

## Comprehensive Analysis of Polar and Apolar Constituents of Butter and Margarine by Nuclear Magnetic Resonance, Reflecting Quality and Production Processes

JAN SCHRIPEMA\*

Analytical Biotechnology, Department of Biotechnology, Delft University of Technology,  
 Julianalaan 67, 2628 BC Delft, The Netherlands, and Grupo Metabolomica, Laboratorio de Ciencias  
 Quimicas, CCT, Universidade Estadual do Norte Fluminense, Av. Alberto Lamego 2000,  
 28015-620 Campos dos Goytacazes, RJ, Brazil

---

The separation of butter or margarine into polar (soluble in water) and apolar fractions (soluble in chloroform) and subsequent analysis of these fractions by  $^1\text{H}$  NMR permits a comprehensive analysis of its constituents. In the polar fraction the preservatives benzoic and sorbic acid, the organic acids citric, lactic, butyric, acetic, and formic acid, and, furthermore, the carbohydrate lactose were quantified. In the apolar fraction the conjugated linoleic acid (CLA) rumenic acid, diglycerides, and linoleic acid were quantified. Rumenic acid is a characteristic component of ruminant fats and was found in all butter samples. The levels varied between 0.50 and 1.08%. Ten brands of Brazilian butter were investigated as was one brand from Norway. Also, two brands of margarine were investigated for comparison. A large variation in especially polar constituents was found between the butter samples, revealing the presence of preservatives in five brands of butter from Brazil, remarkable because these additives are legally not allowed. Furthermore, the levels of organic acids and lactose permitted conclusions about the production process and quality; for example, the presence of higher levels of free butyric acid indicate lipolysis, leading to a lower quality, and low levels of lactose indicate that after churning the residual milk fluids have been removed by an additional washing step in the production process.

---

**KEYWORDS:** Fatty acids; sorbic acid; benzoic acid; citric acid; rumenic acid; CLA; butter; margarine

### INTRODUCTION

Butter is essentially the fat from milk; it contains about 80–82% milk fat, 16–17% water, and 1–2% milk solids other than fat, including salt. Margarine, developed as a butter substitute, is similar in composition, but differs from butter in the fats used for its production, which were originally animal fats but currently almost exclusively vegetable fats are used (1).

The taste of butter is determined by a complex mixture of compounds, originating not only from the milk and the production process but also from storage conditions. Exposure of butter to air easily leads to a rancid taste due to oxidation. In addition, microbiological spoilage is possible due to the relatively high water content (1); for example, contamination of butter by *Listeria* bacteria has led to a listeriosis outbreak in Finland causing the death of six people (2, 3).

Many studies have been devoted to the fat composition of butter; in most cases the fatty acids were analyzed after hydrolysis by GC-MS. About 98% of the fat was found to consist of triglycerides, and the most common fatty acids are

palmitic (22–35%), oleic (20–30%), stearic (9–14%), myristic (8–14%), and butyric (2–5%) acid (4). Around 1980 Pariza and co-workers established that conjugated linoleic acids (CLA) exerted an anticarcinogenic activity (5–7). Food derived from ruminant animals, beef, milk, and derived products, were found to be the major source of these CLA in human food. The *cis*-9,*trans*-11 CLA isomer, known as rumenic acid, is the major isomer found in ruminant fat, normally representing 80–90% of the total CLA in milk fat. The anticarcinogenic effects were especially related to the rumenic acid. Recently, the range of positive health effects associated with CLA in experimental models has been extended to include reduction in body fat accretion and altered nutrient partitioning, antidiabetic effects, reduction in the development of atherosclerosis, enhanced bone mineralization, and modulation of the immune system (8–11). Due to these effects of CLA, many studies have also investigated CLA levels in dairy products and how these can be influenced (see, e.g., refs 12–14).

In the present paper a comprehensive method is described that permits the rapid analysis of both the water-soluble and water-insoluble fatty components of butter and margarine. Among the readily quantifiable compounds are the common

---

\* E-mail j.schripsema@tudelft.nl or jan@uenf.br.

**Table 1.** Content of Polar Components in Butter and Margarine Samples of Different Brands (Micrograms per Gram of Butter)<sup>a</sup>

brand	type	taste <sup>b</sup>	formic acid	benzoic acid	sorbic acid	lactic acid	citric acid	acetic acid	butyric acid	lactose (mg/g)
A		++	21	0	0	0	0	11	13	1.06
B1		+++	35	0	0	1017	444	159	0	12.54
B2		++	31	0	0	178	437	33	0	11.51
B3		++	2	0	0	546	221	197	0	7.19
B4	salted	++	3	0	0	0	408	9	0	10.41
B5	salted	++	2	0	0	0	298	7	0	7.87
C	salted	—	24	0	538	0	0	8	0	0.60
D	salted	—	4	0	0	1788	0	271	222	0.84
E	salted	++	3	0	0	0	241	6	0	7.13
F	salted	+	9	0	54	82	47	21	0	1.51
G	salted	+	49	0	0	349	48	29	0	1.06
H	salted	+	29	0	261	295	0	42	0	1.28
I	salted	—	36	41	41	628	0	169	336	2.26
J	salted	--	64	0	355	294	0	110	23	0
K		++	10	0	0	115	618	30	0	9.28
L	margarine		3	636	521	402	120	19	120	2.81
M	margarine		8	227	200	0	156	16	0	0.79

<sup>a</sup> Brands A–J are brands of butter from Brazil. Brand K is a butter sample from Norway, and L and M are margarine brands from Brazil. <sup>b</sup> taste: +++ very good taste, ++ good taste, + not good/not bad, — bad taste, -- bad rancid taste.

preservatives sorbic acid and benzoic acid, and, furthermore, the organic acids citric, acetic, lactic, butyric, and formic acid and also the important rumenic acid, a CLA, to which has been attributed anticancer activity.

