

Application of Near-Infrared Spectroscopy as an Alternative to Chemical and Color Analysis To Discriminate the Production Chains of Asiago d'Allevo Cheese

Giulio Cozzi,*,[†] Jacopo Ferlito,[†] Gabriella Pasini,[‡] Barbara Contiero,[†] and Flaviana Gottardo[†]

[†]Dipartimento di Scienze Animali and [‡]Dipartimento di Biotecnologie Agrarie, Università di Padova, Viale dell'Università 16, 35020 Legnaro, Italy

A near-infrared spectroscopy (NIRS) application was developed to discriminate Asiago d'Allevo cheese coming from different production chains (alpine farms, mountain and lowland factories). One hundred wheels were collected in different seasons from all productive sites of Asiago d'Allevo: 14 alpine farms and 8 mountain and 13 lowland factories. Samples were analyzed for chemical composition and color and scanned by NIRS (1100–2500 nm). A factorial discriminant analysis based on chemical and color data showed a clear separation between alpine and factory products due to their different fatty acids profile and color. However, cheeses from lowland and mountain factories were undistinguishable. A discriminant analysis using NIRS spectra alone or combined with chemical and color data showed similar results. A final calibration based on NIRS spectra was developed and validated by a set of 7 external samples to discriminate alpine from factory products. This real-time analysis is a reliable alternative to expensive and time-consuming lab determinations.

KEYWORDS: Near-infrared spectroscopy; Asiago d'Allevo cheese; production chain; discriminant analysis

INTRODUCTION

"Asiago" is a typical Italian cheese produced from raw dairy milk according to a regulation approved by the European Union (1). Asiago cheese actually comes in two forms, a young Asiago called "Pressato" and an aged form called "Asiago d'Allevo" with a minimum ripening time of 6 months. Its geographical area of production, in the northeastern part of Italy, includes both lowland and alpine zones, and in this scenario the same cheese-making procedure is applied to milk coming from very diverse husbandry conditions.

It is well-known that cheese nutritional and organoleptic profiles are affected by the milk quality resulting from a given feeding plan of the cows. In particular, forage quality, in terms of botanic composition and storage system, and the forage to concentrate ratio of the diet may result in a different end product within the same cheese-making chain. Pasture grazing compared to preserved forages has shown to modify milk fatty acids with an increase of the unsaturated fraction and conjugated linoleic acid (CLA) (2). These fatty acids can be transferred to cheese, leading to a softer texture (3). Studies were carried out to differentiate mountain cheese from lowland products, by examining lipophilic aliphatic compounds brought by specific herbaceous species such as terpenes and sesquiterpenes (4, 5). Favaro et al. (6) indicated sesquiterpens, revealed by gas chromatography, as an effective marker for the traceability of Asiago mountain cheese. However, more rapid and low-cost screening techniques are needed and should be developed in order to help consumers recognize cheese obtained from different chains of production.

Near-infrared spectroscopy (NIRS) is a recognized efficient tool for real-time control of food production lines. This spectroscopic technique is nondestructive, simple, and rapid because it does not need any sample preparation. The use of NIRS coupled with chemometry to determine quality and identity of dairy products has been reviewed by Karoui and de Baerdemaeker (7). In cheese making, NIRS calibrations have been developed for monitoring rennet coagulation (ϑ) and for the quantification of chemical constituents during ripening (ϑ). Recently NIRS has been used for the prediction of fatty acids (10) and other chemical and physical components of fresh and freeze-dried cheeses (11).

The first aim of the present work is to compare the chemical composition and color of Asiago d'Allevo cheese produced in different production chains. The second aim is to discriminate the cheese from the different production sites by chemical and color analyses, and the third aim is to verify if a real-time NIRS application might be used as an alternative to these expensive and time-consuming lab determinations.

MATERIALS AND METHODS

Description of the Production Chains. The study considered all existing productive sites of Asiago d'Allevo cheese. Each site was assigned to one of the three different chains of production, alpine farm (AF), mountain factory (MF), and lowland factory (LF), on the basis of location and elevation of the area in which milk was collected (lowland < 300 m;

^{*}Author to whom correspondence should be addressed. Tel: ++39-049-8272662. Fax: ++39-049-8272679. E-mail: giulio.cozzi@unipd.it.

