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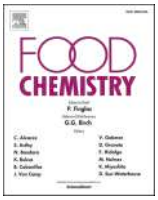

Cheese characterization and authentication through lipid biomarkers obtained by high-resolution ^{1}H NMR profiling

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ABSTRACT

Food quality and safety are at the heart of consumers' concerns across the world. Dairy products, because of their large consumption, are fertile ground for fraudulent acts. This fact justifies the development of effective, accessible, and rapid analytical methods for their authentication. A high-resolution spectral treatment method previously developed by our team was applied to ^{1}H NMR spectra of cheese triacylglycerols. 178 Peaks were thus quantitated and successfully used in the construction of multivariate models for the quantitation of individual fatty acids and for the classification of cheese samples according to the producing species, to their origin and variety. Besides, several peaks related to the amount and position of anteisopentadecanoic, butyric, α-linolenic, myristoleic, rumenic, and vaccenic acids were, among others, specific biomarkers of cheese groups. For the first time in ^{1}H NMR, we were able to identify and to quantitate signals related to minor fatty acids within cheese triacylglycerols.

1. Introduction

Milk and dairy products are among the most consumed and nutritionally complete foods with 811 million tonnes of milk produced in 2017 (Dairy Market Review-April-2018) and an increase to nearly 906 million tonnes in 2020 (Dairy Market Review-April-2021). The global dairy market was valued at 673.8 billion U.S. dollars in 2019 and was projected to grow to 1032.7 billion U.S. dollars by 2024 (Statista, 2020). The authenticity of these high-demand products is thus of great importance to both producers and consumers. On the other hand, dairy products with a protected geographical status (e.g., Protected Designation of Origin: PDO) are distinguished from other similar products of the same category by the link between their characteristics and the geographical environment (natural and human factors) of the regions they originate from. In this way, the geographical environment influences the gut microbiota of the producing animal that in turn affects the organoleptic characteristics of the dairy product (Liu et al., 2021). Moreover, local know-how, which involves a specific processing or ripening procedure, can influence the chemical signature of a cheese sample through lipolysis (Gobbetti et al., 2002). Similarly, animal feeding is also considered a strong factor in the connection between the characteristics of PDO products and their origins (Mordenti, Brogna, & Formigoni, 2017). For instance, in Asiago PDO Cheese, higher contents of conjugated linoleic acids and anteisopentadecanoic acid were found in cheeses made from cows fed on pastures (Segato et al., 2017). The specific composition of milk fat of small ruminants makes it valuable from a nutritional and health point of view (Sampelayo, Chilliard, Schmidely, & Boza, 2007). Beneficial activities of milk lipids include anticancer, antimicrobial, anti-inflammatory, and immunosuppression properties (German & Dillard, 2006). Therefore, a change in the lipid profile of milk can modulate its beneficial effects. In this respect, factors decreasing the amount of saturated medium-chain (C12, C14, and C16) fatty acids in triacylglycerols and increasing those of butyric, vaccenic, and rumenic acids improve the health impact of dairy products (German & Dillard, 2006). The fatty acid (FA) composition of triacylglycerols (TAG) in cheese is also affected by the farming system. According to Giaccone et al. (2016), summer cheeses obtained from extensive farming systems showed a better FA composition for human nutrition then cheeses obtained from the intensive farming system. As a result, the FA profile of TAG in dairy products can be a nutritional and organoleptic

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quality indicator and a source of biomarkers that allow authenticating the animal and geographical origins of the product, the animal feeding, the farming system, and the processing adopted. Several techniques exist for food authentication such as liquid chromatography (LC) (Hoffmann, Münch, Schwägele, Neusüß, & Jira, 2017), isotope ratio monitoring by Mass Spectrometry (irm-MS) (Nečemer, Potočnik, & Ogrinc, 2016), mid-infrared (MIR) spectroscopy (Karoui, Mazerolles, Bosset, Debaerdemaeker, & Dufour, 2007), Raman spectroscopy (Li, Shan, Zhu, Zhang, & Ling, 2012), and nuclear magnetic resonance spectroscopy (NMR) (Hajjar, Haddad, Rizk, Akoka, & Bejjani, 2021; Jung et al., 2010; Merchak et al., 2017). Moreover, molecular biology techniques can be used such as Randomly Amplified Polymorphic DNA (RAPD) (Cunha et al., 2016). Among the possible analytical techniques, NMR spectroscopy has proven to be an indispensable tool for food authentication due to the rich chemical information that this technique affords in non-targeted metabolomics. ^{1}H NMR allowed the discrimination of milk (Fernandez, Astier, Rock, Coulon, & Berdagué, 2003), beef (Jung et al., 2010) and olive oil (Merchak et al., 2017) according to their geographical origins. Additionally, ^{1}H NMR permitted the identification of vegetable fat in imitation cheese and ice cream samples (Monakhova, Godelmann, Andlauer, Kuballa, & Lachenmeier, 2013). However, ^{1}H NMR spectrum of lipids is complex with overlapping signals. Application of high-resolution processing, previously developed by our team to analyze ^{1}H NMR spectra of lipid mixtures, allowed to overcome this limitation and led to a better classification of olive oil samples (Hajjar et al., 2020) as well as egg samples (Hajjar et al., 2021). The aim of this study was to develop a high-resolution ^{1}H NMR method for the authentication and characterization of cheeses since they constitute foodstuffs of great nutritional and economic value. In this respect, cheese samples from different geographical origins, producing species, and varieties were collected and their TAG extracted and analyzed by ^{1}H NMR following the high-resolution method. Multivariate analyses of spectral variables were conducted to identify origin biomarkers and to construct prediction models for percentages of individual fatty acids within triacylglycerols.

2. Materials and methods

2.1. Samples

Fifty two cheese samples were collected from local grocery stores. Cheese samples were selected according to the producing species, to their geographical origin and variety. Samples included 32 cow, 10 sheep, and 10 goat cheeses. Cow cheese samples were from the following countries: Bulgaria ($n = 6$), France ($n = 7$), Germany ($n = 1$), Hungary ($n = 6$), Italy ($n = 6$), and Netherlands ($n = 6$). Goat cheese samples were from France ($n = 5$) and Spain ($n = 5$). Sheep cheese samples were from Bulgaria ($n = 5$) and Italy ($n = 5$).

2.2. Chemicals

Extra pure ethanol and petroleum ether (boiling range 308–333 K, ACS basic) were purchased from Scharlab; diethylether (GPR rectapur) was purchased from VWR chemicals and deuterated chloroform was purchased from Eurisotop. Whatman Purasil silica gel (60A, 230e400 Mesh ASTM) was used for column chromatography. Merck TLC Silica Gel 60 F254, aluminium sheets, 20 × 20 cm was used for thin layer chromatography. Standard fatty acid methyl esters were purchased from Sigma Aldrich and included methyl butyrate, methyl hexanoate, methyl caprilate, methyl decanoate, methyl laurate, methyl myristate, methyl palmitate, methyl stearate, methyl oleate, and methyl conjugated (9Z,11E)-linoleate.

