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Relevance of Isotopic and Molecular Biomarkers for the Authentication of Milk According to Production Zone and Type of Feeding of the Cow

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The first objective of the present paper was to assess the potential of both isotopic ($^{18}O/^{16}O$ in milk water) and molecular biomarkers (terpenes, fatty acids, carotenoids, and vitamins) and milk color to discriminate the production zone (lowland or upland areas) from which 49 tanker bulk milks were collected over one year from a total of 204 farms. The milk water ^{18}O enrichment was higher in lowland (<500 m altitude) than in upland (>700 m altitude), but the $\delta^{18}O$ values failed to discriminate systematically the production zone at the scale of the year because of its high variability related to the sampling period. In contrast with vitamins A and E, carotenoids, and milk color measurements, terpenes and fatty acids were confirmed to be relevant tracers of the production zone. The milk compounds with the strongest discriminative potential were fatty acids, which were determined by high-resolution gas chromatography. The calculation of fatty acid ratios, which permits the limitation of using fatty acid relative quantity expressed in percentage of total fatty acids to be overcome, was shown to be particularly relevant in discriminating upland from lowland milk ratios. The selection of two pairs of ratios, namely, *iso*-C17:0/C18:3 n-3 and *iso*-C15:0/*iso*-C14:0, enabled the authentication of 100% of the highland versus lowland milks whatever the season. The second objective was to evaluate the relevance of fatty acid composition to discriminate milks according to the proportion of corn silage in the diets of dairy cows. The selection of two fatty acids ratios, namely, *trans*11*cis*15-C18:2/*trans*11-C18:1 and *cis*9-C16:1/*iso*-C16:0, enabled the correct classification of 100% of the milk samples according to the proportion of corn silage in the basic fodder rations (<25% vs >30%). The relationship between the milk production zone and the type of forage fed to the cows is discussed.

KEYWORDS: Tanker milk; authentication; fatty acids; terpenes; isotopes; upland/lowland; animal diet

INTRODUCTION

After several recent food crises, consumers are increasingly seeking food products having conditions of production that are friendlier to the environment and/or warrant the product quality from a sensory, nutritional, or safety point of view. In addition to traditional traceability systems based on documents, there is an increasing need for instrumental tools enabling the authentication of the key quality specifications of the product. Some conditions of milk production such as cow grass feeding or upland that are known to confer specific organoleptic and nutritional qualities to the milk products (*1*–*4*) provide an added value to the product and justify its higher price. As milk is a relatively expensive raw material in Europe, it could be attractive to falsify key information regarding its production conditions. As they may be dissuasive against fraud, authentication methods represent an important strategic issue for the dairy industry (*5*) and also for bodies operating product quality certification systems that need to be able to guarantee that specification commitments are fully met.

Efforts have recently been made to develop analytical tools to quantify specific compounds in animal tissues or fluids that can act as tracers of the conditions of production. In the dairy sector, stable isotope techniques have proved to be useful in obtaining information on the cow diet and in identifying the geographical origin of milk (*6*–*8*). Focusing on the discrimination between milk produced in lowland or upland zones, Renou et al. (*9*) showed that $^{18}O/^{16}O$ and $^{2}H/^{1}H$ ratios in the milk water determined by isotopic ratio mass spectrometry are relevant biomarkers when the milk is obtained in controlled experimental conditions. Similarly, some volatile compounds such as terpenes have been reported as effective biomarkers of milk and cheese

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production area (upland vs lowland) (*10–12*) due to their rapid transfer from dicotyledon-rich upland grass to milk fat (*13*). Moreover, the feeding of cows with upland grass has been shown to result in specific fatty acid composition of the dairy products compared to lowland milks (*14, 15*), suggesting that these compounds are potential tracers of the production zone. Several reports reviewed by Martin et al. (*16*) suggest that other biomarkers of the type of feeding of cows such as carotenoids, vitamins, or milk color determinants could be relevant for discriminating lowland from upland milks.

The research of isotopic or molecular biomarkers as mentioned above has been performed so far on milk samples from individual cows or small groups of cows fed contrasted diets or from contrasted areas and collected over short periods of time. The relevance of these biomarkers on the bulk milks used by dairy factories and collected by tankers over the year has not yet been demonstrated. The first objective of the present paper was to assess the potential isotopic and molecular biomarkers to discriminate tanker bulk milks collected in lowland and upland areas over one year. The second objective was to evaluate the relevance of these biomarkers to discriminate milks according to the type of feeding of cows and, more particularly, the presence of corn silage in the diet.

MATERIALS AND METHODS

Experimental Design. The study took place in 10 areas of the Haute-Loire department (located in the Massif Central area, France) that differed mainly by the altitude (from 440 to 1150 m) and the forage system used (diets based on grass-fresh or -preserved or corn silage). Within each of these 10 areas, one round (group of dairy farms for the collection of milks) had been chosen so that the 10–36 farms within each round (204 farms in total) were similar regarding altitude and forage system. Milk from the 10 collection round tankers was sampled five times in the course of the year 2002 at key times in animal feeding: twice in the overwintering period with diets based on preserved forage (February and March) and three times during the grazing period: turnout to grass with abundant young grass in May, summer drought in July, and regrowth at the end of September. A total of 50 milk samples were collected in the tankers. Due to a problem of transportation, only 49 of them were analyzed. In addition, one sample of drinking water was collected in one representative herd of each round.

To characterize the milk production conditions on the day it was collected, four surveys (a main one in winter and three additional ones in the grazing period) were carried out with each of the 204 farmers. Questions were asked about (1) farm characteristics (area, altitude, milk production quota, quantities of milk delivered by each farm), (2) herd characteristics (number of dairy cows, calving distribution, breed), (3) forage management (forage characteristics, forage harvesting and conservation techniques, cutting and grazing periods), and (4) feeding of the herd (types of feed used, including pasture, preserved forage, and concentrates). Regarding the latter point, special attention was paid to type of forage (corn silage, grass), type of grassland (permanent or temporary grassland), altitude of grazed paddocks, preservation mode (pasture, hay, silage, wrapped forage), and the estimated respective levels of these feeds.

Instrumental Analyses. The 49 milks analyzed were sampled from farm tankers containing from 4082 to 32998 L of milk. The milk collected corresponded to four or six consecutive milkings. A 1 L sample was collected after milk homogenization and carried at 4 °C to the laboratory.

Fat, Protein, Lactose. A 50 mL subsample of fresh milk was used to determine fat, protein, and lactose contents by infrared spectrophotometry (Milkoscan 4000; Foss Electric, Hillerød, Denmark).

Color. Milk color (L^*, a^*, b^*) was measured by reflectance between 400 and 700 nm (10 nm steps) on fresh milk using a Minolta CM 2002 spectrocolorimeter (Minolta France S.A., Carrières-sur-Seine, France) applied on the bottom of an optical glass (5 cm diameter) containing 20 mL of milk. The color index was calculated as the upper area of the reflectance spectra between 450 and 530 nm (*16*).

Vitamins and Carotenoids. Retinol (vitamin A), α-tocopherol (vitamin E), β-carotene, and xanthophyll (lutein and zeaxanthin) contents in milk were simultaneously measured on frozen milks preserved at −80 °C by HPLC using a UV–visible photodiode array detector after saponification and hexane extraction adapted from the method of Lyan et al. (*17*) as described by Lucas et al. (*4*).

Fatty Acids. Analyses were performed on frozen samples kept at −20 °C. One aliquot (3 mL) was lyophilized, and fatty acids in lyophilized milk were directly methylated according to the method of Loor et al. (*18*). Samples were injected by autosampler into a Trace-GC 2000 series gas chromatograph equipped with a flame ionization detector (Thermo Finnigan, Les Ulis, France). Methyl esters from all samples were separated on a 100 m × 0.25 mm i.d. fused-silica capillary column (CP-Sil 88, Chrompack, Middelburg, The Netherlands). The injector temperature was maintained at 250 °C and the detector temperature at 255 °C. The initial oven temperature was held at 70 °C for 1 min, increased at 5 °C/min to 100 °C (held for 2 min), then increased at 10 °C/min to 175 °C (held for 40 min), and increased at 5 °C/min to a final temperature of 225 °C (held for 15 min). Hydrogen was the carrier gas. Identification of *trans*-C18:1, nonconjugated C18:2, and CLA isomers was as described in Loor et al. (*18*). A butter reference standard (CRM 164, Commission of the European Communities, Community Bureau of Reference, Brussels, Belgium) was used to estimate correction factors for short-chain FA (C4:0 to C10:0).

