *rubra* in vegetation B and *Trifolium* spp. in vegetation A (Table 1). All of these species showed a high content of *n*-alkanes C_{29}, C_{31}, and C_{33};[36,37,41,42] as a consequence, the difference observed for C_{29} was probably due to the contribution of other minor species. For example, the significant presence of *A. millefolium* in vegetation B, already accounted for the differences observed in the essential oil composition, could be responsible for the higher content of nonacosane in the same pasture, according to Palic et al.[43]

No references were found to explain the different amounts of squalene ($p < 0.05$) in the two vegetation types.

Characterization of the Cheese Samples. A total of 24 shapes of Asiago cheese were produced during the experimental period, 12 from milk of cows grazing on pasture A and 12 from milk of cows grazing on pasture B. After 90 days of ripening, cheese was sampled and analyzed. No significant differences were found in the gross composition between the two vegetation types (Table 4). The fat and protein values reflected the usual composition of the PDO Asiago cheese,[46] even though, because of the particular type of experimentation, the mark of origin was not applied on the cheese wheels.

The FA composition of the samples (Table 5), independent from the type of vegetation, was in accordance with the literature data of cheeses obtained from milk of cows grazing on mountain pastures.[11,17]

dx.doi.org/10.1021/jf304756w | J. Agric. Food Chem. 2013, 61, 447−455

Table 4. Mean Values and SEM of the Main Constituents (Weight Percent) of Cheese Samples Produced from Milk Derived from the Two Vegetation Types (A and B)

	vegetation type			
	A	B	SEM	*p*
DM	65.15	65.49	0.414	0.689
fat	29.80	30.16	0.398	0.657
protein	28.64	28.31	0.257	0.531
ash	5.46	5.55	0.080	0.565

According to Leiber et al.,[47] the SFA content, particularly palmitic acid, was lower than that found in milk derived from an intensive breeding system; at the same time, except for linoleic acid (LA), MUFA and PUFA, particularly linolenic acid (ALA), were higher. Finally, the mountain milk fat was characterized by the high content of conjugated linoleic acids (CLAs) and their precursors, principally vaccenic acid (*trans*-11 18:1). Among the other *cis*/*trans* isomers, the contribution of *trans*-11,*cis*-13 18:2 (about 5%) to the total CLA content was close to the range of 5−8%, reported by Collomb et al.[5] and Kraft et al.[4] for fat derived from mountain dairy products, although its concentration was lower than that detected in other mountain cheese samples derived from milk of cows grazing in the Alps.[17] Lower values were also observed for the content of *trans*-11,*cis*-15 18:2, when it was compared to the data reported by Povolo et al.[17] The similar behavior of these two octadecadienoic acids seems to support the hypothesis, reported by Kraft et al.,[4] that *trans*-11,*cis*-13 18:2 directly derived from *trans*-11,*cis*-15 18:2, although the pathway is as yet unclear.

The detailed evaluation of the GC profile showed two minor peaks eluting between palmitoleic (*cis*-9 16:1) and heptadecanoic (17) FAs. The analysis of the authentic standard by both GC and GC−MS, together with the comparison to the literature data,[48] allowed for the identification of these peaks as two isomers of phytanic acid. It derives from hydrogenation of the double bond and subsequent oxidation of the alcoholic group of phytol, and it was recently proposed as a potential marker for organic milk.[49]

Table 5. Mean Values [g 100 g^{-1} of Fatty Acid Methyl Esters (FAMEs)] and SEM of FAs of Asiago Cheese Samples Produced from Milk Derived from the Two Vegetation Types (A and B)