2.3. Triacylglycerol extraction

A specific procedure for the extraction of triacylglycerols from cheese samples was developed and published in a previous paper (Haddad et al., 2021). Unless otherwise specified, working temperature was 295 ± 1 K. Cheese samples (30 g) were taken from the middle of the cheese blocks. Samples were cut into small pieces and dissolved in absolute ethanol (60 mL on average, depending on samples) at temperatures between 313 K and 323 K. The mixture was then filtered after 15 min of protein precipitation and the residue was successively washed with petroleum ether (70 mL), ethanol:diethyl ether (1:1 v/v, 30 mL), and diethyl ether (60 mL). Complete extraction of lipids was assessed by Thin Layer Chromatography (TLC) using normal-phase silica as the stationary phase and a mixture of diethyl ether in petroleum (1:3 v/v) as eluent. Solvents were evaporated under vacuum at 318 K. The precipitate appearing during the concentration of the solution was separated by decantation of the liquid phase containing total lipids. The precipitate was washed with absolute ethanol (3 × 10 mL) and then with diethyl ether (10 mL). Washing fractions and the liquid phase containing extracted lipids were combined and evaporation of solvents continued. Total lipids (8.5 g) thus obtained were subjected to a solid-phase extraction (SPE) over silica gel (7.8 g) to separate triacylglycerols and cholesterol. Lipids were dissolved in 40 mL of diethyl ether:petroleum ether (1:9 v/v) and pulled through the column. Additional 85 mL were necessary to elute triacylglycerols (TAG).

2.4. NMR experiments

2.4.1. Acquisition

^{1}H NMR spectra of cheese TAG were recorded on a Bruker Avance II spectrometer operating at 400.13 MHz (SI, Fig. S1). TAG (167 mg) was dissolved in 560 μL of $CDCl_3$ and filtered into a 5 mm NMR tube. The longest longitudinal relaxation time T_1 (3.19 s) was observed for the methyl group of the α-linolenic acid (LnA) as measured by the inversion-recovery method. For each sample, seven spectra were recorded using the following conditions: probe temperature 298 K, time-domain size 64 K, pulse angle 30°, pulse width 27.4 μs, spectral width 9 ppm, acquisition time 9.1 s, relaxation delay 1 s, 4 dummy scans, and 32 scans. The global experiment time for the seven acquisitions was 43 min.

2.4.2. Spectral processing

Bruker TOPSPIN 4.0.5 software was used for spectra processing. Recorded Free Induction Decays (FIDs) were zero-filled to 128 K. Before Fourier transformation, an exponential apodization function was applied, inducing a line broadening of 0.3 Hz. Spectra (SI, Fig. S1) were manually phased and a fifth-order polynomial baseline correction was automatically applied. Spectra were also subjected to reference line-shape adjustment (RLA) based on the residual $CHCl_3$ signal. The $CHCl_3$ signal was used as reference to compute the error function $\varepsilon^r(t)$ that will be used to adjust the experimental FID according to the following equation (Metz, Lam, & Webb, 2000; Morris, Barjat, & Home, 1997):

$$FID_{adj}(t) = \frac{FID_{exp}(t)}{\varepsilon^r(t)}$$

where $FID_{exp}(t)$ is the experimental FID and $FID_{adj}(t)$ is the adjusted one that now replaces $FID_{exp}(t)$. $CHCl_3$ peak shape was adjusted so as to obtain a perfect Lorentzian signal with a line width at half-height between 0.5 and 0.6 Hz. After this adjustment, the line width at half-height of the most intense peak in the methyl region was between 1.4 and 1.5 Hz. Each region of the spectrum was then calibrated and deconvoluted by adding the minimum number of peaks allowing the best fit (Hajjar et al., 2020) (SI, Fig. S2). Deconvoluted peaks were obtained for the following spectral regions of triacylglycerols: aliphatic, allylic, diallylic, vinylic, methyl groups, glycerol, methylene at position 3 of FAs ($CH_{2\beta}$), and methylene at position 2 of FAs ($CH_2\alpha$). Intensities and areas of deconvoluted peaks were calibrated against the $CH_2\alpha$ signal of FAs (its overall area or the sum of its peak intensities were set at 600). Signals of caproleic acid (C10:1, 9) between 4.80 and 4.94 ppm, and those of

rumenic acid (RA, isomer C18:2, *c*9*t*11 of conjugated linoleic acid) at 5.55, 5.85, and 6.20 ppm were weak and thus not able to be deconvoluted. Instead, they were manually integrated and integrals calibrated against the $CH_2\alpha$ signal of FAs, which integral was set at 100 (SI, Fig. S3).

2.5. Gas chromatographic analysis

Fatty acid methyl esters were prepared by shaking 100 mg of TAG in 1.5 mL of heptane with 0.2 mL of 2 N methanolic potassium hydroxide (European Commission, 1977). A 1:10 dilution in heptane was performed. An Agilent Technologies chromatograph equipped with a flame ionization detector (GC-FID) and a polyethylene glycol (PEG) modified with nitrophthalic acid/polyethylene glycol 2-nitroterephthalate column (Optima FFAP-Plus 10 °C–250 °C (260 °C): 30 m × 250 μm × 0.25 μm) was used with helium as carrier gas at flow of 1.2 mL/min. Oven was programmed as follows: 50 °C for 3 min after 10 °C/min until reaching 220 °C. Injection volume was 1 μL with a split ratio of 15:1 with an injector temperature of 250 °C. Two injections were performed for each sample with a total elution time of 30 min. Standard fatty acid methyl esters were used to determine the retention time of methyl butyrate, methyl hexanoate, methyl caprylate, methyl decanoate, methyl laurate, methyl myristate, methyl palmitate, methyl stearate, methyl oleate, and methyl conjugated (9Z,11E)-linoleate.

2.6. Chemometrics

TANAGRA data mining software was used for chemometrics analysis (Rakotomalala, 2005). Data obtained from ^{1}H NMR and gas chromatography (GC) analyses were subjected to statistical treatments. One-way analysis of variance (ANOVA) was used to assess the relative size of variance among group means compared to the average variance within groups. Principal component analysis (PCA) was used as an exploratory analysis. Canonical Discriminant Analysis (CDA) and Linear Discriminant Analysis (LDA) were used for the construction of classification models of samples according to their species, geographical origin, and variety. In each case, the performance and robustness of the model were assessed using LDA-Error rate (LDA-Er) and Leave-One-Out Error rate (LOO-Er).

Partial Least Square Regression (PLSR) was used to construct individual FA quantitation models based on the variables obtained from ^{1}H NMR spectra as predictors and percentages of FA from GC as targets. The number of components (h) was determined following cross-validation (internal validation by randomly leaving out 10% of the training samples) by considering the parameter Q^2 as an indicator of predictability. Maximum cumulative Q^2 (Q^2_{cum}) and Predicted Residual Error Sum of Squares (PRESS) were used as robustness indicators (Hawkins, Basak, & Mills, 2003). The coefficient of determination R^2 was used to assess how well the relative percentage of a given FA can be predicted using the ^{1}H NMR variables. Adjusted R^2 was used to compare models constructed with different numbers of predictors. Prediction models were subjected to external validation using test samples that had not been considered in the construction of models. Pred-R^2 was used as indicator (Hajjar et al., 2020).

