Significance of the Nonvolatile Minor Compounds of the Neutral Lipid Fraction as Markers of the Origin of Dairy Products

MILENA POVOLO,* VALERIA PELIZZOLA, DANIELA RAVERA, AND GIOVANNA CONTARINI

CRA—Centro di Ricerca per le Produzioni Foraggere e Lattiero-Casearie, Via A. Lombardo 11, 26900 Lodi, Italy

In the past few years several researchers have approached the problem of traceability of dairy products mainly by examining the volatile compounds that could be transferred from forage into milk and cheese. Our research focused on the study of the composition of the nonvolatile minor components of the neutral lipid fraction in mountain dairy products, obtained from animals feeding on pasture, and in milk and cheese samples produced from cows under intensive breeding, fed with concentrates and silages. Hydrocarbons were separated by silica gel column chromatography from the whole lipid matrix and analyzed by GC/MS. Among all the compounds detected, 1-phytene, 2-phytene, neophytadiene, and to a lesser extent the esters of phytol with C16 and C18 fatty acids seem to be promising tools for the recognition of the feeding system. The value of the sum of isoprenoid hydrocarbons ($\sum$-hyd) of mountain dairy products (12.3−34.0 mg/kg) was always higher than that obtained from plain samples (1.3−6.4 mg/kg).

KEYWORDS: Hydrocarbons; esters; milk; cheese; mountain

INTRODUCTION

Food traceability and knowledge of the relationship between production processing and food composition have become subjects of great interest during the past decade because of the increasing demand for genuineness, high quality, and origin assurance of food. In the past few years several researchers have approached the problem of food authenticity (*1*) and in the dairy field many papers dealing with the traceability of the origin of milk and cheese have been published. In particular, studies have been carried out to differentiate between mountain cheeses, produced by animals feeding on pasture, and cheeses of lowland origin. In fact, the animal feed has an influence on milk composition regarding microbiological, chemical, and sensory characteristics (*2−4*). The characteristics of milk are affected significantly by moving the animal herd from stabling to grazing, and in particular, compounds like terpenes and conjugated linoleic acid tend to increase their concentration in milk produced by cows grazing on pasture (*5−9*). Other studies showed that season and type of forage affected the composition of milk and cheese volatile compounds, in particular by enriching this fraction in terpenes (*10, 11*).

Besides volatile compounds, extensively considered in literature as biochemical markers of origin, even nonvolatile hydrocarbons molecules could be of interest to evaluate cheese traceability. Hydrocarbons are minor components of the unsaponifiable fraction of oils and fats. Most fat matrixes contain small quantities of linear, branched, saturated, unsaturated, and terpenic hydrocarbons.

Because the hydrocarbon profile is characteristic of each vegetable species, it has been used to assess authenticity of vegetable fats; e.g., olive oil varieties can be detected by means of their *n*-alkane profile (*12, 13*).

Actual knowledge of hydrocarbon composition of milk fat is limited and dated. Literature reports studies about their content in butter, goats' milk, and milk powder (*14−17*), but there are no studies on cheese. Squalene is the main constituent, and 1-phytene, 2-phytene, phytane, neophytadiene, phytol, dihydrophytol are other isoprenoid hydrocarbons present in fat of dairy products (*17, 18*).

The aim of our research was to study the minor compounds of the neutral fraction of milk and cheese lipids in order to verify if in these fractions there were possible markers of the origin of dairy products when different feeding systems are adopted. This item could be particularly important when, within the same type of cheese, different productions with different economical values are present and need to be protected.

MATERIALS AND METHODS

Milk and Cheese Sampling. Milk and cheese from both plain and mountain origin were analyzed. The samples from plain origin were collected directly from companies producing commercial dairy products. The samples from mountain origin were from dairy productions for specific research projects and local manufacturers.

Six samples of nonhomogenized bulk cows' milk (plain milks, PM) were sampled twice from the storage tanks of three dairy companies collecting milk from farms located in the lowland area of the Po Plain. The Po Plain is the major plain of the North of Italy. It extends 600 km in an east−west direction, and its altitude varies from sea level to about 500 m high. Each dairy company receives the milk from about 100 farms of 100−150 head of

*To whom correspondence should be addressed. Phone: +39 0371 45011. Fax: +39 0371 35579. E-mail: milena.povolo@entecra.it.

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cows each and adopts an intensive breeding system throughout the year. A seventh milk sample was collected directly from the storage tank of a small farm breeding 60 cows.

As regards highland milk, 11 samples of nonhomogenized bulk cows' milk (mountain milks, MM) were sampled directly from the storage tanks of three farms breeding about 60 cows each in a highland area of the West Alpine Italian region located at 2100 m.

For all the milk sampling an amount of 2 L of sample was put into glass bottles after having carefully mixed the bulk milk. All the milk samples were collected on different days during the month of July of the same year.

Eleven cheeses produced by four Italian manufacturers, collecting milk from about 200 farms of the Po Plain and adopting an intensive breeding system, were sampled (plain cheeses, PC). The cheeses selected were representative of different types of production technology: hard cheese (three samples of Grana Padano, PDO), fresh and ripened pasta filata cheese (Mozzarella and Provolone, two and three samples, respectively), and fresh soft cheese (three samples of Crescenza). Three-hundreds slices were sampled for Grana Padano and Provolone cheeses, whereas as regards Mozzarella and Crescenza, the whole portion of 100 g was collected. Taking into account the ripening time of the different types of cheese, the samples were collected at a time corresponding to the summer (July) milk production.