	vegetation type			
	A	B	SEM	*p*
4	3.99	3.88	0.149	0.942
6	2.60	2.56	0.064	0.764
8	1.26	1.24	0.030	0.744
10	2.54	2.52	0.058	0.846
10:1	0.34	0.34	0.008	0.808
12	2.84	2.85	0.053	0.964
12:1	0.05	0.05	0.001	0.983
13 iso	0.08	0.08	0.002	0.810
13	0.14	0.14	0.002	0.831
14 iso	0.16	0.16	0.001	0.253
14	10.51	10.79	0.114	0.225
14:1	1.01	1.00	0.003	0.733
15 iso	0.30	0.30	0.006	0.163
15 anteiso	0.58	0.57	0.018	0.867
15	0.99	1.01	0.008	0.097
16 iso	0.30	0.30	0.003	0.923
16	26.18	27.07	0.163	0.012
cis-7 16:1	0.21	0.20	0.002	0.689
cis-9 16:1	1.18	1.27	0.013	0.003
phytanic	0.17	0.11	0.006	<0.0001
17 iso	0.55	0.55	0.004	0.683
17 anteiso	0.39	0.39	0.003	0.446
17	0.62	0.61	0.004	0.384
17:1	0.21	0.20	0.003	0.550
18 iso	0.05	0.05	0.001	0.309
18	9.23	9.40	0.127	0.512
trans-(6 + 7 + 8) 18:1	0.33	0.30	0.006	0.015
trans-9 18:1	0.24	0.22	0.005	0.039
trans-10 18:1	0.30	0.30	0.008	0.882
trans-11 18:1	3.27	2.88	0.056	0.002
trans-12 18:1	0.40	0.33	0.008	0.001
trans-16 18:1	0.31	0.27	0.004	<0.0001
cis (9 + 10) + *trans* (13 + 14 + 15) 18:1	20.68	20.35	0.236	0.495

	vegetation type			
	A	B	SEM	*p*
cis-11 18:1	0.50	0.47	0.009	0.098
cis-12 18:1	0.18	0.16	0.006	0.072
cis-13 18:1	0.06	0.05	0.001	0.243
cis-15 18:1	0.15	0.13	0.002	<0.0001
trans-9,*trans*-12 18:2	0.03	0.03	0.002	0.867
trans-9,*cis*-13 18:2	0.23	0.19	0.004	0.002
trans-8,*cis*-12 18:2	0.11	0.10	0.002	0.186
trans-8,*cis*-13 18:2	0.09	0.08	0.002	0.006
cis-9,*trans*-12 18:2	0.05	0.05	0.001	0.448
trans-9,*cis*-12 18:2	0.03	0.02	0.001	0.057
trans-11,*cis*-15 18:2	0.30	0.23	0.006	<0.0001
cis-9,*cis*-12 18:2 (LA)	1.88	1.85	0.024	0.612
20	0.13	0.12	0.002	0.062
18:3*n*-6	0.02	0.02	0.001	0.658
cis-9,*cis*-12,*cis*-15 18:3 (ALA)	0.71	0.69	0.006	0.047
cis-9,*trans*-11 18:2 (CLA)[a]	1.48	1.30	0.030	0.009
trans-11,*cis*13 18:2 (CLA)[b]	0.08	0.07	0.002	<0.0001
∑*cis*/*trans* + *trans*/*cis* CLA[c]	0.06	0.06	0.002	0.650
∑*trans*/*trans* CLA[d]	0.03	0.02	0.001	0.006
20:2*n*-6	0.03	0.03	0.001	0.318
22	0.05	0.05	0.001	0.568
20:3*n*-6	0.05	0.05	0.001	0.146
20:4*n*-6 (ARA)	0.09	0.08	0.001	0.426
20:4*n*-3	0.03	0.02	0.001	0.205
20:5*n*-3 (EPA)	0.04	0.04	0.001	0.053
24	0.03	0.03	0.001	0.699
22:4*n*-3	0.01	0.01	0.001	0.128
22:5*n*-3 (DPA)	0.06	0.06	0.001	0.064
SFA	64.71	65.91	0.322	0.077
MUFA	29.41	28.53	0.271	0.118
PUFA	5.41	5.02	0.062	0.004
branched FA	2.58	2.51	0.013	0.054
TFA (CLA excluded)	5.69	5.02	0.082	<0.0001

[a]This peak can include *trans*-7,*cis*-9 and *trans*-8,*cis*-10, accounting for about 3% of *cis*-9,*trans*-11 CLA, according to Collomb et al.[5] [b]This peak can include some *cis*/*cis* CLA isomers, according to Kramer et al.[50] [c]Sum of the peaks eluting in the region of the *cis*/*trans* and *trans*/*cis* isomers of CLA. [d]Sum of the peaks eluting in the region of the *trans*/*trans* isomers of CLA.