3. Results and discussion

3.1. Spectral variables used as potential predictors in classification and quantitation models

As mentioned in Section 2.4.2, deconvolution was applied to the following spectral regions: aliphatic, allylic, diallylic, vinylic, methyl groups, glycerol, methylene at position 3 of FA ($CH_2\beta$), and methylene at position 2 of FA ($CH_2\alpha$). As a result, 178 peaks were deconvoluted and their corresponding intensities and areas were determined (SI, Tables S1 and S2). The global within-lab reproducibility (S_{Rwg}) of the whole analytical method, expressed as relative standard deviation, was determined for each spectral variable by analyzing five aliquots of the same cheese sample, starting from TAG extraction until data processing using topspin. For each spectral variable, S_{Rwg} was used to assess its ability to be used in multivariate analyses. This was done by considering S_{Rwg} –representing the precision of the measurement– and by comparing it with the variability (S_v) between the cheese samples. S_v for a given peak intensity or area is the relative standard deviation of the 52 samples used in the study. Spectral variables having S_{Rwg} and S_{Rwg}/S_v ratio lower than 10% and 0.33, respectively, were used as predictors in classification and quantitation models. Each spectral variable with one of these two parameters higher than the set value was merged with nearby ones in order to reach acceptable S_{Rwg} and S_{Rwg}/S_v for the package. (SI, Tables S1 and S2).

3.2. Classification of cheese samples

3.2.1. Classification of samples according to the producing species

The potential of ^{1}H NMR variables in the discrimination of cheese samples according to the producing species was investigated. Using CDA, a classification model based on two variables was constructed with LDA-Er and LOO-Er of 0% (Fig. 1a and SI, Table S3a).

Besides, TAG variables that vary significantly ($p \leq 0.05$) between cheese samples from different groups are shown in (Table 1). Such variables can be used to characterize cheese samples according to the producing species. For instance, variable Bu-s (SI, Fig. S2a and Table S2), related to butyric acid, was lower in goat than in sheep and cow cheese samples (Table 1 and Fig. 1b). Variable AL10s (or AL10i) (SI, Fig. S2c, Tables S1 and S2), which is the area (or intensity) of a deconvoluted peak in the allylic region of the spectra, was higher in cow than in goat and sheep cheese samples (Table 1 and Fig. 1b). We discovered that the deconvoluted peak AL10 (SI, Fig. S2c) corresponds to myristoleic acid (C14:1, *c*9), as shown in Section 3.3 ($R^2 = 0.832$). Percentages of this acid in TAG from cow cheese samples were thus higher than those of sheep and goat ones, which is in accordance with the literature (Ceballos et al., 2009).

Similarly, we discovered that variable AL18-19s (or AL18-19i), which is a combination of deconvoluted peaks AL18 and AL19 in the allylic region, corresponds to vaccenic acid (C18:1, *t*11, VA) (see Section 3.3). This acid showed significantly lower percentages in cow than in goat and sheep cheeses (Table 1). Other variables allowing such discrimination between cheeses from different species are reported in Table 1. Moreover, we found that sheep cheese samples were richer in α-linolenic (C18:3, *c*9*c*12*c*15, LnA) and polyunsaturated FAs (PUFA) than those from cow and goat. Also, rumenic acid (RA) –isomer C18:2, *c*9*t*11 of conjugated linoleic acid, known to have anticarcinogenic, antiobese, antidiabetic and antihypertensive properties (Koba & Yanagita, 2014)– was higher in sheep than in cow and goat cheese samples (Table 1).

According to Stoop et al. (Stoop, Van Arendonk, Heck, Van Valenberg, & Bovenhuis, 2008), the within-breed genetic variation and the differences in feeding regimens have a considerable effect on FA composition. For C4:0 to C18:0, genetic factors were dominant. Whereas, for unsaturated C18 FAs, including VA and RA, herd factors involving the feeding regimen and other management practices were larger than genetic ones. However, in the discrimination discussed in this section, the genetic factors should be the prominent ones since different species were involved and not different breeds. Moreover, herd factors were not likely since samples of each species were from different geographical origins (i.e., different countries), different producers within the same country, and different seasons.

3.2.2. Classification of samples according to their geographical origin

Aiming to eliminate interference of the animal origin factor with the classification of samples according to their geographical origin, only cheese samples from the same producing species were considered at a

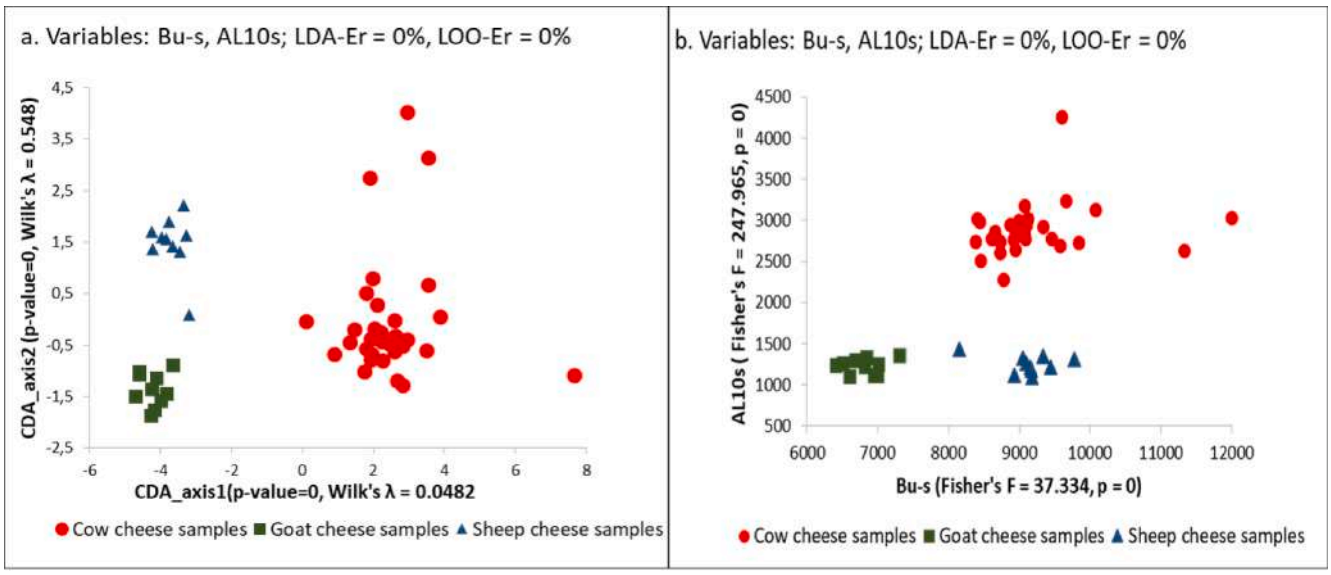

Fig. 1. Classification of goat, sheep, and cow cheeses according to the producing species using CDA axes (a) or variables Bu-s and AL10s (b).

time. First, cow cheese samples were considered. The collected samples came from the following countries: Bulgaria, France, Hungary, Italy, and Netherlands. It was possible to discriminate between all groups using CDA with 10 predictors, as assessed by LDA-*Er* (0%) and LOO-*Er* (6.45%) (Fig. 2a and SI, Table S3b).