In the present study 14 different samples of butter available in Brazil were investigated as were 1 sample of a Norwegian butter and 2 samples of margarine available in Brazil.

## MATERIALS AND METHODS

**Samples.** Butter and margarine samples were obtained from commercial establishments and maintained in the refrigerator until the analysis was performed. All samples were analyzed well before the date of validity of the product.

**Preparation of Samples for Analysis.** A quantity of 120–130 mg of butter was weighed. To the butter was added 0.80 mL of deuterated water (Cambridge Isotope Laboratories, 99.9% D) containing an exact quantity (52.0 mg/100 mL) of the internal standard (tetradeuterio-trimethylsilylpropionate, sodium salt) and 0.80 mL of deuterated chloroform (CIL, 99.9%). After the butter had dissolved in the mixture and the mixture had been allowed to stand for a few minutes for a good separation of the layers, 0.50 mL of the water layer was transferred to one NMR tube and 0.50 mL from the chloroform layer to another tube. The pH of the water layer was verified, and in all cases it was between 5.5 and 6.0.

**NMR Measurements.** NMR spectra were obtained on a JEOL Eclipse+ 400 spectrometer, operating at 400 MHz for protons and 100 MHz for carbon-13. All spectra were obtained at 25 °C, with a spectral width of 10 ppm and a relaxation delay of 4 s, acquiring 16K data points, leading to a recycle time of 8 s. An exponential line-broadening was applied (0.3 Hz) and zero filling with a factor 4. For acquisition of the <sup>1</sup>H NMR spectra in D<sub>2</sub>O, 1000 scans were recorded and the water signal was suppressed by presaturation using the pulse program H2SAT from the Delta NMR Processing and Control Software (JEOL USA, Inc., version 4.3). <sup>1</sup>H NMR spectra of the CDCl<sub>3</sub> samples were recorded with 100 scans. For identification of the observed signals, 2D NMR spectra were obtained. 2D-COSY, HSQC, and HMBC spectra were used. These were all recorded with the standard Delta software.

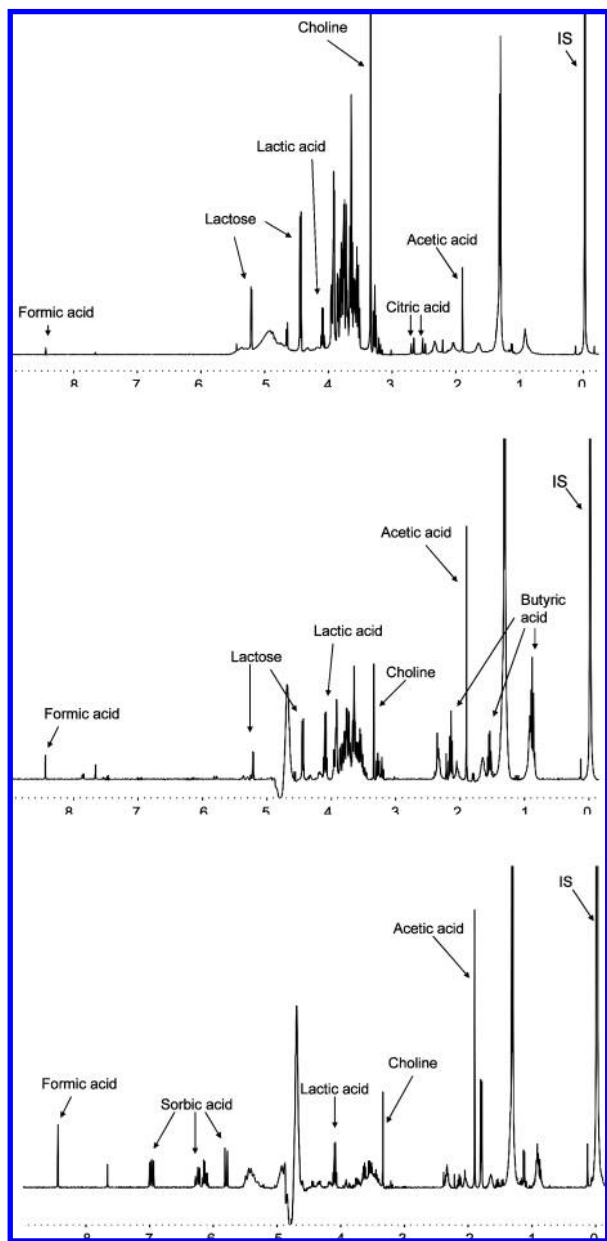
**Quantitative Analysis.** In the spectra recorded from the polar fractions in D<sub>2</sub>O individual signals were integrated and the quantities of the compounds were calculated through comparison with the signal from the internal standard. No corrections were made for differences in relaxation times (see Results and Discussion). In the spectra recorded from the apolar fractions in CDCl<sub>3</sub> the signals were integrated and the integrals were related to the integral from the signals of the four protons at the *sn*-1,3 position of glycerol at 4.30 and 4.15 ppm. The quantity of rumenic acid was calculated using the integrals of the signals at