Table 6. Mean Values (mg kg^{-1} of Fat) and SEM of the Main Non-volatile Hydrocarbons Detected in Asiago Cheese Samples Produced from Milk Derived from the Two Vegetation Types (A and B)

compounds	identification method[a]	vegetation type A	vegetation type B	SEM	p
hexadecane (C_{16})	MSa	0.7	0.5	0.07	0.014
heptadecane (C_{17})	MSa	1.1	0.6	0.08	0.320
1-phytene	MSr/PI	7.9	9.8	0.41	0.031
octadecane (C_{18})	MSa	3.3	3.0	0.11	0.143
phytane	MSa	1.2	1.0	0.05	0.152
neophytadiene	MSr/PI	2.8	3.1	0.14	0.259
2-phytene	MSr/PI	8.5	7.5	0.22	0.030
nonadecane (C_{19})	MSa	0.7	0.9	0.10	0.426
heneicosane (C_{21})	MSa	2.3	1.5	0.18	0.042
tricosane (C_{23})	MSa	4.9	4.1	0.55	0.467
pentacosane (C_{25})	MSa	7.6	7.4	0.58	0.862
hexacosane (C_{26})	MSa	5.3	4.6	0.49	0.428
heptacosane (C_{27})	MSa	9.0	7.9	0.32	0.103
squalene	MSa	37.0	43.1	0.94	0.004
nonacosane (C_{29})	MSa	10.8	9.3	0.34	0.052
hentriacontane (C_{31})	MSa	6.2	5.3	0.22	0.060
phytyl C_{16}	MSa	15.8	17.8	0.97	0.303
phytyl C_{18} sat/unsat[b]	MSa	7.6	7.5	0.53	0.888
∑phytenes[c]		19.2	20.4	0.67	0.393
1-phytene/2-phytene		0.9	1.3	0.04	<0.0001

[a]Confirmation of the identification: MSa, mass spectra of authentic compounds (authentic compounds had the same RIs as the molecules detected in the samples); MSr, comparison to mass spectra reported in the literature; and [44] PI, published indexes, comparison of KI calculated with published indexes.[45] [b]sat/unsat = saturated/unsaturated. [c]Sum of 1-phytene + 2-phytene + neophytadiene.

Table 6 shows the non-volatile hydrocarbons detected in cheese samples. Most of them were *n*-alkanes, with the carbon atom number ranging from 16 to 31, and C_{16}, C_{21}, and C_{29} showed statistically significant differences ($p < 0.05$). *n*-Alkanes are only partially absorbed from the animal digestive tract and, therefore, are often used as markers in animal nutrition studies by determining their composition in feces. Literature data[51] demonstrated that the recovery of the odd chain *n*-alkanes in feces increased linearly with the *n*-alkane chain length, suggesting that those having a lower carbon atom number are more absorbed. This is confirmed by the comparison between the *n*-alkane composition of pastures and that of cheeses. Independent from the type of vegetations, C_{31}, followed by C_{29} and C_{33}, was the most abundant *n*-alkane in pastures (Table 3), whereas, in cheese samples (Table 6), C_{33} was not detected and C_{29}, C_{27}, and C_{25} were the most abundant *n*-alkanes.

As far as the isoprenoid hydrocarbons derived from phytol are concerned (1-phytene, 2-phytene, and neophytadiene), their total concentration (∑phytenes) was already used to distinguish dairy products derived from cows grazing on fresh pastures in the mountain from those derived from cows fed under an intensive breeding system throughout the year, in lowland areas.[26] The values of ∑phytenes obtained for all of the samples of Asiago cheese varied from 13.5 to 25.7 mg kg^{-1} of fat and were, therefore, always higher than the range of variability for lowland dairy products (2.5−11.1 mg kg^{-1} of fat).[26] However, the Asiago cheese samples showed a lower ∑phytenes in comparison to "Toma" cheese samples (20 and 80 mg kg^{-1}) derived from cows always grazing in the Alps but on pastures principally dominated by *Festuca* and *Trifolium*.[13,17] The result of these comparisons confirmed the influence of the type of feeding on the concentration of phytenes and suggest extending the studies on this subject for its possible use as an authenticity index.

As for the phytyl esters, the most substantiated hypotheses on their origin include the direct transfer from plant and/or the esterification in rumen or mammary cells.[26] In the case of long ripened cheese, it should not be excluded that their concentration can also be influenced by the availability of free FAs, derived from the lipolytic processes naturally occurring during the cheese ripening.

Influence of the Vegetation Type on the Cheese Composition. The following *trans*-FAs: *trans*-(6 + 7 + 8) 18:1, *trans*-9 18:1, *trans*-11 18:1, *trans*-12 18:1, *trans*-16 18:1, *trans*-9,*cis*-13 18:2, *trans*-8,*cis*-13 18:2, *trans*-11,*cis*-15 18:2, *cis*-9,*trans*-11 18:2 (CLA), *trans*-11,*cis*-13 18:2 (CLA), and *trans*/*trans* CLA showed a significantly lower percentage in cheeses produced from vegetation B than those produced from vegetation A (Table 5).