Of the mountain cheeses, 15 samples of semihard, 3 month ripened cheeses (MC), manufactured in different days in the month of July, were analyzed. For each sample slices of 300 g were cut. Six cheeses were manufactured from milk produced on a farm breeding 150 cows, located in the East Alpine region (1647 m altitude). Four cheeses were collected directly from local producers of the same Alpine region. Five cheeses were manufactured from milk produced on a farm breeding 115 cows, located in the West Alpine region (2100 m altitude).

Cows bred in the Po Plain received a diet composed of silage, grain, and concentrate accounting for about 63%, 15%, and 22%, respectively. Cows bred in the highland area (East and West Alpine region) grazed on pasture composed mainly of Leguminosae, Gramineae, Rosaceae, Plantaginaceae, Compositae plant families, with no more than 3 kg/(cow·day) of concentrate.

Milk and cheese samples were stored at −20 °C until the analyses, which were done in duplicate.

Reagents. High purity standards of squalane (99%), squalene (≥98%), *n*-alkanes from C11 to C34 (99%), β-cariophyllene (≥98.5%), methyl esters (purities ranging from 99.5% to 99.8%), and ethyl esters (purities ranging from 98% to 99%) of fatty acids, except for ethyl palmitate and ethyl oleate, were supplied by Sigma-Aldrich (Milan, Italy). Phytol, a mixture of (*Z*)- and (*E*)-isomers, was purchased from Merck supplied by VWR (Milan, Italy). Phytane (99%) was purchased from Ultra Scientific (Bologna, Italy). Palmitoyl (98%), stearoyl (97%), oleoyl (approx 99%) chlorides, and pyridine were bought from Sigma-Aldrich (Milan, Italy). Cholesteryl esters of acetic, butyric, caprylic, myristic, and palmitic acids were supplied from Nu-Check Prep. Inc. (Elysian, MN), whereas cholesteryl esters of caproic, lauric and stearic acids were supplied from Sigma-Aldrich. All the cholesteryl esters had a purity of 99%.

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

Synthesis of Phytyl Esters (*19*). Approximately 5.0 mmol of phytol and palmitoyl (C16), stearoyl (C18), oleyl (C18:1) chlorides were dissolved in 2 mL of carbon tetrachloride in the presence of a few drops of pyridine. The solution was left at room temperature overnight. The reaction mixture was transferred into a 50 mL separatory funnel and diluted with 2 mL of cold water. The organic layer was allowed to separate, dried over anhydrous Na_2SO_4, and evaporated to dryness in a rotary evaporator (Buchi, Switzerland). The residue was purified by silica gel column chromatography prepared in *n*-hexane/diethyl ether (99:1). Each synthesized ester was eluted from the column with 10 mL of *n*-hexane/diethyl ether (99:1) to give a pure sample of the desired compound.

Fat Extraction. The methods reported below were developed at the laboratory in order to obtain the fat extraction without using solvents.

Frozen milk sample (1 L) was thawed at 37 °C in a water bath, divided into four 250 mL screw-cap polycarbonate bottles, and centrifuged in a J2-21M/E Centrifuge (Beckman Coulter Inc., Fullerton, CA) for 15 min

at 6000 rpm to separate the cream. An amount of 20 g of cream was transferred to a 60 mL screw-cap glass tube, with 0.5 mL of an aqueous lactic acid solution (85%) added in order to decrease the pH to < 5, and the mixture was shaken by vortex for 2 min. Then an amount of 3 mL of distilled water was added in the glass tube and the mixture was shaken by vortex for 3 min. The fat fraction was separated by centrifugation at 60 °C for 20 min at 1250 rpm (Gerber Instruments AG, Effretikon, Switzerland) and transferred into a glass vial.

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 described above for milk samples.

Saponification of Fat and Thin Layer Chromatography (TLC). Fat saponification was performed according to the procedure described in the ISO 12078:2006 (*20*). The hydrocarbon fraction was separated from the unsaponifiable by thin-layer chromatography on silica gel precoated plates (Merck, Darmstadt, Germany) using *n*-hexane/diethyl ether (60:40) as mobile phase. The unsaponifiable fraction was dissolved in 1 mL of standard solution (0.02 mg/mL squalane), and an amount of 400 μL of the solution was loaded on the silica plate by a microsyringe. After the development, the plate was sprayed with a solution of rhodamine G in ethanol (0.15% w/v) and examined under UV light at 254 nm. The hydrocarbon band was scraped off and extracted with chloroform and diethyl ether alternately. Solvents were evaporated under vacuum.

Column Chromatography (CC). The hydrocarbon fractions were separated from the whole lipid matrix by column chromatography into a glass chromatographic column (40 cm length, 15 mm i.d.) containing 15 g of silica gel 60, extra pure (Merck, Darmstadt, Germany), prepared as described by Mariani and Fedeli (*21*).