Terpenes. Milk terpene analysis was performed on a 180 mL aliquot stored at −20 °C. Lipids were extracted according to a method adapted from Viallon et al. (*19*), to perform a single centrifugation step. The frozen milk samples were left overnight to thaw at 20 °C. About 40 g of the creamy upper layer was weighed in a 40 mL screw-capped polyallomer bottle (Beckman, 357003) and centrifuged for 2 h at 75000*g* at 25 °C in a Beckman Avanti J-301centrifuge (Beckman Inc., Palo Alto, CA). The supernatant liquid fat phases (clear yellow) were taken up using a Pasteur pipet and stored at −20 °C. Volatile compounds were extracted by the dynamic headspace method using a Tekmar LSC 2000 apparatus (Cincinnati, OH). The fat (0.2 g) was deposited on 0.2 g of glass wool placed in a glass extraction cartridge (diameter = 28 mm, height = 70 mm). The extraction conditions were as follows: purge, 30 min at 110 °C with helium, 47 mL min^{-1}; trap on Tenax at 30 °C; preheating of the trap, 175 °C; desorption at 180 °C, 5 min; transfer line temperature, 200 °C; cryofocusing at −150 °C in the chromatograph injection port; injection by heating for 2 min at 225 °C; gas chromatograph oven temperature, 40 °C; baking of trap before next injection, 30 min at 180 °C. Volatile compounds were separated by gas chromatography using a Hewlett-Packard 5890 chromatograph. The separation conditions were as follows: capillary column (60 m × 0.32 mm) (Supelco, Gland, Switzerland); stationary phase, SPB-5 (1 μm); carrier gas, helium (1 mL min^{-1}); oven program, 5 min at 40 °C, rise to 230 °C at 3 °C/min and then 2 min at 230 °C. Detection, semiquantification, and identification were performed using an HP5971S electron impact (70 eV) mass spectrometer (Hewlett-Packard France). The terpenes were detected in the selected ion mode by monitoring their characteristic ions at *m*/*z* 93, 136, 161, and 204 (*12*). Semiquantification was performed by integrating the 93 ion peaks between 25 and 50 min for monoterpenes and the *m*/*z* 161 ion between 50 and 70 min for sesquiterpenes, using the MS Chemstation software (Hewlett Packard France). No correction was applied for coelution or mass fragment ratios. The results were expressed in arbitrary area units (aau). A sample chosen among the richest in terpenes was analyzed again, monitoring ions between *m*/*z* 33 and 230. Terpene identifications were proposed on the basis of mass spectra and experimental retention indices, by comparison with those found in published databases (*20–22*).

$\delta^{18}O$. Samples were stored at −20 °C before further analysis. Milk for ^{18}O enrichment was prepared by rennet addition to a 20 mL full milk aliquot. The sample was left overnight at room temperature, after which milk water was collected by filtration and frozen at −20 °C until analysis. For both milk water and cow drinking water, ^{18}O enrichment values were determined by gas chromatography–isotope ratio mass spectrometry (VG Isochrom-μgas, VG Isotech, Cheshire, U.K.). Samples (1 mL) were introduced into 10 mL vacutainers (100

× 16 mm, Becton Dickinson sterile vacutainers with no additive) previously filled with a 5% CO_2/He gas mixture at atmospheric pressure. The vacutainers were then placed in a shaker and equilibrated at 25 °C for a minimum of 10 h, by which time at least 99% equilibrium had been reached. Results were expressed as the isotope ratio in parts per million relative to that of International Standard Vienna Mean Ocean Water (SMOW): $\delta = (R_S/R_{SMOW} - 1) \times 10^3$, where R_S and R_{SMOW} are the heavy and light isotope ratios in the sample and SMOW, respectively. The $^{18}O/^{16}O$ isotope ratio in SMOW is 2005.2 ppm.

Data Treatment. Data were processed using the Statistica Software release 6.1 package (Statsoft, Maisons-Alfort, France).

Indicators describing the milk production conditions for the 49 milks were built using the results of the surveys weighted by the relative contribution of each farm to the filling of the tanker the day of sampling (0.1–27.6%). We calculated for each of the 49 milk samples the mean altitude of the herd and the proportion of grass and corn silage in the fodder ration. The samples were considered to belong to the lowland and upland groups when the altitude of their production site ranged from 442 to 465 m (453 ± 16 m on average, $n = 9$) and from 723 to 1152 m (990 ± 148 m, $n = 40$), respectively. Moreover, corn silage was considered to be practically present or absent in the diet when its proportion was found to be >30% ($n = 13$) or <25% ($n = 36$) of animal intake, respectively.

For isotope ratios, the effect of the production zone and the sampling period (February, May, July, and September) were tested using a two-way ANOVA including the interaction.

For each sample, the relative quantity of each fatty acid (normalized fatty acid) was expressed as percentage of the sum of the areas of the quantified fatty acids of the sample. According to the question treated, discriminative fatty acids were determined by one-way ANOVA according to the models: normalized fatty acid = production zone (lowland/upland) or animal feeding (>30%/<25% of corn silage in the diet), respectively. All possible ratios between significant fatty acids were calculated. Significant fatty acid ratios were determined by one-way ANOVA according to the models: fatty acid ratio = production zone (lowland/upland) or animal feeding (>25% or <30% of corn silage in the diet), respectively. Apart from fatty acids, discriminative compositional variables and color measurement were filtered by one-way ANOVA according to the model variable production zone or animal feeding, respectively ($p < 0.05$). To assess the robustness of the discriminative variables, a leave-one-out cross-validation procedure was undertaken on one-way ANOVA models obtained for each discriminative variable or ratio of variables on the complete data set ($n = 49$ samples). A discriminative variable was considered to be validated when it was significant ($p < 0.05$) for all of the 49 ANOVAs performed on the n–1 data set (*23*).

Discrimination of Production Zone and Animal Feeding. The corresponding ANOVAgrams, graphic representation of the factor contribution to the variation of the abundance of each fragment/specific ion, were constructed for both production zone and animal feeding questions. Discriminant analyses (DA) were carried out on discriminative variables to select the best subset of variables enabling the discrimination of the productions zones or the animal diet. Only the two most relevant variables were selected according to the "best subset" algorithm.

RESULTS AND DISCUSSION

Isotopic Biomarkers of Production Zone. In accordance with Renou et al. (*9*), the milk water ^{18}O enrichment was higher in lowland than in upland ($p < 0.05$, $R^2 = 0.15$), but the $\delta^{18}O$ values could not discriminate systematically the production zones because their variability related to the sampling period was too high (**Figure 1**). As already reported by Kornexl et al. (*6*), the lowest values were measured in winter and the highest in spring and summer, the autumn values being intermediate ($p < 0.001$). The influence of the sampling period is not linked to a drinking water ^{18}O enrichment, which was constant on average over the different periods (**Figure 1**). The drinking water $\delta^{18}O$ was correlated negatively with the altitude ($p < 0.0001$, R^2=0.78, result not shown), and the depletion was −0.19 for every 100 m in elevation, confirming previous geological data obtained for groundwater in upland areas (*24*). The seasonal variations of the milk water $\delta^{18}O$ may be explained by a variable isotope intake with food. Actually, the animals were fed diets that differed according to the sampling period and changes in food composition—or food type—are known to modify milk water $\delta^{18}O$ values (*9*). Most of the water intake by the cows at pasture is from fresh plants, whereas it is from drinking water in winter. According to Kornexl et al. (*6*), fresh plants contain, relative to groundwater, ^{18}O-enriched water due to evapotranspiration of the plants. In addition, perspiration of the animals while grazing at pasture during the warmest months also leads to an ^{18}O enrichment of their body water and to a further increase in the $\delta^{18}O$ values in milk. As a consequence of the seasonal variations observed, the milk water $\delta^{18}O$ values are suitable for the discrimination of the production zone only within a short period but not over a whole year.