As reported by Leiber et al.,[47] changes in the ruminal ecosystem because of energy shortage and specific plant metabolites are possible hypotheses for the particular composition of FAs of milk fat of mountain origin. Moreover, it was demonstrated that some molecules of plant essential oil, particularly monoterpene alcohols and aldehydes, are able to inhibit the growth and metabolism of rumen microbes.[52]

As cited above, vegetation B was characterized by a higher amount of α-pinene, β-pinene, 1,8-cineol, camphor, borneol, bornylacetate, and germacrene D with respect to vegetation A. Thus, it seems reasonable to suppose that the presence of a higher concentration of these molecules affected the rumen environment, leading to a reduction of both the intermediates and the final products of the biohydrogenation. Moreover, literature data demonstrated that other constituents belonging to the class of plant secondary compounds, such as tannic polyphenols, can have an effect on the milk FA composition, because of their bacteriostatic and bactericidal effect on rumen microbes.[53,54] The different rate of rumen biohydrogenation was also confirmed by the concentration of phytanic acid that resulted in significantly higher samples from vegetation A (0.17%) with respect to those from vegetation B (0.11%).

The composition of the hydrocarbon fraction of the A and B cheeses statistically differed for hexadecane, 1-phytene, 2-phytene, heneicosane, and squalene (Table 6). In our previous paper, where the effect of two pastures of Italian southwestern alpine region (*Trifolium alpinum* and *Festuca nigrescens*) were studied,[17] the ratio of C_{29}/C_{27} demonstrated to be effective in distinguishing between the cheeses obtained from milk from those two areas. In this research, both the vegetation areas belonged to the same pasture classification (*Agrostis*−*Festuca*) that was undoubtedly different from that dominated by *T. alpinum* but showed a certain similarity to that dominated by *F. nigrescens*. Thus, the ratio of C_{29}/C_{27} was tested on the data of Asiago samples. As expected, the ratio values did not discriminate between the cheese samples derived from vegetations A and B (Figure 2), but all of the values obtained, except for sample A2 (−0.02), were higher than 0, as previously obtained for Toma cheese samples produced on a pasture dominated by *F. nigrescens*.[17]

Unlike the results obtained on the hydrocarbon constituents of Toma cheeses,[17] 1-phytene and 2-phytene showed statistically significant differences, according to the type of vegetation

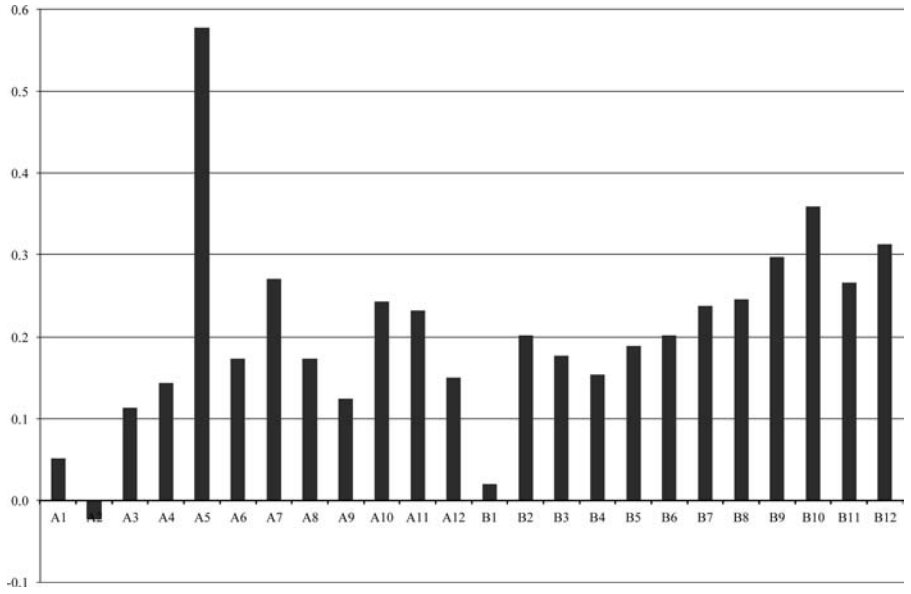

Figure 2. Ratio between the amount of C_{29} and C_{27}, expressed as 1.4 (C_{29}/C_{27}), in Asiago cheese samples produced from milk of cows grazing on vegetations A and B.