A total of 400 mg of fat was dissolved in 1 mL of squalane internal standard solution (0.02 mg/mL *n*-hexane) and loaded into the chromatographic column. The solvent mixture *n*-hexane/diethyl ether (99:1) was used as eluent phase. A total of 200 mL of this mixture was loaded into the column, and an amount of 140 mL of the eluate phase was collected in a 250 mL flat-bottom flask. The hydrocarbon fraction, after evaporation of the solvents under vacuum, was dissolved in 0.3 mL of *n*-hexane and analyzed by GC/MS.

Gas Chromatography−Mass Spectrometry (GC/MS). The GC/MS analysis was performed on a TraceGC coupled with a TraceMS Plus mass spectrometer (ThermoElectron Corporation, Woburn, MA). A HP-5 MS cross-linked 5% phenylmethylpolysiloxane capillary column (Agilent Technologies, Palo Alto, CA) (30 m length, 0.32 mm i.d., 0.25 μm film thickness) was used. On-column injection (1 μL) was adopted, and helium was used as carrier gas at a flow rate of 1.5 mL/min. Oven temperature was held at 60 °C for 3 min, programmed to 280 °C at a rate of 10 °C/min, held at 280 °C for 1 min, programmed to 320 °C at a rate of 3 °C/min, and held at 320 °C for 20 min. Interface was held at 320 °C and MS source at 250 °C. Acquisition was performed in EI mode (70 eV) at 0.4 scans/s, and the mass range used was 35−300 *m/z* from 5 to 20 min and 35−600 *m/z* from 20 to 60 min.

The identification of the compounds was made by using the NIST library (*22*), the MS data of literature, the injection of authentic standards, when available, and the comparison of the retention indices with published data.

The amount of each compound was calculated by relating its peak abundance (TIC) to that of squalane (internal standard). At this stage of the research, any correction factor with respect to the internal standard was calculated, and consequently the results, even though expressed as mg/kg, should be considered a semiquantitative evaluation. Because of the small data set, the significance of the differences between the mountain and plain samples was calculated by applying the Kolmogorov−Smirnov's test (*23*).

RESULTS AND DISCUSSION

Setting Up of the Analytical Procedure. In order to avoid, as much as possible, the contamination of samples due to the hydrocarbons that could have been present in the solvent used for the fat extraction, the first part of the research was dedicated to the setting up of different fat extraction procedures for both milk and cheese samples.

Because of its chemical composition and physical proper-ties (*24*), the milk fat globule membrane represents a natural obstacle to the release and separation of lipids from milk. The problem was overcome by simulating the physical—chemical conditions adopted during the butter manufacturing and respon-sible for the inversion of the phases. Cream was first separated from milk by centrifugation, then added with an aqueous lactic acid solution and shaken. Finally, the mixture was centrifuged again in order to separate the lipids.

The fat extraction from the cheese samples, in which the milk fat globule membrane is partially disrupted by the technological process, was performed by centrifugation at 60 °C of cheese without any addition of solvent.

Two aspects were taken into account in the choice of the minor compounds of the neutral lipid fraction separation technique: the possible external contamination of the sample and the loss of molecules of interest caused by the complexity of the procedure.

Two techniques were tested for the separation: thin layer chro-matography (TLC) and column chromatography (CC). Even though the TLC separation ability allowed a preliminary dis-crimination of the compounds according to their chemical class, this technique showed non-negligible disadvantages. The low

Table 1. Repeatability of the Hydrocarbon Analysis Expressed as Relative Standard Deviation of Four Replicates of the Same Milk Sample from the Plain

compd	mean (mg/kg fat)	SD[a]	RSD (%)[a]
heptadecane	0.5	0.06	12.1
1-phytene	4.0	0.24	6.1
octadecane	2.0	0.02	0.9
2-phytene	3.5	0.45	12.9
squalene	21.3	3.09	14.5
phytyl C16	6.2	0.82	13.2