Table 1. Description of the Production Chains of Asiago d'Allevo Cheese

	F	production chains	
	alpine farm	mountain factory	lowland factory
no. of productive sites	14	8	13
location	mountain	mountain	lowland
elevation of the area where milk is produced (m above sea level)	>1000	>600	<300
average production of wheels (no./year)	530	5300	20000
production period	seasonal (summer)	all seasons	all seasons
cattle breeds (% total herd)			
Holstein	16	36	82
Brown	35	44	18
dual purpose	49	20	
dairy cows feeding plan			
forage:concentrate ratio (% total dry matter)	80:20	65:35	60:40
forage included in the diet			
maize silage (no. of sites)		2	12
meadow and lucerne hay (no. of sites)	1	8	13
grazing pasture (no. of sites)	14	7	

mountain > 600 m), size (number of wheels/year), and period of production (seasonal or throughout the year). In each site, a specific questionnaire was submitted to the cheese making manager to gather descriptive information about the prevailing management of dairy farms in which milk was collected. Specific questions considered the herd composition in terms of cattle breeds distinguishing between high-producing (Holstein, Brown) and dual purpose breeds (Rendena, Alpine Gray, and Simmental). The cows' feeding plan was described considering either the average forage:concentrate ratio or the type of roughage included in the diet.

Fourteen out of the 35 sites were small-scale AF operating only during the summer season according to an extensive system of production. The milk processed in these sites comes from a single herd made of cows mainly belonging to local dual purpose breeds (**Table 1**). According to a traditional farming system, these herds are transferred to the alpine farm located at high elevation in early June. All of the lactating cows have seasonal calving, and at the beginning of the summer grazing they are in a late stage of lactation. Pasture grazing is the main feed source for these animals, and it is supplemented with a small portion of energy concentrates provided at the two daily milkings.

The remaining 21 sites were classified as industrial cheese factories, with 8 located in the mountains and 13 in the lowland. Mountain cheese factories are of medium size in terms of number of wheels produced per year, and they collect milk in the mountain area from several dairy producers mainly raising high producing and dual purpose dairy cows under semi-intensive rearing systems (Table 1). These herds are fed diets with an average forage to concentrate ratio close to that adopted by the lowland farms. Meadow and alfalfa hay are the prevailing roughage sources included in these diets, since in the largest part of the mountain areas where Asiago d'Allevo cheese is produced it is impracticable and inconvenient to grow corn for silage making. During the summer, some milk processed by these sites may come from grazing herds, but it represents always a small portion of the total. Cheese factories located in the lowland are the biggest in terms of wheels produced per year, and they process milk from intensive dairy farms in which high-producing Holstein and Brown cows are raised (Table 1). High concentrate diets are provided to these animals, which do not graze during the summer season. In most of these diets, maize silage provides 35-45% of the total dry matter, and lucerne and meadow hay are the other source of forage included in the rations.

Cheese Sampling. Target of the study was Asiago d'Allevo cheese with a ripening time of 12 months. The sampling protocol considered 100 wheels of cheese (weight 8-12 kg, diameter 30-36 cm, height 9-12 cm) that were collected at the different production sites during the period June 2003 to January 2005 as reported in **Table 2**. The sampling scheme was designed to represent all seasons of production of the cheese. In this regard, it must be noted that alpine farms produced only during the summer;

Table 2. Sampling Scheme Adopted in the Study Expressed as Number of Wheels

	2003				2004					2005	
production chain	June	July	Sept	Oct	Jan	March	June	July	Sept	Jan	total
alpine farm mountain factory	7	7	6 2	5	3	4	8	7 2	7 2	5	42 23
lowland factory	3	3	2	5	7	6	2	1	1	5	35

the limited number of wheels collected from the cheese factories during this season was due to the reduced production of this ripened cheese in this period of the year. However, in order to avoid an overlapping between season and production chain effects, the sampling scheme adopted in the study considered for all the summer months the collection of at least 3 wheels of cheese from industrial factories (**Table 2**).

Cheese Chemical and Color Analysis. Cheese samples were analyzed in duplicate for several chemical constituents. Moisture content was measured by the oven drying method at 102 °C (*12*), total lipids by the Gerber van Gulik method (*13*), and total protein and water-soluble nitrogen by the Kjeldahl method (*14*). Soluble nitrogen in 12% trichloroacetic acid was determined according to Gripon et al. (*15*), and sodium chloride by the potentiometric method (*16*).