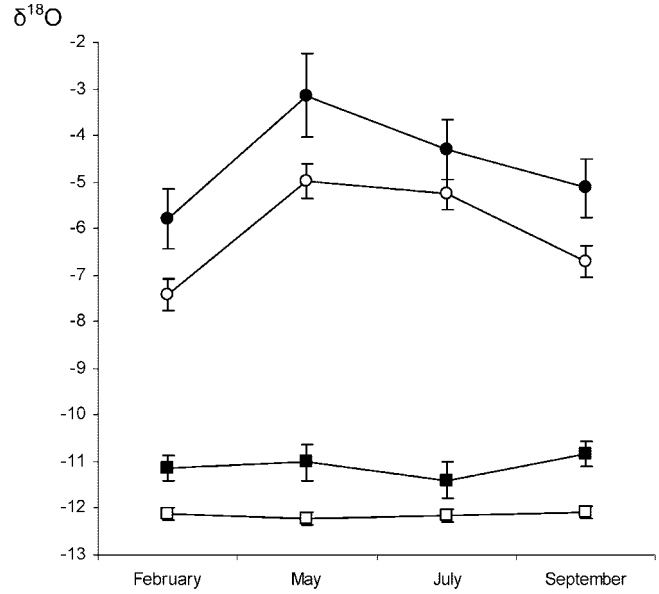

Figure 1. $\delta^{18}O$ values for drinking and milk water according to the period, in lowland and upland, for the two types of dairy cow feedings: (solid circles) lowland milk water; (open circles) upland milk water; (solid square) lowland drinking water; (open square) upland drinking water.

Molecular Biomarkers of Production Zone. According to **Figure 2**, 10 terpenes, 30 fatty acids, and 1 carotenoid enabled the discrimination of milks according to their production zone ($p < 0.05$), showing that these measurements are still relevant when they are performed on milk subsamples obtained by bulking milk samples collected over the year.

Terpenes. As shown in **Figure 2a**, discriminative terpenes were a group of 10 sesquiterpenes that were found to be more abundant in upland compared with lowland. This is in agreement with previous results obtained by Cornu et al. (*25*). Five of the nine identified sesquiterpenes, β-bourbonene, β-caryophyllene, γ-muurolene, and γ- and δ-cadinene, were already identified in milk as potential tracers of a cow diet based on natural grassland plants (*26*). Moreover, germacrene-D was found among the volatile components of natural grassland plants (*27*). These observations suggested that the lowland–upland discrimination based on terpenes is related, at least partly, on the predominance of natural grassland plants in the diet of cows grazing in upland areas.

According to several papers, terpene intake of ruminants can change with the season due to the modifications affecting the

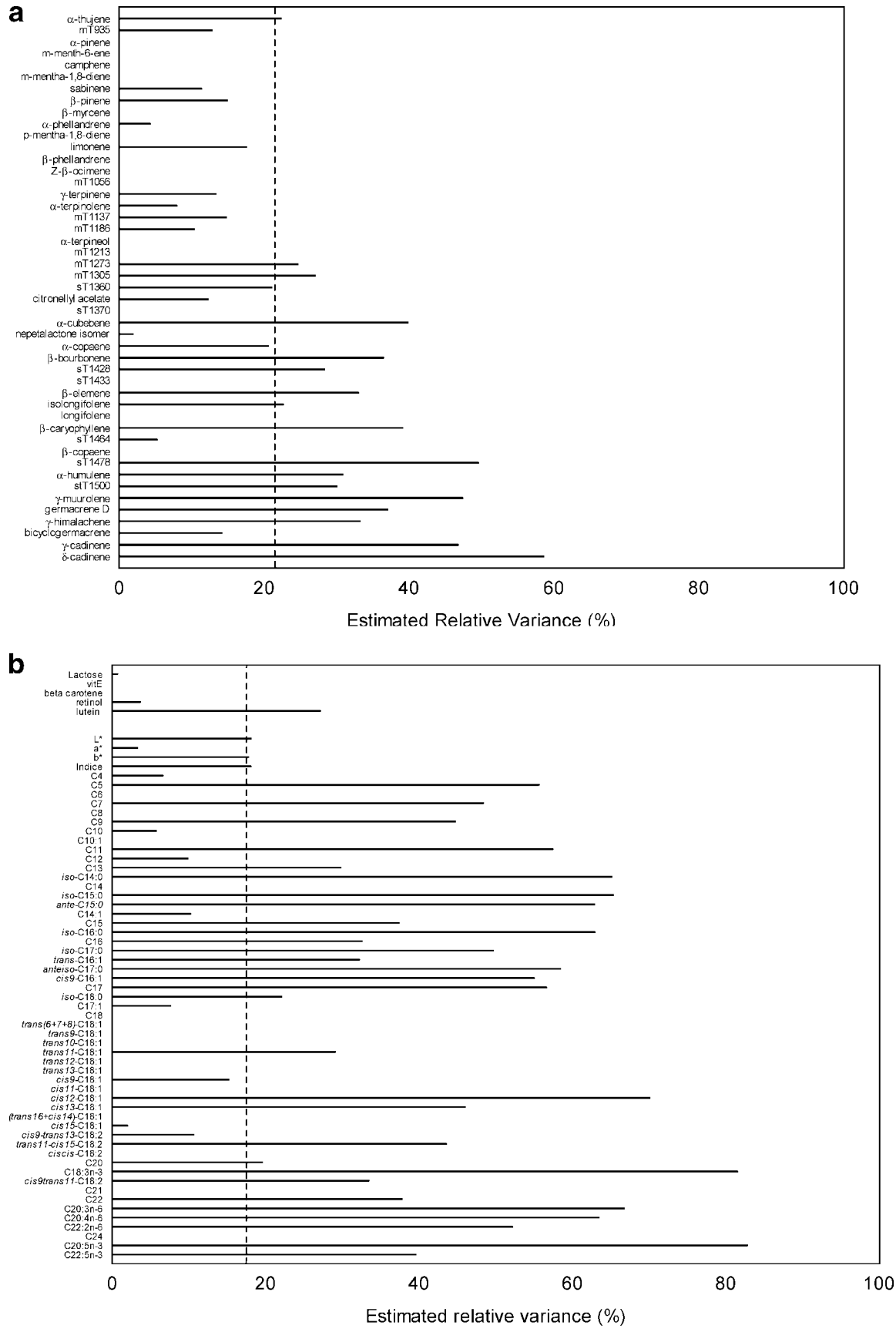

Figure 2. Ability of the constituents and color parameters of milk to discriminate milk production zone: (**a**) terpenes; (**b**) nonvolatile compounds. For each compound or color parameter, the discriminative power is represented by the estimated relative variance of the production zone factor (upland or lowland) after one-way ANOVA. The minimum estimated relative variance value is indicated by the dotted line. It corresponded to the threshold value above which the production zone factor is significant ($p < 0.05$) and validated according to the "leave-one-out" procedure. mT or sT followed by a number indicates, respectively, a monoterpene or a sesquiterpene and its retention index.

forage composition during its storage for winter feeding (*12, 28*). To evaluate the potential effect of the season factor on the robustness of an authentication based on terpenes, two-way ANOVAs were processed on each discriminative terpene, the two sources of variation being "production zone" (lowland/upland) and "season" (winter/summer). No significant interaction was found. The significance of the production zone was confirmed for all terpenes, pointing out the relevance of these compounds as tracers of upland cow milk versus lowland cow milk. The influence of the season on milk composition was more terpene-dependent. In contrast with the other six sesquiterpenes, the content of which was higher during the grazing period, the abundances in γ-muurolene, δ-cadinene, γ-cadinene, and an unidentified sesquiterpene eluting at a retention index of 1478 were not significantly affected by season. According to Fernandez et al. (*12*), losses of grass chemical components resulting from the wilting process affect the lighter terpenes and monoterpenes more than the sesquiterpenes. The relatively high retention index of the sesquiterpenes remaining unaffected by the season is consistent with this hypothesis.

