



Assessment of milk fat content in fat blends by ^{13}C NMR spectroscopy analysis of butyrate

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ABSTRACT

Butyric acid (butyrate) is a candidate marker of milk fat in complex fat blends, since it is exclusive of milk triacylglycerols (TAGs) from different ruminant species. In this work, we determined the amount of milk fat used for the preparation of fat blends by ^{13}C Nuclear Magnetic Resonance (^{13}C NMR) spectroscopy-based quantification of butyrate. When tested on fat samples spiked with known amounts of reference bovine milk fat (BCR-519 certified material), the relative composition of the mixtures was reliably assessed through the integration of the diagnostic ^{13}C NMR carbonyl (C1) or α -carbonyl methylene (C2) resonances of butyrate. NMR data exhibited strict correlation with high resolution-gas chromatography (GC) of fatty acid methyl esters ($R^2 = 0.99$), which was used as an independent and well-established method for the determination of butyrate. Thus, ^{13}C NMR can be used for the direct assessment of milk fat content in fat mixtures, at a limit of detection lower than 5%, with clear advantages over the traditional GC methods in terms of speed, robustness and minimal sample handling. The natural variability of butyrate in milk has been taken into account to estimate the uncertainty associated with the milk fat content in unknown fat blends.

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1. Introduction

According to the current European legislation (EC Reg., 1994; 2007), the milk fat content in commercial fat mixtures must be clearly indicated on the label. Since milk fat has the highest price among fats, there is a realistic possibility that milk fat could be partly or totally replaced with other fats, such as lard, beef tallow or vegetable oils, to obtain industrial blends.

The International Dairy Federation (IDF) developed an accurate and reliable method to determine the composition of mixed

spreads based on the fatty acid profile of milk and non-milk fat ingredients (Muuse & Martens, 1993). The Official EU method of analysis for the authenticity assessment of butter (EC Reg., 2001) is based on the gas-liquid chromatography (GC) evaluation of triacylglycerols (TAGs) (Precht, 1992). Several other strategies or methods have been proposed to assess the content of milk fat in blended fats. Details of these methods and related drawbacks have been recently reviewed by Derewiaka, Sosińska, Obiedziński, Krogulec, and Czaplicki (2011).

Butterfat TAGs can be effectively separated, characterized and quantified by capillary GC coupled to electron ionization (EI) mass spectrometry (MS) (Kemppinen & Kalo, 2006). Foreign fats, such as for instance tallow, have been quantified by fatty acid analysis coupled to multivariate statistical techniques (Ulbert, 1995). Yoshinaga, Nagai, Mizobe, Kojima, and Gotoh (2013) applied liquid chromatography coupled with atmospheric-pressure chemical ionization tandem mass spectrometry (LC-APCI-MS/MS) to quantify milk fat in blends and butter biscuits, using 1,2-dipalmitoyl-3-butyrolyl-glycerol as a specific analytical target. Indeed, milk fat

Abbreviations: IDF, International Dairy Federation; GC, High Resolution Gas-liquid Chromatography; TAGs, Triacylglycerols; EI, Electron Ionization; MS, Mass Spectrometry; LC-APCI-MS/MS, Liquid Chromatography-Atmospheric Pressure Chemical Ionization-Mass Spectrometry; BA, Butyric Acid; NMR, Nuclear Magnetic Resonance; FAMES, Fatty Acid Methyl Esters; NOEs, Nuclear Overhauser Effects; BB, Broad Band; TMS, tetramethylsilane.

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contains major amounts of short-chain fatty acids, biosynthesized from acetic (as acetyl-CoA) and β -hydroxy-butyric acids derived from rumen fermentation. In particular, butyric (or butanoic) acid (BA) exclusively occurs in milk fat TAGs of ruminants (Alais, 2000). Thus, BA can be monitored as a robust marker of milk fat content in fat mixtures (Glaeser, 2002; Molkentin & Precht, 2000; Pocklington & Hautfenne, 1986) as well as in a variety of foodstuff, such as chocolate, cakes, pastries, ice-creams (Molkentin & Precht, 1997; Ulberth, 1998).