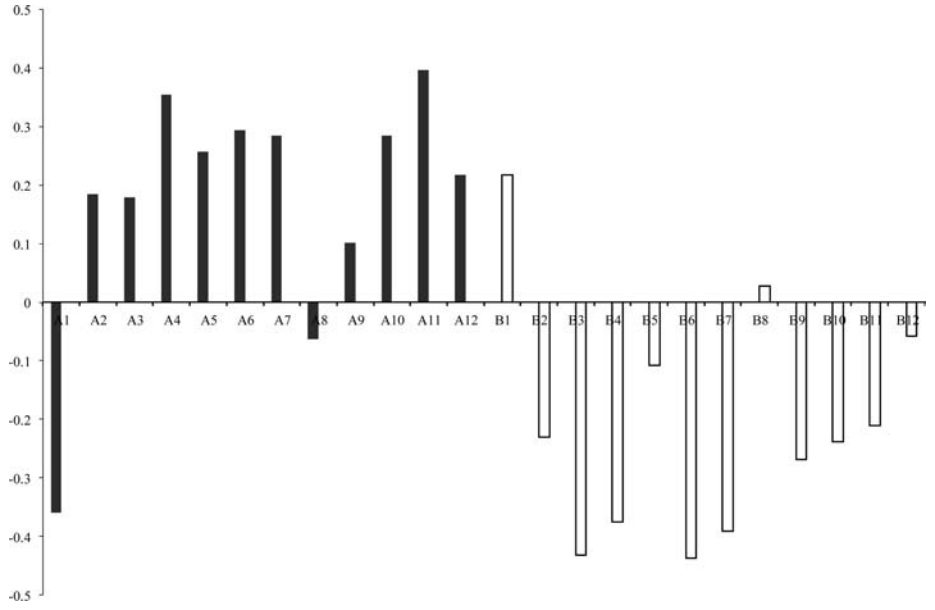

Figure 3. Ratio between the amount of 1-phytene and 2-phytene in Asiago cheese samples, expressed as 1.1 (1-phytene/2-phytene). Vegetation A, solid bars; vegetation B, empty bars.

(Table 6). 1-Phytene was higher in samples obtained from vegetation B, whereas 2-phytene was higher in those from vegetation A. With regard to their origin, 1-phytene derives from hydrogenation of neophytadiene, which in its turn originates from phytol, whereas 2-phytene is probably formed from the migration of the double bond of 1-phytene.[55] Because these molecules are products of different steps of the phytol metabolism, it can be hypothesized that the rumen environment, created by the pastures adopted, affected the chemical reactions on phytol, as previously observed for *trans*-FA and phytanic acid. The ratio between 1-phytene and 2-phytene was then calculated, and all values were subtracted from the overall mean value (1.1 mg kg^{-1} of fat), to better clarify the behavior of each sample group. The values obtained were positive for cheese samples derived from vegetation A and negative for those derived from vegetation B (Figure 3). Two cheese samples, at the same sampling period for each vegetation type (A1, A8, B1, and B8), provided values in contrast with those of samples derived from the same vegetation type. No clear evidence for the reason for these results was found, although, at least for samples A1 and B1, it can be supposed that the rumen microflora was not completely adapted to the type of feeding. Indeed, these cheese samples were produced with milk collected the first day after the entrance of cows in the experimental paddock.

The results obtained in this research, together with those of the previous research,[17] clearly indicate that the botanical composition of the mountain pasture influenced the concentration and ratio of some constituents of the lipid constituents of the dairy products. The multidisciplinary collaboration, including experts in different fields, allowed for a detailed evaluation of most factors

influencing the results. This approach could be considered a key factor also for future studies aiming at the definition of markers of authenticity, which would contribute to the sustainability of the extensive milk production systems, traditionally adopted in the alpine areas and in other highland regions.

AUTHOR INFORMATION

Corresponding Author

*Telephone: +39-0371-45011. Fax: +39-0371-35579. E-mail: giovanna.contarini@entecra.it.

Funding

This research was supported by interdepartmental funding from MEF, MIUR, MiPAAF, and MATT within the FISR Project Pro-Alpe "Importance of the Alpine *Terroir* for the Characterization and Preservation of Mountain Dairy Products" (Coordinator: Efisio Piano).

Notes

The authors declare no competing financial interest.

REFERENCES

(1) Jutzeler van Wijlen, R. P.; Colombani, P. C. Grass-based ruminant production methods and human bioconversion of vaccenic acid with estimations of maximal dietary intake of conjugated linoleic acids. *Int. Dairy J.* **2010**, *20*, 433−448.