Also, to ensure milk traceability based on the analysis of terpene fractions, a validation of the discriminative power of terpenes is necessary. Fernandez et al. (*12*) could discriminate upland and lowland milks produced in winter or summer using a model including six terpenes. β-Caryophylene, β-bourbonene, and γ-cadinene, which have already been identified in natural grassland plants (*27*), were part of the discriminative compounds. Even if the previous identification of these terpenes by previous studies (*12, 27*) strengthens the tracer status of these compounds, the limited geographical dispersion of the upland regions sampled in Auvergne may explain the consistency of the results. Finally, before conclusions can be drawn on the general relevance of these compounds as tracers of upland milk, it will be necessary to sample upland milk in other regions and to check the between-year variability of the plant content in these compounds due to climate fluctuations.

Carotenoids and Vitamins. **Figure 2b** shows that the lutein content differs significantly in upland and lowland regions. This carotenoid was found at a higher content in most milk samples from upland regions compared with milk samples from lowland regions. In contrast, no difference between lowland and upland milks could be found in the abundance in fat-soluble vitamins, in β-carotene, or in the parameters used to measure milk color (**Figure 2b**). The vitamin A and E contents in milk depend directly on their levels in the cow diet, which in turn are governed by the forage contents in β-carotene and vitamin E, respectively. However, they can also be influenced by the inclusion of supplemental synthetic vitamins A and E in the diets (*29*), which could explain the poor discriminative power of milk vitamins A and E evidenced in our data. The carotenoid contents of milk are highly dependent on their intake by the cows (*30*), which varies according to the type of forage. In contrast with corn silage, fresh grass and grass silage are carotene-rich. In our data, the proportions of forage (pasture and grass silage) in both lowland and upland areas are not sufficiently contrasted to exhibit the discriminative potential of β-carotene and subsequently milk color measurements (L^*, a^*, b^* and index). Whereas the β-carotene content of grass forage is known to decrease strongly during sun-drying (*30*), the lutein losses occur less rapidly. Additionally, previous data showing that lutein content in dairy products is higher when cows are fed hay-based diets compared with corn silage based diets suggest that the lower lutein content of lowland milk results from the higher proportion of corn silage in the lowland diet (*4*).

Table 1. Significant Differences in Fatty Acid Mean Content between Lowland and Upland Milks (Grams per 100 g of Total Fatty Acids)

fatty acid[a]	lowland ($N = 9$)	upland ($N = 40$)
C5:0	0.032	0.024
C7:0	0.029	0.022
C9:0	0.035	0.027
C11:0	0.063	0.048
C13:0	0.201	0.182
iso-C14:0	0.110	0.158
iso-C15:0	0.265	0.352
anteiso-C15:0	0.498	0.619
C15:0	1.224	1.323
iso-C16:0	0.271	0.345
C16:0	33.229	29.627
iso-C17:0	0.377	0.448
*trans*9-C16:1	0.067	0.117
anteiso-C17:0	0.570	0.667
*cis*9-C16:1	1.581	1.353
C17:0	0.596	0.673
iso-C18:0	0.048	0.054
*trans*11-C18:1	1.059	1.968
*cis*12-C18:1	0.156	0.101
*cis*13-C18:1	0.056	0.043
*trans*11*cis*15-C18:2	0.060	0.222
C20:0	0.123	0.137
C18:3 n-3	0.381	0.734
*cis*9*trans*11-C18:2	0.479	0.908
C22:0	0.060	0.072
C20:3 n-6	0.073	0.060
C20:4 n-6	0.108	0.088
C20:5 n-3	0.051	0.078
C22:2 n-6	0.025	0.037
C22:5 n-3	0.074	0.090

[a] The relative quantity of each fatty acid (normalized fatty acid) is expressed as a percentage of the sum of the areas of the quantified fatty acids of the sample.

Fatty Acids. According to **Figure 2b**, the estimated relative variance of nine fatty acids was higher than that of the other variables (<60%), suggesting that these compounds have a particularly strong discriminative potential. As shown in **Table 1**, the milk fat percentages of C18:3 n-3 (α-linolenic acid), *cis* 9*trans*11-C18:2 (rumenic acid, the main isomer of milk conjugated linoleic acids, CLA), C20:0, C22:2 n-6, *iso*-C14:0, *iso*-C15:0, *iso*-C16:0, *iso*-C17:0, and C20:5 n-3 were higher in upland milk, confirming previous data (*14, 31, 32*). However, our results show that lowland milks were richer in C7:0, C16:0, and *cis*9-C16:1, which agrees with the findings of Collomb et al. (*14*). In addition to some of the major fatty acids, the presence of which in upland milk has been widely investigated in the literature due to their putative beneficial (n-3, CLA) or detrimental (C16:0, C18:2 n-6) effects on health (*32, 33*), minor fatty acids that can now be quantified by high-resolution gas chromatography were confirmed to be relevant tracers of the production zone. In accordance with Collomb et al. (*14*), who observed that *iso*-C14:0, *iso*-C15:0, *iso*-C16:0, and *iso*-C17:0 were more abundant in milks from uplands than in milks from lowlands, *iso*-fatty acids ranging from C14:0 to C17:0 could be considered as tracers of upland origin (**Table 1**). The C14–C17 *iso*-acids are mostly synthesized by rumen bacteria and are then transferred to milk. Increasing forage to concentrate ratio in the diet is likely to enhance dietary neutral detergent fiber content and thus to result in a higher percentage of milk *iso*-fatty acids (*34*). The predominant part of the cow diet taken by permanent grassland forages, and consequently the high proportions of dietary fiber (*35, 36*), in upland areas, thus could explain the higher percentages of *iso*-acids in the milks from these areas.

The present work agrees with the findings of Collomb et al. (*14*) that lowland milks are more generally characterized by a