The analytical determination of BA generally relies on GC analysis of fatty acid methyl esters (Molkentin & Precht, 2000). For instance, BA has been specifically targeted to detect milk fat in cocoa butter and chocolate fats (Buchgraber, Androni, & Anklam, 2007).

Based on the stereospecific esterification of BA on the *sn*-3 position of glycerol backbone in milk fat TAGs, the study of BA *sn*-regioisomerism using Nuclear Magnetic Resonance (NMR) spectroscopy also enables the detection of low percentages of synthetic or inter-esterified TAGs in authentic butterfat (Picariello et al., 2013).

In the last decades, NMR has been recognized as a very powerful tool for food analysis (Alberti, Belton, & Gil, 2002). NMR boasts several advantages over other techniques, including its non-destructive nature, the possibility to preserve food structure, the high informative level obtainable from complex food systems, minimal sample handling required, speed of analysis and good reproducibility (Alberti et al., 2002; Sacchi & Paolillo, 2007).

Milk fat lipids can be distinguished by other hard fats, such as lard, using ^1H or ^{13}C NMR, the latter maximizing the resolution, because of the wider range of chemical shifts (Fadzillah et al., 2017). The ^{13}C NMR analysis of glycerolipids, which normally occur in relatively high amounts in foodstuff, provides a reliable quantitative determination of butter milk TAG fatty acids (Belloque & Ramos, 1999). In contrast, Gouk, Cheng, Hock Ong, and Hock Chuan (2012) reported some discrepancy about the positional fatty acid composition obtained through NMR analysis and conventional enzymatic or chemical methods, in particular leading to overestimation of the saturated fatty acids content at *sn*-1 or *sn*-3 positions with respect to *sn*-2 position. ^1H and ^{13}C NMR spectroscopy has been widely applied to the direct analysis of different oils and fats, including olive oil (Brescia & Sacco, 2008; Sacchi et al., 1992, 1997). Recently, NMR spectroscopy has been successfully applied directly to milk analysis without any pretreatment step (Hu, Furihata, Ito-Ishida, Kaminogawa, & Tanokura, 2004; Sundekilde, Larsen, & Bertram, 2013). Several organic compounds in whole milk were also simultaneously quantified by one- (^1H , ^{13}C) and two-dimensional NMR spectroscopy (Hu, Furihata, Kato, & Tanokura, 2007).

The well-resolved ^{13}C NMR resonances of C1 (carbonyl carbon) of BA with respect to C1 of long-chain fatty acids (Pfeffer, Sampugna, Schwartz, & Shoolery, 1977; Andreotti, Trivellone, Lamanna, Luccia, & A. Motta, 2000, 2002), were also suggested as a diagnostic probe to discriminate genuine butter from mixtures with synthetic TAGs with many advantages related to the robustness and minimal sample handling (Picariello et al., 2013). However, the quantification of milk fat in unknown fat blends based on BA determination can be biased by the natural fluctuation of fatty acids in milk TAGs, depending on a variety of biotic and abiotic factors.

In this work, we aimed at developing a direct and reliable NMR method to assess milk fat in fat blends. Therefore, butyrate as a marker of milk fat was monitored by ^{13}C NMR in blends containing butter along with lard and vegetable margarine, which simulate commercial “mixed fats” and other spreads used as cheaper butter surrogates. To this purpose, we compared the direct NMR

determination of butyrate in fat blends containing known amount of milk fat to the capillary GC analysis of trans-methylated fatty acids, chosen as a robust and well-established control analytical method.

2. Material and methods

2.1. Standards and reagents

Chloroform-*d* (with 0.03% v/v internal tetramethylsilane, TMS) was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). HPLC-grade solvents and all other chemicals of the highest purity available were purchased from Fluka (Buchs, Switzerland).