Spectral variables showing an obvious tendency to be higher or lower in samples from a given geographical origin than in others are reported in Table 1. Related variations between groups are also reported. Variable CH3-20s (SI, Table S2 and Fig. S2a) was in general lower in samples from Netherlands than in other samples (Table 1). Variable G6-8s (SI, Fig. S2g) showed a tendency to be higher in Italian than in other samples (Table 1). Most likely, this variable is affected by the presence or absence of short-chain fatty acids in the TAG molecule. Feeding systems are the main factor leading to the observed differences in the lipid profile of cheeses from different geographical regions (Elgersma, Tamminga, & Ellen, 2006; Gaspardo, Lavrenčič, Levart, Del Zotto, & Stefanon, 2010; Segato et al., 2017). Moreover, diet and environment are also among the main factors affecting the rumen microbiota of the herd that, in turn, imprints the FA profile of milk fat (Liu et al., 2021). On the other hand, breeds of herds in a given country may also contribute to the observed discrimination. For some FAs, this factor is the most influencing (Stoop et al., 2008).

For sheep cheeses, variable V13i (SI, Table S1) was sufficient to discriminate Bulgarian from Italian samples (Fig. 2b). V13i is the intensity of a deconvoluted peak from the vinyl region of the spectra (SI, Fig. S2h). It is one of the peaks within the signal of RA at 5.35 ppm. However, Sum of intensities V13i, V14i, and V15i (SI, Fig. S2h) had a stronger correlation ($R^2 = 0.9172$, $n = 52$, $p < 0.00001$) with percentage of RA (determined by GC) than V13i alone ($R^2 = 0.8012$, $n = 52$, $p < 0.00001$). Plausibly, this indicates that peak V13 corresponds to a specific position of RA on the glycerol moiety of TAG and not to total RA. Although the RA percentage was, in general, higher in Bulgarian than in Italian cheeses, the best discrimination was not due to this variable but to V13i. Variables related to the distribution of individual FAs at the *sn*-1, *sn*-2, and *sn*-3 positions of the glycerol backbone of TAG could be essential nutritional biomarkers. This is due to the activity of pancreatic lipase that is highly specific to positions *sn*-1 and *sn*-3. Thus, FAs released from the *sn*-1 and *sn*-3 positions are often metabolized differently from fatty acids retained in the *sn*-2 position (Hunter, 2001).

Similarly, classification of goat cheese samples according to their geographic origins was possible with variable V9i (Fig. 2c). V9i is the intensity of a deconvoluted peak from the vinylic region of the spectra (SI, Table S1 and Fig. S2h).

3.2.3. *Classification of cow cheese samples from the same country according to their variety*

Classification of samples according to the cheese variety was also investigated. Varieties from the same country were compared, aiming to minimize environmental effects. Obvious differences between cheese varieties are shown in (Table 1). The French cheese "Comté" was distinguishable from the other cow cheeses. The "Comté" samples were richer in RA (Fig. 3a) and Table 1. RA in milk TAG is synthesized in two ways: (i) as an intermediate of the microbial biohydrogenation of linoleic acid to stearic acid in the rumen, (ii) and mainly from VA by the action of delta-9 desaturase in the mammary gland. It should be kept in mind that VA is formed during biohydrogenation of oleic, linoleic, and α-linolenic acids by the rumen microbiota (Palmquist, Lock, Shingfield, & Bauman, 2005). Thus, the rumen microbiota is the primary factor in synthetic routes of both VA and RA. It was highly remarkable that percentages of bioactive fatty acids RA, VA, and LnA were the highest in "Comté" compared to all other cow cheese samples in this study. A similar increase of these three FAs was observed in milk from cows grazing cool-season pasture compared to cows grazing pearl millet (Bainbridge et al., 2018). It is known that fractional contribution of LnA to total FAs is higher in grasses than in forbs (Clapham, Foster, Neel, & Fedders, 2005). This can explain the highest percentage of this FA in TAG of "Comté" since this PDO cheese results from an extensive farming system in a restricted geographical area where cows ("Montbéliarde" and "Simmental française" breeds) are mainly fed fresh grass in the fields or hay from the same fields in winter. Moreover, "Comté" specifications indicate a limitation to one cow per hectare of fodder surface-area, a ban on silage, and a limitation of feed concentrate to 30% dry matter in the total feed (Colinet, Desquilbet, Hassan, Dilhan, Orozco, & Requillart, 2006). Besides, a strong positive correlation ($R = 0.88$, $n = 52$, $p < 0.00001$) was found between spectral variables corresponding to VA (variable AL18-19i) and RA (variable RA) when all cow, goat and sheep cheese samples were considered. This correlation was in accordance with that reported by Jahreis et al. (Jahreis, Fritsche, & Steinhart, 1997) ($R = 0.85$). Similarly, a strong positive correlation ($R = 0.79$, $n = 52$, $p < 0.00001$) was found between spectral variables corresponding to LnA (variable LnA-i) and RA (variable RA).

For Bulgarian cheeses, white cheese samples were discriminated from "Kashkaval" samples using G2-9s (Fig. 3b), a variable extracted from the *sn*-2 signal of the glycerol moiety in TAG (SI, Fig. S2h and

Table 1
Variations of cheese TAG variables in response to changes of producing species, geographical origin, cheese variety, and producer.