Fatty acids were extracted from 10 g of cheese ground with a mixture of dichloromethane/methanol solution (2:1 v/v) as described by Nourooz-Zadeh and Appelqvist (17). The samples were then transesterified according to the Christie procedure (18), and fatty acid methyl esters were analyzed using a gas chromatograph (Shimadzu GC-17A, Shimadzu Corporation, Kyoto, Japan) equipped with flame ionization detector, using a capillary column (Omegawax 250, 30 m × 0,25 nm; Supelco Inc., Bellefonte, PA, USA).

Cheese color was measured using a portable Minolta CM-2002 spectrocolorimeter (Minolta Camera Co., Osaka, Japan), and the CIELAB color system (19) was chosen to numerically describe the color parameters.

Near-Infrared Spectroscopic Analysis. A slice of cheese (at least 150 g) was ground with a Retsch Grindomix (Retsch GmbH, Haan, Germany) at 4000 rpm for 10 s under liquid nitrogen after removal of 2 cm of crust all around. Approximately 20 g of grated fresh cheese was placed in a 50 mm diameter ring cup and scanned in the reflectance mode at 2 nm intervals from 1100 to 2500 nm using a Foss NIRSystems model 5000 scanning NIR spectrometer (Foss NIRSystems, Silver Spring, MD, USA) controlled by WinISI II software version 1.5 (Infrasoft International, Port Matilda, PA, USA). Each spectrum was time averaged from 32 scans. Reflectance values were converted into absorbance values using the formula absorbance= log(1/reflectance). Calibrations were developed using WinISI II version 1.5 (Infrasoft International). The modified partial least-squares (PLS) regression was used to obtain the calibrations, and data were subjected to a scattering correction by standard normal variate and detrending (20) followed by a 1,4,4 mathematical pretreatment where the first digit is the derivative order, the second is the number of data points over which the derivative is calculated, and the third is the number of data points used in the smoothing.

Statistical Analysis. Cheese chemical and color data were submitted to ANOVA within PROC-GLM (21), adopting a linear model that considered the fixed effects of production chain and sampling month. This latter factor was included in the model to allow a comparison among the production chains purified for any season effect. The two degrees of freedom of the production chain factor were used to perform the following set of orthogonal contrasts: AF vs cheese factories [(MF + LF)/2] and MF vs LF

A factorial discriminant analysis (FDA) was performed within PROC-CANDISC (21) using a pooled data set of chemical and color parameters to discriminate the three production chains of Asiago d'Allevo cheese. The data set included only variables for which the previous ANOVA showed a significant production chain effect, and it was subsequently reduced by deleting those variables that showed a high multicolinearity. Relationship between the initial variables and the discriminant factors were displayed in a correlation circle, and Fisher's distances were calculated to display differences among chains of productions. Samples scores were plotted on the factors space, and 95% confidence ellipses for the three chains of cheese production were displayed. The final confusion matrix with the overall percentage of well-classified samples was calculated and a 10×10 cross

		production chains	orthogonal con			
chemical composition	alpine farm (AF)	mountain factory (MF)	lowland factory (LF)	AF vs (MF + LF)/2	MF vs LF	SE ^a
moisture (%)	28.2	27.4	27.5	ns	ns	0.35
total lipids (%)	34.2	35.4	35.5	0.04	ns	0.45
total protein (%)	30.9	30.7	30.5	ns	ns	0.29
water-soluble nitrogen (%)	1.5	1.3	1.3	0.02	ns	0.06
12% TCA soluble nitrogen (%)	1.1	0.9	0.9	0.02	ns	0.05
NaCl (%)	2.7	2.4	2.5	ns	ns	0.09

^a Standard error.

fold validation was applied. The robustness of the classification was evaluated by sensitivity and specificy indexes and by validation error of the three chains of production.

Samples were classified according to the three chains of production using NIR spectra in a PLS discriminant analysis carried out by WinISI II (version 1.5. Infrasoft International). In a following step, a general discriminant analysis computed by Statistica version 6.1 (StatSoft Inc. Tulsa, OK, USA) combined spectra with chemical and color data to evaluate if there was any improvement in the results of the confusion matrix. In this procedure, spectral information was accounted using the scores of the first three principal components, which explained more than 90% of the total spectral variance.

