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ORIGINAL PAPER

^{1}H- and ^{13}C-NMR Characterization of the Molecular Components of the Lipid Fraction of Pecorino Sardo Cheese

P. Scano · R. Anedda · M. P. Melis · M. A. Dessi' · A. Lai · T. Roggio

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Abstract In this work the molecular fatty components of Pecorino Sardo Protected Designation of Origin (PS PDO) cheese were characterized through an exhaustive investigation of the ^{1}H- and ^{13}C-NMR spectra of the extracted lipids. Several fatty acids (FA), such as long chain saturated, oleic, linoleic, linolenic, butyric, capric, caprylic, caproic, *trans* vaccenic, conjugated linoleic acid (*cis*9, *trans*11–18:2), and caproleic (9–10:1) were unambiguously detected. The positional isomery of some acyl groups in the glycerol backbone of triacylglycerols (TAG) was assessed. Furthermore, the NMR signals belonging to *sn*-1,2/2,3, *sn*-1,3 diacylglycerols (DAG), and free fatty acids (FFA) were analysed as a measure of lipolytic processes on cheese. Lastly, ^{1}H-NMR resonances of saturated aldehydes and hydroperoxides were detected, their very low intensity indicating that the lipid oxidation process can be considered to be of minor relevance in Pecorino Sardo cheese.

P. Scano and R. Anedda contributed equally to this work.

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P. Scano (✉) · A. Lai
Dipartimento di Scienze Chimiche, Università degli Studi di Cagliari, Cittadella Universitaria di Monserrato, S. S. 554-Bivio per Sestu, 09042 Monserrato, Cagliari, Italy
e-mail: scano@unica.it

R. Anedda · T. Roggio
Porto Conte Ricerche Srl, S. P. 55 Porto Conte/Capo Caccia, 07041 Tramariglio-Alghero, SS, Italy

M. P. Melis · M. A. Dessi'
Dipartimento di Biologia Sperimentale, Sezione di Patologia Sperimentale, Università degli Studi di Cagliari, Cittadella Universitaria, Monserrato, CA, Italy

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Introduction

It is well known that hundreds of fatty acids (FA) are present as acyl chains in triacylglycerols (TAG) of ruminant milk, but approximately only fifteen occur in a percentage above 1%. Among these, short chain FA (C < 10), in particular butyric (C4:0) and caproic (C6:0) that are esterified mainly in the *sn*-3 position in TAG [1], are of fundamental importance in the development of cheese aroma. Furthermore, ruminants' fat, and particularly milk and milk-derivatives, are the only food products that naturally contain unsaturated FA in *trans* configuration, mainly represented by the *trans* vaccenic acid (VA) and the *cis*9, *trans*11–18:2 conjugated linoleic acid (CLA). This latter FA occurs in a concentration that ranges approximately from 27 mg/g of fat in sheep milk to 7 mg/g in cow milk [2, 3], even though compositional fluctuations, strongly linked with diet and seasonal variations, exist [4]. Although *trans* FA from partially hydrogenated oils are well known for having negative effects on human health [5], recent studies have reported that milk *trans*-FA have beneficial effects and this issue is still subject to extensive studies [6, 7]

During the ripening of cheese, milk lipids are subjected to enzymatic lipolysis. The hydrolysis of the ester linkage between an acyl group and the glycerol backbone of TAG produces free fatty acids (FFA) and diacylglycerols (DAG) in the *sn*-1,2, *sn*-1,3 and sn-2,3 isomeric forms. The released FFA and their catabolic products have been found to be the main elements that are responsible for the aroma in cheese, especially in Italian hard cheeses where lipolysis

reaches high levels [8]. In Pecorino Sardo Protected Denomination of Origin (PS PDO), where rennet paste is used to coagulate the milk, the prevailing lipase is the pregastric esterase (PGE), that, being specific to the *sn*-3 position of TAG, releases mainly short chain FA [9]. As a consequence of PGE action, among DAG, the isomeric *sn*-1,2 form is the first to be formed [1]. During the last stage of ripening and during shelf life the *sn*-1,2 isomer is usually converted to the *sn*-1,3 isomer, which is thermodynamically more stable [10].

The comprehensive characterization of the lipid fraction of milk and dairy products represents a challenge, and several investigation techniques have been employed. Recently, ^{1}H- and ^{13}C-NMR spectroscopy have been successfully applied to the study of the fatty acid composition of milk from different animal species [11–13] and to discriminate Asiago cheese produced in alpine farms and in industrialized factories [14]. NMR spectroscopy has the main advantage of not requiring extensive manipulation of the sample, thus preserving molecular integrity, and allowing detection of all the substances present in the sample at the same time. In particular, ^{1}H-NMR spectroscopy allows a rapid analysis of complex mixtures due to the high NMR susceptibility and natural abundance of this isotope. On the other hand, the ^{13}C nucleus, a "diluted spin", sweeps a wider range of chemical shifts, therefore improving resolution and facilitating assignments and quantitative analysis. The ^{1}H–^{13}C-NMR correlation spectroscopy takes advantage of both the high sensitivity of ^{1}H NMR and the spectral resolution achievable by ^{13}C NMR.

The aim of the present work was to extend and complement by NMR the information obtained so far on dairy products. To this goal an exhaustive characterization of ^{1}H- and ^{13}C-NMR spectra of the lipid fraction of PS PDO cheese was carried out, paying particular attention to the study of *trans* FA and of *sn*-1,3 and *sn*-1,2/-2,3 DAG.

The investigated lipid samples were obtained from PS PDO, a semi-cooked hard cheese produced in Sardinia (Italy) using exclusively raw milk from Sarda sheep grazing on natural pasture. In 1996 the PS cheese received the Protected Designation of Origin status, which strictly identifies its production areas within the island of Sardinia. PS PDO is characterized by a dense, hard, grainy paste, and intense, aromatic, spicy flavors. It can be produced in two different typologies, sweet (Dolce) and mature (Maturo), depending on the size and weight of the wheels and their ripening status. It is rich in fine aromas and is well suited for sophisticated cuisine. We believed that a thorough investigation into the lipid composition of PS PDO and possible modifications during ripening would be useful to improve its quality.

Materials and Methods

Samples

Six samples of PS PDO Maturo cheese, coming from different producers located in Sardinia, were examined. The extraction procedure of the lipid fraction was carried out according to the Folch method [15]. Briefly, a weighted amount (approximately 1 g) of cheese paste from the inner part of the form was extracted with 20 ml of a chloroform:methanol (2:1, v/v) solution for 20 min, and filtered through filter paper. In order to remove the non-lipid contaminants, a salt solution of 0.9% NaCl was added to the filtrate. The mixture was then centrifuged to recover the hydrophobic (lipid extract) fraction separately. Solvents were eliminated by means of a rotary evaporator at reduced pressure in order to avoid loss of volatile compounds. The dried sample was kept at −80 °C until analysis.

