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Original article

Methods Assessment for a Sustainable Preparation of the Animal Fat Samples from Dairy Matrix for ^{1}H-NMR Analysis Used to Check Dairy Products Conformity[1]

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Abstract

In the context of globalization and the free movement of foodstuffs, conformity assessment has become a condition for detecting fraud affecting their quality. Therefore, fast and reliable methods of analysis are mandatory. NMR proved that can provide a fatty acid profile that characterizes the apolar matrix.

Sample preparation is a very important step that influences the results. Any used method should have none to minimum impact in the profile of the NMR spectrum.

The study was carried out to examine the influence of fat sample preparation in 1H-NMR analyses. Four methods were assessed to obtain necessary fat. We included the referential ISO 17189:2003 in this investigation in order to have a base in comparison of the results. This standard is used to calculate total fat content from butter and it is quite complex in terms of sample preparation. Only the part regarding fat separation and extraction was used. The other variants were centrifugation, direct extraction via phases destabilizing and fat drying by high temperature. Butter was produced in pilot plant by churning commercial 30% fat cream. 1H-NMR spectra were obtained using a Bruker 400 MHz spectrometer.

Sustainability, economical approach and environmental factors were the most important criteria followed to choose the right method. No important differences in the fatty acids profile of the butter fat extract were observed in NMR spectra, this conclusion offer the base of using phase destabilisation as a preparation method for this type of analyse.

Keywords: Food Quality, Dairy Products Conformity, NMR Sample Preparation.

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INTRODUCTION

In the new light of worldwide comprehensive changes, free movement of foodstuffs became a daily activity. In these terms, a sine qua non condition to detect fraud affecting food quality is formulated as conformity assessment. There is high need to develop quick and reliable analysis methods and Nuclear Magnetic Resonance (NMR) can provide a fatty acid profile that characterizes the apolar matrix in specific dairy products.

NMR is a physical method which gives the most complete structural, configuration and conformational information regarding organic compounds. Initially applied in ^{1}H protons was extended to other nuclides as ^{13}C, ^{19}F, ^{31}P, ^{17}O etc. NMR spectroscopy is based on electromagnetic energy absorption when parallel orientations goes to antiparallel orientation, passing from one energetic level to another in the same time with spin inversion according to external applied magnetic field. Phenomena appear when nucleus placed in a homogenous magnetic field is irradiated with specific frequency electromagnetic waves (Balaban et al., 1983).

Butter is the mainly fat obtained from milk and contains 80-82% milk fat, 16-17% water and 1-2% milk solids other than fat, including fat. Butter contains one of the most valuable edible fats having more than 500 different fatty acids comprising of saturated fatty acids, monounsaturated fatty acids and small amounts of polyunsaturated fatty acids. Because of this reason, we have chosen to check fatty acids profile of fat extracted from butter.

NMR is used in fast quantification in very small sample quantities. This is possible because of signal intensity which is connected to protons quantity in case of ^{1}H-NMR. Quantification is possible with ^{13}C-NMR, too, signal intensity being correlated with ^{13}C atoms from followed chemical group.

NMR signal specificity allows absolute quantifications without interference of sample impurities. Most often, it is not necessary to use reference compounds. There are studies demonstrating feasibility and limits of NMR, by exemplifying with algae toxins and butter polar fraction. Schripsema et al. (2008) assessed the polar fraction components of the butter by ^{1}H-NMR, preparing samples with deuterium oxide. This method is very useful in detection of forbidden additives, microbial deterioration by detection of increasing levels of butyric acid and fingerprinting by comparison of levels of rumenic acid. On the other side, apolar fraction of butter was studied by Fadzillah et al. (2017) in order to identify and quantify lard adulteration by ^{1}H-NMR correlated with ^{13}C-NMR. They admit that TAG (triacylgliceride) composition of lard could act as a chemical marker for Halal authentication. Sample preparation was made in deuterated methanol.