6.28, 5.94, and 5.65 ppm. Diglycerides were quantified by integration of the signals at 5.08 and 3.71 ppm. The quantity of linoleic acid was obtained from the integral of the signal at 2.80 ppm after subtraction of the quantity of rumenic acid, which also gives a signal at this position. The results are summarized in **Table 2**.

## RESULTS AND DISCUSSION

Fifteen samples of butter (14 from Brazil and 1 from Norway) and 2 samples of margarine (from Brazil) were investigated. The samples are listed in **Table 1**, together with the results from the analysis of the polar components. The 14 butter samples from Brazil were of 10 different brands, indicated by the letters A–J. From one brand of butter five different samples were analyzed (B1–B5). Also indicated is the type of butter, salted or not. Butter is a water-in-fat emulsion, and before analysis, a separation was made between the polar and apolar components by dissolving the butter in a water/chloroform mixture (see Materials and Methods).

**Polar Components.** **Figure 1** shows some representative spectra from samples B1, I, and J. They show the great variability in the spectra. Sample B1 was a sample from butter with a pleasant taste and good texture. Sample J, on the contrary, was rather rancid tasting. In the spectrum from this sample a rather high quantity of sorbic acid was observed, a preservative not usually present in butter. In fact, it is forbidden by Brazilian legislation to add preservatives to butter (15). Also, free butyric acid was detected. Sample I, also a sample with a bad taste, did show a high concentration of butyric acid, and furthermore both sorbic and benzoic acid were detected, both at the rather low concentration of 40 µg/g of butter. To get absolute quantifications of the compounds present in the butter, a known amount of a suitable standard was added to the solutions. The signals in the <sup>1</sup>H NMR spectrum of the standard should not interfere with the ones from the compounds to be analyzed and the ones from the solvent (in this case, water). The derivative of TMS normally used as an internal standard for the chemical shift values, (2,2,3,3-*d*<sub>4</sub>)-trimethylsilylpropionate (TMSP), was found to be excellent for this purpose (16); its spectrum contains only one signal at 0.00 ppm, which does not overlap with any other signal. For the experiment 52 mg of TMSP was accurately weighed and dissolved in an exact quantity of deuterated water. This solution was then used for the dissolution of the polar components of butter.



**Figure 1.**  $^1\text{H}$  NMR spectra from the polar fraction of butter samples B1 (top), I (middle), and J (bottom), measured at 400 MHz in  $\text{D}_2\text{O}$ .

Quantitative measurements by  $^1\text{H}$  NMR need some specific precautions, aside from the obvious need for adequate signal-to-noise ratio in the spectrum (17). These are the avoidance of differential saturation effects and the need to characterize the NMR resonance line-shape properly (using at least 4 points/Hz). To completely avoid the differential saturation effects the spins should fully relax between pulses, demanding recycle times of at least 5 times  $T_1$  (longitudinal relaxation or spin-lattice relaxation time) of the slowest relaxing nuclei. In our experiments a recycle time of 8.0 s was used, not sufficient to achieve a complete relaxation of all nuclei, considering that formic acid, the slowest relaxing compound, has a  $T_1$  of 8.0 s. Also, TMSP has a rather long  $T_1$  of 3.2 s. To obtain the best accuracy for the quantitations a correction can be made for the differences in  $T_1$  using a mathematical correction factor for quantitation (18), but it depends on the purpose of the measurements if this is necessary. If a very accurate quantitation (+1%) of a single compound is required, the correction factor can be used, and it would also be advisable to make a calibration curve of that compound. Care was also taken with the start and finish of the

integral: for a NMR signal (Lorentzian line shape), the integral should ideally cover 20 times the half-height line-width on each side of the peak if it is to include 99% of it (10–20 Hz each side) (17).