Compared cheese groups[a]	Variable[b]	Variability[c] (%)	Compared cheese groups[a]	Variable[b]	Variability[c] (%)
Cow (32) to Goat (10)	B2i* *(0)* ≈ B1i* *(0)* ≈ B3i* *(0)* ≈ Bu-s *(0) [C4:0]*	+33.9	**Cow: Netherlands (6) to other countries (25)**	CH3-20s *(0.00090)*	−19.1
	A11i *(0)*	+95.2		DAL18-20i *(0.00089)*	−44.4
	AL10s *(0) [C14:1c9]*	+80.5	**Sheep: Bulgaria (5) to Italy (5)**	V13i* *(0)* ≈ *RA (0.0094)* ≈ *V9i (0.033) [C18:2 c9, t11]*	+49.9
	G9i *(0)*	+117		DAL9-11i *(0.0097) [C18:3c9,12,15]*	+20.3
	AL8s *(0)*	−119.3		G2-10s *(0.011)*	+17.0
	AL17i *(0)*	−30.4		Caproleic *(0.027)*	−16.8
	AL18-19s *(0) [C18:1t11]*	−79.6		G2-9i *(0.014)*	−9.4
	AL16s *(0)*	−130.6		V16-19s *(0.014)*	+13.2
	G13-14s *(0)*	+72.6	**Goat: France (5) to Spain (5)**	V9i *(0.0011)*	+55.7
	V19i *(0)*	+49	**France cow: "Comté" (3) to other cheeses (4)**	AL18-19i *(0) [C18:1 t11]*	+59.1
	AL15s *(0)*	+94.4		DAL12-14i *(0)*	+53.1
	AL11-13i *(0)*	−19.2		RA (0.0070) ≈ V13i*(0.0073) ≈ V8-11i (0.011) ≈ V14i* *(0.015)* ≈ V1-7i *(0.0084) [C18:2c9,t11]*	+104.8
	G2-9s *(0)*	−28.5		LnA-i *(0.011)* ≈ DAL9-11i *(0.013) [C18:3c9,12,15]*	+67.4
	G10-12i *(0)*	−10.0		A12s *(0.018)*	–22.2
Cow (32) to Sheep (10)	A11i *(0)*	+96.0		V22i *(0.020) [ω 5,6,7,8 &9]*	+13.0
	V19i *(0)*	+ 46.7		V15i *(0.031)*	+40.5
	AL10i *(0) [C14:1c9]*	+69.5		CH3-25-26i *(0.031)*	−5.8
	AL14s (0)	+53.4		AL15-17i *(0.038)*	+30.9
	DAL9-11i *(0)* ≈ LnA-i *(0)* ≈ *V6i (0) [C18:3c9,12,15]*	−70.8	**Italy cow: "Parmesan" (3) to other cheeses (3)**	AL12s *(0.0060)*	+21.5
	DAL12-14i *(0)*	−53.8		DAL9-11i *(0.013) [C18:3c9,12,15]*	+40.3
	AL8s *(0)*	−91.2		DAL12-14i *(0.015)*	+35,3
	G1i *(0)*	+110.5		V24i *(0.015)*	+20.3
	G9i *(0)*	+63.8		DAL21-23i *(0.019)*	+34.2
	V21s *(0)*	+26.1		DAL18-20i *(0.022)*	+32.0
	V8-11i (0) *[C18:2c9,t11]*	−65.6		AL4-5s (0.038)	−32.3
	G11i *(0)*	+34.4		V21s *(0.046)*	+6.8
	AL1-3i *(0) [PUFA]*	−64.6	**Bulgaria cow: "kashkaval" (2) to Bulgarian white cheese (4)**	G2-10s *(0.0038)*	−18.5
	AL18-19i *(0) [C18:1,t11]*	−60.6		G2-9s *(0.0078)*	+18.8
				V6i *(0.016) [C18:3c9,12,15]*	+31.8
Goat (10) to Sheep (10)	G20s *(0)*	−40.3		B3s* *(0.019) [C4:0]*	−9.5
	Bu-s *(0)* ≈ B2i* *(0)* ≈ B1i* *(0) [C4:0]*	−29.1	**Cow: "Kashkaval" (8) to other cheeses (14)**	V14s* *(0) [C18:2c9,t11]*	+29.3
	G2-10i *(0)*	−56.8	**Cow: Bulgarian white cheese (4) to other cheeses (18)**	AL14s *(0)*	+37.9
	G17i *(0)*	−36.0		B3s* *(0.00051) [C4:0]*	+10.5
	CH3-27-28i *(0)[Anteiso C15:0]*	−41.4		AL9i *(0.0016)*	+13.3
	G26i *(0)*	−35.5		G2-9s *(0.0085)*	−15.2
	G23s *(0)*	−51.6	**Cow: "Comté" (2) to other cheeses (20)**	RA (0) ≈ V13i* (0) ≈ V8-11i (0) ≈ V1-7i (0.00062) ≈ V14s (0.0078) *[C18:2c9,t11]*	+116.2
	G2-12i *(0)*	−51.6		AL18-19i *(0) [C18:1 t11]*	+51.5
	G2-9i *(0)*	+29.7		AL20i *(0)*	+51.0
	G2-5i *(0)*	+11.8		V12s (0)	+26.9
	G18-19i *(0)*	+6.1		Ln-Ai *(0)*	+33.8
	V8-11i *(0.00023)[C18:2c9, t11]*	−53.1		DAL14i *(0.001)*	+60.6
	G2-7i *(0)*	−14.6		V15i *(0.00014)*	+35.6
	G21-22s *(0)*	+4.9		AL1-3i *(0.00022) [PUFA]*	+47.2
	G2-10s *(0)*	−60.2	**Cow: "Parmesan" (3) to other cheeses (19)**	DAL12i *(0.0070)*	+33.7
	G27-28i *(0)*	+5.9		DAL9-11i *(0.040) [C18:3c9,12,15]*	+27.7
	CH3-25-26i *(0)*	+7.1	**Cow: "Emmental" (2) to other cheeses (20)**	V12i *(0.0072)*	–22.3
	G2-3i *(0)*	+39.6		V16-19i *(0.0077)*	−17.4
	G2-8i *(0)*	+10.1		V23i *(0.014)*	−14.8
	LnA-i *(0)* ≈ DAL9-11s *(0) [C18:3c9,12,15]*	−68.8		AL1-3i *(0.030) [PUFA]*	−45.3
	B12s *(0)*	+20.8		G2-6s (0.0021)	−6.33
	V18i *(0)*	+35.3		CH3-27-28i *(0.018) [Anteiso C15:0]*	−12.6
	AL1-3i *(0) [PUFA]*	−58.6		G17i *(0.030)*	+7.31
Cow: Bulgaria (6) to other countries (25)	AL17i *(0)*	+13.2	**Cow Hungary "Kashkaval": Producer 1 (2) to Producer 2 (4)**	RA *(0.030)*	–23.03
	CH3-17i *(0)*	+16.0		G2-13s *(0.034)*	+7.3
	V18i *(0.0015)*	+34.7		CH3-20s *(0.035)*	+10.7
Cow: Italy (6) to other countries (25)	G6-8s *(0)*	+19.2		G18-19i *(0.050)*	+1.7
	V21s *(0.0012)*	−11.4			
	G5i *(0)*	–32.5			

[a] Between brackets is the number of samples in each group. [b]Number in brackets represents the p value obtained by means of ANOVA, $p = 0$ means that it is<0.00001. Variables are placed according to their increasing p-values. The symbol ≈ means that the variables correspond to the same FA. If known, the FA corresponding to the variable(s) figures in square brackets. When present, the symbol * means that the variable is probably related to the position of a given FA or group of FAs on the glycerol backbone. It can also mean that the variable corresponding to a given FA is probably affected by the two other FAs present on the glycerol backbone.

[c]Variability represents the percentage of variation of the corresponding variable (the first one if several are reported): [((average group 1 − average group 2)/((av. gr. 1 + av. gr. 2)/2)) * 100]. The sign (+) means that the average value of the variable is higher in group 1 than in group 2. The sign (−) means the opposite.