Mihalache et al. (2012) built on ^{1}H-NMR fatty acid profile of vegetable oils and developed a chemometric quantitative calculation of four classes of fatty acids: tri-unsatured, di-unsatured, mono-

unsaturated and satured. This strategy was extended to cheese fat, which is the most concentrated butter based fat.

Using chemometric equations, saturated fatty acids and short chain fatty acids ratio could be calculated very fast. We can emphasise compositional differences in maturated cheese and white fermented cheese extracted from different types of dairy products. Using NMR in this specific case of profile determination was validated with similar results obtained by standard GC analyses (Tociu et al., 2018).

Combining chemometric equation systems with ^{1}H-NMR analyse is an already applied technique in fast screening of the four groups of fatty acids from lipid profile. These quantifiable compounds are:

- saturated fatty acids (mainly C16:0; palmitic acid and C18:0; stearic acid);
- mono-unsaturated fatty acids (mainly C18:1; oleic acid);
- di-unsaturated fatty acids (mainly C18:2; linoleic acid);
- tri-unsaturated fatty acids (mainly C18:3; linolenic acid) (Chira et al., 2011).

In this respect, fat sample preparation (apolar fraction) is a very important step that influences the final fatty acids profile. Any used method should have none to minimum impact in the final NMR profile in order to be feasible and easy to apply. A very important characteristic of NMR analyse is the ability to provide information without affecting the sample, which makes it applicable for small amounts of sample requiring more than one characterisations.

Materials and Methods

This study was carried out to examine the influence of fat sample preparation in ^{1}H-NMR spectrum profile.

Butter for samples was prepared in National Research & Development Institute for Food Bioresources – IBA pilot plant. This procedure was followed in order to mitigate the risk of contamination with additives from commercial butter. Preparation started by churning commercial 30% fat cream. The typical method of butter producing kept the steps used in the traditional way of churning, see Table 1.

Table 1. Steps for churning cream to butter

Step	**Activity**
Step 1	Cream was kept in freezer for approximatively 30-40 minutes
Step 2	Cream was mixed continuously with an industrial whisk for about 20 minutes, until it showed a glossy appearance. From that point, we stopped agitation to clean the vessel walls and include all amount in the mix
Step 3	After 30-40 minutes, butter started to separate from butter milk. When butter fat started to accumulate around whisk, we stopped the agitation
Step 4	Butter was washed with iced water for 3-4 times

From 1000g of cream, 375g butter and 500ml butter milk were obtained. Butter samples were kept in freezer until experiments were conducted.

Four methods were assessed to obtain the necessary fat. These are presented in Table 2.

Table 2. Methods for ^{1}H-NMR fatty acids profile assessment

Method	**Comments**
Reference method	ISO 17189:2003 was used in this investigation in order to have a base in comparison of the results. This standard gives total fat content from butter and it is quite complex in terms of sample preparation. We took from it only the interested part about fat separation and extraction. Basically, fat is extracted from butter with n-hexane solvent followed by successive centrifugation/ washing operations
Centrifugation method	The second method was emulsion destabilising by thermic treatment (40°C), followed by separation of phases via centrifugation
Direct method	Other option was to total separate phases by thermal treatment in water bath (60°C) and carefully extraction of upper separated plan – apolar fraction with pipette
Clarifying method	The last tested method was fat drying in high temperature (90-95°C). This is the typical method used in Asian countries to obtain ghee dairy based product

Centrifuge used is an Ohaus Frontier 5706. Samples were processed in it with 4500rot/min at 40°C for 15 min. For mixing with solvent we used a vortex stirrer.