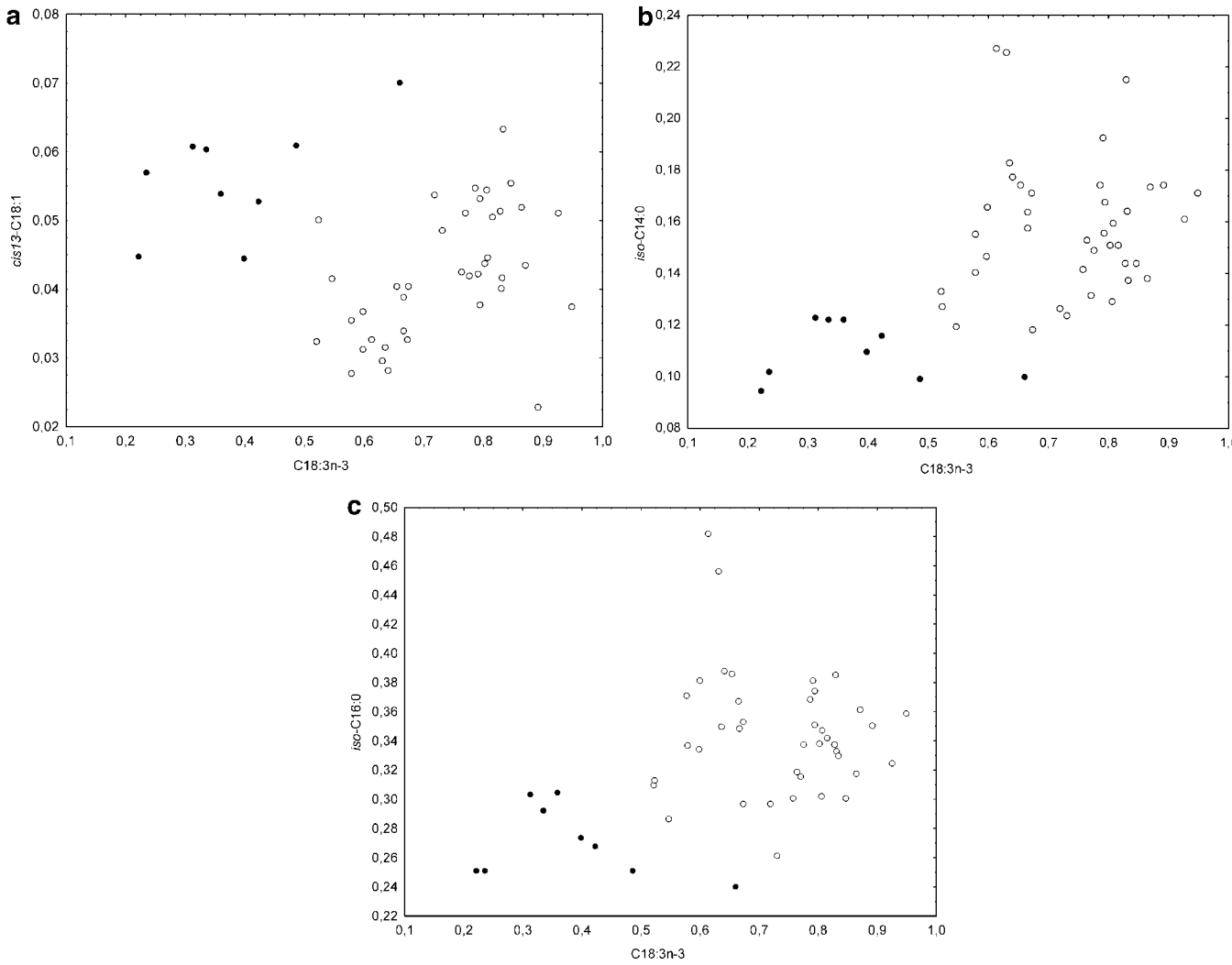

Figure 3. Discrimination of milk from lowland and upland based on their composition in fatty acids: (**a**) C18:3 n-3, *cis*13-C18:1; (**b**) C18:3 n-3, *iso*-C14:0; (**c**) C18:3 n-3, *iso*-C16:0. The relative quantity of each fatty acid (normalized fatty acid) is expressed as a percentage of the sum of the areas of the quantified fatty acids of the sample. The two types of production zones are (solid circles) lowland milk and (open circles) upland milk.

higher content in odd linear short- and medium-chain fatty acids (ranging from C5 to C13). These odd linear chain fatty acids are derived from incorporation of propionate in fatty acid of rumen bacterial lipids or to a lesser extent as a substrate for de novo mammary synthesis, which could be increased with corn silage rich diets (*36*). Because milks from uplands are richer in polyunsaturated fatty acids than those from lowlands, we can hypothesize that the dietary C18:3 n-3 intake is also higher in upland than in lowland diets. Dietary C18:3 n-3 and/or *trans*-fatty acid arising from C18:3 n-3 biohydrogenation could also have an inhibitory effect on de novo synthesis of these fatty acids (*33*, *35*) or could change the activity of ruminal bacteria (*34*).

To select among the 30 tracers identified above (**Figure 2b**) the best subset of compounds enabling the authentication of the production zone whatever the season, fatty acid data were processed by linear discriminant analysis. Only subsets of two tracers were considered for limiting overfitting problems, which may arise from a too high ratio between the number of observations and the number of predictive variables used to build the discriminative model. A leave-one-out cross-validation procedure pointed out that three subsets of two predictive variables (fatty acid percentages) could be used to classify correctly 48 of the 49 milks according to their production zone.

Figure 3 shows the discrimination obtained by plotting the milk samples according to the quantity of the two compounds selected in the subsets. Linolenic acid was systematically selected as one of the two variables, whereas the associated fatty acid was either *cis*13-C18:1, *iso*-C14:0, or *iso*-C16:0, confirming the interest of the determination of percentages of *iso*-C14:0 and *iso*-C16:0 for discriminating upland and lowland milks. Nevertheless, the misclassification pointed out in the three cases shows that the knowledge of the relative content in these fatty acids was insufficient to correctly discriminate all milks according to their production zone.

To improve the discrimination, normalized fatty acids were replaced in the data set by all of the possible ratios of the 30 fatty acids found to be discriminative ($p < 0.05$) according to **Figure 2b**. After a one-way ANOVA filtering according to the production zone, the data were processed by linear discriminant analysis. Several pairs of ratios enabling the correct ranking of 100% of the milks sampled according to their production zone were determined thanks to a leave-one-out cross-validation procedure. The better discrimination obtained with the fatty acid ratios shows that their calculation allowed some limitations of internal normalization, that is, the ratio between the area of a given fatty acid and the sum of the area of the whole fatty acid, to be overcome. Whereas the information related to the relative

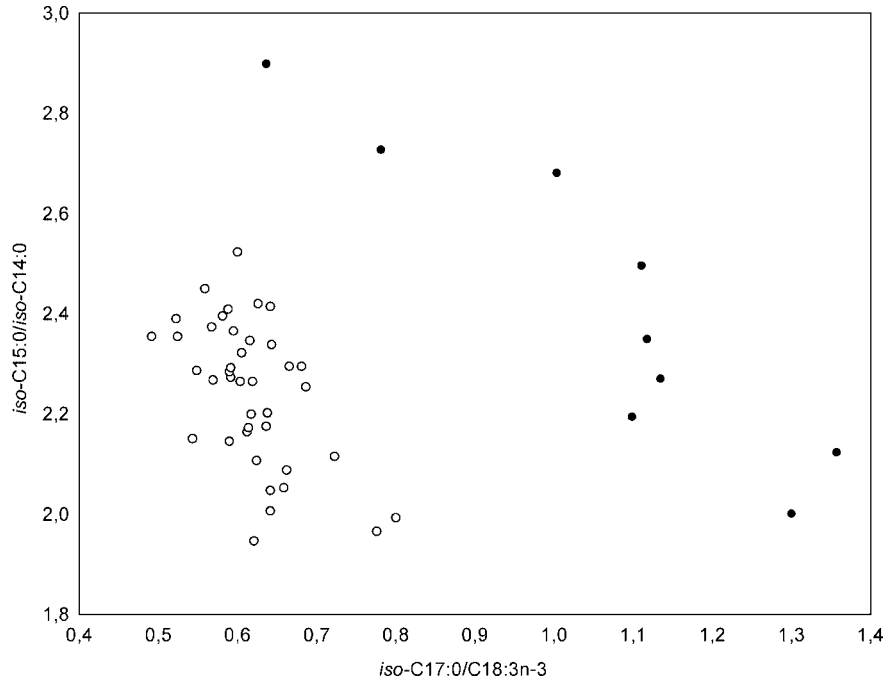

Figure 4. Discrimination of milk from lowland and upland based on the determination of two fatty acid ratios. The two types of production zones are (solid circles) lowland milk and (open circles) upland milk.

quantity of fatty acid is preserved, the addition of the uncertainties associated with the quantification of each fatty acid and its inclusion in the denominator through internal normalization (*23*, *37*) is indeed suppressed by the division operation.

Figure 4 shows the discrimination obtained with the best pairs of ratios, namely, *iso*-C17:0/C18:3 *n*-3 and *iso*-C15:0/*iso*-C14:0. Interestingly, linolenic acid and *iso*-C14:0 comprised one of the three best subsets (**Figure 3b**) obtained from data