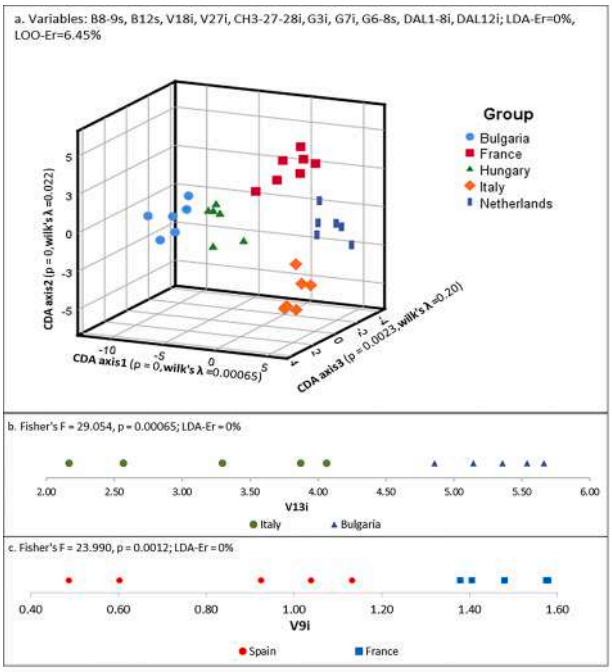

Fig. 2. Classification of cheese samples according to their geographical origin: (a) cow cheese samples, (b) sheep cheese samples, and (c) goat cheese samples.

Table S2). Other variables (G2-10s, B3s, and V6i) reported in Table 1 were also discriminators for these two cheese varieties. Variables G2-10s (SI, Table S2 and Fig. S2h) and B3s (SI, Table S2 and Fig. S2f), related to the butyrate group in TAG, were both lower in kashkaval than in white Bulgarian cheeses. Most likely, this was due to lipolysis of TAG during the ripening step in kashkaval production that is known to increase free butyric acid and thus, to reduce TAG-containing butyrate at the *sn*-3 position (Gobbetti et al., 2002). Moreover, V6i, a variable in the vinylic region (SI, Table S1 and Fig. S2h) correlated to LnA ($R = 0.88$, $n = 52$, $p < 0.00001$), was higher in "kashkaval" samples. However, variables LnA-i and LnA-s, which represent total LnA within TAG, were not discriminators for these samples. Similarly, in the case of Italian cheeses, while variable DAL9-11i (SI, Table S1 and Fig. S2e) –which is correlated to LnA ($R = 0.86$, $n = 52$, $p < 0.00001$)– allowed us to discriminate between the "Parmesan" and the other samples, LnA-i and LnA-s did not do so. Variables V6i and DAL9-11i are most probably related to LnA at a given position in TAG (*sn*-1,3 or *sn*-2) or LnA in TAG molecules containing a given type of FAs (e.g., short- or long-chain FAs).

3.2.4. *Classification of cow cheese samples according to their variety and regardless of their geographical origin*

In this step, we tried to train a model that allows classifying at a time all cow cheese samples according to their variety. Samples from the same variety were considered to be in the same group, even if they were from different origins. The following samples were considered: 4 samples of Bulgarian white cheese (B); 8 samples of "Kashkaval" (K), 2 from Bulgaria and 6 from Hungary; 2 samples of "Comté" (C); 3 samples of "Emmental" (E) from France, Germany, and Netherlands; 2 samples of "Mimolette" (M) from France and Netherlands; and 3 samples of "Parmesan" (P). A model based on 11 predictors was able to classify samples with no LDA and LOO errors (SI, Table S3c and Fig. 3d). Moreover, a successful external validation was achieved with a "Comté" (EV-C) and a "Mimolette" (EV-M) sample from Netherlands (Fig. 3d).

Besides, several variables reported in Table 1 were individually able to discriminate a given variety of cheese from others. We cite herein AL14s (SI, Table S2 and Fig. S2c), a discriminator for Bulgarian samples group; V14s (SI, Table S2 and Fig. S2h), related to RA and affected by the position or the distribution of FAs in TAG, which was generally higher in

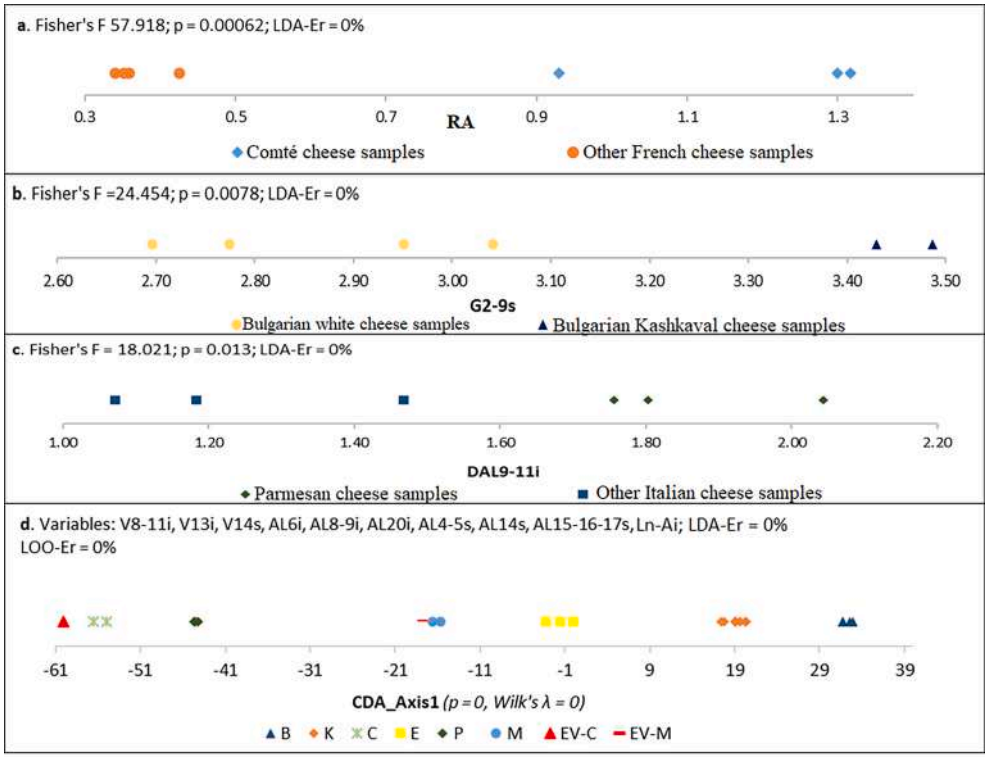

Fig. 3. Classification of cow cheese samples according to their variety: (a) within France ("Comté" form other varieties), (b) within Bulgaria (Bulgarian white cheese from Kashkaval), (c) within Italy ("Parmesan" from other varieties), and (d) regardless of their origin. In Fig. 3d, designations of groups is as follow: B for Bulgarian White cheese ($n = 4$); K for Kashkaval from Bulgaria ($n = 2$) and Hungary ($n = 6$); C for "Comté" ($n = 2$); E for "Emmental" from France ($n = 1$), Germany ($n = 1$), and Netherlands ($n = 1$); P for "Parmesan" ($n = 3$); M for "Mimolette" from France ($n = 1$) and Netherlands ($n = 1$); EV-C for a sample of "Comté" used in external validation; and EV-M for a sample of "Mimolette" used in external validation.

the "kashkaval" than in the other samples; RA and LnA-I, obvious markers of the "Comté" cheeses (cf. Section 3.2.3); and AL1-3i (SI, Table S1 and Fig. S2c), which was lower in the "Emmental" than in almost all the other samples. Variable AL1-3i was correlated to LnA-i ($R = 0.91$, n = 52, $p < 0.00001$). However, LnA-i, which represents total LnA, did not show the same trend as AL1-3i. It seems that AL1-3i is related to LnA at the *sn*-1 and/or the *sn*-3 positions of TAG. Low values of this variable in the "Emmental" samples could be due to the release of LnA from the before-mentioned positions of TAG during the cheese ripening phase.