NMR sample was prepared with deuterated chloroform (CDCl3, min. 99.8%) to the dilution of 2:8 (v/v). Four samples were analysed in 5mm NMR tubes (Wilmad, Vineland, NJ, USA). The ^{1}H-NMR spectra of the fats extracted from butter were run on a Bruker Avance III, 400 MHz spectrometer, operating in a 9.4T electromagnetic field. This corresponds to a resonance frequency of 400.13 MHz for ^{1}H nucleus. Magnet is equipped with a direct detection four nuclei probe head and field gradient on z axis. We used for ^{1}H-NMR spectra typical parameters as 45^{0} pulse without attenuated power, 2.05s acquisition time, 6.4 KHz spectral window, 16 scans, 26K data points, 1s relaxation time. FID was not processed prior to Fourier transformation. The ^{1}H-NMR spectra acquisition time was approximatively 2 minutes and taken with IconNMR, Bruker. All spectra were processed with TopSpin and MestreNova.

Results and Discussions

Separated fats obtained through above presented methods where analysed by ^{1}H-NMR spectroscopy. Lipid profile and long chain saturated fatty acids/ short chain saturated fatty acids are presented in Table 3 and Table 4. We processed spectra with Topspin and MestreNova for comparison, where k is a constant, s are the short-chain saturated fatty acids, y poly-unsaturated fatty acids, z mono-unsaturated fatty acids and x long-chain saturated fatty acids.

Table 3. Integrated with TopSpin

Sample	**k**	**s**	**y**	**z**	**x**	**R**
ISO 17189:2003	1.09	0.11	0.04	0.23	0.63	5.94
40Cdgr/ centrifugation/ sampling	1.08	0.11	0.04	0.23	0.63	5.92
60Cdgr/ sampling	1.07	0.11	0.04	0.23	0.63	5.96
90-90Cdgr/ sampling	1.09	0.11	0.04	0.21	0.64	6.09
Average	***1.08***	***0.11***	***0.04***	***0.22***	***0.63***	***5.98***
Standard deviation	**0.01**	**0.00**	**0.00**	**0.01**	**0.01**	**0.08**

Table 4. Integrated with MestreNova

Sample	**k**	**s**	**y**	**z**	**x**	**R**
ISO 17189:2003	1.22	0.06	0.04	0.22	0.68	10.77
40Cdgr/ centrifugation/ sampling	1.22	0.07	0.04	0.22	0.68	10.38
60Cdgr/ sampling	1.21	0.07	0.04	0.21	0.68	9.89
90-90Cdgr/ sampling	1.21	0.07	0.03	0.22	0.67	9.41
Average	***1.21***	***0.07***	***0.04***	***0.22***	***0.68***	***10.11***
Standard deviation	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.59**

As it can be noticed, even if quantitatively there are differences between Topspin and MestreNova, in terms of standard deviation it can be assumed that these 4 methods provide sampling materials with similar to identical lipid profile.

This statement is better proved in the graphs below.