NMR Experiments

An aliquot of the lipid extract was dissolved in 0.8 ml of deuterated chloroform and the solution put into a 5-mm NMR tube. A Bruker Avance 600 MHz instrument, equipped with a 5-mm BBO probe and a 5-mm quadruple-resonance QXI inverse-detection with xyz gradients, was used to acquire all spectra. The first probe was used to collect 1-D ^{13}C spectra, while 1-D ^{1}H and 2-D maps were acquired with QXI probe. Proton-decoupled, NOE-suppressed ^{13}C-NMR spectra were acquired in order to perform quantitative analysis while in qualitative experiments, in order to help in the assignments, the decoupling pulse was kept on during ^{13}C acquisition, thus allowing an enhanced signal to noise ratio. Both typologies of ^{13}C spectra were acquired using 90 ° carbon pulses of 9 μs, 30 s relaxation time (2 s were used for NOE-enhanced spectra), and 8 K scans. Acquisition time was set to 4 s. Assignments were made based on literature reports and by spiking the sample with the standard compounds. 2-D ^{13}C-^{1}H HSQC ($J = 145$ Hz) and ^{1}H-^{1}H COSY spectra were acquired in order to facilitate and/or confirm assignments.

Quantitative NMR Spectral Analysis

^{13}C-NMR spectra were phased and then baseline corrected by using the software MestRe Nova v.5.2.4 (Mestrelab Research S.L.). Apodization of 0.3 Hz and 600 k points zero filling were used prior to Fourier transformation. The area of each peak of interest was calculated by the integration software, and the sum of the integrated areas was used as a normalization parameter. Regions with extensively overlapping signals were resolved by a

deconvolution procedure, as implemented in the software Origin V.6 (OriginLab): the spectral region under analysis was simulated by a number of Lorentzian functions corresponding to the expected signals; a fitting procedure was then applied letting all the curve parameters free to vary.

HPLC Analysis

For HPLC analysis, an aliquot of the chloroform phase was mildly saponified using a procedure described by Banni et al. [16] in order to hydrolyze the ester bond between glycerol and acyl groups. Lipid extracts were dissolved in 5 ml of ethanol, 100 µl of Desferal (25 mg/ml H_2O), 1 ml of a 25% water solution of ascorbic acid, 0.5 ml of 10 N KOH, left in the dark at room temperature for 14 h. 10 ml of *n*-hexane and 7 ml of H_2O were added and then acidified with 37% HCl, to a pH 3–4. Samples were centrifuged for 1 h at 900×*g*. The hexane phase was collected, the solvent evaporated, and the residue dissolved in 0.5 ml of CH_3CN/0.14% of CH_3COOH (v/v). Aliquots of the samples, containing a mixture of FA, coming from the original FFA and acyl groups, were injected into the HPLC system. Analysis of unsaturated FA in cheese samples was carried out with an Agilent Technologies 1100 liquid chromatograph, using a Chrompack column, Inertsil 5 ODS-2 (150 × 4.6 mm, 5 µm particle size) with a mobile phase of $CH_3CN/H_2O/CH_3COOH$ (70/30/0.12, v/v/v) at a flow rate of 1.5 ml/min. Unsaturated non-conjugated FA were detected at 200.4 nm, conjugated diene FA at 234.4 nm [16, 17]. The presence of caproleic acid in cheese samples was confirmed by HPLC injection of the standard compound dissolved in CH_3CN with 0.14% (v/v) CH_3COOH.

Results and Discussion

The collected ^{1}H- and ^{13}C-NMR spectra of the lipid fraction of PS PDO contained qualitative-quantitative information regarding the molecular components of the mixture. This information was deciphered by the analysis of two kinds of spectral features: the chemical shifts and their relative intensities. The former parameters permitted the identification of the molecular classes of the mixture while the latter allowed their relative quantification. In particular, for quantitative purposes, due to the great number of resonances present, subsequent to a careful assignment, only those peaks that better fulfil the needs of the present work were chosen.

^{1}H-NMR Spectra

A representative ^{1}H-NMR spectrum of the lipid fraction of PS PDO is shown in Fig. 1 and an expansion in Fig. 2. Peak assignments are reported in Table 1. The dominant signals are due to the preponderant presence of TAG, but at a higher vertical scale (Fig. 2) it is possible to detect other

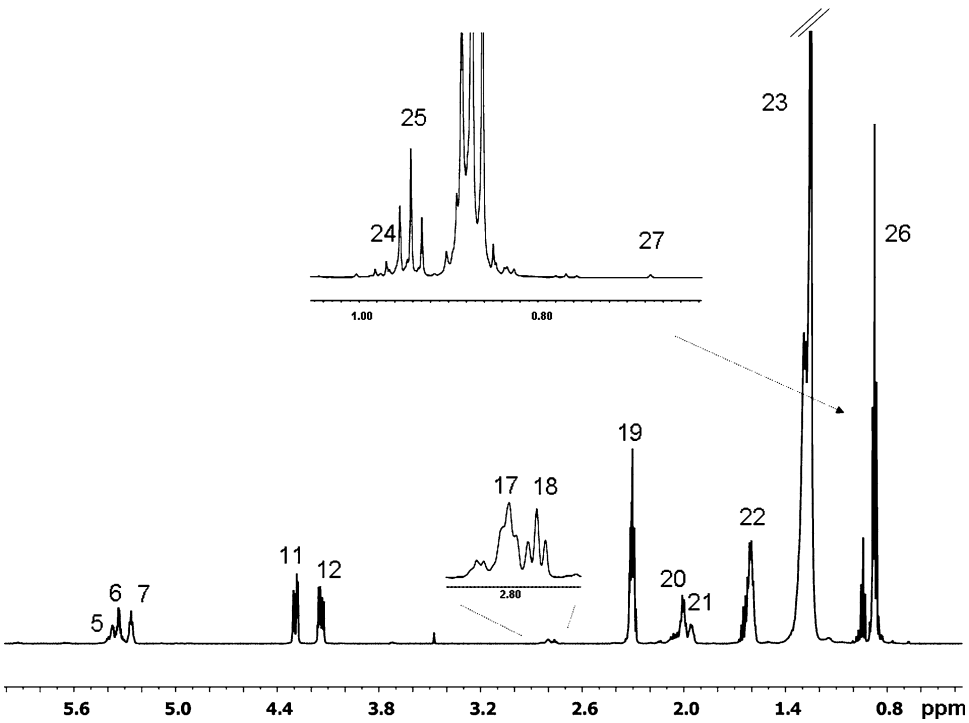

Fig. 1 0.6–6.0 ppm region of a 600 MHz ^{1}H spectrum of PS PDO extracted lipids. Numbering is reported as in Table 1. Vertical expansion is shown for the up field aliphatic region from 0.7 to 1.2 ppm