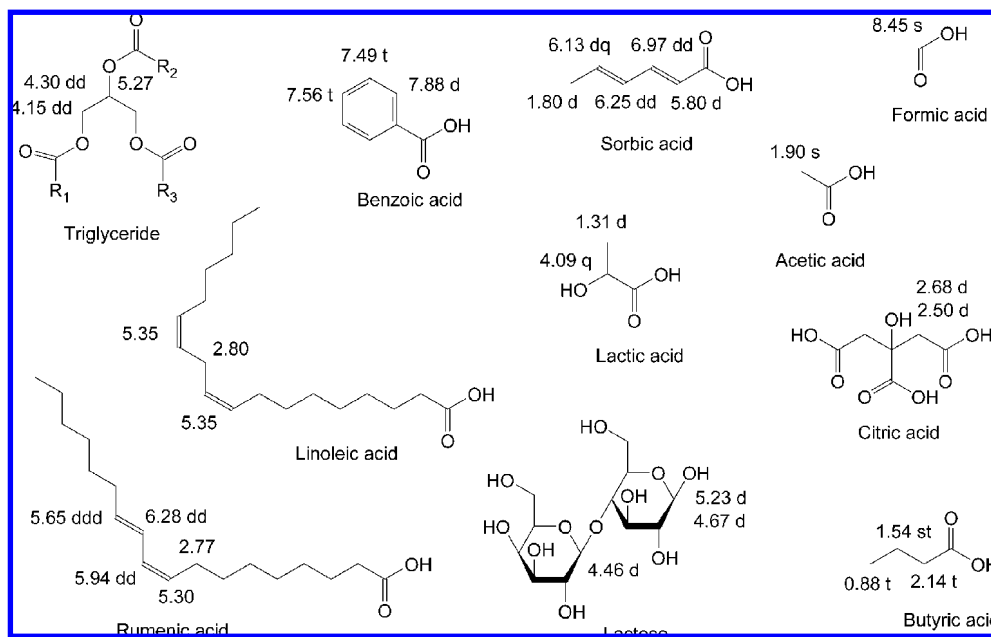
The amount of the compounds in the solution was calculated from the integrals of the  $^1\text{H}$  NMR signals as described under Materials and Methods. The signals chosen for quantification had as little as possible interference from other signals. For example, in the case of lactic acid, the spectrum of this compound has two signals, one at 4.09 ppm and one at 1.31 ppm. The signal at 1.31 ppm did in most spectra show interference with an underlying signal of probably free fatty acids, making this signal unsuitable for quantification. The other signal, however, was in all spectra essentially free from interference. It was possible to quantify accurately microgram amounts of the compounds, leading to a detection limit in the low micrograms per gram of butter range for the compounds. The exact detection limit depends on the specific signals used for the quantification; for example, for acetic acid, one singlet is obtained for all three protons in the molecule, leading to a low detection limit, whereas for, for example, sorbic acid, multiplets are obtained for any proton in the molecule, leading to a higher detection limit. The main limitation in the precision of the measurements (5%) was the signal-to-noise relation of the signals. Consequently, a higher precision can be obtained and even much smaller quantities can be quantified, when a higher number of scans are obtained or higher field NMR spectrometers or special probes are used.

All spectra were obtained from samples of butter qualified as “Manteiga Extra” and which thus should fulfill the highest requirements for quality and taste (19). Types of butter with a lower quality are qualified as “Manteiga de primeira qualidade” or “Manteiga de segunda qualidade”. The latter is commonly known as “Manteiga comum”. These were not investigated in this paper. In **Figure 2** a listing is given of the compounds detected and quantified in the analyzed samples. Compounds belonging to different classes are detected (see **Table 1**), and it was surprising to encounter the sometimes completely different profiles of the aqueous extracts (**Figure 1**). Remarkable was the fact that in five brands of butter preservatives (benzoic and/or sorbic acid) were detected, which according to Brazilian legislation should not be present (19). Sorbic acid was detected in five brands, and the levels were between 41 and 538  $\mu\text{g/g}$  of butter. Benzoic acid was detected in only one brand at a level of 41  $\mu\text{g/g}$ . In an investigation of butter samples from Taiwan similar levels of these preservatives were found (20). In butter samples from The Netherlands very low levels of benzoic acid were reported, between 0.8 and 2.2  $\mu\text{g/g}$ , which may correspond to the natural level (21).

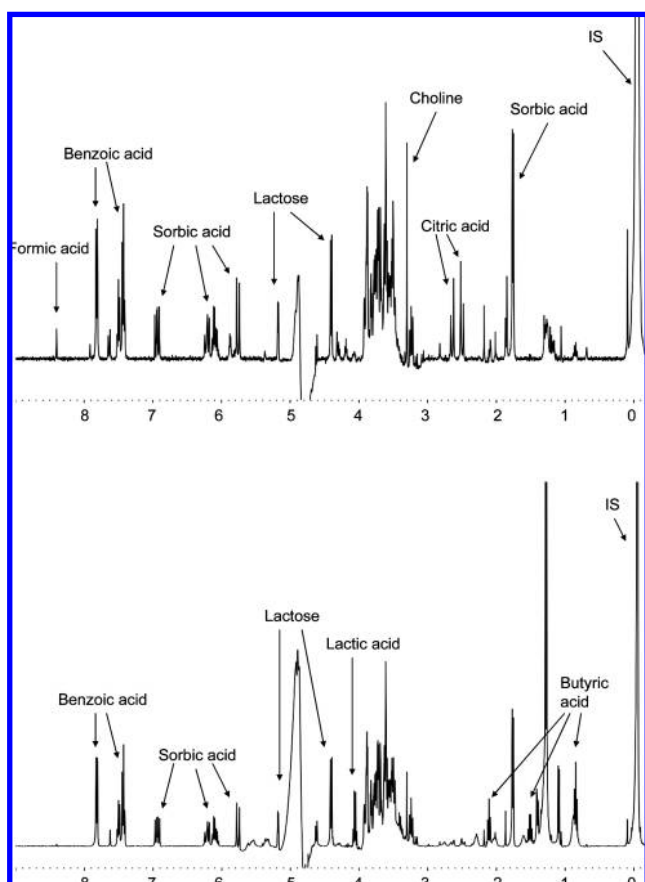
In margarine the addition of preservatives is permitted, and in both investigated brands benzoic acid and sorbic acid were found (**Figure 3**).