2.2. Samples

Lard and margarine (fractionated and inter-esterified vegetable fats) samples used for the experiments were purchased on the local market. Anhydrous bovine milk fat (BCR-519 certified material) was from the Institute for Reference Materials and Measurements (Geel, Belgium). Prior to blending, fat samples were melted at 40 °C for 1 h under N_2 to prevent possible auto-oxidation and homogenized. Melted lard and margarine were dehydrated using sodium sulfate. Fat blends were prepared by spiking a 1:1 (w/w) mixture of melted lard and margarine with BCR-519 milk fat at 5, 10, 25 and 50% (w/w) relative amount. Although butyrate can be even detected at 1% (w/w) and quantified at 2.5% (w/w) of butterfat in complex fats (Picariello et al., 2013, see also herein below), blends containing less than 5% (w/w) of milk fat were not investigated, since they are scarcely relevant under a commercial standpoint.

2.3. Capillary gas-liquid chromatography (GC)

GC analysis of fatty acid methyl esters (FAMES) was carried out by cold trans-methylation in KOH/methanol (Christie, 2003; Ichiyama 1996). Fat aliquots (100 mg to the nearest 0.1 mg) were dissolved in 10 mL n-hexane, mixed with 0.5 mL of 2 N KOH in methanol and shaken vigorously for 1 min using a vortex mixer. The resulting solution was centrifuged for 1 min. The clear supernatant was used for GC analysis. FAME analysis was performed by using a Shimadzu GLC17A gas chromatography (Shimadzu Italia, Milan, Italy) equipped with split/splitless injection port, flame ionization detector and a 60 m fused-silica capillary column (Quadrex Corporation, New Heaven, U.S.A.) coated with cyanopropyl methyl silicone (0.25 μm film thickness). Samples (1 μL) were introduced using a Shimadzu AOC-20i automatic injector (Shimadzu Italia). The temperature of both the injector and detector was 250 °C. The initial oven temperature was set at 70 °C for 4 min. The temperature rate was set on 10 °C min^{-1} up to 170 °C for 10 min, and an increase at a rate of 10 °C min^{-1} was followed to finally reach a temperature of 220 °C that was maintained for 10 min. Helium was the carrier gas at a 1.8 mL min^{-1} flow rate. The split ratio was 1:60. The GC method was calibrated using a mixture of 37 FAME standards fatty acids (Sigma), C4–C24, and the calibration for butyrate had $R^2 = 0.992$. Samples were analyzed in triplicate and results were averaged.

2.4. High resolution ^{13}C NMR spectroscopy

For the ^{13}C NMR analysis, 50 mg of fat samples were dissolved in 0.5 mL of chloroform-*d* containing 0.03% (v/v) TMS and transferred in a 5 mm i.d. NMR-tube. The ^{13}C NMR spectra were recorded on a Bruker AV 400 spectrometer (Bruker, Karlsruhe, Germany) operating at a ^{13}C -frequency of 100.62 MHz. Full spectra were recorded with 32 K data points, spectral width 200 ppm, pulse width 45°,

acquisition time 0.81 s, relaxation delay 4 s (with a digital resolution of 2 Hz/pt), 128–3000 scans up to an S/N ratio > 5 for the lowest BA signal (C4) (Sacchi, Addeo, & Spagna Musso, 1995). The experimental time was 20–120 min in relation to the milk fat amount in the samples. High resolution carbonyl spectra were recorded at 30 ± 1 °C with 32 K data points, spectral width 10 ppm, pulse width 90°, acquisition time 23.2 s and relaxation delay 2 s. The resulting digital resolution was 0.04 Hz/pt. Nuclear Overhauser Effects (NOEs) were measured only for those signals relevant in the quantitative analysis by comparing spectra recorded in Broad Band (BB) decoupling mode (with NOE enhancement) with those recorded by using the inverse gated decoupling sequence (with NOE suppression). Inverse gated spectra were recorded with the same acquisition parameters and with the same number of scans used in the BB experiments, with an additional delay of 20 s. Carbon-13 spin-lattice relaxation times (T1) were measured using the inversion-recovery (180- τ -90) pulse sequence. Spectra recorded at different delays between pulses were transformed in absolute intensity mode without any phase correction. In order to obtain the T1 values, peak intensities were fitted to an exponential curve via a three-parameter minimization (Jacobsen, 2007). Chemical shift values were referenced to internal TMS and assigned by comparison with standard compounds and literature data (Andreotti, Lamanna, Trivellone, & Motta, 2002, 2000; Pfeffer et al., 1977; Van Calsteren, Barr, Angers, & Arul, 1996).