Thus, the ripening phase and its duration, the cattle feeding, the farming system, the geographical origin, the environmental conditions, and the cattle breed are factors affecting the FAs profile of cheese TAG, leading to their varietal specificity.

3.2.5. Classification of hungarian cow cheese samples according to the producer

Samples from two producers of "kashkaval" cheese in Hungary were considered: Prod-1 ($n = 2$) and Prod-2 ($n = 4$). TAGs in Prod-2 samples were higher in RA, and variable G2-6s (SI, Table S2 and Fig. S2h) made it possible to discriminate samples from the two producers (Fig. 4). Moreover, variable CH3-27-28i (SI, Table S1 and Fig. S2a) showed higher values for Prod-2 samples. For the first time in ^{1}H NMR, we discovered that this variable is related to anteisopentadecanoic acid (cf. Section 3.3). Other variables able to discriminate between Prod-1 and Prod-2 samples are reported in Table 1. Thus, samples can be distinguished according to the producer, even within the same country and cheese variety. Such discrimination could be due to differences regarding the cattle breed, the pasture, and the environment. These factors also affect the rumen microbiota that impacts the FAs profile in TAG (cf. Section 3.2.3).

3.3. Quantitation of individual FAs within cheese TAGs using ^{1}H NMR

Aiming to improve the characterization of cheese samples by ^{1}H NMR, three approaches were used to quantitate individual FAs. First, known signals were used to quantitate α-linolenic, butyric, rumenic, and caproleic acids. For LnA, the sum of its methyl protons peak intensities, denoted LnA-i (SI, Fig. S2a and Table S1), was used to predict its relative mass percentage while taking GC values as targets: predicted LnA (%) = 0.0485 × LnA-i − 0.119, $R^2 = 0.926$, $n = 51$, $p < 0.00001$. Similarly, butyric acid was quantitated using the sum of its methyl protons peak intensities, denoted Bu-i (SI, Fig. S2a and Table S1): predicted butyric acid (%) = 0.0167 × Bu-i − 0.344, $R^2 = 0.873$, $n = 41$, $p < 0.00001$; the observed discrepancy between predicted and GC values was most likely due to losses of methyl butyrate in the methanolic phase during TAG transesterification before GC analysis. RA was quantitated using the average of its signal integrals (RA-intg, SI, Fig. S3b and Table S2), calibrated against the integral of $CH_2\alpha$ signal of FAs (set at 100). Prediction of RA relative mass percentage based on GC values as targets gave: predicted RA (%) = 1.31 × RA-intg + 0.0957, $R^2 = 0.961$, $n = 51$, $p < 0.00001$. Caproleic acid was quantitated only by ^{1}H NMR using corresponding signal integrals between 4.80 and 4.94 ppm (vinylic methylene protons, SI, Fig. S3a and Table S2). Integrals in question were calibrated similarly to those of RA. Correlation between amounts of caproleic acid determined by ^{1}H NMR and GC was excellent in the case of sheep cheese samples ($R^2 = 0.983$, $n = 9$, $p < 0.00001$). However, these amounts were remarkably less correlated in the cases of goat ($R^2 = 0.584$, $n = 10$, $p = 0.01$) and cow ($R^2 = 0.317$, $n = 32$, $p = 0.0008$) cheese samples. The discrepancy observed for goat cheese samples was most probably due to another compound having ^{1}H NMR signals overlapping with those of caproleic acid used in the quantitation. For cow cheese samples, it was most likely due to an isomer of caproleic acid with the same retention time in GC analysis.

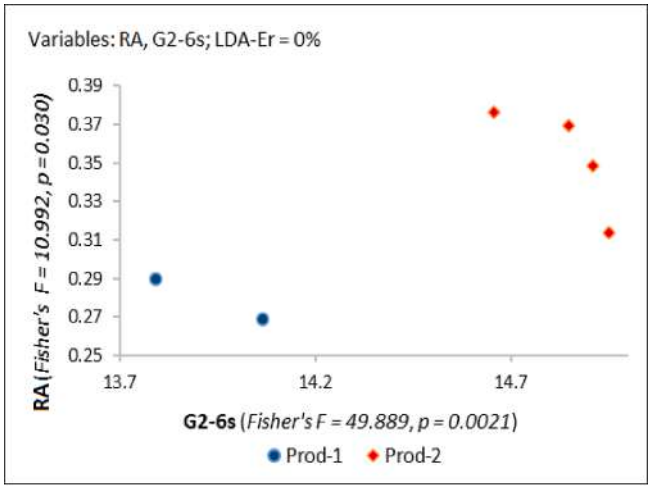

Fig. 4. Classification of cheese samples from the same variety and country according to the producer in Hungary.

In a second FA quantitation approach, the linear correlations between the ^{1}H NMR variables (the deconvoluted peak intensities or areas) and the relative mass percentages of FAs determined by GC were investigated. This approach allowed us to discover peaks corresponding to specific FAs. Thus, vaccenic (VA), anteisopentadecanoic (Anteiso-C15:0), and myristoleic acids were quantitated. Variable AL18-19i (SI, Fig. S2c and Table S1) was found to be correlated to VA percentage determined by GC: predicted VA (%) = 0.396 × AL18-19i − 0.267, $R^2 = 0.854$, $n = 51$, $p < 0.00001$. Variable CH3-27-28i (SI, Fig. S2a and Table S1) allowed predicting GC-determined mass percentages of Anteiso-C15:0: predicted Anteiso-C15:0 (%) = 0.0294 × CH3-27-28i − 0.174, $R^2 = 0.846$, $n = 51$, $p < 0.00001$. We mention herein that variable CH3-27-28i represented the percentage of all branched FAs (antieso- and isoFAs) since the coefficient of determination (R^2) reached 0.922 ($n = 51$, $p < 0.00001$) when all the GC-quantitated FAs of this category were considered in the correlation. Similarly to variables AL18-19i and CH3-27-28i, variable AL10i (SI, Fig. S2c) and × allowed quantitating myristoleic acid: predicted myristoleic acid (%) = 0.117 × AL10i − 0.722, $R^2 = 0.832$, $n = 51$, $p < 0.00001$. It should be noted that myristoleic acid percentages in TAG from goat and sheep cheeses were approximately the same but, on average, 6.71 times lower than those in TAG from cow cheeses. Myristoleic acid was the only unsaturated FA showing this trend and AL10i was the only ^{1}H NMR variable in accordance with this trend. However, AL10i was, on average only 1.84 times lower in TAG of goat and sheep cheeses than in cow ones. This most likely means that myristoleic acid and another unsaturated FA have signals that overlap at AL10. However, the amount of the second FA does not follow the same trend as that of myristoleic acid when comparing goat and sheep cheeses with cow ones.