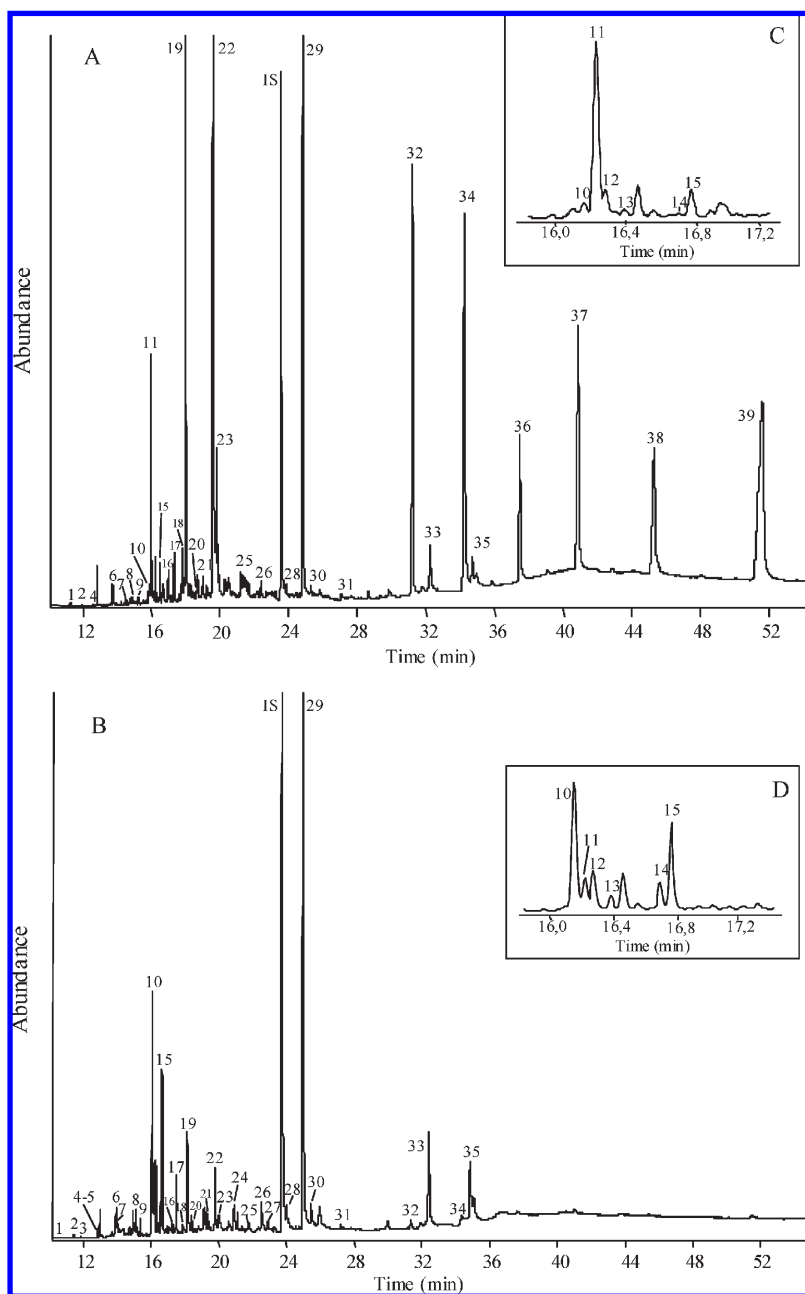[a] Abbreviations: SD, standard deviation. RSD, relative standard deviation.

Figure 1. GC/MS profiles of the hydrocarbon fraction of plain (**A**) and mountain (**B**) cheeses. Peaks are numbered as in **Table 2**. Boxes **C** and **D** show the enlarged views of the time range where the isoprenoid hydrocarbons elute for the profiles **A** and **B**, respectively.

Table 2. Compounds Detected in the Neutral Lipid Fraction of Milk and Cheese Fat

	peak no.	compd (KI)	identification method[a]
hydrocarbons	2	tetradecane	MSa
	4	pentadecane	MSa
	7	hexadecane	MSa
	8	heptadecane	MSa
	12	octadecane	MSa
	16	nonadecane	MSa
	20	heneicosane	MSa
	24	tricosane	MSa
	25	tetracosane	MSa
	26	pentacosane	MSa
	27	hexacosane	MSa
	28	heptacosane	MSa
	30	nonacosane	MSa
	31	hentriacontane	MSa
ethyl esters	1	ethyl decanoate (1396)	Msa/PI
	6	ethyl laurate (1595)	Msa/PI
	11	ethyl myristate (1795)	Msa/PI
	18	ethyl palmitoleate (1976)	
	19	ethyl palmitate (1996)	Msa/PI
	22	ethyl oleate (2172)	PI
	23	ethyl stearate (2197)	Msa/PI
methyl esters	9	methyl myristate (1727)	Msa/PI
	17	methyl palmitate (1927)	Msa/PI
	21	methyl stearate (2130)	Msa/PI
sesquiterpenes	3	β-cariophyllene (1426)	Msa/PI
	5	unknown (1501)	
isoprenoid hydrocarbons	10	1-phytene (1790)	MSr/PI
	13	phytane (1811)	MSa
	14	neophytadiene (1842)	MSr/PI
	15	2-phytene (1849)	MSr/PI
	29	squalene (2857)	MSa
cholesteryl esters	32	cholesteryl butyrate	MSa
	34	cholesteryl hexanoate	MSa
	36	cholesteryl octanoate	MSa
	37	cholesteryl decanoate	MSa
	38	cholesteryl laurate	MSa
	39	cholesteryl myristate	MSa
phytyl esters	33	phytyl C16	MSa
	35	phytyl C18 saturated/unsaturated	MSa

[a] Confirmation of the identification: MSa = mass spectra of authentic compounds (authentic compounds had the same retention indexes as the molecules detected in the samples); MSr = comparison with mass spectra reported in the literature (*18*); PI = published indexes, comparison of KI calculated with published indexes (*37,38*).

loading capacity of the TLC technique, together with the very low amount of hydrocarbon and ester molecules in milk fat, required the milk fat saponification, followed by the unsaponifiable matter extraction as preseparation and concentration steps. As a consequence, this procedure involved the use of a high amount of different solvents, which increased the risk of contamination. Moreover, it was more time-consuming and prevented the determination of esters, which were hydrolyzed during the saponification. In contrast, CC consisted of fewer steps and required only hexane as eluent phase and allowed the whole lipid matrix to be loaded into the column. The neutral eluted fraction included different compounds belonging to the different chemical classes, but the good performances of the gas chromatographic column provided an efficient separation. Thus, the CC separation was adopted.