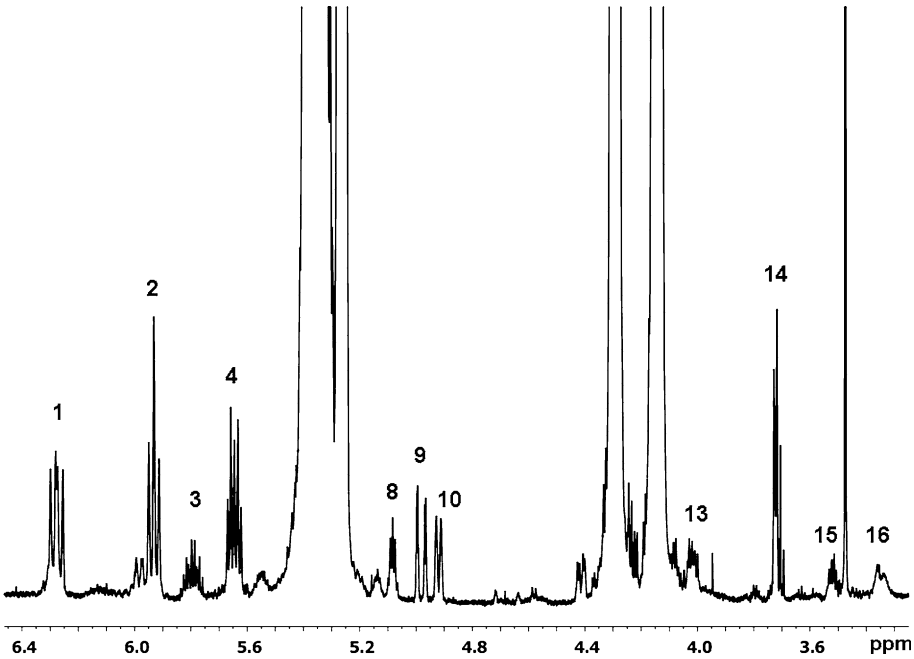

Fig. 2 3.3–6.5 ppm region of a 600 MHz ^{1}H spectrum of PS PDO extracted lipids, resonances with lower intensities are shown. Numbering is reported as in Table 1

Table 1 Peak assignments for ^{1}H-NMR spectrum of lipid extracted from PS PDO dissolved in $CDCl_3$

Peak	Compound	Atom	Functional group	δ (ppm)	Multiplicity[a]	HSQC correlations[b]
1	CLA	H11	–CH=	6.28	dd	125.8
2	CLA	H10	–CH=	5.93	t	128.6
3	Caproleic	H9	–CH=CH_2	5.79	m	138.6
4	CLA	H12	–CH=	5.65	dt	134.3
5	Unsaturated *trans* FA		–CH=CH–	5.37	m	
6	Unsaturated *cis* FA		–CH=CH–	5.33	m	
7	Glycerol in TAG		–CH–OOC–	5.26	m	
8	Glycerol in 1,2 DAG		–CH–OOC–	5.08	m	71.8
9	Caproleic FA	H10b	=CH	4.98	dd	114.0
10	Caproleic FA	H10a	=CH	4.92	dd	114.0
11	Glycerol in TAG		–CH_2–OOC–	4.28	dd	
12	Glycerol in TAG		–CH_2–OOC–	4.15	dd	
13	Glycerol in 1,3 DAG		–CH_2–OOC–	4.03	m	67.8
14	Glycerol in 1,2 DAG		HO–CH_2–CH–	3.71	d	62.0
15	Cholesterol	H1	–CH–OH	3.51	m	
16	Choline in PL		–$N(CH_3)_3$	3.35	m	
17	PUFA (linolenic FA)	H11, H14	=CH–CH_2–CH=	2.80	m	
18	PUFA (linoleic FA)	H11	=CH–CH_2–CH=	2.76	m	
19	All FA	H2	–OOC–CH_2–CH_2–	2.30	t	
20	Unsaturated *cis* FA		–CH_2–CH=CH–	2.01	m	
21	Unsaturated *trans* FA		–CH_2–CH=CH-	1.95	m	
22	All FA	H3	–OOC–CH_2–CH_2–	1.60	m	
23	All FA		–$(CH_2)_n$–	1.25	m	
24	All n-3 FA	ω1	–CH_3	0.97	t	
25	Butyric FA	H4	–CH_3	0.94	t	
26	All FA except n-3 and butyric	ω1	–CH_3	0.87	t	
27	Cholesterol		–CH_3	0.67	s	

[a] *m* multiplet, *d* doublet, *dd* doublet of doublets, *t* triplet

[b] ^{13}C δ (ppm)

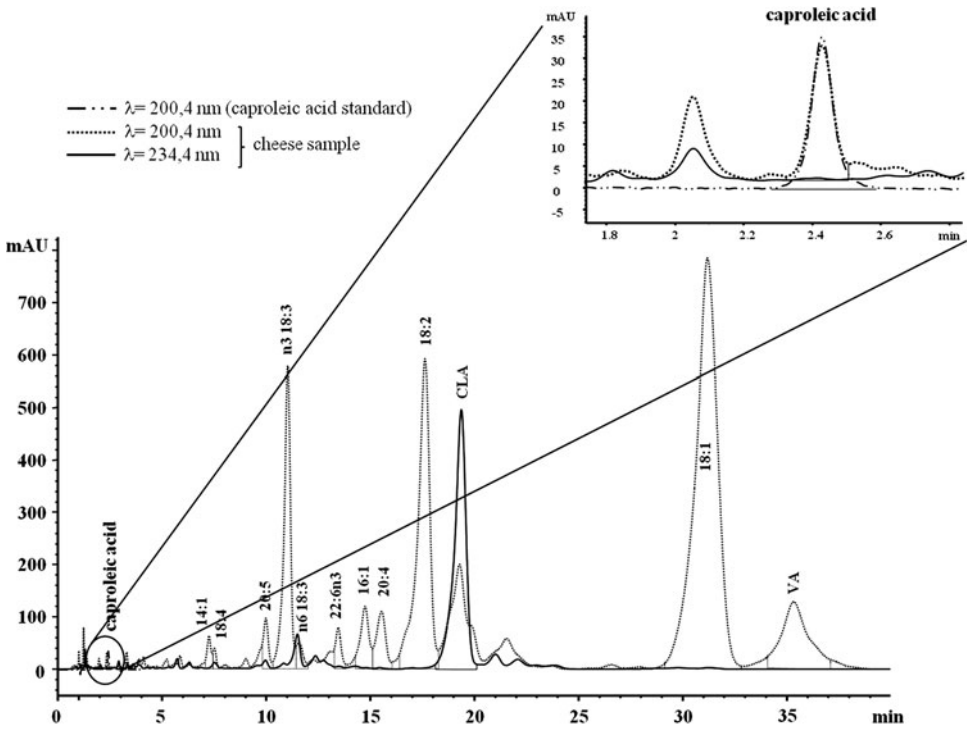

Fig. 3 A typical HPLC chromatogram, recorded at 200.4 nm (*dotted line*) and 234.4 nm (*continued line*) for unsaturated FA and CLA isomers of PS PDO, respectively. The superimposed chromatogram (*line with bars*), recorded at 200.4 nm, represents the caproleic acid standard, equivalent to 1 μg of the fatty acid. In the insert an expanse of the chromatogram is shown. As can be seen, the peak at around 2.4 min of the caproleic acid standard perfectly overlaps one of the cheese FA peak