RESULTS AND DISCUSSION

Cheese Chemical and Color Analysis. Chemical composition data of cheese are shown in Table 3. Significance differences were detected for total lipids and non-protein nitrogen fractions only for the contrast between AF and the cheese factories. The lower fat content of AF samples could have arisen from the cow feeding plan. Extensive dairy systems frequently show a decrease in milk yield associated with a reduction of fat and protein content due to the incapacity of pasture to meet the nutritional requirements of lactating cows (22). A second hypothesis to explain the same result could be related to the economic value of the butter. Butter produced by AF has a top price due to the great demand by local consumers and tourists, and therefore in these sites the preskimming of the milk prior the cheese making is supposed to be more forced. The increase of the soluble nitrogen fractions observed in AF cheese is instead an indication of a more intense maturation process likely due to the less standardized procedures of cheese making and ripening adopted by small-scale producers.

Consistent with the previous results, fatty acids concentration of the cheese samples showed a clear distinction between AF and factories products, regardless of their location (Table 4). Alpine farm cheese had a lower content of short chain fatty acids and C16:0 with an increase of unsaturated fatty acids and ω -3. It is well-known that milk fatty acids composition is affected by the cow's feeding plan and particularly by the amount and the quality of the forage included in the diet (23). Confirming previous findings of Chilliard et al. (2), MF and LF cheese made by processing milk of dairy cows fed preserved forages and/or corn silage increased the concentration of short chain fatty acids, C14:0 and C16:0. In contrast, pasture-grazing promoted the endogenous synthesis of C18:1 t11 (24) which is the main precursor of CLA in milk and cheese (25, 26). On the basis of the fatty acids profile, AF cheese showed interesting dietary properties not only related to positive effects of CLA but also to the lower content of C12:0 and C16:0, which are recognized risk factors for coronary heart diseases (27).

The different chain of production also affected cheese color (**Table 5**). Cheese made in AF showed significant higher redness and yellowness values than factory products, while no difference was detected between MF and LF samples. The increased

Table 4.	Effect of Production Chains on Fatty Acids Concentration (g/100 g	g of
Fattv Aci	ds) of Asiago d'Allevo Cheese ^a	-

	I	production cha	orthogonal	contrasts		
fatty acids	alpine farm (AF)	mountain factory (MF)	lowland factory (LF)	AF vs (MF+LF)/2	MF vs LF	SE
saturated	56.51	60.67	62.28	<0.001	0.04	0.60
C4:0	2.01	2.11	2.10	0.05	ns	0.03
C6:0	1.31	1.59	1.59	< 0.001	ns	0.06
C8:0	0.75	1.10	1.07	0.001	ns	0.07
C10:0	1.69	2.84	2.70	0.001	ns	0.23
C12:0	2.08	2.96	2.94	<0.001	ns	0.11
C14:0	8.53	10.29	10.32	<0.001	ns	0.17
C15:0	1.07	1.10	1.12	ns	ns	0.02
C16:0	24.94	28.38	29.09	<0.001	ns	0.41
C17:0	0.82	0.70	0.72	0.001	ns	0.02
C18:0	12.73	11.17	11.07	<0.001	ns	0.26
C20:0	0.20	0.18	0.19	ns	ns	0.01
monounsaturated	31.60	26.46	26.59	<0.001	ns	0.52
C14:1 c9	0.70	0.81	0.84	0.002	ns	0.03
C15:1 c10	0.28	0.29	0.27	ns	0.07	0.01
C16:1	1.37	1.30	1.43	ns	0.01	0.04
C17:1 c10	0.31	0.29	0.31	ns	ns	0.02
C18:1 c9	25.20	21.25	21.65	<0.001	ns	0.41
C18:1 t11	3.58	2.38	1.92	<0.001	ns	0.21
polyunsaturated	5.48	4.88	4.36	<0.001	< 0.001	0.11
CLA	1.37	1.10	0.97	ns	ns	0.16
ω-3	0.90	0.74	0.66	<0.001	ns	0.04
ω-6	3.09	3.13	2.98	ns	ns	0.07

^a Abbreviations: SE, standard error; CLA, Σ(*cis*-9-*trans*-11-C18:2; *trans*-10-*cis*-12-C18:2); ω-3, Σ(C18:3n3; C20:3n3; C20:5n3; C22:6n3); ω-6, Σ(C18:2n6; C18:3n6; C20:3n6; C20:4n6).

