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Determination of water self-diffusion coefficient in complex food products by low field ^{1}H PFG-NMR: comparison between the standard spin-echo sequence and the T_1-weighted spin-echo sequence

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Abstract

In 1990, Van Den Enden et al. proposed a method for the determination of water droplet size distributions in emulsions using a pulsed-field-gradient nuclear magnetic resonance (PFG-NMR) T_1-weighted stimulated-echo technique. This paper describes both the T_1-weighted spin-echo sequence, an improved method based on this earlier work, and, the standard PFG spin-echo sequence. These two methods were compared for water self-diffusion coefficient measurement in the fatty protein concentrate sample used as a 'cheese model.' The transversal and longitudinal relaxation parameters T_1 and T_2 were determined according to the temperature and investigated for each sample; fat-free protein concentrate sample, pure anhydrous milk fat, and fatty protein concentrate sample. The water self-diffusion in fat-free protein concentrate samples followed a linear behavior. Consequently, the water self-diffusion coefficient could be easily characterized for fat-free protein concentrate samples. However, it seemed more complicated to obtain accurate water self-diffusion in fatty protein concentrate samples since the diffusion-attenuation data were fitted by a bi-exponential function. This paper demonstrates that the implementation of the T_1-weighted spin-echo sequence, using the different T_1 properties of water and fat phases, allows the accurate determination of water self-diffusion coefficient in a food product. To minimize the contribution of the ^{1}H nuclei in the fat phase on the NMR echo signal, the fat protons were selectively eliminated by an additional 180° pulse. This new method reduces the standard errors of diffusion data obtained with a basic spin-echo technique, by a factor of 10. The effectiveness of the use of the T_1-weighted spin-echo sequence to perform accurate water self-diffusion coefficients measurement in fatty products is thus demonstrated.

Keywords: ^{1}H NMR; Low-field NMR; Self-diffusion; Relaxation; Food products

1. Introduction

Pulsed-field-gradient spin-echo NMR (PFG-NMR) is a powerful method for studying molecular diffusion [1–3]. The NMR pulsed-field gradient technique represents a versatile tool for studying transport phenomena of molecules such as water, lipids or sugars in porous media such as food gels, wheat starch gels [4–6], gellan gum gels [7], cheeses [8], and bread matrixes [9]. Moreover, structural information can be obtained from a careful analysis of the system in which the water is diffusing such as a micro-emulsion [10].

The classic method for self-diffusion coefficient determination was firstly proposed by Stejskal and Tanner [11]. The determination is carried out by the acquisition of an echo, either of spin or stimulated. If acquisition is done with a high field NMR spectrometer, then the acquisition of the echo is followed by the Fourier Transform in order to identify the molecule according to the chemical shift. The self-diffusion coefficient is then directly estimated from the variation of the surface (or the intensity) of the peak according to the gradient

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amplitude. The main advantages of using high field spectrometers are high sensitivity and selectivity. Then the self-diffusion of several molecules can be determined simultaneously [8,12,13]. If most of the self-diffusion studies are done with high-field spectrometers, some are performed with low-field permanent magnet NMR spectrometers, which present the advantages of being low-cost and easy-to-handle on a laboratory bench [14]. Nevertheless, this system poses intrinsic limitations on sensitivity and resolution. Because of the low intensity and low homogeneity of the magnet field, the chemical shift cannot be used anymore to discriminate the different molecules. The only solution, in this case, is to use the T_1 and T_2 relaxation parameters to select the signal from the different molecules. This could be easily achieved for a high water content product [15,16] or for a high fat content product [9] because the relaxation of water or fat protons could be easily distinguished or neglected. For more complex mixtures, characterized by a multi-exponential relaxation decay, careful attention should be paid to the echo time choice. Either modification of the standard spin-echo or stimulated-echo sequence could be chosen. Let us consider a binary system with each component characterized by its own self-diffusion coefficient and relaxation time parameters. According to the self-diffusion coefficient values, two cases should be considered. If the self-diffusion coefficients differ by a factor of more than 10, then the variation of the signal intensity versus the pulsed-field gradient strength would be analyzed by a bi-exponential function. So the self-diffusion coefficient of each component should be calculated. If the self-diffusion coefficient values are close, then more sophisticated pulse sequences should be implemented [17,18]. For example, Van den Eden et al. [19], removed the contribution of the oil protons in water-in-oil (w/o) emulsions by an additional 180° pulse before the first 90° pulse of the standard stimulated-echo sequence. For sucrose-water mixture, the contribution of the sucrose proton relaxation to the echo intensity could be neglected by an increase of the echo-time [16]. Surprisingly, if the T_1-weighted spin-echo sequence has been generally accepted for measuring selectively the water self-diffusion coefficient in fatty products, the efficiency of this sequence versus the standard spin-echo sequence has never been discussed. For example, the delay between the first 180° pulse (τ_{null}) is generally calculated from the spin–lattice relaxation time T_1 of the fat proton according to the relationship $\tau_{null} = T_1 \times \ln 2$ [20]. This relationship is only valid if the fat proton relaxation could be fitted with a mono-exponential function. This situation is not often met in food products, because of the complex chemical composition of the fat, the relaxation is better described by a multi-exponential distribution of relaxation time. Consequently, a direct estimation of the delay (τ_{null}) is not possible.