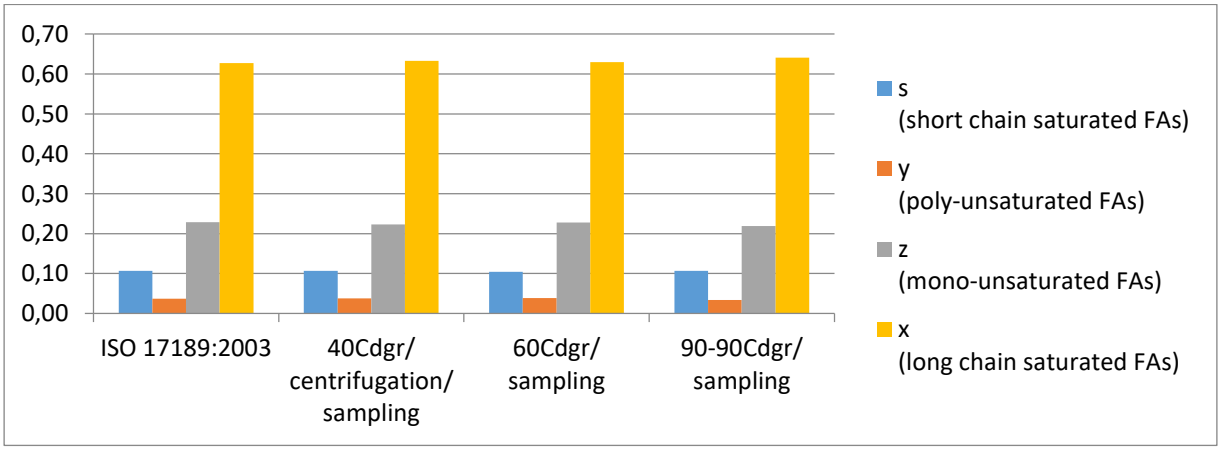

Figure 1. Fatty acids profile processed with TopSpin

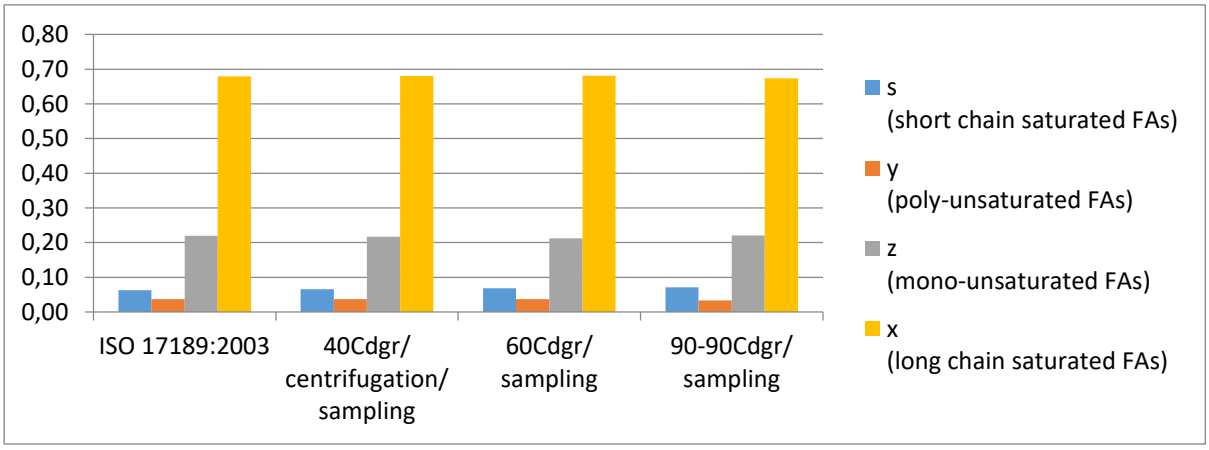

Figure 2. Fatty acids profile processed with MestreNova

Chemometric results were obtained by integrating ^{1}H-NMR spectra and applying specific equations for butter (Tociu et al., 2018), where R is ration between long chain saturated fatty acids and short chain saturated fatty acids. This ratio could give information regarding specific fatty acids for non-milk fats.