Table 5. Effect of Production Chains on Color of Asiago d'Allevo Cheese^a

		production cha	orthogonal co			
chemical composition	alpine farm (AF)	mountain factory (MF)	lowland factory (LF)	AF vs (MF + LF)/2	MF vs LF	SE
lightness (L)	73.0	73.2	74.4	ns	ns	0.71
redness (a _L)	2.0	0.2	0.2	<0.001	ns	0.18
yellowness (b_L)	18.6	14.9	14.2	<0.001	ns	0.84

^a Abbreviations: SE, standard error.

yellowness of cheese made with milk coming from pasturegrazing cows has been reported in previous studies (28, 29). Yellow colors originate from β -carotene and related carotenoid compounds, which are transferred from fresh plants to milk and cheese. These compounds are sensitive to oxidation, photolysis, and temperature and suffer serious depletion during forage preservation and storage (30).

The total canonical structure of FDA performed using the pooled data set of chemical and color parameters showed a Wilks' Lambda test value of 0.15 (P < 0.0001). Two discriminant factors were detected (**Figure 1**). The first explained 95% of the total variability and was positively correlated with C12:0, C14:0, C14:1, and C16:0 and negatively correlated with C18:1 c9,

C18:1 t11, and redness. The second factor explained the remaining 5% of the total variability and positively correlated with C16:1. Canonical coefficients of the samples were plotted on the factors space and 95% confidence ellipses for the three chains of cheese production were displayed (**Figure 2**). Fisher's distances among production chains were calculated, and significant values were detected only for AF vs MF (10.3; P < 0.001) and AF vs LF



Figure 1. Factorial discriminant analysis of the Asiago d'Allevo samples using the pooled data set of chemical and color parameters: main variables correlated with the 2 discriminant factors.



Figure 2. Factorial discriminant analysis of the Asiago d'Allevo samples using the pooled data set of chemical and color parameters: plot of the samples on factors space and 95% confidence ellipses.

(15.4; P < 0.001). On the contrary, there was a substantial overlapping between MF and LF sample scores (Fisher's distances= 1.0; P < 0.43) as a consequence of the similar management of these two production chains. Consistent with these results, the confusion matrix generated by FDA showed a high percentage of correct classification only for AF samples with 0% of false positive rate (1 - specificity), 4.8% of false negative rate (1 - sensitivity) and a validation error of 5.83%. The classification of MF and LF samples showed instead lower values for sensitivity and specificity and higher validation errors (Table 6). The uncertain classification of the samples coming from industrial factories must not be considered a failure of the analysis, but it is a clear consequence of the similar farming system adopted by intensive dairy producers located either in the lowland or in the mountains. In the past decade, the increase of the milk price along with the convenient cost of the energy concentrates has oriented many dairy farmers operating in the mountain environment to embrace intensive ways of farming introducing high producing dairy cattle breeds and high concentrates diets. This strategy led to an increase in milk yield, but as clearly shown by FDA results, made cheese quality conform to the standard of lowland products.

Near-Infrared Spectroscopic Analysis. Consistent with previous results obtained in fresh cheeses by Lucas et al. (11), maxima in the averaged spectra were located at 1208, 1456, 1728, 1762, 1940, 2308, and 2348 nm for all three production chains of Asiago d'Allevo samples. The absorption bands at 1456 and 1940 nm are attributed to water (31) and originate, respectively, from the first overtone of the O-H stretch (1456 nm) and the combination band of the asymmetric and scissor stretch O-H vibrations (1940 nm). All remaining maxima are related to lipids (11, 32) and arise from the second overtone of C-H stretch (1208 nm), the first overtone of a C-H stretch (1728 and 1762 nm), and combination bands coming from C-H stretch and deformation in a CH₂ group (2308 and 2348 nm) in lipid molecules. Results of the discriminant analysis computed by using the NIR spectra alone are presented in Table 7. More than 90% of the AF samples were correctly assigned to the class they belong to with a false positive rate lower than 20%. The NIR region does not include the visible region; therefore it is likely that the good discrimination of AF samples by NIRS mainly arose from their different fatty acid profile.