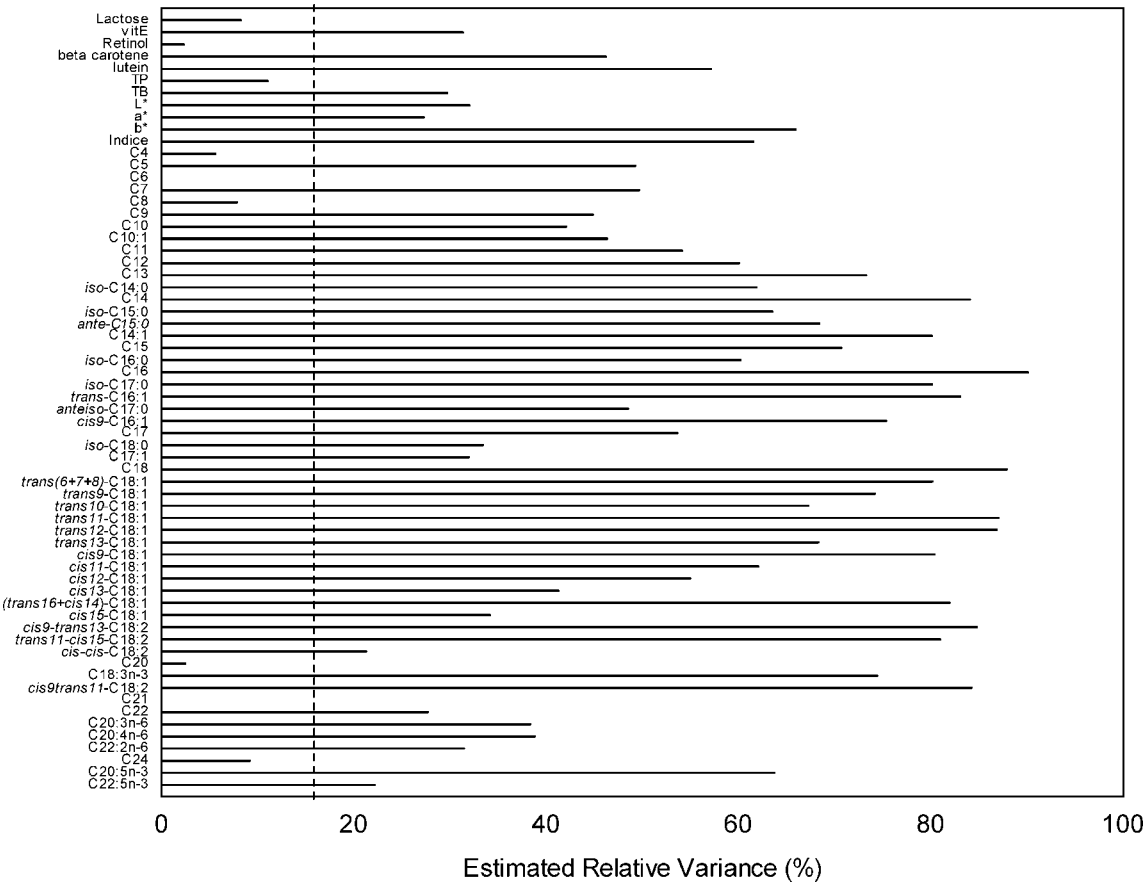

Figure 5. Ability of the constituents and color parameters of milk to discriminate the type of feeding of cows. For each compound or color parameter, the discriminative power is represented by the estimated relative variance of the type of feeding factor (<25% corn silage, >30% corn silage) after one-way ANOVA. The minimum estimated relative variance value is indicated by the dotted line; it corresponded to the threshold value above which the production zone factor is significant ($p < 0.05$) and validated according to the leave-one-out procedure.

preprocessed by internal normalization. This result confirms the relevance of these two FA as potential tracers of the production zone and suggests that ratios are built with the most relevant variables under constraint of a relatively poor correlation between numerator and denominator. This could be explained by the fact that these milk fatty acids have different origins. The C18:3 *n*-3 concentration in milk depends on dietary C18:3 *n*-3 intake and on factors affecting rumen hydrogenation (*18, 38*). Grass is the primary source of C18:3 *n*-3, which explains why milk produced from upland diets contains more C18:3 *n*-3 than milk produced from lowland diets, which contain higher proportions of corn silage (*39, 40*). *iso*-fatty acids (*iso*-C14:0, *iso*-C16:0, *iso*-C15:0, *iso*-C17:0, *iso*-C18:0) are mainly synthesized by cellulolytic bacteria from iso-volatile fatty acids arising primarily from ruminal amino acid degradation. These bacteria could be increased by forage/concentrate ratio or by NDF amount in the diet (*34*). Thus, *iso*-FAs are known to vary mainly according to the dietary neutral detergent fibre content of the diet, which is lower in a corn silage-based diet compared with a grass-based diet. Replacing grass-based diets by corn silage-based diets decreased *iso*-C14:0, *iso*-C15:0, and *iso*-C17:0 percentages in milk (*36*). These literature data suggest that the differences between upland and lowland milks evidenced in the present study originated at least in part from the contrast existing between both contexts of production, that is, the higher use of corn silage in lowland (46% of the fodder ration) and the almost exclusive use of grass fodders in upland (94% of the fodder ration).

Molecular Biomarkers of the Type of Feeding. According to **Figure 5**, 33 fatty acids and lutein enabled the discrimination of milks produced with either <25 or >30% corn silage in the cow diet. **Table 2** presents the discriminative fatty acids. The results agree with previous data dealing with the comparison of grass- and corn silage-based diets (*34, 40–43*). Apart from *anteiso*-C15:0, *cis*9-C18:1, and *cis*9*trans*13-C18:2, the fatty acids for which percentages were higher in milks produced by cows fed low proportions of corn silage were also those fatty acids that were more abundant in upland milks (**Table 1**). Additionally, the fatty acids that were more abundant in milk produced from corn silage rich diets were found to be more abundant in lowland milk. These statements are consistent with the lower corn silage proportions observed in diets of cows located in upland areas compared with those observed in lowland areas. Thus, they confirm that the differentiation of the two production zones was related at least in part to the type of feeding of the cows.

A linear discriminant analysis was carried out on the data to determine the best subset of fatty acids enabling the discrimination of milks according to the proportion of corn silage in the cow diet. When raw data were expressed in percentages of total fatty acids, the best subset selected according to a cross-validation procedure comprised two compounds, C18:3 n-3 and *iso*-C16:0 (**Figure 6a**). Their higher content in milks produced by cows fed low proportions of corn silage and high forage-based diets is consistent with literature data (*34, 39, 40, 43*). Nevertheless, **Figure 6a** shows that when it is based on this subset of normalized fatty acids the discrimination in two groups remained unclear for some of the milks studied.

A linear discriminant analysis was then performed on fatty acid ratios selected as previously described for the discrimination between lowland and upland milks. **Figure 6b** shows that the best subset of ratios, *trans*11*cis*15-C18:2/*trans*11-C18:1 and *cis* 9-C16:1/*iso*-C16:0, enabled the correct classification of 100% of the milk samples according to the proportion of corn silage in cow diet. In the same time that it compressed the data and overcame the limitation of internal normalization, the fatty acid ratio pointed out more subtle information related to differences in metabolic pathways involved in the biosynthesis of the respective discriminant fatty acids and could differ between contrasted production zones or contrasted feeding conditions. Regarding the best subset of two ratios, the literature data confirm the relative independence between the variations of fatty acids featuring on numerator and denominator. First, milk *trans*11*cis*15-C18:2 is a ruminal biohydrogenation intermediate of dietary C18:3 n-3, whereas *trans*11-C18:1 can originate from ruminal biohydrogenation either of *trans*11*cis*15-C18:2 or of *cis*9*trans*11-C18:2 arising from dietary C18:2 n-6 or C18:3 n-3. Thus, the proportions in milk of both *trans*11*cis*15-C18:2 and *trans*11-C18:1 increased when C18:3 n-3 intake increased with grass-based diets, whereas only *trans*11-C18:1 increased with corn silage diets, that is, diets rich in C18:2 n-6 (*35, 44*). Second, *cis*9-C16:1 is endogenously produced from C16:0 by Δ9-desaturase activity in the mammary gland (*33*), whereas *iso*-C16:0 fatty acid is mostly synthesized by rumen bacteria (see previous section). *cis*9-C16:1 and *iso*-C16:0 have thus different origins, and dietary factors may have inverse effects on their concentrations. Indeed, the milk *cis* 9-C16:1 percentage was increased by corn silage-based diets when compared to grass-based diets (*36, 45*), whereas the *iso*-C16:0 percentage was generally decreased by corn silage compared to grass-based diets (*34*).