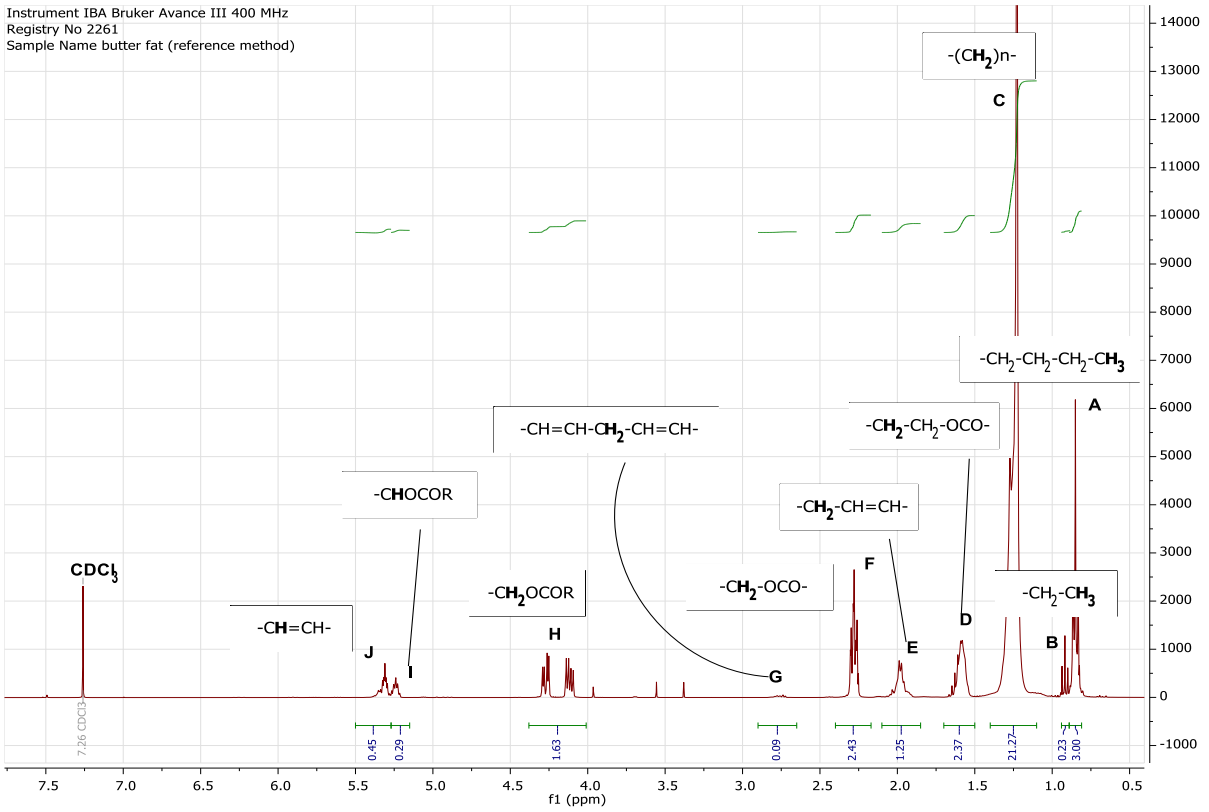

Figure 3. Spectra of butter fat obtained via reference method ISO 17189:2003 with peak assignments

Nuclear Magnetic Resonance could be used to quantify lipids from foodstuff in a non-destructive way. It is one of the most popular standardized AOAC methods for measuring total fat content, by

measuring melting curves of these, more specific of density variation of solid fat vs. liquid fat. Total fat content is made by low resolution NMR (Nielsen, 2010).

In case of milk, a very complex constituent considered a complete biological fluid for human diet, ^{1}H-NMR wasn't applied directly as a complete scan. Milk contains emulsified fats, proteins in colloidal state, minerals and sugars solubilised in a big quantity of water, over 85%. Signals given by the protons from interested compounds go to baseline because water signal is very big. On the other hand, milk is a very complicated emulsion and can't be obtained a complete dissolving of all components, many of them being trapped in emulsion. So, almost all ^{13}C-NMR and ^{31}P-NMR experiments were started with a pre-treatment (triacylglycerol extraction, remove of fats and metallic ions and/or specific adjusting of pH). ^{31}P-NMR allowed study of phospholipids composition in different storage conditions and ^{13}C-NMR gave differentiation of milk by donor species. More and more NMR is applied in geographical fingerprinting coupled with chemometric methods. A good discrimination of milk samples was realised by building of fatty acids profile, sugars, amino acids and organic acids from polar fraction (water soluble). There is only one example of ^{1}H-NMR applied directly, without pre-treatment on milk with assignment of signals of butyric acid, mono and poly-unsaturated fatty acids, lactose, citrate, N-acetyl-carbohydrates and lecithin in bidimensional experiments (Mannina et al., 2012).

Our results show very small differences in lipid profile obtained by the four extraction methods.