The accuracy of classification based on NIR spectra alone was instead very low for the MF samples, which showed a sensitivity of 8.7%. Five out of the 8 MF samples misclassified as AF were summer wheels likely produced in sites where some milk coming from grazing herds is processed. The correct classification of these samples was improved by the discriminant analysis combining spectral data with chemical and color determinations (**Table 7**). The misclassification between MF and LF samples by the calibration based on NIR spectra alone was much more frequent, and it regarded samples collected at all of the different sampling times. This unsatisfactory performance confirms the outcomes of FDA based on chemical and color data (**Table 6**) and is consistent with the conclusions of parallel research that tried to identify the production chain of a subset of the same cheese samples by using nuclear magnetic resonance spectroscopy (*33*). The correct

Table 6. Confusion Matrix Generated by Factorial Discriminant Analysis Based on Chemical and Color Data and Its Validation Indexes

			predicted					
real	п	alpine farm	mountain factory	lowland factory	sensitivity (%)	specificity (%)	validation error (%)	
alpine farm	42	40	1	1	95.2	100.0	5.83	
mountain factory	23	0	17	6	73.9	93.5	47.00	
lowland factory	35	0	4	31	88.6	89.2	23.33	

Table 7. Confusion Matrix of Discriminant Analysis Carried Out To Correctly Identify Samples of the Three Chains of Production by Using NIR Spectra Alone or Pooled with Chemical and Color Data

		NIR spectra					NIR spectra + chemical and color data				
		predicted					predicted				
real	n	alpine farm	mountain factory	lowland factory	sensitivity (%)	specificity (%)	alpine farm	mountain factory	lowland factory	sensitivity (%)	specificity (%)
alpine farm	42	38	1	3	90.5	82.8	40	2	0	95.2	96.5
mountain factory lowland factory	23 35	8 2	2 3	13 30	8.7 85.7	94.8 75.4	2 0	16 4	4 31	69.6 88.6	92.2 93.7

Table 8. Confusion Matrixes of Discriminant Analysis Carried Out To Correctly Identify Samples of Alpine Farms from Those Produced by Cheese Factories Using NIR Spectra Alone or Pooled with Chemical and Color Data

		NIR spectra					NIR spectra + chemical and color data				
		pre	predicted			predicted					
real	п	alpine farm	cheese factory	sensitivity (%)	specificity (%)	alpine farm	cheese factory	sensitivity (%)	specificity (%)		
alpine farm cheese factory	42 58	39 5	3 53	92.9 91.4	91.4 92.9	40 1	2 57	95.2 98.3	98.3 95.2		

classification of MF and LF samples was only partially improved by the discriminant analysis combining NIR spectra with chemical and color data, which halved from 16 to 8 the misclassified samples coming from the industrial factories (**Table 7**). Therefore location and elevation cannot be considered reliable markers of a different cheese quality unless they are expression of a real diverse dairy farming system in terms of cattle breeding and feeding management, as in the case of AF.

On the basis of these findings, a final set of calibrations has been developed to discriminate AF samples from all others coming from industrial factories, and the corresponding confusion matrixes are presented in Table 8. The correct identification of sample based on NIR spectra alone was satisfactory for both classes with sensitivity and specificity values higher than 90%. The accuracy of the discrimination was slightly improved by combining the spectral data with chemical and color values (Table 8), but this better performance does not justify the cost and time budget required by the wet chemistry and color determination. The robustness of the discriminant equation based on NIRS spectra alone to segregate AF cheese samples from industrial factory ones was assessed by an external validation. The validation set was made of 7 independent Asiago d'Allevo samples with a similar ripening time as the training set. All cheese samples were collected in the year 2008, 2 from AF and 5 from LF. The prediction for the production chain correctly classified all of the validation samples.

Outcomes of this study indicate that cheeses produced in different altimetric locations can be distinguished by both NIRS and lab analysis only when they represent real diverse dairy production systems. The application of NIRS to discriminate the production chain of Asiago d'Allevo cheese has shown performance similar to that obtained by using chemical and color analysis, and therefore it has proved to be a reliable alternative to more expensive and time-consuming lab determinations. The inclusion of the visible region together with the NIR region as input for the discriminant models along with the increase in the number of samples belonging to each cheese category could bring a further improvement in accuracy of classification. However, the real-time analysis allowed by the use of NIRS could help consumers to identify alpine cheese from industrial products obtained in the same environment under intensive rearing systems. Moreover the possible certification of AF products through the NIRS application may represent a promising tool to promote this type of farming system, which has been shown to be the only sustainable solution for the protection of the mountain environment.

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