resonances. Some of these minor peaks belong to protons of glycerol in DAG; in particular, the multiplet at 5.08 ppm (peak 8) and the doublet at 3.71 ppm ($J = 5$ Hz) (peak 14) arise from the –CH and HO–CH_2- functional groups of *sn*-1,2/2,3 isomers, respectively; the *sn*-1,3 DAG isomer gives rise to the multiplet at 4.03 ppm (peak 13). Furthermore, following the literature [18, 19] and by the aid of 2D NMR experiments (COSY and HSQC), peaks 1, 2 and 4 have been assigned to the protons involved in the olefinic bonds of *cis*9, *trans*11-CLA. In particular, the doublet of doublets at 6.28 ppm ($J = 10$ Hz; 15 Hz), and the apparent ^{1}H triplet ($J = 10.8$ Hz) at 5.93 ppm have been assigned to the "inner"-positioned H11 and H10 olefinic protons. The "outer" positioned H12 and H9 atoms of the same CLA isomer resonate at 5.65 ($J = 7$ Hz; 15 Hz) and 5.33 ppm (superimposed in 1D ^{1}H), respectively. Furthermore, peaks 3, 9 and 10 of Fig. 2 were assigned to the terminal vinyl group of caproleic FA (9-decenoic acid), a compound naturally occurring in milk derivatives [20]. In particular, the multiplet at 5.79 ppm (peak 3) was assigned to H9, while the two doublets of doublets at 4.92 ppm ($J =$ 10 Hz; 0.9 Hz) and at 4.98 ppm ($J = 17$ Hz; 0.9 Hz) arise from the H10 atoms in *cis* (H10a) and in *trans* (H10b) vicinal positions with respect to H9 of the terminal vinyl (–CH=CH_2) group. Confirmation of NMR assignments was obtained by spiking the sample with the standard caproleic acid, by performing 1D-selective-TOCSY experiments (see Electronic Supplementary Material) and by the 2D experiments. Since the assignment of this spin system to caproleic FA is in contrast with previous works that ascribed these resonances to primary (peroxides, [11]) and secondary (1-pentene, [14]) products of lipid peroxidation, it needs further discussion: this detrimental process, leading to rancidity, does not usually occur to a significant extent in cheese [8, 9], not even in a 120 day ripened pecorino [21]. In this regard, and in agreement with previous works [22], we assigned some very low-intensity resonances in the low-field region of our ^{1}H-NMR spectra to lipid oxidation products. In particular, the broad doublet at 8.07 ppm was assigned to the –OOH group of hydroperoxides, while the apparent singlet at 9.76 ppm was ascribed to the unresolved triplet due to the aldehydic proton of saturated alkanals (see Electronic Supplementary Material). These signals, however, are almost hidden in the background noise, thus demonstrating that oxidation products in PS PDO cheese reach a very low concentration and their presence can be safely considered to be of minor relevance. In conclusion, due to the much higher intensity of the signals at 4.92, 4.98, and 5.79 ppm, we believe that they should be reasonably ascribed to caproleic FA rather than to primary and/or secondary oxidation products. Moreover, the presence of caproleic acid was also assessed by HPLC analysis. In Fig. 3 the HPLC chromatogram of unsaturated FA, obtained from a sample of PS PDO, is shown with a superimposition of the chromatogram of the standard caproleic acid. As can be seen, the caproleic acid

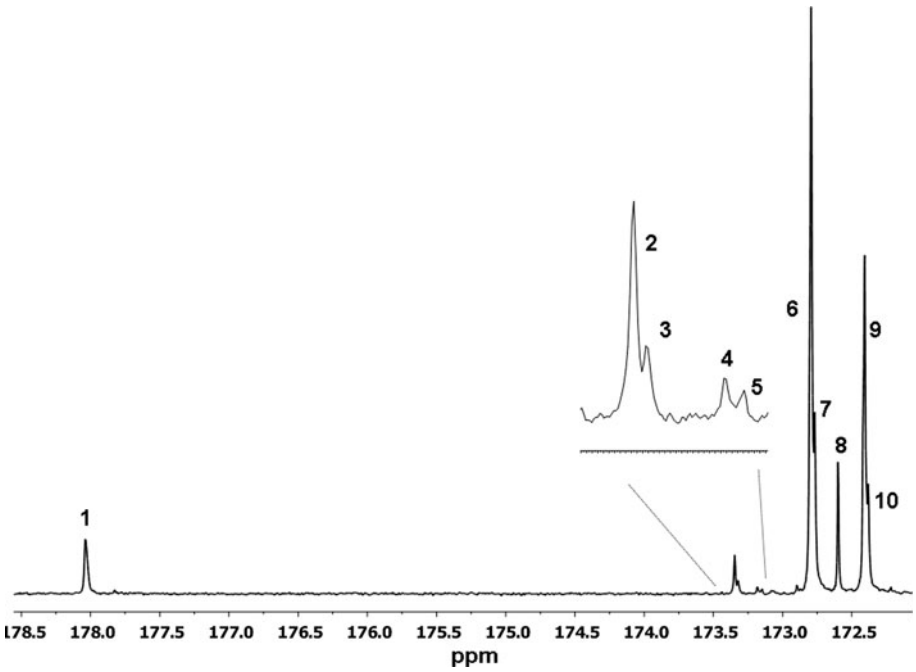

Fig. 4 Carboxylic region of a 600 MHz ^{13}C spectrum of PS PDO extracted lipids. Numbering is reported as in Table 2. Vertical expansion is shown from 173 to 174 ppm

Table 2 Peak assignments for the carboxyl region of ^{13}C-NMR spectrum (Fig. 3) of lipid extracted from PS PDO dissolved in $CDCl_3$

Peak	Compound	Carbon	Functional group	δ (ppm)
1	FFA	C1	OOC–CH_2–	178.04
2	Saturated FA in *sn*-1,3 of DAG	C1	–CH_2–OOC–CH_2–	173.35
3	Unsaturated FA in *sn*-1,3 of DAG	C1	–CH_2–OOC–CH_2–	173.32
4	FA in *sn*-1 (*sn*-3) of 1,2 (2,3) DAG	C1	–CH_2–OOC–CH_2–	173.18
5	FA in *sn*-2 of 1,2 (2,3) DAG	C1	–CH–OOC–CH_2–	173.08
6	Saturated FA in *sn*-1,3 of TAG	C1	–CH_2–OOC–CH_2–	172.79
7	Unsaturated FA in *sn*-1,3 of TAG	C1	–CH_2–OOC–CH_2–	172.70
8	Butyric FA in *sn*-1,3 of TAG	C1	–CH_2–OOC–CH_2–	172.60
9	Saturated FA in *sn*-2 of TAG	C1	–CH–OOC–CH_2–	172.41
10	Unsaturated FA in *sn*-2 of TAG	C1	–CH–OOC–CH_2–	172.38

standard peak overlaps perfectly with one of the peaks present in the cheese FA.