The recovery rate of the extraction was verified by collecting, after the first 140 mL, two other aliquots of the eluent phase (30 mL each). Except for traces of squalene, in the first 30 mL aliquot, all the other compounds were not found in these

supplementary fractions. Unfortunately, the first 30 mL aliquot included also non-negligible amounts of triglycerides that made the GC separation difficult. Consequently only the first 140 mL of solvent was recovered. Blank tests of CC procedure, i.e., analyses obtained by using the same amount of reagents and internal standard, without the sample, were performed, and the GC profile showed only negligible peaks of linear or branched hydrocarbons, having a total concentration of about 0.4 mg/kg.

Squalane was used as internal standard, since it eluted in the middle zone of the chromatogram and did not overlap with other peaks.

In order to verify the repeatability of the method and the yield of extraction, one sample of fat extracted from lowland milk was analyzed four times to calculate the repeatability of the hydrocarbon extraction by the CC technique. Six compounds, present in high (squalene), medium (1-phytene, 2-phytene, phytyl C16), and low (octadecane, heptadecane) amounts were taken into account and quantified (**Table 1**). RSD ranged from 0.9% to 14%. The result can be considered satisfactory, mainly taking into account the low concentration of these molecules and the complexity of the milk fat matrix. Moreover, the repeatability results obtained in our research were comparable with those reported by other authors applying the same separation techniques (*25*).

Evaluation of Milk and Cheese Samples. Six main groups of compounds were detected in milk and cheese: linear hydrocarbons, fatty acid ethyl esters, fatty acid methyl esters, isoprenoid hydrocarbons, cholesteryl esters, and phytyl esters. In addition traces of terpenes were also found. **Figure 1** shows the GC/MS chromatograms of plain (**A**) and mountain (**B**) cheese samples, whereas **Table 2** reports the compounds detected and the type of recognition performed. For some of the compounds detected it was not possible to find authentic standards useful to confirm their identity. In these cases the spectra obtained were compared with those found in literature or obtained by analyzing compounds synthesized, on purpose.

As expected, squalene, which is the cholesterol precursor, was the most abundant compound. Together with squalene, linear, branched, and the other isoprenoid hydrocarbons (1-phytene, 2-phytene, and neophytadiene) were detected, and this result was in accordance with the findings of Flanagan and Ferretti (*16*) and Urbach and Stark (*18*) in dairy fat.

The presence of methyl and ethyl esters of medium and long chain fatty acids was observed particularly in cheese. They can originate by esterification reaction between free fatty acids and alcohols and alcoholeysis reaction directly from glycerides and alcohols (*26, 27*).

Unlike free cholesterol, which is a polar molecule, cholesteryl esters have a chemical behavior similar to that of the neutral compound and consequently eluted together with the hydrocarbon fraction; the presence of these molecules in milk fat had been already reported by other authors (*28−30*).

The following EI fragmentations m/z (relative abundance) was obtained from peak no. 33 [43 (54), 55 (57), 57 (76), 68 (69), 81 (100), 95 (87), 109 (30), 123 (58), 137 (13), 151 (2), 165 (2), 179 (3), 193 (2), 239 (2), 253 (1), 278 (9)] and from peak no. 35 [43 (53), 55 (62), 57 (56), 68 (68), 81 (100), 95 (86), 109 (32), 123 (51), 137 (14), 151 (4), 165 (4), 179 (4), 193 (2), 253 (1), 263 (2), 267 (1), 278 (8), 281 (3)]. The preliminary identification was performed by using the NIST library (*22*) and literature data. The fragment m/z 278 in their spectra suggested the presence of phytol in these molecules. The paper of Cranwell et al. (*31*) supported this hypothesis, since it reported the fragment $[M - R^1CO_2H]^+$ (m/z 278) as the diagnostic ion of the phytyl group in the spectra of phytyl esters in plants. The presence of fatty acids was demonstrated