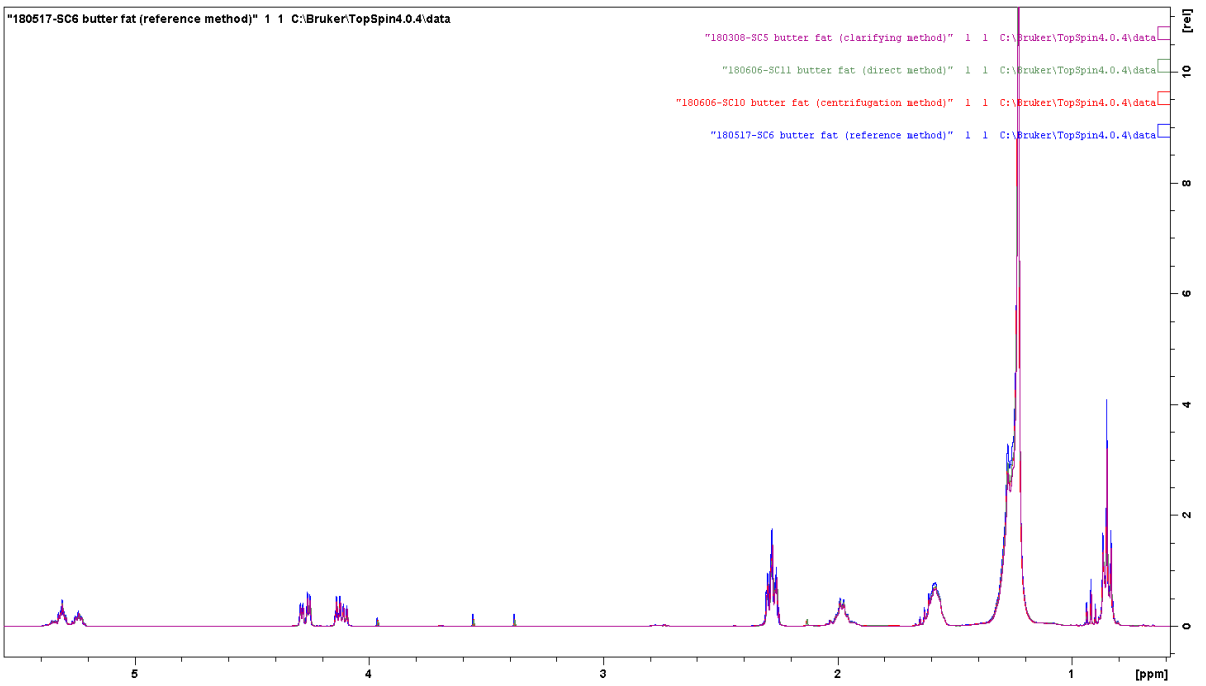

Figure 4. Stacked spectra of butter fat (reference, centrifugation, direct and clarifying methods)

We can observe a very good super-positioning of spectra, which goes to a high acceptance of all four methods for build the same lipid profile. The few differences are not in the interested peaks area and come from $CDCl_3$ additives or impurities.