^{13}C-NMR Spectra

The ^{13}C-NMR spectrum of lipid extract of PS PDO was divided into the following four spectral regions.

1. Carboxylic region

Assignments of resonances in this spectral region, shown in Fig. 4, are reported in Table 2. The following attributions were performed: carbonyl carbons of long chain saturated and unsaturated acyl groups in *sn*-1,3 TAG give rise to the cluster at 172.79–172.70 (peaks 6 and 7), while those in *sn*-2 TAG give rise to the cluster at 172.41–172.38 ppm (peaks 9 and 10), respectively [23]. Peak 8, at 172.60 ppm, belongs to butyrate in *sn*-1,3 position of TAG and the lack of resonance of butyrate in *sn*-2, which is expected to be found at 172.22 ppm, confirms its location solely in the external positions of TAG [9]. Analogously to TAG, saturated and unsaturated acyl groups in *sn*-1,3 DAG give rise to the cluster at 173.35–173.32 ppm (peaks 2 and 3), respectively. Acyl chains in the external position in *sn*-1,2/-2,3 DAG resonate at 173.18 (peak 4), while those in *sn*-2 are found at 173.08 ppm (peak 5). At lower fields, the resonance at 177.30 ppm (peak 1) was ascribed to FFA. Integration of this peak for quantifying the degree of hydrolysis of TAG is not advisable since, especially at the initial stage of ripening, the first FA to be hydrolyzed are the short chain FA, i.e. butyric and caproic, due to their *sn-3* position in TAG. Because of the physical–chemical properties of these short chain FA, they

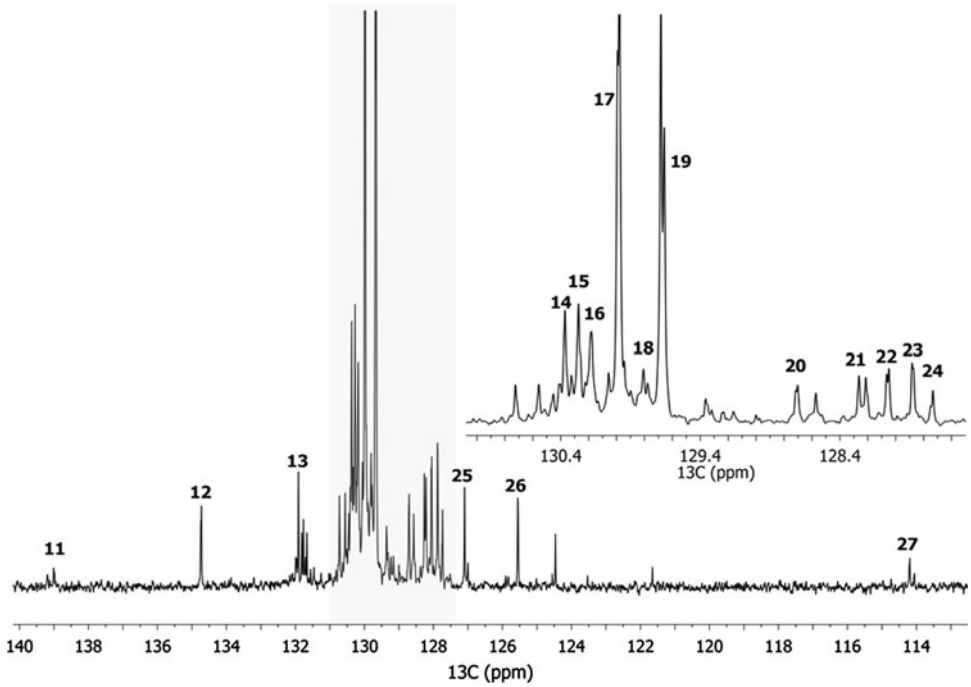

Fig. 5 Olefinic region of a 600 MHz ^{13}C spectrum of PS PDO extracted lipids. Numbering is reported as in Table 3. Vertical expansion (*gray shaded*) is shown from 127 to 130 ppm

Table 3 Peak assignments for the olefinic region of ^{13}C-NMR spectrum (Fig. 4) of lipid extracted from PS PDO dissolved in $CDCl_3$

Peak	Compound	Carbon	Functional group	δ (ppm)	HSQC correlation[a]
11	Caproleic FA	C9	–CH=CH_2	138.70	5.74
12	CLA	C12	–CH=CH	134.45	5.58
13	All n-3 FA	ω3	–CH=CH	131.66	
14	VA FA	C12	–CH=CH	130.09–130.08	
15	VA FA	C11	–CH=CH	130.00–129.98	
16	Linoleic + linolenic FA	C13 + C9	–CH=CH	129.89	
17	MUFA	C10	–CH=CH	129.70–129.69	
18	Linoleic FA	C9	–CH=CH	129.51–129.49	
19	MUFA	C9	–CH=CH	129.39–129.37	
20	CLA	C10	–CH=CH	128.42	5.88
21	Linolenic FA	C13–C12	–CH=CH	127.97–127.92	
22	Linoleic FA	C10	–CH=CH	127.77–127.76	
23	Linoleic FA	C12	–CH=CH	127.59–127.58	
24	Linolenic FA	C10	–CH=CH	127.46–127.44	
25	All n-3 FA	ω4	–CH=CH	126.77	
26	CLA	C11	–CH=CH	125.26	6.28
27	Caproleic FA	C10	–CH=CH_2	114.05	4.93, 4.87

[a] ^1H δ (ppm)

can be found dispersed, during the extraction procedure, either in the aqueous phase or in the headspace.

2. Olefinic region

As shown in Fig. 5, this spectral region is rich in resonances and can give a picture of the variety of unsaturated FA which constitute the lipid extract of PS PDO. Assignments of resonances to oleic, linoleic, linolenic, VA, either FFA or acyl groups, are reported in Table 3.

In order to confirm assignments in this spectral area to the olefinic carbons of caproleic acid and CLA, 2D ^{13}C-^1H-HSQC NMR, experiments were carried out. In particular the HSQC 2D spectrum, reported in Fig. 6, shows a clear connectivity between proton signals at 4.98 and 4.92 ppm of H10 of caproleic acid and the ^{13}C signal at 114.1 ppm. Moreover, the ^1H signal at 5.79 ppm, due to H9 of caproleic acid, correlates with the carbon at 138.7 ppm. As far as CLA is concerned, the following