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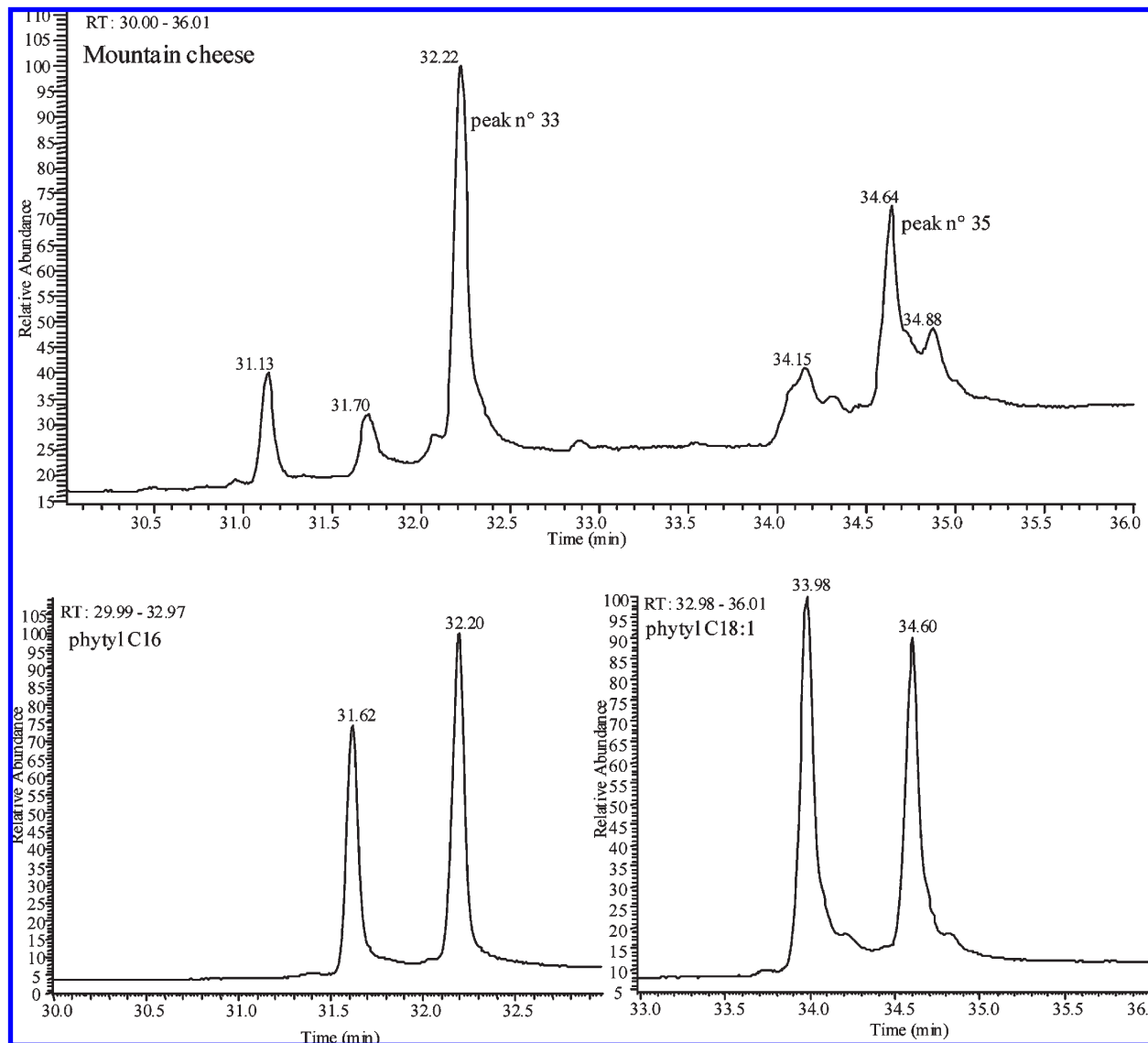*J. Agric. Food Chem.,* Vol. 57, No. 16, 2009   **7391**

Figure 2. Enlarged view of the GC/MS profile of a mountain cheese sample and of standards phytyl C16 and phytyl C18:1 synthesized.

by the acyl fragments m/z 239 for C16 acid (peak no. 33) and m/z 267−263 for C18 saturated/unsaturated acid (peak no. 35). The latter result suggested that two compounds coeluted as one peak. A further aid in the recognition of these two peaks was then provided by the comparison of the mass spectra obtained with those reported in literature (*32*, *33*).

In order to achieve a definite identification of these peaks as esters of long chain fatty acids with phytol and because of the absence of commercially available authentic standards, phytyl esters of C16, C18, and C18:1 acids were synthesized. **Figure 2** reports the enlarged view of the GC/MS profiles of a mountain cheese sample where peaks 33 and 35 elute plus the synthesized C16 and C18:1 phytyl esters. **Figure 3** reports the mass spectra obtained by peaks 33 and 35 of a mountain cheese sample plus synthesized C16 and C18:1 phytyl esters. Since the commercially available standard of phytol was a mixture of the (Z)- and (E)-isomers, two peaks of each ester were found when the synthetic molecules were analyzed. Under the GC conditions applied in our research the (Z)-isomer eluted before the (E)-isomer. The synthetic C18:0 phytyl ester, characterized by the fragment m/z 267, eluted at a retention time (34.75 min) very close to that of the C18:1 phytyl ester. This result, particularly if phytyl C18:1 is highly abundant, can explain the coelution

occurring in the sample. The correspondence between the mass fragmentations and the (E)-isomers retention times confirmed the recognition of these molecules as esters of the fatty acids C16, C18 (saturated and unsaturated) with phytol in trans configuration. The small peaks eluting at the retention time corresponding to those of (Z)-isomers did not match the fragmentation of these molecules.

To our knowledge, no papers reporting the presence of these molecules in milk fat are available in literature. Two reasonable hypotheses can be formulated: direct transfer from grass, since they had been detected in vegetable matrixes (*32*) and/or endogenous (rumen or mammary cells) esterification product between fatty acids and free (E)-phytol, which is the main isomer naturally occurring in the chlorophyll molecule (*34*).