(2) Dewhurst, R. J.; Shingfield, K. J.; Lee, M. R. F.; Scollan, N. D. Increasing the concentrations of beneficial polyunsaturated fatty acids in milk produced by dairy cows in high-forage systems. *Anim. Feed Sci. Technol.* **2006**, *131*, 168−206.

(3) White, S. L.; Bertrand, J. A.; Wade, M. R.; Washburn, S. P.; Green, J. T.; Jenkins, T. C. Comparison of fatty acid content of milk from Jersey and Holstein cows consuming pasture or a total mixed ration. *J. Dairy Sci.* **2001**, *84*, 2295−2301.

(4) Kraft, J.; Collomb, M.; Möckel, P.; Sieber, R.; Jahreis, G. Differences in CLA isomer distribution of cow's milk lipids. *Lipids* **2003**, *38*, 657−664.

(5) Collomb, M.; Sieber, R.; Bütikofer, U. CLA isomers in milk fat from cows fed diets with high levels of unsaturated fatty acids. *Lipids* **2004**, *39*, 355−364.

(6) Collomb, M.; Bütikofer, U.; Sieber, R.; Jeangros, B.; Bosset, J. O. Composition of fatty acids in cow's milk fat produced in the lowlands, mountains and highlands of Switzerland using high-resolution gas chromatography. *Int. Dairy J.* **2002**, *12*, 649−659.

(7) Collomb, M.; Bisig, W.; Butikofer, U.; Sieber, R.; Bregy, M.; Etter, L. Fatty acid composition of mountain milk from Switzerland: Comparison of organic and integrated farming system. *Int. Dairy J.* **2008**, *18*, 976−982.

(8) Sickel, H.; Bilger, W.; Ohlson, M. High levels of α-tocopherol in Norwegian alpine grazing plants. *J. Agric. Food Chem.* **2012**, *60*, 7573−7580.

(9) Lourenco, M.; Van Ranst, G.; Vlaeminck, B.; De Smet, S.; Fievez, V. Influence of different dietary forages on the fatty acid composition of rumen digesta as well as ruminant meat and milk. *Anim. Feed Sci. Technol.* **2008**, *145*, 418−437.

(10) Collomb, M.; Butikofer, U.; Sieber, R.; Jeangros, B.; Bosset, J. O. Correlation between fatty acids in cows' milk fat produced in the lowlands, mountains and highlands of Switzerland and botanical composition of the fodder. *Int. Dairy J.* **2002**, *12*, 661−666.

(11) De Noni, I.; Battelli, G. Terpenes and fatty acid profiles of milk fat and "Bitto" cheese as affected by transhumance of cows on different mountain pastures. *Food Chem.* **2008**, *109*, 299−309.

(12) Tornambé, G.; Cornu, A.; Pradel, P.; Kondjoyan, N.; Carnat, A. P.; Petit, M.; Martin, B. Changes in terpene content in milk from pasture-fed cows. *J. Dairy Sci.* **2006**, *89*, 2309−2319.

(13) Falchero, L.; Lombardi, G.; Gorlier, A.; Lonati, M.; Odoardi, M.; Cavallero, A. Variation in fatty acid composition of milk and cheese from cows grazed on two alpine pastures. *Dairy Sci. Technol.* **2010**, *90*, 657−672.

(14) Coppa, M.; Martin, B.; Pradel, P.; Leotta, B.; Priolo, A.; Vasta, V. Effect of a hay-based diet or different upland grazing systems on milk volatile compounds. *J. Agric. Food Chem.* **2011**, *59*, 4947−4954.

(15) Consonni, R.; Cagliani, L. R. Ripening and geographical characterization of Parmigiano Reggiano cheese by ^{1}H NMR spectroscopy. *Talanta* **2008**, *76*, 200−205.

(16) Bontempo, L.; Larcher, R.; Camin, F.; Hölzl, S.; Rossmann, A.; Horn, P.; Nicolini, G. Elemental and isotopic characterization of typical Italian alpine cheeses. *Int. Dairy J.* **2011**, *21*, 441−446.

(17) Povolo, M.; Pelizzola, V.; Lombardi, G.; Tava, A.; Contarini, G. Hydrocarbons and fatty acid composition of cheese as affected by the pasture vegetation type. *J. Agric. Food Chem.* **2012**, *60*, 299−308.