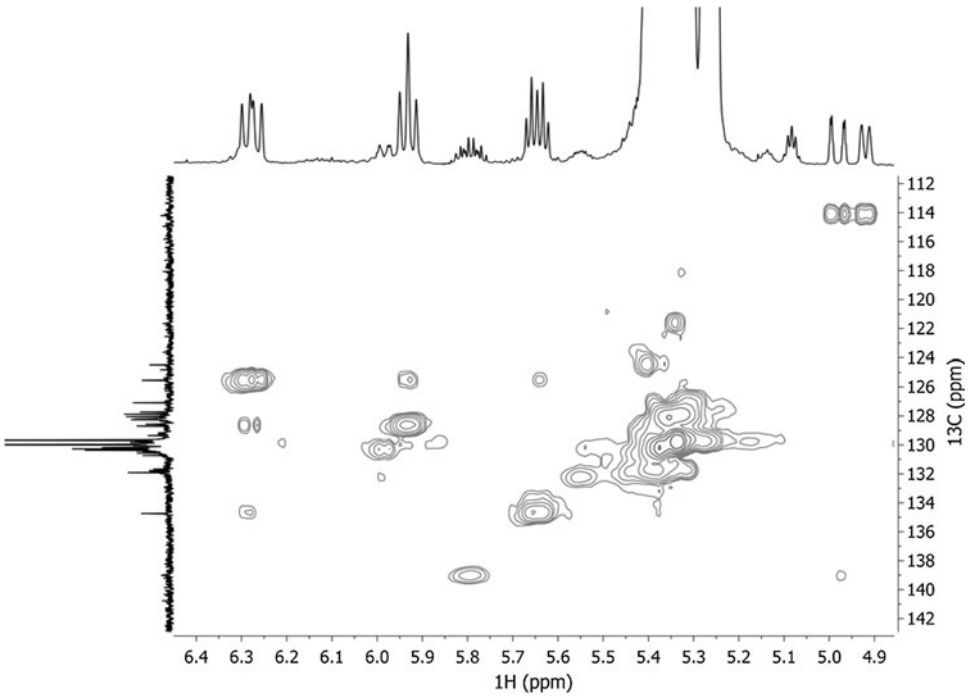

Fig. 6 Expanded (4.9–6.4 and 112–142 ppm) spectral region of a 600 MHz ^{1}H–^{13}C-HSQC spectrum of PS PDO extracted lipids

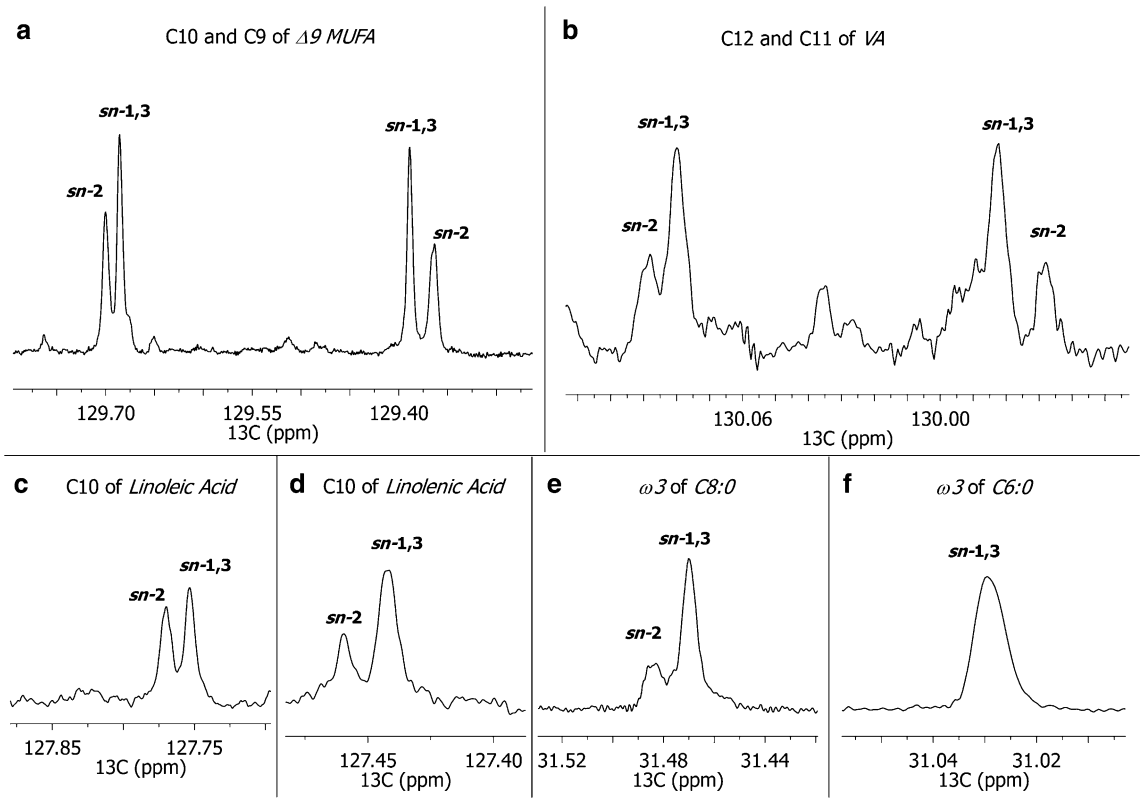

Fig. 7 Expanded olefinic **a**, **b**, **c**, **d** and aliphatic **e**, **f** spectral regions of a 600 MHz ^{13}C spectrum of PS PDO extracted lipids. The intensities were adjusted in order to show the different distribution of the acyl groups between *sn*-1,3 and *sn*-2 positions in TAG

assignments were made from the analysis of Fig. 6: the H11 (dd at 6.28 ppm) correlates with the carbon at 125.3 ppm that was therefore assigned to C11 of (*cis*9, *trans*11–18:2) CLA. Similarly, the triplet at 5.93 ppm (H10) correlates in the HSQC spectrum with the carbon at 128.4 ppm (C10). Protons at 5.65 (H12) and 5.34 ppm

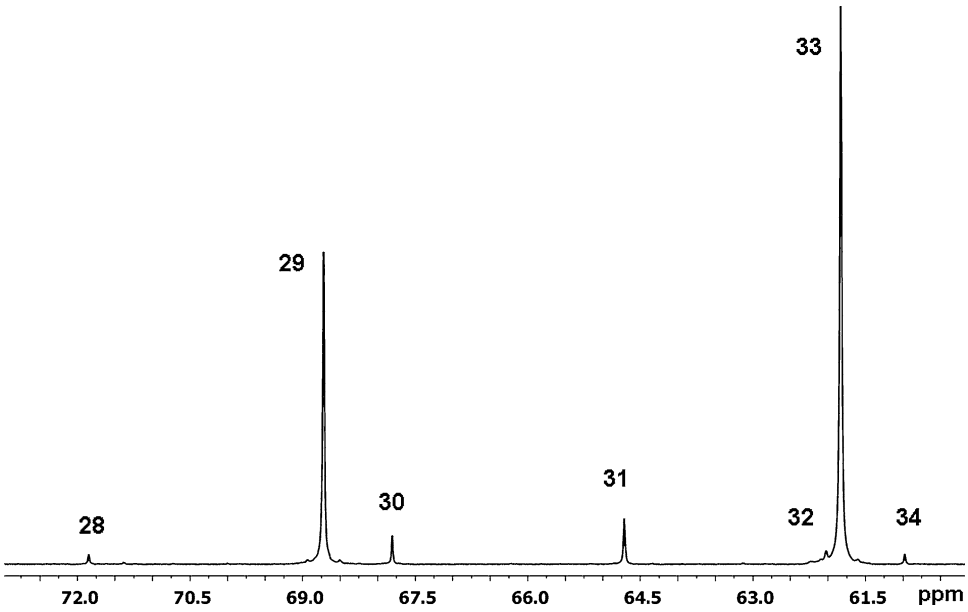

Fig. 8 Glycerol region of a 600 MHz ^{13}C spectrum of PS PDO extracted lipids. Numbering is reported as in Table 4