Two data sets, including the compounds detected in milk and cheese, were obtained. Thirty-nine compounds deriving from different sources, as previously explained, were detected in amounts ranging from 0.1 to about 60 mg/kg. According to the aim of this work, i.e., the detection of possible markers of traceability, the attention was focused on the compounds having a proved relationship with the feeding and showing significant differences between plain and mountain dairy samples. Within the compounds assessing these criteria, three isoprenoid
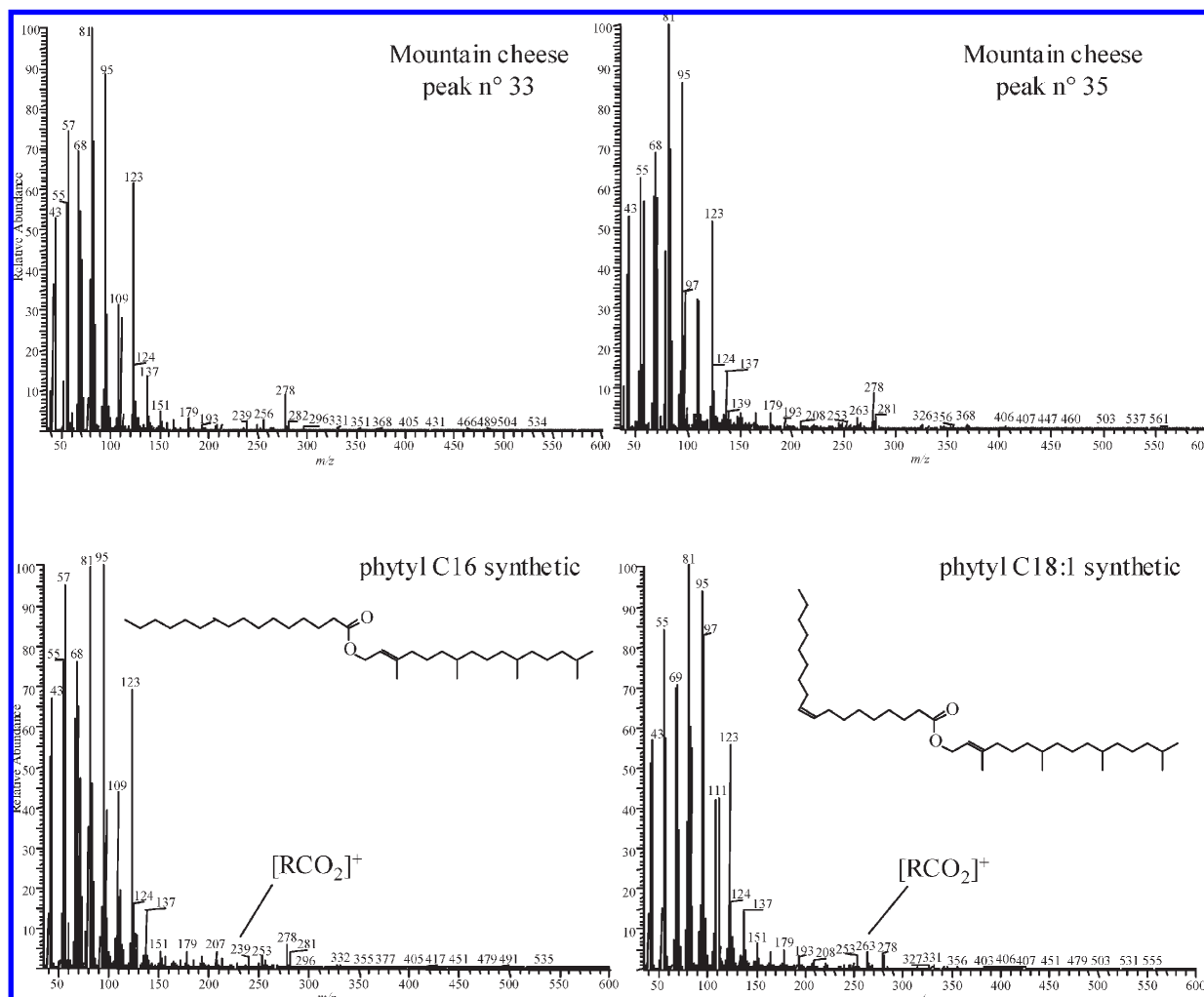
Figure 3. Electron impact spectra of peak nos. 33 and 35 and of synthesized phytyl C16 and phytyl C18:1.

Table 3. Mean (mg/kg fat) and Standard Deviation (SD) Values of Isoprenoid Hydrocarbons and Phytyl Esters in Milks and Cheeses of Different Origin[a]

| | milk | | | | cheese | | | |
| | plain (PM) | | mountain (MM) | | plain (PC) | | mountain (MC) | |
	mean	SD	mean	SD	mean	SD	mean	SD
1-phytene	1.8	1.20	10.3	3.81	2.5	0.85	9.6	3.53
neophytadiene	0.4	0.29	2.2	1.07	0.8	0.38	3.0	1.62
2-phytene	2.5	0.88	7.1	1.49	3.8	1.58	9.7	3.28
phytyl C16	5.2	1.11	14.6	5.35	6.2	2.04	18.3	10.42
phytyl C18 sat/unsat[b]	2.4	1.01	7.7	3.49	3.2	1.22	14.7	9.82

[a] Within the same matrix the mean values of each compound were always statistically different ($p < 0.01$). [b] sat/unsat = saturated/unsaturated.