2.5. Quantitative spectral analysis

NMR signals were fitted to a sum of Lorentzian curves using a nonlinear least-squares algorithm and intensity of peaks was quantified using the Linesim (Bruker) software. The relative concentration of BA was calculated as detailed in section 3.1. ^{13}C NMR spectra were acquired in triplicate and signal integration values were averaged. The ^{13}C -NMR-based limit of detection (LOD) and limit of quantification (LOQ) of butterfat in complex fats were 1% (w/w) and 2.5% (w/w), respectively. LOD and LOQ were determined as previously detailed (Picariello et al., 2013).

3. Results and discussion

3.1. NMR analysis

The ^{13}C NMR spectral regions relevant to carbonyls (spectra acquired in high resolution mode) and methylene/methyl groups (spectra acquired in low resolution mode) of milk fat are shown in Fig. 1. The carbon signals of the butyryl backbone were completely resolved and ^{13}C NMR chemical shifts were assigned as following: C1 173.13 ppm, C2 35.94 ppm, C3 18.37 ppm and C4 13.63 ppm. As expected, no butyrate was detected in lard and margarine NMR spectra (data not shown). Although butyrate exclusively occurs at the *sn*-3 positions of the glycerol backbone of milk TAGs (Alais, 2000; Pfeffer et al., 1977), the *sn*-1,3 positions are undistinguishable using the ordinary NMR methods; for this reason, thereafter butyrate is indicated as esterified at the *sn*-1,3 glycerol positions (Fig. 1). The C1 and C2 resonances of butyrate could be alternatively selected as suitable diagnostic indicators of milk TAGs because they: i) can be easily referred to the C1 and C2 resonances corresponding to the acyl chains of fatty acids other than BA, ii) are resolved enough from interfering signals, and iii) provide an additional information about the *sn*-regioisomerism of BA in TAGs, allowing the discrimination between authentic butterfat and possible trans-esterified fats (Picariello et al., 2013). Therefore, the signals of C1 or C2 *sn*-1,3-butyryl can be integrated for the relative determination of BA content, which can be expressed as either molar fraction percentage of total fatty acid chains or referred to the