(H9) correlating with carbons at 134.4 and 129.6 ppm respectively, were assigned to the "outer" positioned carbons (C12 and C9, respectively) of the same CLA isomer. It should be pointed out that, due to the presence of very intense resonances arising from the olefinic carbons of oleic and VA FA, the region between 129.3 and 130.1 ppm appears quite crowded in all the spectra and especially in HSQC, which, in this region, shows a single, poorly- resolved, broad cross peak.

Positional isomery of unsaturated acyl groups in TAG can be inferred from the analysis of this spectral region. In fact, each olefinic carbon give rise to two distinct peaks, relative to the acyl chains in *sn*-1,3 and in *sn*-2 positions of the glycerol backbone. The chemical shift differences (Δ ppm) between these resonances become smaller as they go further from the carbonyl group [23]. As shown in Fig. 7, this behaviour is clearly visible for C9 and C10 of oleate (Fig. 7a), C11 and C12 of VA (Fig. 7b), C10 for linoleate (Fig. 7c), and linolenate (Fig. 7d).

Finally, other small peaks are observable in Fig. 5 but we were unable to assign them. It is worth pointing out that a diet based on natural pasture, as is that of Sardinian sheep, increases the variety and quantity of polyunsaturated FA and their metabolites [17, 24] in the milk fat. This can explain the number of unidentified signals detected in the olefinic region of our spectra.

3. Glycerol region

In this spectral region (Fig. 8) we found the aliphatic groups of glycerol. Assignments are reported in Table 4. Besides the major resonances ascribed to glycerol in TAG (peaks 29 and 33), other minor resonances appear. These, with the aid of the literature and by inspection of HSQC spectra were assigned as follows: peaks 30 and 31 at 67.81 (correlated in 2D maps to the ^{1}H multiplet at 4.03 ppm) and 64.75 ppm arise from the $-CH_2$ and –CH carbons of the glycerol backbone of *sn*-1,3 DAG, peaks 28 and 34 at 71.85 (correlated to the ^{1}H-NMR signal at 5.08 ppm) and 60.83 ppm to glycerol carbon atoms of *sn*-1,2/-2,3 DAG.

By quantitative analysis of this spectral region we found that *sn*-1,3 DAG and *sn*-1,2/2,3 DAG represented the 1.0 ± 0.3 and the 0.6 ± 0.2 mol% of the total acylglycerols, respectively. The *sn*-1,2 DAG are the first products to be formed in cheese, as a consequence of the action of lipolytic enzymes on TAG [9], while *sn*-1,3 DAG can be formed in a second instance [1, 9] also through acyl migration mechanism [10]. Therefore, the presence of *sn*-1,2 DAG in PS PDO can be seen as an index of recent lipolytic activity.

4. Aliphatic region

This spectral region is shown in Fig. 9, and peak assignments are reported in Table 5. Length of saturated FA and acyl chains, the proximity of the last double bond of unsaturated to the methyl end, that results in n-3, n-6, or n-9 classification of FA, strongly affect the chemical shifts, in particular those of the $\omega 3$ carbons (carbon number 3 starting from the methyl end); as a result, this spectral region is often carefully analysed for quantitative purpose. In addition to the attributions of the $\omega 3$ carbons reported in previous works on milk lipids [12, 13], we also report assignments of CLA and VA, both n-7 *trans* FA, at 31.57 and 31.56 ppm (peaks 45 and 46), respectively. The presence of the latter two FA was also confirmed by an inspection of the region between 32–33 ppm, where the allylic carbons next to the *trans* bonds resonate: peak 38 at

Table 4 Peak assignments for the glycerol region of ^{13}C-NMR spectrum (Fig. 7) of lipid extracted from PS PDO dissolved in $CDCl_3$

Peak	Compound	Functional group	δ (ppm)	HSQC correlations[a]
28	Glycerol in 1,2/2,3 DAG	–CH–OOC–	71.85	5.04
29	Glycerol in TAG	–CH–OOC–	68.72	
30	Glycerol in 1,3 DAG	HO–CH–$(CH_2)_2$	67.81	4.03
31	Glycerol in 1,3 DAG	–CH_2–OOC–	64.75	
32	Glycerol in 1,2/2,3 DAG	HO–CH_2–CH–	62.02	3.67
33	Glycerol in TAG	–CH_2–OOC–	61.83	
34	Glycerol in 1,2 DAG	–CH_2–OOC–	60.83	

[a] ^{1}H δ (ppm)

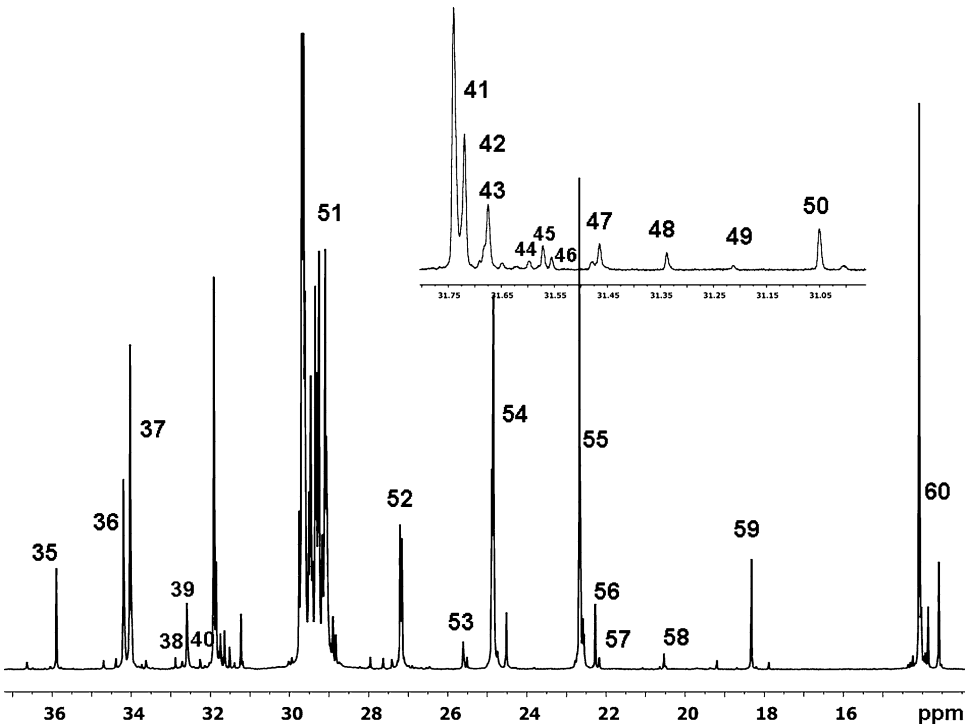

Fig. 9 Aliphatic region of a 600 MHz ^{13}C spectrum of PS PDO extracted lipids. Numbering is reported as in Table 5. Vertical expansion is shown from 31.0 to 31.8 ppm