Table 2. Significant Differences in Lutein and Milk Fatty Acid Composition between Diets

milk constituent[a]	corn < 25% ($N = 13$)	corn > 30% ($N = 36$)
lutein	0.02	0.01
C5:0	0.02	0.03
C7:0	0.02	0.03
C9:0	0.03	0.03
C11:0	0.05	0.06
C13:0	0.18	0.20
iso-C14:0	0.16	0.11
C15:0	1.32	1.25
iso-C15:0	0.36	0.27
anteiso-C15:0	0.63	0.50
C16:0	29.06	33.70
iso-C16:0	0.35	0.28
*cis*9-C16:1	1.33	1.56
*trans*9-C16:1	0.12	0.06
C17:0	0.68	0.60
*cis*9-C17:1	0.27	0.26
iso-C17:0	0.46	0.37
anteiso-C17	0.67	0.58
iso-C18:0	0.06	0.05
*cis*12-C18:1	0.10	0.15
*cis*13-C18:1	0.04	0.05
*trans*11-C18:1	2.11	0.95
*cis*9-C18:1	19.13	17.34
*trans*11*cis*15-C18:2	0.24	0.05
*cis*9*trans*11-C18:2	0.97	0.44
*cis*9*trans*13-C18:2	0.15	0.11
C18:3 n-3	0.75	0.44
C20:3 n-6	0.06	0.07
C20:4 n-6	0.09	0.10
C20:5 n-3	0.08	0.05
C22:0	0.07	0.06
C22:2 n-6	0.04	0.03
C22:5 n-3	0.09	0.08

[a] The relative quantity of each fatty acid (normalized fatty acid) is expressed as percentage of the sum of the areas of the quantified fatty acids of the sample. Lutein is expressed micrograms per milliliter of milk.

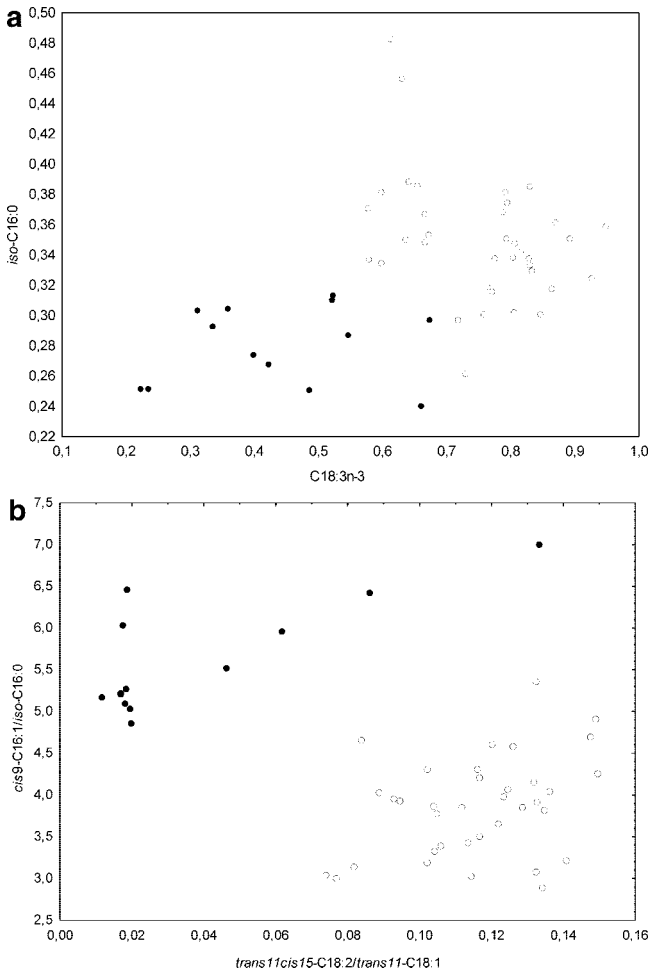

Figure 6. Discrimination of milk according to the richness of the cow fodder ration in corn silage. The discrimination is based on milk composition in fatty acids (**a**) or on the determination of two fatty acid ratios (**b**). In **a**, the relative quantity of each fatty acid (normalized fatty acid) is expressed as a percentage of the sum of the areas of the quantified fatty acids of the sample. The two types of cow feeding are (solid circles) >30% corn silage in the fodder ration and (open circles) <25% corn silage in the fodder ration.

By enabling the determination of key tracers such as the *iso*-FAs, the present findings demonstrate the potential of fatty acid when determined by HRGC and the efficiency of the fatty acid ratio for the authentication of both milk production zone and animal diet. Because of the structure of our data set, the discriminations of milks according to these two factors/criteria were strongly related: the authentication of the production zone based on fatty acid determination was based at least in part on the differences in the animal feeding systems. However, the poor discrimination of animal feeding with ratios selected for the discrimination of production zone (data not shown) showed that, even if the corn silage percentage of the diet and the upland–lowland distinction were related to the same group of fatty acids (**Tables 1** and **2**), their discrimination was finally achieved by two different sets of four different fatty acids. Further research is being undertaken to test the robustness of the biochemical tracers evidenced in this work on a broader range of milk samples, namely, samples collected in different European countries. To dissociate the two issues of milk production zone and type of feeding of the cow, the experimental design also includes both milks from cows fed corn silage and located in upland areas and milks from cows fed a grass-based diet and located in lowland areas.

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LITERATURE CITED

(1) Bosset, J. O.; Jeangros, B.; Berger, T.; Butikofer, U.; Collomb, M.; Gauch, R.; Lavanchy, P.; Scehovic, J.; Troxler, J.; Sieber, R. Comparison of Gruyere-type hard cheese, produced in highland and lowland areas. *Rev. Suisse Agric.* **1999**, *31*, 17–22.

(2) Coulon, J. B.; Delacroix-Buchet, A.; Martin, B.; Pirisi, A. Relationship between ruminant management and sensory characteristics of cheeses: a review. *Lait* **2004**, *84*, 221–242.

(3) Martin, B.; Verdier-Metz, I.; Buchin, S.; Hurtaud, C.; Coulon, J. B. How do the nature of forages and pasture diversity influence the sensory quality of dairy livestock products. *Anim. Sci.* **2005**, *81*, 205–212.

(4) Lucas, A.; Agabriel, C.; Martin, B.; Ferlay, A.; Verdier-Metz, I.; Coulon, J. B. Relationships between the conditions of cow's milk production and the contents of components of nutritional interest in raw milk farmhouse cheese. *Lait* **2006**, *86*, 177–202.

(5) De La Fuente, M. A.; Juarez, M. Authenticity assessment of dairy products. *Crit. Rev. Food Sci. Nutr.* **2005**, *45*, 563–585.

(6) Kornexl, B. E.; Werner, T.; Rossmann, A.; Schmidt, H. L. Measurement of stable isotope abundances in milk and milk ingredients - a possible tool for origin assignment and quality control. *Z. Lebensm. Unters. Forsch. A* **1997**, *205*, 19–24.

(7) Pillonel, L.; Badertscher, R.; Froidevaux, P.; Haberhauer, G.; Hölzl, S.; Horn, P.; Jakob, A.; Pfammatter, E.; Piantini, U.; Rossmann, A.; Tabacchi, R.; Bosset, J. O. Stable Isotope ratios, major, trace and radioactive elements in Emmenthal cheeses of different origins. *Lebensm.-Wiss. -Technol.* **2003**, *36*, 615–623.

(8) Manca, G.; Franco, M. A.; Versini, G.; Camin, F.; Rossmann, A.; Tola, A. Correlation between multielement stable isotope ratio and geographical origin in peretta cow's milk cheese. *J. Dairy Sci.* **2006**, *89*, 831–839.

(9) Renou, J. P.; Deponge, C.; Gachon, P.; Bonnefoy, J. C.; Coulon, J. B.; Garel, J. P.; Vérité, R.; Ritz, P. Characterization of animal products according to geographic origin and feeding diet using nuclear magnetic resonance and isotope ratio mass spectrometry: cow milk. *Food Chem.* **2004**, *85*, 63–66.

(10) Dumont, J. P.; Adda, J. Occurrence of sesquiterpenes in mountain cheese volatiles. *J. Agric. Food Chem.* **1978**, *26*, 364–367.

(11) Buchin, S.; Delague, V.; Duboz, G.; Berdague, J. L.; Beuvier, E.; Pochet, S.; Grappin, R. Influence of pasteurization and fat composition of milk on the volatile compounds and flavor characteristics of a semi-hard cheese. *J. Dairy Sci.* **1998**, *81*, 3097–3108.

(12) Fernandez, C.; Astier, C.; Rock, E.; Coulon, J. B.; Berdagué, J. L. Characterisation of milk by analysis of its terpene fractions. *Int. J. Food Sci. Technol.* **2003**, *38*, 445–451.

(13) Viallon, C.; Verdier-Metz, I.; Denoyer, C.; Pradel, P.; Coulon, J. B.; Berdagué, J. L. Desorbed terpenes and sesquiterpenes from forages and cheeses. *J. Dairy Res.* **1999**, *66*, 319–326.