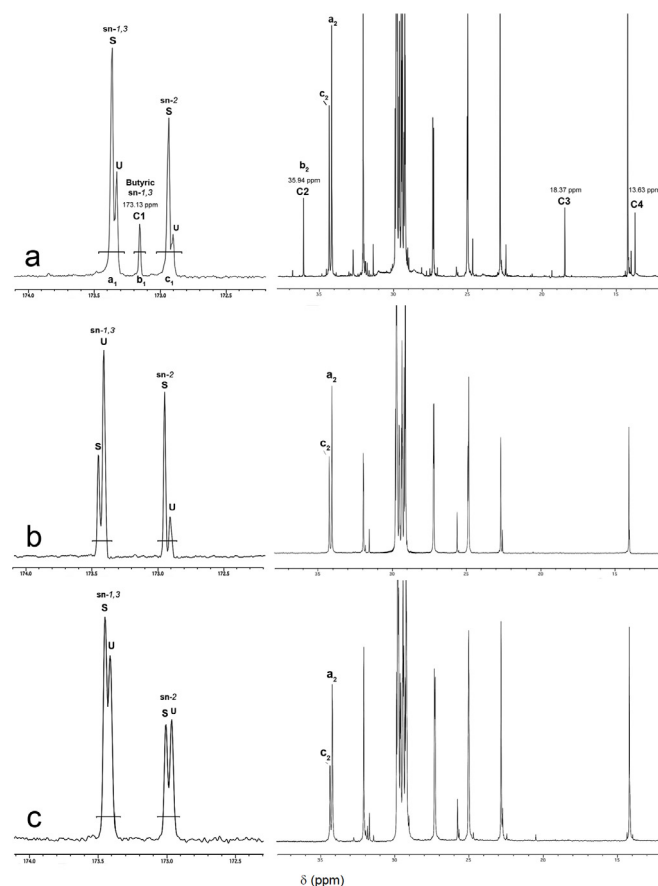


Fig. 1. ^{13}C NMR spectra of milk fat (a), lard (b) and margarine (c) triacylglycerols in chloroform- d at 25 °C. *Left panel:* expansions of spectral regions relevant to carbonyls (spectra acquired in high resolution mode); *right panel:* expansion of the spectral region relevant to methylene/methyl groups (spectra acquired in low resolution mode). Saturated (S) and unsaturated (U) fatty acids signals are assigned to the *sn*-1,3 and *sn*-2 positions on the glycerol backbone. Carbon signals of the butyric acyl chain are labeled as C1, C2, C3 and C4. The carbonyl signals of acyl groups were indicated as a_1 (long acyl chain in *sn*-1,3-position), b_1 (butyrate in *sn*-1,3 position) and c_1 (long acyl chain in *sn*-2 position). The α -carbonyl methylene carbon signals were indicated as a_2 (long acyl chain in *sn*-1,3-position), b_2 (butyrate in *sn*-1,3 position) and c_2 (long acyl chain in *sn*-2 position).

sn-1/3-acyl chains alone.

The quantitative analysis of butyrate can be performed by referring the area of the C1 or C2 peaks at 173.13 ppm and 35.94 ppm to the corresponding signals of long, medium and short chain fatty acids (C6–C22), using alternatively one of the following expressions:

$$\text{Butyrate \% (mole fraction)} = b_1 \cdot 100 / (b_1 + a_1 + c_1) \quad (1)$$

where b_1 = intensity of C1 butyrate signal (173.13 ppm); a_1 = intensity of C1 *sn*-1,3-long chain fatty acids (C6–C20) (173.36 ppm); c_1 = intensity of C1 *sn*-2-long chain fatty acids (C6–C20) (172.95 ppm).

$$\text{Butyrate \% (mole fraction)} = b_2 \cdot 100 / (b_2 + a_2 + c_2) \quad (2)$$

where b_2 = intensity of C2 butyrate signal (35.94 ppm); a_2 = intensity of C2 *sn*-1,3-long chain fatty acids (C6–C20) (34.18 ppm); c_2 = intensity of C2 *sn*-2-long chain fatty acids (C6–C20) (34.35 ppm).

In order to obtain a reliable quantitative response, ^{13}C NMR

spectra have to be acquired under experimental conditions ensuring a satisfactory digital resolution and a linear recovery of NMR resonances. The linearity between the NMR signal intensity and the concentration of the components is, generally, distorted by different relaxation rates and NOE effects of different carbons (Ng, 2000; Wollenberg, 1990).

In routine ^{13}C NMR qualitative analysis, most spectra are usually recorded using broad band (BB) proton decoupling mode (complete saturation of the proton transitions in order to produce decoupled spectra, eliminating the multiplicity of carbon signals) and using a short delay time (D1) between two subsequent pulses. For quantitative ^{13}C NMR analysis, when carbons have different relaxation behavior, the longitudinal relaxation times (T1) have to be known for all carbons to ensure that all carbons are fully relaxed between two following pulses. For this reason, spectra for quantitative purpose were acquired using experimental conditions that permit a complete relaxation of carbon nuclei between two subsequent pulses, taking into account the known T1 values for different acyl carbons (Ng, 2000; Wollenberg, 1990). T1 values ranged between 9 and 11 s for carbonyls and were shorter than 1 s for all methylene carbons. Based on these T1 values, and using a 45° pulse, a short relaxation delay of 2 s was used for recording quantitative full spectra.