The purpose of this study was to compare (i) the standard spin-echo sequence described by Stejskal and Tanner and (ii) the T_1-weighed spin-echo sequence for determination of the water self-diffusion coefficient in complex food products. Both sequences were evaluated on model food products prepared from protein concentrate and anhydrous milk fat emulsion. The water and fat concentrations were adjusted in order to mimic real food product composition such as cheese.

This study was done with two water protein concentrated solutions, one with fat content and one without. The effect of the temperature was included in order to modify the contribution of fat protons in the NMR signal intensity without modifying the initial structure of the product.

2. Materials and methods

2.1. Materials

Anhydrous milk fat (AMF) and whey protein powder (Protarmor 80 C5866) were provided by Fléchard (La Chapelle d'Andaine, France) and the ARMOR Protéines S.A.S. company (Saint Brice en Cogles, France), respectively. The dairy concentrated protein solution was obtained by ultrafiltration and provided by Les Fromageries Bel (Vendôme, France). The moisture and protein contents are given in Table 1. This protein solution is referred to as 'fat-free protein concentrate.' Sodium azide (Merck-Schuchardt, France) was added as a preservative for all solutions used.

The solutions were prepared in distilled water–sodium chloride (NaCl) solution (Merck Eurolab, France). A hydrochloric solution (HCl) (PRS Panreac Quimica SA, Espagne) was used for pH adjustment. The solvents, hexane and isopropanol (Carbo Erba Reagenti, Val de Rueil, France) were used for fat extraction.

The preparation of the fatty sample is divided into two steps: first the preparation of an emulsion stabilized by dairy proteins and second the incorporation of this emulsion to the fat-free protein concentrate in order to obtain a reconstituted fatty dairy solution, referred to later as "fatty protein concentrate."

Table 1
Chemical composition of the fat-free protein concentrate and of the fatty protein concentrate

Composition[a]	Fat-free protein concentrate (g/100 g of product)	Fatty protein concentrate (g/100 g of product)
Dry matter (DM)	21.7	29
Total proteins (TNM)	15.4	10.2
Fat	0	14.7

[a] TNM, total nitrogen matter; DM, dry matter.

2.2. Emulsion preparation

A whey protein solution was prepared by adding 2.5 wt% whey protein powder to a NaCl–water solution (80 mM) and 0.02 wt% sodium azide was added. For a complete dispersion of the proteins, the solution was stirred slowly overnight at 4 °C. Afterwards, the pH was adjusted to 6.7 with 1 M HCl.

AMF was heated at 60 °C for 20 min in order to melt all existing crystals. AMF and protein solutions were mixed at 60 °C to give 40% (v/v) fat in the final emulsion. Emulsion premix was prepared using the rotor stator system Polytron PT 600 (Kinematica, Littau, Switzerland), equipped with a 30 mm diameter head (PT-DA 3030/4T) working at 20,000 rpm for 2.5 min. To limit the emulsion heating and to avoid air incorporation into this emulsion, the emulsion premix was carried out in a high-turbulence pot plunged into cold water. Homogenization of the coarse emulsion was then achieved using a pressure of 50 bars for 30 min, with a high-pressure valve homogenizer (Stansted Fluid Power A0812W Stansted, United Kingdom).

2.3. Fatty protein concentrate preparation

Fat-free protein concentrate and emulsion were mixed at 60 °C to give 38% (v/v) emulsion in the final fatty protein concentrate model using a rotor stator system (Ultra Turax T25 basic, IKA Werke, Stouphen, Germany) at 11,000 tr min^{-1} for 1.5 min.