Organic acids are natural constituents from all dairy products, including butter. Formic acid was detected in all samples at levels between 2 and 64  $\mu\text{g/g}$  of butter. Lactic acid was not found in five brands of butter. The levels encountered varied between 82  $\mu\text{g}$  and 1.79 mg per gram of butter. Citric acid was not found in six brands, and in the other brands the levels varied from 47 to 618  $\mu\text{g/g}$ . Acetic acid was present in all samples, and the levels varied from 6 to 271  $\mu\text{g/g}$ . Finally, butyric acid was detected in four brands in levels from 13 to 336  $\mu\text{g/g}$ .

In the margarine samples the organic acids were also seen, but in sample M no lactic acid and no butyric acid could be detected.



**Figure 2.** Structural formulas of compounds observed in the  $^1\text{H}$  NMR spectra, with the chemical shifts of the most important signals (s, singlet; d, doublet; t, triplet; q, quartet; st, extet).



**Figure 3.**  $^1\text{H}$  NMR spectra from the polar fraction of margarine samples M (top) and L (bottom), measured at 400 MHz in  $\text{D}_2\text{O}$ .

Lactose was detected in all but one sample of butter and in both samples of margarine. The levels were from 0.60 to 12.54 mg/g of butter.

The concentrations of organic acids and lactose do reflect details of the production process of butter. The normal concentration of lactose in milk varies from 3.8 to 5.5% (22), and by fermentation the lactose is converted to lactic acid. Citric

acid, normally present in milk in a concentration of about 0.2% (23), is converted to acetic acid by souring of the milk. Butyric acid is a major component of the triglycerides of milk fat, present at a level of about 3.5%, and distinguishes it from other fats. This characteristic has been used to determine the milk fat percentage of mixed fats (24). However, the levels of free butyric acid should be low in milk, normally 0.3–1 mM (25). By lipolysis free fatty acids are produced, and the liberation of short-chain fatty acids leads to a rancid flavor of milk or milk products (25). The quantity of free short-chain fatty acids is thus an important parameter for the quality evaluation.

It is clear that from the analysis of the aqueous extracts, data are obtained on a large number of key compounds. Without NMR a series of different HPLC or GC analyses would be required to obtain similar data (26).

Correlations of the taste of the butter with the NMR profiles were observed. Good taste seemed to correlate with higher lactose and citric acid levels, whereas rancid taste correlated with higher levels of free butyric acid. However, the taste of butter is determined by many factors, specific flavor compounds that are present in very small quantities (21).

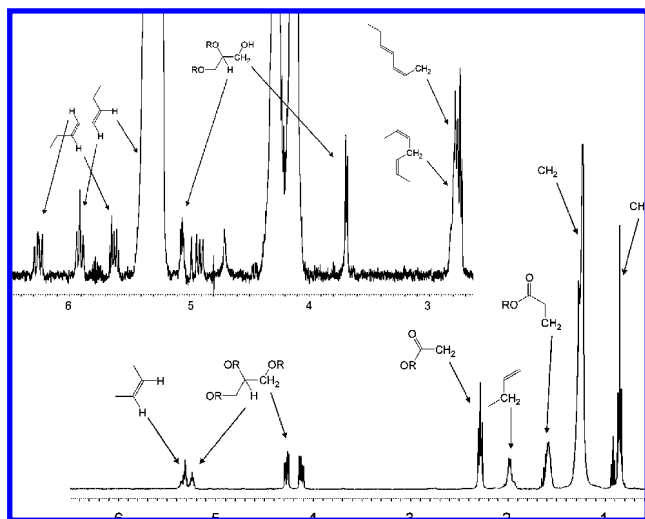
The low levels of lactose in a number of butter samples, usually accompanied by equally low levels of citric acid, point to a probable difference in production procedure. After churning, the excess of milk is removed and the remainder is homogenized, leading to the inclusion of about 15–18% of milk liquid in the butter. Considering an average lactose concentration of about 5% in milk (7), this would correspond to a lactose concentration of 7.5–9.0 mg/g of butter. After the separation, it is possible to do a washing step of the butter particles, leading to substitution of the residual milk. This seems to have occurred with many butter brands, leading to an about 10-fold decrease in lactose and citric acid levels. This also seems to lead then to a decrease of taste.