For methylene carbon C2 signals both BB and inverse-gated full spectra were acquired. The advantage of the BB mode is related to its increased sensitivity because of both the NOE enhancement, yielding higher S/N ratio, and the faster recycle delay between pulses (2.37 s) with respect to those used in inverse gated NOE suppressed spectra (22.37 s). For this latter reason, a higher number of scans per minute (25.3 scans/min) is recorded in the BB accumulation mode with respect to the inverse gated recording (2.7 scans/min), giving rise to spectra with higher signal-to-noise (S/N) ratio. To obtain the same S/N for methylene carbons, 20–30 times shorter accumulation time is required in BB mode than the inverse-gated mode. The complete relaxation of C1 carbonyls was guaranteed by the high acquisition time (12–20 s) requested for high digital resolution (0.04 Hz/pt) (Wollenberg, 1990). Considering the small NOE effect on carbonyl signal intensities (Ng, 2000; Wollenberg, 1990), the BA content was also inferred from the carbonyl high-resolution ^{13}C NMR spectra acquired using the BB mode.

3.2. Comparison between NMR and GC

The quantitative data obtained from the NMR measurements were compared with those obtained by high resolution-gas chromatography (GC) determinations. In Table 1, the amount of butyric acid found in fat blends by NMR (expressed in mole fraction %) and the relative amount of butyrate obtained by GC (expressed as weight %) are reported. The quantitative NMR- and GC-based quantitative data displayed a very high regression coefficient

($R^2 = 0.9996$, Fig. 2a), supporting the correspondence between two independent analytical techniques. NMR spectra also exhibited satisfactory repeatability from triplicate analyses (standard deviation of signal intensities: 0.08).

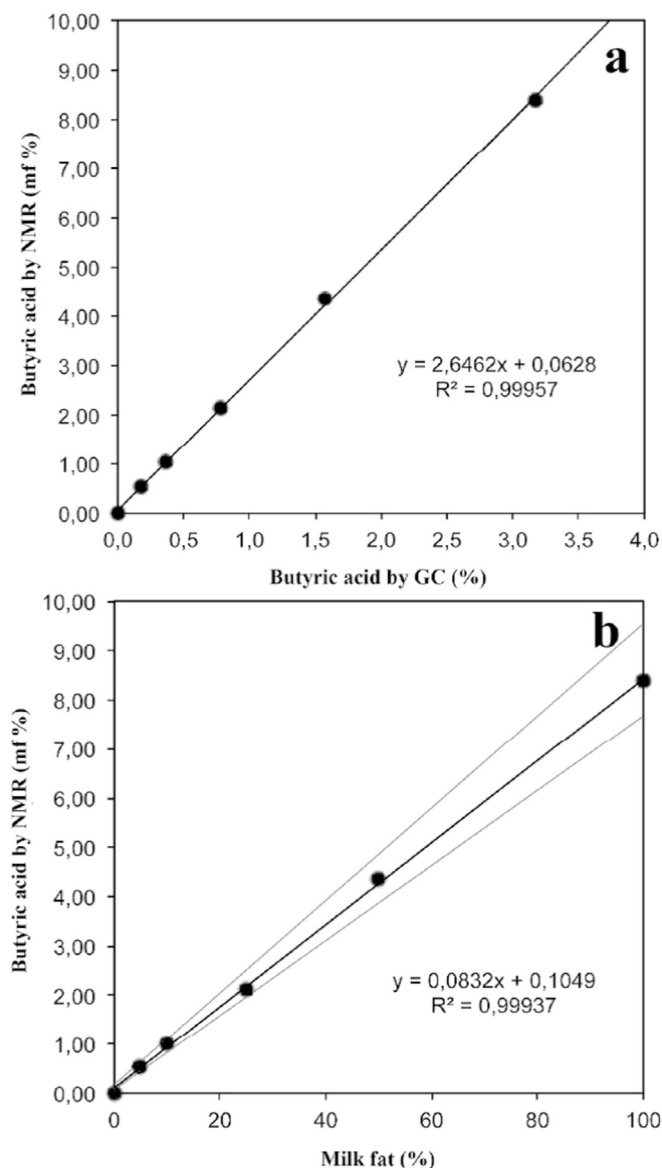


Fig. 2. ^{13}C NMR data of butyrate (mole fraction %) obtained for fat blends spiked with known amount of milk fat, plotted against butyric acid (%) quantified by GC on the same mixtures (a) and against milk fat percentage in the fat blends (b).