In a third approach, PLSR was used to construct individual FA quantitation models based on ^{1}H NMR variables as predictors (only statistically significant variables were kept in models) and relative mass percentages of FAs determined by GC as targets. Following this approach, quantitation models for caproic, caprylic, capric, oleic, palmitic, margaric, anteisomargaric, and linoleic acids were constructed (Fig. 5 and SI, Table S4). The performance and robustness parameters of the models are reported in Fig. 5. Models of caprylic, capric, and anteisomargaric acids were constructed using variables belonging to other FAs. It was due to correlations between FAs within the TAG of the milk matrix.

Aiming to compare the sensitivity of FA quantitation by ^{1}H NMR and GC-FID, Limit of Detection (LOD) and Limit of Quantitation (LOQ) were estimated for both techniques in the case of α-linolenic acid. This was done based on within-Lab reproducibility (S_{Rwg}, expressed herein as standard deviation; cf. Section 3.1) with LOD = 3.S_{Rwg} and LOQ = 10. S_{Rwg} (Magnusson & Örnemark, 2014). For GC-FID, the LOD and LOQ

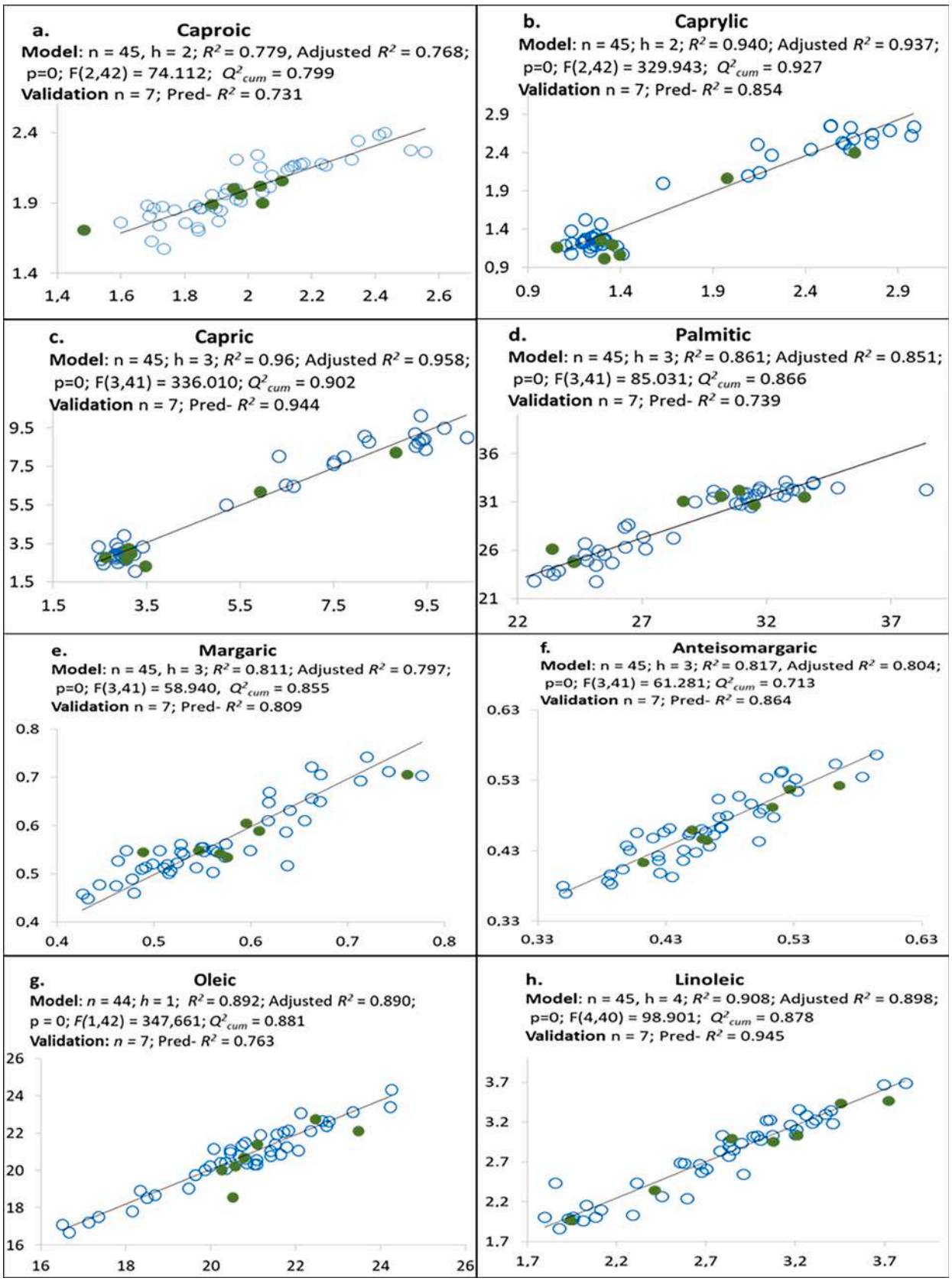

Fig. 5. Correlations between fatty acid percentages obtained by GC (x-axis) and those predicted using variables from high-resolution ^{1}H NMR spectra processing (y-axis). ∘ Training samples, • test samples for model validation, $p = 0$ means that it is less than 0.00001.

values were 0.028% and 0.093%, respectively. For ^{1}H NMR, lower but of the same order of magnitude sensitivity was observed (LOD = 0.065% and LOQ = 0.22%). However, "high-resolution" ^{1}H NMR has the advantage of affording a whole set of variables related not only to the FAs profile of TAG but also to their distribution on the glycerol skeleton.

4. Conclusion

The high-resolution ^{1}H NMR method has proved highly effective in discovering authentication biomarkers for food of animal origin. This method was successfully applied to classify cheese samples according to their producing species, geographical origin, variety, and producer. Variables related to the position or distribution of fatty acids in triacylglycerols were among the biomarkers used in classifications. The obtained results suggested a significant effect of the rumen microbiota on the differences observed between samples. On the other hand, spectral peaks specific to vaccenic, anteisopentadecanoic, and myristoleic acids were discovered. These minor fatty acids were thus quantitated in cheese triacylglycerols. Besides, the characterization of cheese samples by ^{1}H NMR was further improved via individual quantitation of several fatty acids using partial least squares regression. The present methodology paves the way for authentication analysis of all dairy products, including organic, PDO, and traditional specialty products.

CRediT authorship contribution statement

Lenny Haddad: Investigation, Data curation, Formal analysis, Writing – original draft. **Joseph Francis:** Investigation, Formal analysis. **Toufic Rizk:** Project administration, Funding acquisition. **Serge Akoka:** Conceptualization, Writing – review & editing. **Gérald S. Remaud:** Conceptualization, Supervision, Writing – review & editing, Funding acquisition. **Joseph Bejjani:** Conceptualization, Methodology, Validation, Supervision, Resources, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.132434.

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