**Apolar Components.** In the apolar extracts the fats from butter were analyzed. Traditionally the fatty acids are analyzed after hydrolysis of the glycerides (4), but with NMR the glycerides are directly analyzed in the extract. Individual fatty acids in the glycerides were quantified by relating specific signals to the signal of the *sn*-1,3 glycerol position from the

**Table 2.** Interpretation of the  $^1\text{H}$  NMR Spectra from the Apolar Fraction of Butter and Margarine (400 MHz,  $\text{CDCl}_3$ )<sup>a</sup>

brand	ruminic acid <sup>b</sup> , %	linoleic acid <sup>c</sup> , %	diglycerides <sup>d</sup> , %	double bond <sup>e</sup> no./FA $\times$ 100%, %	double bond <sup>f</sup> FA with $\geq 1$ , %	fatty acids <sup>g</sup> , %	av no. of H <sup>h</sup>
A	1.08	1.32	1.72	31.1	29.3	97.7	17.3
B1	0.88	1.69	2.02	30.0	26.7	98.2	17.1
B2	0.81	1.46	1.69	29.8	26.3	95.4	16.9
B3	0.97	1.43	2.21	31.1	28.6	97.8	17.4
B4	0.76	1.83	1.71	29.6	26.8	98.3	17.2
B5	0.68	1.87	1.68	31.2	29.1	99.4	17.2
C	0.65	1.91	1.96	30.1	27.4	98.0	17.2
D	1.07	0.94	3.41	32.1	31.5	100.3	18.6
E	0.50	1.74	1.66	27.9	26.4	97.7	17.3
F	0.87	1.59	1.51	31.9	30.4	98.5	17.3
G	1.01	1.01	1.62	30.1	28.5	97.0	17.5
H	0.96	2.24	1.79	34.1	31.8	97.6	17.5
I	0.87	1.29	2.77	29.5	27.8	98.3	17.4
J	0.90	1.21	2.03	29.4	28.0	98.4	17.6
K	0.59	2.16	1.48	32.7	31.0	101.4	17.7
L	0	36.19	0.46	118.2	79.6	99.3	17.9
M	0	35.19	0	115.8	72.8	94.6	17.5

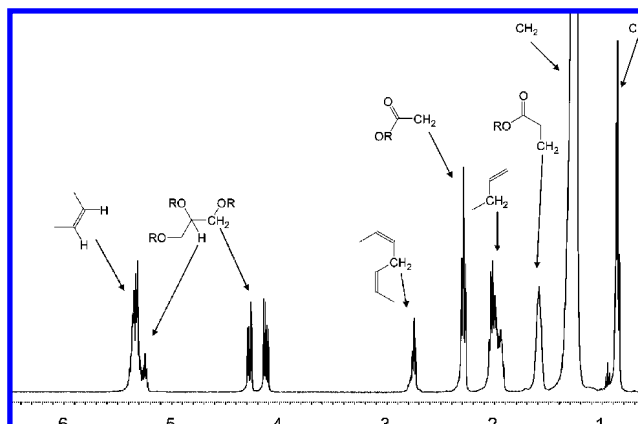
<sup>a</sup> All quantifications are in relation to the signal of the hydrogens at the *sn*-1,3 position of glycerol in triglycerides at around 4.2 ppm and reflect the percentage in relation to the maximum possible number of fatty acids linked to glycerol. <sup>b</sup> Ruminic acid was quantified by integration of the signals at 6.28, 5.94, and 5.65 ppm. <sup>c</sup> Linoleic acid was quantified by integration of the signal at 2.80 ppm and subsequent subtraction of the quantity of ruminic acid. <sup>d</sup> Diglycerides were quantified by integration of the signals at 5.08 and 3.71 ppm. <sup>e</sup> Quantity of double bonds calculated through integration of the signals around 5.30 ppm and corrected for the contribution of the *sn*-2 signal of triglycerides. The number represents the number of double bonds divided by the total number of fatty acids  $\times$  100%. <sup>f</sup> Quantity of double bonds calculated through integration of the signals at 2.0 ppm. The number represents the percentage of fatty acids with at least one double bond. <sup>g</sup> Quantity of fatty acids bound to glycerol, calculated through the signal at 2.30 ppm. <sup>h</sup> Average number of hydrogen atoms per fatty acid chain contributing to the integral of the signal at 1.2 ppm.



**Figure 4.**  $^1\text{H}$  NMR spectrum from the apolar fraction of butter sample B4, measured at 400 MHz in  $\text{CDCl}_3$ . The region showing the signals from ruminic acid and diglycerides has been amplified.

triglycerides at about 4.2 ppm (see **Table 2**). Ruminic acid can be detected directly in the spectra due to its specific signals (**Figure 4**). The identification of this compound was verified with two-dimensional NMR spectra. In all butter samples this fatty acid was found, and the levels were between 0.50 and 1.08%. In the margarine samples no ruminic acid was detected (**Figure 5**).

Ruminic acid is a specific unsaturated fatty acid, which is derived from grass and excreted by the cow to the cow milk. Its biosynthesis has been reviewed by Bauman et al. (27). To this fatty acid an anticancer effect is attributed (5–9), which was confirmed in some recent large-scale studies (28). The quantification of this compound in butter is traditionally done by GC-MS, but it is a difficult and laborious analysis (4, 13). Due to the relatively low quantities the accuracy of the measurement of this compound with NMR is not very high



**Figure 5.**  $^1\text{H}$  NMR spectrum from the apolar fraction of margarine sample L, measured at 400 MHz in  $\text{CDCl}_3$ .