Table 1

High resolution ^{13}C NMR spectroscopy (intensity of butyrate C2 carbon, mole fraction %) and GC (% of fatty acids methyl esters, w/w)* determination of butyric acid found in fat blends obtained by spiking pork fat/vegetable margarine blends with BCR-519 pure milk fat.

Milk fat (%)	Butyric acid by NMR (mf %)		Butyric acid by GC (w/w %)		NMR prediction (w/w %)	CV NMR	CV GC
0	nd		nd				
5	0.55	± 0.05	0.21	± 0.02	0.205 ± 0.017	0.091	0.095
10	1.02	± 0.01	0.44	± 0.01	0.383 ± 0.001	0.010	0.023
25	2.13	± 0.02	0.84	± 0.02	0.803 ± 0.005	0.009	0.024
50	4.36	± 0.06	1.65	± 0.04	1.645 ± 0.020	0.014	0.024
100	8.38	± 0.08	3.21	± 0.07	3.164 ± 0.028	0.010	0.022

*Results are expressed as average values followed by the standard deviation of three replicates.

nd: not detected.

NMR predicted values of butyric acid (w/w %) are calculated from the calibration equation to express the value as weight percentage.

CV = coefficient of variation (relative standard deviation).

Some advantages of the NMR method here proposed with respect to the GC procedure can be pointed out: i) samples of mixed fats are directly analyzed by NMR without any trans-methylation and/or chemical handling, ii) a rapid and non-destructive analysis is performed by NMR, iii) the method can be simply applied by recording NMR spectra of fat dissolved in chloroform-*d*, without the need of any chemical standard for qualitative-quantitative calibration.

3.3. Assessment of milk-fat content

In our experiment, the NMR values of BA plotted against the known amounts of milk fat in mixtures (w/w %) resulted in an excellent linearity and a very good regression coefficient ($R^2 = 0.9994$) as shown in Fig. 2b. The ^{13}C NMR analysis of butyrate enables a reliable detection of milk fat in spreads at relative concentrations of 5% (w/w) or even lower (Picariello et al., 2013).

The accurate ^{13}C -NMR-based quantification of milk fat with foreign fats, however, could be biased by the natural variability of fatty acids in milk, as the milk fatty acid profile is affected by genetic and environmental factors, especially the animal diet, as well as by seasonal changes. High variability in the composition of milk fat even from cows fed the same diet has been previously reported (Bobe et al., 2003). The natural fluctuation of fatty acids determines a window of variability of the BA content in milk fat (Table 2), which has been drawn in Fig. 2 taking into account the values reported in the literature (Butler 2011; Collomb et al., 2002, 2008; Gastaldi et al., 2011; Glaeser, 2002; Kliem, Shingfield, Livingstone, & Givens, 2013; Lourenço, Van Ranst, Vlaeminck, De Smet, & Fievez, 2008; Månsson, 2008; Talpur, Bhanger, Khooharo, & Memon, 2008).

Such a variability implies a possible range of error in the evaluation of milk fat in mixed fats, which is proportional to the milk fat content. For instance, in a hypothetical unknown mixture of fats, for a value of 2% (molar fraction, mf) BA determined by NMR, one could estimate a milk fat content ranging between about 22 and 26%. For a measurement of 7% (mf) BA, the amount of milk fat would be comprised within the 77–85% range. It is obvious that an accurate check of the correspondence between the declared and actual butter content in a fat mixture is possible only if a sample of the butterfat batch used by the industry to produce the fat blend is available for analysis.

Herein, to prepare blends spiked with milk fat we used anhydrous BCR-519 certified material, which is representative of the most common cow breeds and averages the fluctuation of barn (winter) and pasture (summer) feeding, containing butyrate at 3.49% (w/w) (Molkentin & Precht, 1997; 1998).