hydrocarbons and two phytyl esters were selected for further evaluation. **Table 3** reports mean and standard deviation values of 1-phytene, 2-phytene, neophytadiene, phytyl esters of C16 and C18 saturated/unsaturated of the samples analyzed. Milk and cheese samples of mountain origin were characterized by higher amounts of these molecules than those of plain origin. The adoption of pasture feeding was responsible for the important increase of 1-phytene, 2-phytene, neophytadiene, and phytyl esters; differences between plain and mountain products were always statistically significant at a high level ($p < 0.01$). The relationship between the concentration of these compounds and the feeding system was demonstrated by literature data, in different matrixes. Urbach and Stark (*18*) observed that the level of 1-phytene and

neophytadiene in butterfat decreased when the animal feeding changed from grass pasture to chopped lucerne hay and concluded that the precursor of 1-phytene and neophytadiene was the neophytadiene present in the pasture. Larick et al. (*35*) observed similar results studying the hydrocarbon fraction of beef fat: feeding the animals with forage produced a beef fat richer in diterpenoids than that obtained from a grain diet. As far as the origin of these molecules is concerned (**Figure 4**), Body (*36*), studying the rumen liquor, hypothesized that they were derived from dietary phytol of chlorophyll by the action of rumen bacteria. The dehydration of phytol produced neophytadiene; 1-phytene was derived from dehydration of dihydrophytol, which was derived from phytol via hydrogenation in the rumen (*18, 35, 36*).

In order to provide an index useful for the traceability of dairy products, independent from the technological process the milk has been submitted to, 1-phytene, 2-phytene, and neophytadiene were summed and the result was indicated as $\sum$-hyd. The isoprenoid hydrocarbons were preferred to the phytyl ester molecules because the latter could be increased by the presence of high amounts of free fatty acids during the cheese ripening. **Figure 5** reports the distribution of the values of each group of samples, as a box plot chart. The value of $\sum$-hyd of mountain dairy products (MM and CM) was always higher than that obtained from plain samples (MP and CP). The differences between $\sum$-hyd of milk and cheese of the same origin were never significant ($p < 0.01$), confirming that this index was unaffected by the technological process.
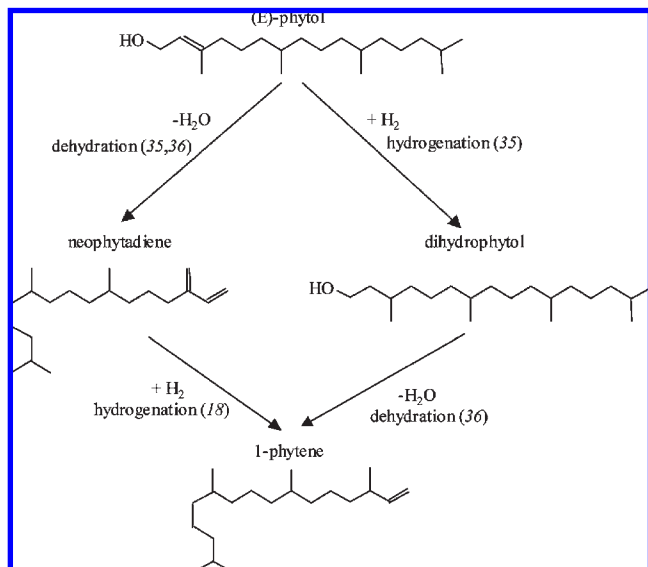
Figure 4. Pathways for the formation of neophytadiene and 1-phytene from (*E*)-phytol in the rumen.
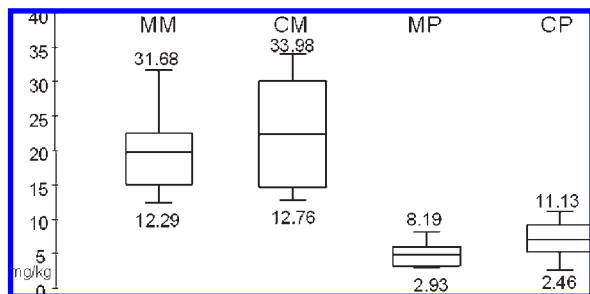

Figure 5. Box-plot of the sum of 1-phytene, 2-phytene, and neophytadiene ($\sum$-hyd) of milk and cheese samples. Each box indicates the smallest observation, lower quartile, mean, upper quartile, and largest observation.

Several authors proposed the volatile terpenes, aliphatic compounds originating from the secondary metabolism of plants, as markers for tracing mountain cheeses. The analysis of volatile compounds is a complex problem due to the chemical physical characteristics of the analytes and usually requires the use of different analytical techniques. Moreover, quantitative data are often very difficult to obtain. In this research a novel approach consisting of the evaluation of the nonvolatile minor components of the neutral lipid fraction seems to be a promising tool for the distinction of dairy products according to two different animal feeding systems (plain intensive breeding and mountain pasture). Within the different compounds detected in this preliminary work, 1-phytene, 2-phytene, and neophytadiene were able to discriminate milk and cheese obtained from the mountain pastures from those produced in the plain, under an intensive breeding system. Moreover, the comparable content of these molecules in milk and cheese fat suggested that these compounds were not affected by either microflora involved in the cheesemaking or the different steps of the technological process. The effectiveness of this approach needs to be supported by a wide number of samples. At the same time a full quantitative determination, achievable by the calculation of the response factors with respect to the internal standard, is an essential requirement in order to apply these markers. This method could find an interesting application in the control of the cheese origin, particularly when, within the same cheese type, two different productions are present and the mountain product, having a higher economical value, needs to be protected.

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