32.68 ppm for C–13 of CLA and peak 39 at 32.35–40 ppm for C10 and C13 of VA. Assignments of the remaining aliphatic resonances are reported in Table 5. Furthermore, positional isomery in TAG of caprylic (C8:0) and C6:0 acyl groups can be determined by the analysis of peaks 47 and 50, respectively, as reported in Fig. 7e and f. The lack of resonance at 30.98 ppm of the ω3 carbon atom of C6:0 in *sn*-2 of TAG is in agreement with previous observations that report the presence of this acyl group only in the external positions [1].

As an example, a quantitative analysis of the FA (mol%) composition of PS PDO is reported in Table 6. Due to the restricted number of samples under investigation we cannot discern whether the observed quantitative differences, actually very small, are imputable to the slightly different cheese-making processes as applied by the diverse producers rather than to different milk composition and/or individual variability. However, the data are in an overall good agreement with those reported in a previous work on cheese from Sarda dairy sheep, fed on Mediterranean pastures [25].

In conclusion, the ^{1}H- and ^{13}C-NMR spectra of PS PDO lipid fraction contain very useful information on the lipid components of cheese and their transformation during ripening. In particular, NMR gives a comprehensive and quantitative description of the lipid composition of PS PDO cheese and provides a clear picture of the molecular effects of seasoning through rapid and non-destructive means. Some of the advantages of NMR over other classical standard analytical methods are its ability to give information, in a single experiment, on lipid classes and on the positional distribution of acyl groups in TAG.

In this work, the extent of hydrolysis on TAG was successfully measured, and the presence of 1,2 DAG

Table 5 Peak assignments for the aliphatic region of ^{13}C-NMR spectrum (Fig. 8) of lipid extracted from PS PDO dissolved in $CDCl_3$

Peak	Compound	Carbon	Functional group	δ (ppm)[a]
35	Butyric FA	C2	–OOC–$\underline{C}H_2$–CH_2–	35.62
36	All FA except butyric in *sn*-2 of TAG	C2	–OOC–$\underline{C}H_2$–CH_2–	33.92
37	All FA except butyric in *sn*-1,3 of TAG	C2	–OOC–$\underline{C}H_2$–CH_2–	33.76
38	CLA	C13	–$\underline{C}H_2$–CH = CH–	32.68
39	VA FA	C10, C13	–$\underline{C}H_2$–CH = CH–	32.40–32.35
40	*trans* MUFA		–$\underline{C}H_2$–CH = CH–	32.07–32.06
41	Saturated C > 10 FA	ω3[b]	–$\underline{C}H_2$–CH_2–CH_3	31.74
42	Monounsaturated n-9 FA	ω3	–$\underline{C}H_2$–CH_2–CH_3	31.72
43	Capric FA C10:0	ω3	–$\underline{C}H_2$–CH_2–CH_3	31.67
44	Palmitoleic[c] FA	ω3	–$\underline{C}H_2$–CH_2–CH_3	31.59
45	VA[d] FA	ω3	–$\underline{C}H_2$–CH_2–CH_3	31.57
46	CLA[d]	ω3	–$\underline{C}H_2$–CH_2–CH_3	31.56
47	Caprylic FA C8:0	ω3	–$\underline{C}H_2$–CH_2–CH_3	31.47
48	Linoleic[e] FA	ω3	–$\underline{C}H_2$–CH_2–CH_3	31.32
49	n-6 *trans*	ω3	–$\underline{C}H_2$–CH_2–CH_3	31.20
50	Caproic FA C6:0	ω3	–$\underline{C}H_2$–CH_2–CH_3	31.03
51	All FA		–$(CH_2)_n$–	29.56–28.73
52	Unsaturated FA		–$\underline{C}H_2$–CH = CH–	26.91–26.88
53	PUFA		–CH = CH–$\underline{C}H_2$–CH = CH–	25.34
54	All FA except butyric		–OOC–CH_2–$\underline{C}H_2$–	24.58
55	All FA except caproic, caprylic, and n-3	ω2	–$\underline{C}H_2$–CH_3	22.48
56	Caprylic FA C8:0	ω2	–$\underline{C}H_2$–CH_3	22.40
57	Caproic FA C6:0	ω2	–$\underline{C}H_2$–CH_3	22.08
58	n-3	ω2	–$\underline{C}H_2$–CH_3	20.13
59	Butyric FA	C3	–$\underline{C}H_2$–CH_3	18.10
60	All FA	ω1	–CH_3	13.85–13.79–13.62–13.33

[a] ^{1}H δ (ppm)

[b] Carbon atom number three from the methyl end

[c] n-7 *cis* FA

[d] n-7 *trans* FA

[e] n-6 *cis* FA

Table 6 Fatty acid composition (mol%) of lipids in PS PDO

Fatty acids	Mean[a]	SD	Peak[b]	δ (ppm)[c]
Butyric	9.55	0.48	35	35.62
Caproic	5.21	0.45	50	31.03
Caprylic	4.82	0.26	47	31.47
Capric	11.83	0.73	43	31.67
n-9	18.59	0.82	42	31.72
n-7 *cis*	1.25	0.37	44	31.59
CLA	1.55	0.24	45	31.56
VA	3.19	0.31	46	31.57
n-6 *cis*	1.45	0.84	48	31.32
n-6 *trans*	0.81	0.58	49	31.20
n-3	1.62	0.44	58	20.13
Saturated long chain (C > 12)	40.14	2.21	42	31.74

[a] Means and standard deviations (SD) over six samples

[b] Number of the peaks (Table 5) used for quantitative purpose

[c] Their ^{13}C chemical shift

suggests that lipolytic processes are still active in a 120-day seasoned cheese, such as mature PS PDO. The very low intensity of NMR resonances from primary and secondary products of lipid oxidation indicated that FA of PS PDO do not easily undergo auto-oxidation. This finding has clear, direct consequences on the nutritional value of this product, and on whatever concerns the practices of seasoning, storage, and shelf life. Novel NMR spectral assignments to *trans* FA, namely VA and CLA, of which sheep milk and cheese are particularly rich, are reported, and their relative presence was quantified. Furthermore, the positional isomery of acyl groups in TAG, which is of great importance in human nutrition and flavor development, was also assessed. Lastly, some attributions reported in previous works on milk and cheese lipid fractions [11, 14] were discussed and revised. The detailed spectral assignments here reported can serve as an NMR data bank for further studies on the lipid fractions of dairy products.

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