( $\pm 10\%$ ), but the method rapidly displays differences between butter samples.

Interestingly, next to the triglycerides, also the content of diglycerides can be determined. They display the characteristic signals from the glycerol part with a free hydroxyl at position 3. At 5.08 ppm the pentet from H-2 is seen and at 3.71 ppm, the doublet from both H-3 protons. In the traditional methods for the analysis of the fats in butter, the glycerides are hydrolyzed before the analysis of the fatty acids. The level varied from 1.48 to 3.41%. In one of the margarine samples also some diglyceride was found, 0.46%.

Linoleic acid has a specific NMR signal due to the signal of the methylene group located between the two double bonds. This appears at 2.80 ppm. Also, one of the methylene groups next to the double bonds of ruminic acid appears at this position; therefore, a correction is necessary to calculate the linoleic acid quantity. The linoleic acid levels were calculated to be between 0.94 and 2.24% in the butter samples. In the margarine samples the levels were around 36%.

Besides the higher levels of linoleic acid in margarine also the much higher percentage of other unsaturated fatty acids (e.g.,

oleic acid) is evident (**Figure 5**). The signal at 5.3 ppm corresponds to the hydrogens linked to double bonds and from this signal the relative quantities of the different types of fatty acids can be estimated from the relative integrals of the observed signals in the  $^1\text{H}$  NMR spectra. In margarine the average quantity of double bonds per fatty acid is 1.2; thus, the quantity is 120% in relation to the quantity of fatty acids (**Table 2**). This is mainly due to the high percentage of linoleic acid. In butter this percentage is about 30%. The signal at about 2.0 ppm is related to the methylene groups next to the double bonds and also indicates the percentage of unsaturated fatty acids, but in this case the percentage reflects the percentage of fatty acids with one or more double bonds.

Also, the other signals observed in the spectra can be interpreted. The signal at 2.3 ppm corresponds to the methylene of the fatty acid located next to the ester bond, whereas the signal at 1.6 ppm corresponds to the methylene  $\beta$  to the ester bond. At 0.9 ppm the signal corresponding to the terminal methyl groups can be observed. The signal at 1.2 ppm corresponds to most other methylene groups in the fatty acids. With the integral of this signal the average number of protons of these methylene groups can be calculated (**Table 2**). In this value butter and margarine are similar, due to the presence of a high percentage of unsaturated fatty acids in margarine and the presence of a high percentage of short-chain fatty acids in butter.

The presented method for the analysis of butter and margarine with  $^1\text{H}$  NMR yields for each sample characteristic fingerprints, which reveal many details about the composition, production process, and quality. Many possibilities exist for the use in quality control and process monitoring; a few examples include the detection of unallowed additives, such as preservatives, the detection of microbial deterioration by increasing levels of butyric acid, and the comparison of levels of ruminic acid in different types of butter.