2.4. Chemical composition

For all samples, the water, protein and fat contents were checked (Table 1). Dry matter for each dairy sample was obtained after drying in an oven at 104 ± 2 °C and estimated by weighing the residues. The proteins content was determined by the Kjeldahl method (FIL20B-IDF 20-1/ISO 8968-1).

The fat content was obtained by cold extraction performed with a mixture (3/2, vol/vol) of hexane and isopropanol. Then, following the separation of the upper phase after centrifugation, solvents and water were eliminated, by maintaining successively the extracted fat under vacuum in a rotovapor and overnight in a lyophilizer, respectively. Then they were weighed.

2.5. NMR measurements

The samples were taken (10 mm height, i.e., approximately 0.5 g, corresponding to the homogeneity area of the probe, 8 mm diam.) and put into sealed NMR tubes.

All NMR analyses were carried out with a 0.47 T NMR spectrometer (The Minispec; Bruker SA, F-67166 Wissembourg, France) operating at 20 MHz for protons and equipped with a Pulsed Gradient Unit (NMS GU200 ($G \leqslant 4\,T\,m^{-1}$)). The NMR probe was heated or cooled by a constant gas flow (air or liquid nitrogen) delivered by the variable temperature unit B-VT3000 equipped with a temperature controller (Eurotherm mode 902). Before NMR measurements, the tubes were placed in a cryostat (Julabo FP50-HP, Julabo Labortechnik GmbH, Germany). The time needed for thermal equilibrium (10 min) and the temperature was controlled with a series of samples equipped with a copper–constantan thermocouple placed at the centre of the sample. The measurement temperatures were 5, 20, 30, and 40 ± 0.5 °C.

The sample temperature was monitored periodically by inserting a copper–constantan thermocouple in the NMR tube filled with same product. This sample was not included in the NMR experimental planning.

2.5.1. Spin–spin and spin–lattice relaxation measurements

The spin–spin relaxation times T_2 were measured using the Carr-Purcell-Meiboom-Gill (CPMG) sequence. The recuperation delay was TR = 1.50 s at 5 °C, 2 s at 20 °C and 3 s for 30 °C and 40 °C for AMF and TR = 4 s at 5 and 20 °C and TR = 5 s at 30 and 40 °C for fat-free protein concentrate and TR = 5 s at 5 and 20 °C and TR = 6 s at 30 and 40 °C for reconstituted fatty sample. The CPMG sequence was composed of 845 echoes, the 90–180° pulse spacing (τ), varied between 0.1 and 1 ms according to the product and temperature. The spin–lattice relaxation times T_1 were obtained using the saturation-recovery (SR) pulse sequence. The SR sequence was constituted by 100 points, between 30 ms and the recuperation time TR, defined according to the temperature and the sample.

2.5.2. NMR relaxation decay adjustment

In order to avoid a badly adjusted decay curve relaxation, two different fitting methods were compared: discrete methods such as the Levenberg Marquardt procedure [21] and the continuous methods such as the maximum entropy method (MEM) [22].

2.5.3. Determination of diffusion coefficients using PFG-NMR

The strengths of the gradient pulses were calibrated with pure water at 25 °C ($D = 2.30 \times 10^{-9}\,m^2\,s^{-1}$). The experiments were conducted at various temperatures: 5, 20, 30, and 40 °C. The echo intensity was the average of three repetitive scans with a recycling delay of 5 s. Self-diffusion experiments were performed using two sequences: a spin-echo sequence described by Stejskal and Tanner [11] and a T_1-null inversion recovery sequence (Fig. 1).