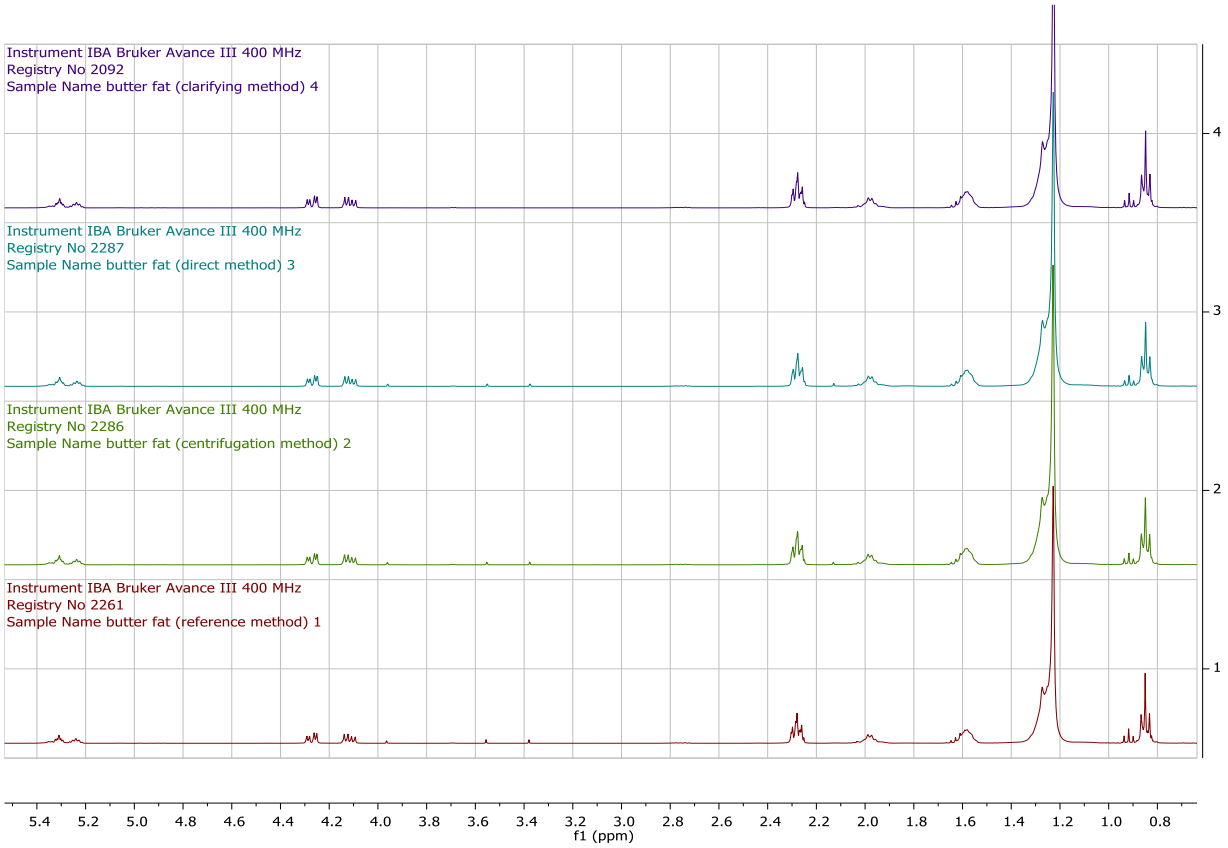

Figure 5. Side-by-side 1H-NMR spectra comparison of the four extraction methods

Taking the four methods side-by-side we can expose the pros and cons of these.

Table 5. Butter fat extraction for ^{1}H-NMR assessment

Method	**Pros**	**Cons**
Reference method ISO 17189:2003	generally accepted as a standard provide sample without impurities	Time consuming Not sustainable (energy consumption via centrifugation and solvent evaporation) Not friendly with environment (uses toxic solvents as hexane)
Centrifugation method 40Cdgr/ centrifugation/ sampling	provide sample without impurities	Shorter time than reference method, but still time consuming Energy consuming by centrifugation High attention when sampling not to contaminate sample with polar phase Not accepted as a standard
Direct method 60Cdgr/ sampling	Fast sampling Low energy consuming Small impurities are not affecting lipid profile	High attention when sampling not to contaminate sample with polar phase Not accepted as a standard
Clarifying method 90-90Cdgr/ sampling	After filtration, can provide sample without impurities Higher time for storage of the sample because of complete removal of proteins and water	Shorter time that reference method, but still time consuming Energy consuming by heating Not accepted as a standard

Conclusions

Sustainability, economical approach and environmental factors were the most important criteria followed to choose the right method. Big differences in the fatty acids profile of the butter fat extract were not observed in NMR spectra, therefore this conclusion gives proof for using phase destabilisation as a preparation method for this analyse.

Results shows very low differences between lipid profile of fats made by the four extraction methods. If case we need only determination of main classes of fatty acids, lipid profile, we can surely use direct method. It eliminates need of organic solvents, being more sustainable and environmentally friendly, faster and involving less energy consumption.

In the case of a more detailed analyse, we should use reference method, generally accepted as international standard.

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