(14) Collomb, M.; Butikofer, 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* (8), 649–659.

(15) Collomb, M.; Bütikofer, U.; Sieber, R.; Jeangros, B.; Bosset, J. O. Correlations 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.

(16) Martin, B.; Cornu, A.; Kondjoyan, N.; Ferlay, A.; Verdier-Metz, I.; Pradel, Ph.; Rock, E.; Chilliard, Y.; Coulon, J. B.; Berdagué, J. L. Milk indicators for recognizing the types of forages eaten by dairy cows. In *Indicators of Milk and Beef Quality*; EAAP

Publication 112; Hocquette, J. F., Gigli, S., Eds.; Wageningen Academic Publishers: Wageningen, The Netherlands, 2005; pp 127–136.

(17) Lyan, B.; Azaïs-Braesco, V.; Cardinault, N.; Borel, P.; Alexandre-Gouabau, M. C.; Grolier, P. Simple method for clinical determination of 13 carotenoids in human plasma using an isocratic high-performance liquid chromatographic method. *J. Chromatogr.* **2001**, *751*, 297–303.

(18) Loor, J. J.; Ferlay, A.; Ollier, A.; Doreau, M.; Chilliard, Y. Relationship among trans conjugated fatty acids and bovine milk fat yield due to dietary concentrate and linseed oil. *J. Dairy Sci.* **2005**, *88*, 726–740.

(19) Viallon, C.; Martin, B.; Verdier-Metz, I.; Pradel, P.; Garel, J. P.; Coulon, J. B.; Berdagué, J. L. Transfer of monoterpenes and sesquiterpenes from forages into milk fat. *Lait* **2000**, *80*, 635–641.

(20) NIST/EPA/NIH. Mass Spectral Database, Standard References Database Program of the National Institute of Standards and Technology (NIST), version 1.5 for PC; 1996; www.nist.gov.

(21) Adams, R. P. *Identification of Essential Oil Components by Gas Chromatography/Mass Spectroscopy*; Allured Publishing: Carol Stream, IL, 1995.

(22) Kondjoyan, N.; Berdagué, J. L. *A Compilation of Relative Retention Indices for the Analysis of Aromatic Compounds*; Laboratoire Flaveur, INRA-Theix: Saint-Genès Champanelle, France, 1996; Vol. F63122

(23) Engel, E.; Ratel, J. Correction of the data generated by mass spectrometry analyses of biological tissues: application to food authentication. *J. Chromatogr., A* **2007**, *1154*, 331–341.

(24) Schürch, M.; Kozel, R.; Schotterer, U.; Tripet, P. P. Observation of isotopes in the water cycle–the Swiss Natioanl Network (NISOT). *Environ. Geol.* **2003**, *45*, 1–11.

(25) Cornu, A.; Martin, B.; Verdier-Metz, I.; Pradel, P.; Coulon, J. B.; Berdagué, J. L. Use of terpene profiles in dairy produce to trace the diet of dairy cows. *Grassland Sci. Eur.* **2002**, *9*, 550–551.

(26) Tornambe, 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* (6), 2309–2319.

(27) Cornu, A.; Carnat, A. P.; Martin, B.; Coulon, J. B.; Lamaison, J. L.; Berdagué, J. L. Solid phase microextraction of volatile components from natural grassland plants. *J. Agric. Food Chem.* **2001**, *49*, 203–209.

(28) Fedele, V.; Rubino, R.; Claps, S.; Sepe, L.; Morone, G. Seasonal evolution of volatile compounds content and aromatic profile in milk and cheese from grazing goat. *Small Rumin. Res.* **2005**, *59* (2), 273–279.

(29) Hartman, A. M.; Dryden, L. P. *Vitamins in Milk and Milk Products*; American Diary Science Association: Champaign, IL, 1965; pp 123.

(30) Nozière, P.; Graulet, B.; Lucas, A.; Martin, B.; Grolier, P.; Doreau, M. Carotenoids in ruminants: from forages to dairy products. *Anim. Feed Sci. Technol.* **2006**, *131*, 418–450.

(31) Bugaud, C.; Buchin, S.; Coulon, J. B.; Hauwuy, A.; Dupont, D. Influence of the nature of alpine pastures on plasmin activity, fatty acid and volatile compound composition of milk. *Lait* **2001**, *81*, 401–414.

(32) Kramer, J. K.; Cruz-Hernandez, C.; Deng, Z.; Zhou, J.; Jahreis, G.; Dugan, M. E. Analysis of conjugated linoleic acid and trans 18:1 isomers in synthetic and animal products. *Am. J. Clin. Nutr.* **2004**, *79*, 1137S–1145S.

(33) Chilliard, Y.; Ferlay, A.; Mansbridge, R. M.; Doreau, M. Ruminant milk fat plasticity: nutritional control of saturated, polyunsaturated, trans and conjugated fatty acids. *Ann. Zootechnol.* **2000**, *49*, 181–205.

(34) Vlaeminck, B.; Fievez, V.; Cabrita, A. R. J.; Fonseca, A. J. M.; Dewhurst, R. J. Factors affecting odd- and branched-chain fatty acids in milk: a review. *Anim. Feed Sci. Technol.* **2006**, *131*, 389–417.

(35) Roy, A.; Ferlay, A.; Shingfield, K. J.; Chilliard, Y. Examination of the persistency of milk fatty acid composition responses to plant oils in cows fed different basal diets, with particular emphasis on trans-$C_{18:1}$ fatty acids and isomers of conjugated linoleic acid. *Anim. Sci.* **2006**, *82*, 479–492.

(36) Couvreur, S.; Hurtaud, C.; Marnet, P. G.; Faverdin, P.; Peyraud, J. L. Composition of milk fat from cows selected for milk fat globule size and offered either fresh pasture or a corn silage-based diet. *J. Dairy Sci.* **2007**, *90*, 392–403.

(37) Deport, C.; Ratel, J.; Berdague, J. L.; Engel, E. Comprehensive combinatory standard correction: a calibration method for handling instrumental drifts of gas chromatography-mass spectrometry systems. *J. Chromatogr., A* **2006**, *1116* (1–2), 248–258.

(38) Loor, J. J.; Ueda, K.; Ferlay, A.; Chilliard, Y.; Doreau, M. Biohydrogenation, duodenal flow, and intestinal digestibility of trans fatty acids and conjugated linoleic acids, in response to dietary forage:concentrate ratio and linseed oil in dairy cows. *J. Dairy Sci.* **2004**, *87*, 2472–2485.

(39) Chilliard, Y.; Ferlay, A. Dietary lipids and forages interactions on cow and goat milk fatty acid composition and sensory properties. *Reprod. Nutr. Dev.* **2004**, *44*, 467–492.

(40) Ferlay, A.; Martin, B.; Pradel, P.; Coulon, J. B.; Chilliard, Y. Influence of grass-based diets on milk fatty acid composition and milk lipolytic system in Tarentaise and Montbeliarde cow breeds. *J. Dairy Sci.* **2006**, *89* (10), 4026–4041.

(41) Elgersma, A.; Ellen, G.; van der Horst, H.; Boer, H.; Dekker, P. R.; Tamminga, S. Quick changes in milk fat composition from cows after transition from fresh grass to a silage diet. *Anim. Feed Sci. Technol.* **2004**, *117* (1), 13–27.

(42) Rego, O. A.; Portugal, P. V.; Sousa, M. B.; Rosa, H. J. D.; Vouzela, C. M.; Borba, A. E. S.; Bessa, R. J. B. Effect of diet on the fatty acid pattern of milk from dairy cows. *Anim. Res.* **2004**, *53*, 213–220.

(43) 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.

(44) Precht, D.; Molkentin, J. Trans unsaturated fatty acids in bovine milk fat and dairy products. *Eur. J. Lipid Sci. Technol.* **2000**, *102*, 635–639.

(45) Nielsen, T. S.; Straarup, E. M.; Vestergaard, M.; Sejrsen, K. Effect of silage type and concentrate level on conjugated linoleic acids, trans-C18:1 isomers and fat content in milk from dairy cows. *Reprod. Nutr. Dev.* **2006**, *46*, 699–712.

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