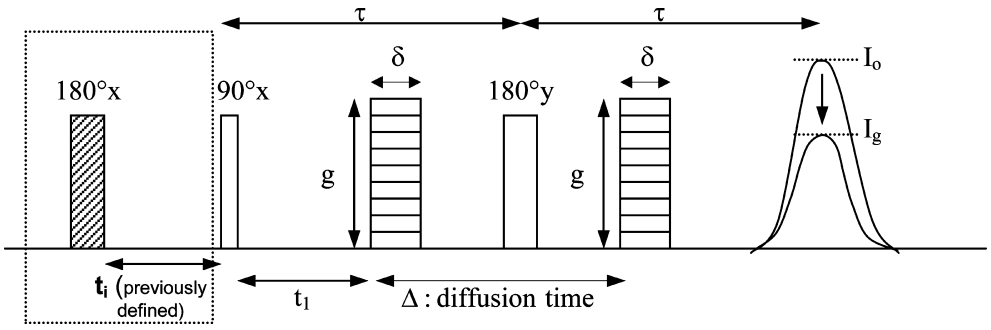

Fig. 1. The standard spin-echo and the T_1-weighted spin-echo sequences. A spin-echo NMR signal is generated from a sequence consisting of 90°x and 180°y radio-frequency pulses and its intensity is modulated by two-field gradient pulses g. TE is the echo time and corresponding to 2τ. Recuperation time TR = 5 s, inter-pulse spacing time $\tau = 7.5$ ms, diffusion time $\Delta = 7.5$ ms, width of the field gradient pulses $\delta = 0.5$ ms and the delay (t_1) between the first pulse RF and the first gradient pulse was fixed at 1 ms. In the experiments, g was incremented from 0.4 to 3.3 T m^{-1}. For the T_1-weighted spin-echo sequence, an additional 180°x radio-frequency pulse was included (diagonally shaded) and the parameters are identical to the spin-echo sequence. The pre-delay t_i is experimentally defined for each temperature.

The diffusion coefficients were obtained using:

$$I(\delta,\Delta,g,T_2,T_1,2\tau,\mathrm{TR}) = I_0\exp\left(-\frac{2\tau}{T_2}\right)\left(1-\exp\left(-\frac{\mathrm{TR}}{T_1}\right)\right)\times\exp\left[-\gamma^2g^2\delta^2\left(\Delta-\frac{\delta}{3}\right)D\right], \quad (1)$$

where $I(\delta,\Delta,g,T_2,T_1,2\tau,\mathrm{TR})$ and I_0 are the echo intensities of NMR signal in the presence of gradient pulses of strength g and in absence of gradient pulses, respectively. γ is the gyromagnetic constant for ^{1}H ($\gamma = 2.6752\times10^8$ rad T^{-1} s^{-1} for protons), δ is the duration of the z gradient pulse, and Δ is the time interval between the gradient pulses. The delay between the first 90° pulse and the first gradient pulse t_1, was fixed at 1 ms. τ is the time interval between the successive RF pulses and TR the recuperation time. T_2 and T_1 were, respectively, the spin–spin and spin–lattice relaxation times. To eliminate the effect of spin relaxation, the diffusion coefficient determination was performed by keeping δ and Δ constant and varying g. In our experiments, g was incremented from 0.4 to 3.3 T m^{-1}. Then the echo intensity in the presence of gradients divided by the echo intensity without application of gradients, i.e., the attenuation of the NMR spin-echo signal intensity, became:

$$\frac{I_g}{I_0} = \exp[-kD], \quad (2)$$

where k is defined as $k = -\gamma^2g^2\delta^2(\Delta-(\delta/3))$. As a result, the self-diffusion coefficient of H_2O (D_{water}) was equal to the slope calculated from a regression analysis of the data sets $(\ln(I_g/I_0),k)$ using Eq. (2). This approach is valid when the echo intensity could be attributed to the water proton relaxation only. If the echo intensity became dependent on both water and fat relaxation, the relaxation parameters of each component should be considered in the equation given the self-diffusion coefficient. In the study of multiple component diffusion, the echo attenuation observed is dependent on $\gamma^2g^2\delta^2\Delta(2)$. So, for a fixed τ, TR, $T_{2\mathrm{water}}$, $T_{2\mathrm{fat}}$, $T_{1\mathrm{water}}$, $T_{1\mathrm{fat}}$, γ, the echo intensity was given by the equation:

$$I(\delta,\Delta,g) = I^*_{\mathrm{water}}\exp[-kD_{\mathrm{water}}] + I^*_{\mathrm{fat}}\exp[-kD_{\mathrm{fat}}], \quad (3)$$

where, for TR $\gg T_{1\mathrm{fat}}$ and $T_{1\mathrm{water}}$, one has:

$$I^*_{\mathrm{water}} = \exp\left(-\frac{2\tau}{T_{2\mathrm{water}}}\right) \text{ and } I^*_{\mathrm{fat}} = \exp\left(-\frac{2\tau}{T_{2\mathrm{fat}}}\right), \quad (4)$$

where $T_{2\mathrm{water}}$, $T_{1\mathrm{water}}$, $T_{2\mathrm{fat}}$, and $T_{1\mathrm{fat}}$ were, respectively, the spin–spin and the spin–lattice relaxation times of water and fat and D_{water} and D_{fat} were the respective water and fat self-diffusion coefficients.