(18) Smit, H. J.; Taweel, H. Z.; Tas, B. M.; Tamminga, S.; Elgersma, A. Comparison of techniques for estimating herbage intake of grazing dairy cows. *J. Dairy Sci.* **2005**, *88*, 1827−1836.

(19) Daget, P.; Poissonet, J. Une méthode d'analyse phytologique des prairies. Critères d'application. *Ann. Agron.* **1971**, *22*, 5−41.

(20) Magurran, A. E. *Ecological Diversity and Its Measurement*; Princeton University Press: Princeton, NJ, 1988.

(21) D'Ottavio, P.; Scotton, M.; Ziliotto, U. Legumes in mountain pastures of Monti Sibillini (Central Apennines, Italy) grazed by sheep. *Grassl. Sci. Eur.* **2000**, *7*, 286−288.

(22) Association of Official Analytical Chemists (AOAC). *Official Methods of Analysis*, 17th ed.; AOAC International: Gaithersburg, MD, 2000.

(23) Tava, A.; Cecotti, R.; Grecchi, M.; Falchero, L.; Coppa, M.; Lombardi, G. Volatile constituents of *Festuca nigrescens*, *Phleum alpinum* and *Poa alpina* from N.W. Italian alpine pastures. *Nat. Prod. Commun.* **2011**, *6*, 101−105.

(24) National Institute of Standards and Technology (NIST). *Mass Spectral Database, Standard References Database Program of the NIST, Version 2.0 for PC*; NIST: Gaithersburg, MD, 2001.

(25) Adams, R. P. *Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry*, 4th ed.; Allured Publishing: Carol Stream, IL, 2007.

(26) Povolo, M.; Pelizzola, V.; Ravera, D.; Contarini, G. Significance of the non-volatile minor compounds of the neutral lipid fraction as markers of the origin of dairy products. *J. Agric. Food Chem.* **2009**, *57*, 7387−7394.

(27) Goodwin, T. W.; Mercer, E. I. *Introduction of Plant Biochemistry*, 2nd ed., Pergamon Press: New York, 1983.

(28) Hatanaka, A. The biogeneration of green odour by green leaves. *Phytochemistry* **1993**, *34*, 1201−1218.

(29) Jennings, W.; Shibamoto, T. *Qualitative Analysis of Flavour and Fragrance Volatiles by Glass Capillary Gas Chromatography*; Academic Press: New York, 1980.

(30) Orav, A.; Arak, E.; Raal, A. Phytochemical analysis of the essential oil of *Achillea millefolium* L. from various European countries. *Nat. Prod. Res.* **2006**, *20*, 1082−1088.

(31) Shawl, A. S.; Srivastava, S. K.; Syamasundar, K. V.; Tripathi, S.; Raina, V. K. Essential oil composition of *Achillea millefolium* L. growing wild in Kashmir, India. *Flavour Fragrance J.* **2002**, *17*, 165−168.

(32) Weyerstahl, P.; Marschall, H.; Seelmann, I.; Rustaiyan, A. Constituents of the essential oil of *Achillea eriophora* DC. *Flavour Fragrance J.* **1997**, *12*, 71−78.

(33) Magiatis, P.; Skaltsounis, A. L.; Chinou, I.; Haroutounian, S. A. Chemical composition and in-vitro antimicrobial activity of the essential oils of three Greek *Achillea* species. *Z. Naturforsch.* **2002**, *57*, 287−290.

(34) Dove, H.; Mayes, R. W. Using *n*-alkanes and other plant wax components to estimate intake, digestibility and diet composition of grazing/browsing sheep and goats. *Small Ruminant Res.* **2005**, *59*, 123−139.

(35) Lacey, S.; Ljerka, K.; Reinhard, J. Sealing plant surface: Cuticular wax formation by epidermial cells. *Annu. Rev. Plant Biol.* **2008**, *59*, 683−707.