## LITERATURE CITED

- (1) <http://webexhibits.org/butter/index.html>.
- (2) Maijala, R.; Lyytikäinen, O.; Johansson, T.; Autio, T.; Aalto, T.; Haavisto, L.; Honkanen-Buzalski, T. Exposure of *Listeria monocytogenes* within an epidemic caused by butter in Finland. *Int. J. Food Microbiol.* **2001**, *70*, 97–109.
- (3) Lyytikäinen, O.; Autio, T.; Maijala, R.; Ruutu, P.; Honkanen-Buzalski, T.; Miettinen, M.; Hatakka, M.; Mikkola, J.; Anttila, V.-J.; Johansson, T.; Rantala, L.; Aalto, T.; Korkeala, H.; Siitonen, A. An outbreak of *Listeria monocytogenes* serotype 3a infections from butter in Finland. *J. Infect. Dis.* **2000**, *181*, 1838–1841.
- (4) Jensen, R. G. The composition of bovine milk lipids: January 1995 to December 2000. *J. Dairy Sci.* **2002**, *85*, 295–350.
- (5) Pariza, M. W.; Ashoor, S. H.; Chu, F. S.; Lund, D. B. Effects of temperature and time on mutagen formation in pan-fried hamburger. *Cancer Lett.* **1979**, *7*, 63–69.
- (6) Pariza, M. W.; Hargraves, W. A. A beef-derived mutagenesis modulator inhibits initiation of mouse epidermal tumors by 7,12-dimethylbenz[*a*]anthracene. *Carcinogenesis* **1985**, *6*, 591–593.
- (7) Ha, Y. L.; Grimm, N. K.; Pariza, M. W. Anticarcinogens from fried ground beef: heat altered derivatives of linoleic acid. *Carcinogenesis* **1987**, *8*, 1881–1887.
- (8) Belury, M. A. Conjugated dienoic linoleate—a polyunsaturated fatty acid with unique chemoprotective properties. *Nutr. Rev.* **1995**, *53*, 83–89.
- (9) Ip, C.; Banni, S.; Angioni, E.; Carta, G.; McGinley, J.; Thompson, H. J.; Barbano, D.; Bauman, D. Conjugated linoleic acid-enriched butter fat alters mammary gland morphogenesis and reduces cancer risk in rats. *J. Nutr.* **1999**, *129*, 2135–2142.
- (10) Houseknecht, K. L.; Vanden Heuvel, J. P.; Moya-Camarena, S. Y.; Portocarrero, C. P.; Peck, L. W.; Nickel, K. P.; Belury, M. A. Dietary conjugated linoleic acid normalizes impaired glucose tolerance in the Zucker diabetic fatty fa/fa rat. *Biochem. Biophys. Res. Commun.* **1998**, *244*, 678–682.
- (11) Park, Y.; Pariza, M. W. Mechanisms of body fat modulation by conjugated linoleic acid (CLA). *Food Res. Int.* **2007**, *40*, 311–323.
- (12) Collomb, M.; Schmid, A.; Sieber, R.; Wechsler, D.; Ryhanen, E.-L. Conjugated linoleic acids in milk fat: variation and physiological effects. *Int. Dairy J.* **2006**, *16*, 1347–1361.
- (13) Seekin, A. K.; Gursoy, O.; Kinik, O.; Akbulut, N. Conjugated linoleic acid (CLA) concentration, fatty acid composition and cholesterol content of some Turkish dairy products. *Lebensm. Wiss. Technol.* **2005**, *38*, 909–915.
- (14) Ledoux, M.; Chardigny, J.-M.; Darbois, M.; Soustre, Y.; Sebedio, J.-L.; Laloux, L. Fatty acid composition of French butters, with special emphasis on conjugated linoleic acid (CLA) isomers. *J. Food Compos. Anal.* **2005**, *18*, 409–425.
- (15) Brazilian legislation: Portaria do Ministério da Agricultura, Abastecimento e Reforma Agrária (MAARA) n° 146 de 07 de Março de 1996. Aprova padrões microbiológicos, físicos e químicos para leite e derivados. *Diario Of. Uniao* **1996**, Section 1 (March 11), 3977–3986.
- (16) Schripsema, J.; Verpoorte, R. Investigation of extracts of plant-cell cultures by proton nuclear-magnetic resonance spectroscopy. *Phytochem. Anal.* **1991**, *2*, 155–162.
- (17) Claridge T. D. W. *High-Resolution NMR Techniques in Organic Chemistry*; Pergamon Press: New York, 1999.
- (18) Saude, E. J.; Slupsky, C. M.; Sykes, B. D. Optimization of NMR analysis of biological fluids for quantitative accuracy. *Metabolomics* **2006**, *2*, 113–123.
- (19) Brazilian legislation: Decreto no. 1812 (Feb 8th 1996). *Diario Of. Uniao* **1996**, Section 1 (Feb 9), 2241.
- (20) Choong, Y. M.; Wang, M. L.; Chou, C. H.; Fan, J. J. Rapid method for the determination of sorbic acid, dehydroacetic acid and benzoic acid in butter, cheese, margarine and salad dressing by gas chromatography. *J. Chin. Agric. Chem. Soc.* **1999**, *37*, 166–178.
- (21) Adahchour, M.; Vreuls, R. J. J.; van der Heijden, A.; Brinkman, U. A. T. Trace-level determination of polar flavour compounds in butter by solid-phase extraction and gas chromatography–mass spectrometry. *J. Chromatogr. A* **1999**, *844*, 295–305.
- (22) Alfa Laval/Tetra Pak. Chapter 2. The Chemistry of Milk. In *Dairy Processing Handbook*; Tetra Pak Processing Systems: Lund, Sweden, 1995; pp 13–36.
- (23) Bosworth, A. W.; Prucha, M. J. The fermentation of citric acid in milk. *J. Biol. Chem.* **1910**, *8*, 479–482.
- (24) Molquentin, J.; Precht, D. Validation of a gas-chromatographic method for the determination of milk fat contents in mixed fats by butyric acid analysis. *Eur. J. Lipid Sci. Technol.* **2000**, 194–201.
- (25) Schmidt, A.; Standfuss-Gabisch, C.; Bilitewski, U. Microbial biosensor for free fatty acids using an oxygen electrode based on thick film technology. *Biosens. Bioelectron.* **1996**, *2*, 1139–1145.
- (26) Karoui, R.; De Baerdemaeker, J. A review of the analytical methods coupled with chemometric tools for the determination of the quality and identity of dairy products. *Food Chem.* **2007**, *102*, 621–640.
- (27) Bauman, D. E.; Baumgard, L. H.; Corl, B. A.; Griinari, J. M. Biosynthesis of conjugated linoleic acid in ruminants. *P. Am. Soc. Animal Sci.* **1999**, 1–15.
- (28) Wahle, K. W. J.; Heys, S. D.; Rotondo, D. Conjugated linoleic acids: are they beneficial or detrimental to health. *Prog. Lipid Res.* **2004**, *43*, 553–587.

Received for review July 28, 2007. Revised manuscript received February 5, 2008. Accepted February 12, 2008.