4. Conclusions

A rapid and accurate ^{13}C NMR spectroscopic method was developed to detect and quantify milk-derived fat in butter-like spreads composed by different kinds of fats, namely vegetable margarine and pork fat. The effectiveness of the method was assessed by comparative GC determination of butyrate, as a molecular marker of milk fat, in milk fat-containing blends. The correlation between the two independent methods was excellent. However, the ^{13}C NMR spectroscopy detection and quantification of butyrate was advantageous for several reasons, primarily because no sample pre-treatment was required. In addition, NMR analysis does not require the use of chemical standard for signal identification and quantitative calibration. The intensity of selected diagnostic signals, in fact, allows the direct quantitative determination of BA content and, hence, the assessment of the milk fat content.

In general, the NMR instrumentation and maintenance costs, as well as the operative skills required, could represent limiting factors to routine application for authenticity assessment of edible fats. However, in a perspective of a rational and integrated control policy, NMR spectroscopy can be considered as a powerful and versatile method, especially suitable for confirmatory purposes. In the last years, the high magnetic fields available (600–800 MHz) and the development of ultra-high resolution NMR, with the spread of cryo-probes, has strikingly enhanced the instrumental sensitivity, reducing the acquisition time to a few minutes. Therefore, it is expected in the future that NMR will be applied widely at both control laboratories and industrial levels for assessing fat authenticity and food quality in general.

The method here discussed can help to control fraudulent practices and to certify the quality of fat blends. However, all methods based on the quantification of butyric acid for the assessment of the amount of milk fat added to other foodstuffs suffer from an intrinsic limitation due to the variability of the BA content in butter, which depends on the animal species, diets, stages of lactation, seasons of the year and processing conditions that influence fatty acid composition of butter. In this work we determined a window of variability of the milk fat in fat blends, compatible with the analytical determination of BA amounts. The accurate check of the amount of milk fat included in a 'mixed fat' would be possible only if manufacturers were required to store butter samples for post-preparation assays.

In principle, the ^{13}C NMR analysis of butyrate in edible fat blends could enable the assessment of milk fat absence, also applying this technique as a tool for consumers' protection when the consumption of milk-derived material is not allowed for ethical or

Table 2
Natural fluctuation of butyric acid content in milk fat from different animal species, as inferred from literature reports.

Species	Minimum	Maximum	Average	SEM [#]	Reference
Cow	3.27	3.9	3.54	0.16	Talpur et al., 2008 ^a
	3.35	3.9	3.46	0.06	Kliem et al., 2013 ^a
	3.22	3.59	2.94	0.55	Molkentin & Precht, 1997, 1998, 2000 ^a
	8.42	8.45	8.44	0.02	Gastaldi et al., 2011 ^b
	4	5.1	4.4	0.1	Månsson, 2008 ^a
	3.39	3.46	3.4	0.2	Collomb et al., 2002 ^a
	3.14	3.48	3.31	0.2	Collomb et al., 2008 ^a
	1.06	2.75	1.91	0.11	Butler, Stergiadis, Seal, Eyre, & Leifert, 2011 ^a
	3.88	4.76	4.29	0.75	Lourenço et al., 2008 ^a
	3.66	4.02	3.9	0.11	Talpur et al., 2008 ^a
Buffalo	3.74	4.17	4.06	0.11	Talpur et al., 2008 ^a
Ewe	2.19	2.88	2.46	0.14	Talpur et al., 2008 ^a
Goat	5.26	5.07	5.17	0.02	Gastaldi et al., 2011 ^b

[#]SEM: Standard Error of the Mean.

^a Expressed as % butyrate on the fat content (w/w x 100).

^b Expressed as % molar fraction (mole butyrate/mole all fatty chains x 100).

nutritional (e.g. food allergy and intolerances) reasons. On the other hand, the possibility to correlate butyrate with the possible presence of milk allergens should be carefully validated in terms of sensitivity, by comparing ^{13}C NMR to specific techniques (e. g. immunochemical methods, mass spectrometry), since allergens could be injuring also at very low amounts. Furthermore, the indirect detection of markers other than protein allergens or molecules responsible of intolerances (e. g. lactose) increases the risk of obtaining false positive determinations (Picariello, Mamone, Addeo, & Ferranti, 2011).

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