(36) Dove, H.; Mayes, R. W. The use of plant wax alkanes as marker substances in studies of the nutrition of herbivores: A review. *Aust. J. Agric. Res.* **1991**, *42*, 913−952.
(37) Fraser, M. D.; Theobald, V. J.; Moorby, J. M. Determining diet composition on complex swards using *n*-alkanes and long-chain fatty alcohols. *Ecol. Appl.* **2006**, *16*, 1901−1910.
(38) Dove, H.; Mayes, R. W. Plant wax components: A new approach to estimating intake and diet composition in herbivores. *J. Nutr.* **1996**, *126*, 13−26.
(39) Martins, H.; Elston, D. A.; Mayes, R. W.; Milne, J. A. Assessment of the use of *n*-alkanes as markers to describe the complex diets of herbivores. *J. Agric. Sci.* **2002**, *138*, 425−434.
(40) Ferreira, L. M. M.; Oliván, M.; Celaya, R.; Garcia, U.; Rodrigues, M. A. M.; Osoro, K. The use of *n*-alkanes to estimate diet composition of ruminants grazing on species diverse plant communities—Effect of feeding selectivity on diet composition estimates. *Livest. Sci.* **2007**, *111*, 114−123.
(41) Dawson, L. A.; Mayes, R. W.; Elston, D. A.; Smart, T. S. Root hydrocarbons as potential markers for determining species composition. *Plant Cell Environ.* **2000**, *23*, 743−750.
(42) Cortes, C.; Damasceno, J. C.; Bechet, G.; Prache, S. Species composition of ryegrass (*Lolium perenne*) and tall fescue (*Festuca arundinacea*) mixtures using various combinations of *n*-alkanes. *Grass Forage Sci.* **2005**, *60*, 254−261.
(43) Palic, R.; Eglinton, T. I.; Benitez-Nelson, B. C.; Eglinton, G.; Velickovic, J.; Stojanovic, G. Alkanes from plants of the genus *Achillea*. *J. Serb. Chem. Soc.* **1999**, *64* (7−8), 443−446.
(44) Urbach, G.; Stark, W. The C-20 hydrocarbons of butterfat. *J. Agric. Food Chem.* **1975**, *23*, 20−24.
(45) Um, K. W.; Bailey, M. E.; Clarke, A. D.; Chao, R. R. Concentration and identification of volatile compounds from heated beef fat using supercritical CO_2 extraction−gas liquid chromatography/mass spectrometry. *J. Agric. Food Chem.* **1992**, *40*, 1641−1646.
(46) European Union (EU). Regulation (CE) No. 510/2006, O. J. series C 321. *Off. J. Eur. Union*, Dec 29, **2006**.
(47) Leiber, F.; Kreuzer, M.; Nigg, D.; Wettstein, H. R.; Scheeder, M. R. A study on the causes for the elevated ω-3 fatty acids in cows' milk of alpine origin. *Lipids* **2005**, *40*, 191−202.
(48) Vetter, W.; Schroder, M. Phytanic acid—A tetramethyl-branched fatty acid in food. *Lipid Technol.* **2011**, *23*, 175−178.
(49) Schroder, M.; Yousefi, F.; Vetter, W. Investigating the day-to-day variations of potential marker fatty acids for organic milk in milk from conventionally and organically raised cows. *Eur. Food Res. Technol.* **2011**, *232*, 167−174.
(50) Kramer, J. K. G.; Cruz-Hernandez, C.; Zhou, J. Conjugated linoleic acids and octadecenoic acids: Analysis by GC. *Eur. J. Lipid Sci. Technol.* **2001**, *103*, 594−632.
(51) Ferreira, L. M. M.; Celaya, R.; García, U.; Rodrigues, M. A. M.; Osoro, K. Differences between domestic herbivores species in alkane faecal recoveries and the accuracy of subsequent estimates of diet composition. *Anim. Feed Sci. Technol.* **2009**, *151*, 128−142.
(52) Benchaar, C.; Calsamiglia, S.; Chaves, A. V.; Fraser, G. R.; Colombatto, D.; McAllister, T. A.; Beauchemin, K. A. A review of plant-derived essential oils in ruminant nutrition and production. *Anim. Feed Sci. Technol.* **2008**, *145*, 209−228.
(53) Khiaosa-ard, R.; Soliva, C. R.; Kreuzer, M.; Leiber, F. Influence of alpine forage either employed as donor cow's feed or as incubation substrate on in vitro ruminal fatty acid biohydrogenation. *Livest. Sci.* **2011**, *140*, 80−87.
(54) Cabiddu, A.; Molle, G.; Decandia, M.; Spada, S.; Fiori, M.; Piredda, G.; Addis, M. Responses to condensed tannins of flowering sulla (*Hedysarum coronarium* L.) grazed by dairy sheep. Part 2: Effects on milk fatty acid profile. *Livest. Sci.* **2009**, *123*, 230−240.
(55) Larick, D. K.; Hedrick, H. B.; Bailey, M. E.; Williams, J. E.; Hancock, D. L.; Garner, G. B.; Morrow, R. E. Flavor constituents of beef as influenced by forage- and grain-feeding. *J. Food Sci.* **1987**, *52*, 245−251.