Finally,

$$\frac{I_g}{I_0} = \%P_{\mathrm{water}}\exp[-kD_{\mathrm{water}}] + \%P_{\mathrm{fat}}\exp[-kD_{\mathrm{fat}}] \quad (5)$$

with $\%P_{\mathrm{water}} = I^*_{\mathrm{water}}/(I^*_{\mathrm{water}}+I^*_{\mathrm{fat}})$, the relative water echo signal intensity weighted by the water relaxation parameters and $\%P_{\mathrm{fat}} = I^*_{\mathrm{fat}}/(I^*_{\mathrm{water}}+I^*_{\mathrm{fat}})$, the relative fat echo signal intensity weighted by the fat relaxation parameters.

The calculation of the water self-diffusion coefficient from Eq. (5) could be performed using a bi-exponential fitting.

However, if only water self-diffusion coefficient is required, an appropriate choice of the sequence parameters should be used. In this work, a T_1-null inversion recovery sequence was evaluated.

So, for the implementation of the T_1-weighed spin-echo sequence, a 180°x pulse was added before the first 90° pulse (Fig. 1). The delay between the 180° and the 90° pulse was defined by t_i, the inversion time.

For this specific sequence, Eq. (4) could be modified to include the signals arising from each fat and water component, and became:

$$I^{\Theta}_{\text{water}} = \exp\left(-\frac{2\tau}{T_{2\text{water}}}\right)\left(1-2\exp\left(-\frac{t_i}{T_{1\text{water}}}\right)\right)$$
$$\text{and}\quad I^{\Theta}_{\text{fat}} = \exp\left(-\frac{2\tau}{T_{2\text{fat}}}\right)\left(1-2\exp\left(-\frac{t_i}{T_{1\text{fat}}}\right)\right). \tag{6}$$

So, if $t_i = T_{1\text{fat}}\ln 2$, the relationship $(1-2\exp(-(t_i/T_{1\text{fat}})))$ became null and Eq. (3) or Eq. (5) was simplified as:

$$I(\delta,\Delta,g) = I^{\Theta}_{\text{water}}\exp[-kD_{\text{water}}]. \tag{7}$$

The water self-diffusion coefficient was determined from a linear regression of the logarithmic echo intensity attenuation versus k using Eq. (7).

The choice of each inversion time t_i was experimentally determined for each temperature and these values of t_i were detailed and discussed in the results part.

The values of the delays Δ and δ used in the water self-diffusion measurements were 7.5 and 0.5 ms, respectively; while δ was 2 ms for the fat self-diffusion measurements. At 5 °C, the delay Δ was decreased to 5.5 ms for fat self-diffusion coefficient determination, because of the combined-effect of the temperature on the relaxation time and on the decrease of the NMR liquid signal intensity.

3. Results and discussion

3.1. Relaxation time measurements

3.1.1. Characterization of fat-free protein concentrate NMR signals

Table 2 summarizes the fat-free protein concentrate NMR spin–spin and spin–lattice relaxation parameters obtained from the Marquardt method at different temperatures. The results obtained from MEM method were in good agreement with the results from Marquardt adjustment, both for the spin–lattice and spin–spin relaxation. Whatever the temperature, the T_2 and T_1 were both well fitted with a single exponential. The T_2 values varied from 30 to 37 ms and the T_1 values varied from 393 to 592 ms between 5 and 40 °C. Note that the water relaxation time increased slightly with the temperature, especially for T_2.

Table 2
Mono-exponential Marquardt's fitting of the spin–spin and spin–lattice relaxation decay curves and water self-diffusion coefficients for fat-free protein concentrate obtained at 20 MHz, respectively, T_2 (ms), T_1 (ms), and D_{water} ($\times 10^9$ m^2 s^{-1}) at different temperatures

Temperature (°C)	T_2 (ms)	T_1 (ms)	D_{water} ($\times 10^{-9}$ m^2 s^{-1})
5	29.7 (±0.1)	393 (±2)	0.807 (±0.011)
20	32.7 (±0.1)	477 (±2)	1.283 (±0.008)
30	34.7 (±0.1)	535 (±3)	1.674 (±0.013)
40	37.5 (±0.2)	592 (±2)	2.131 (±0.004)

Each NMR value was the mean of three separate acquisitions. Standard errors are given between brackets.

As demonstrated previously [23], this component could be attributed to the water proton and exchangeable proton from protein and lactose. However, the contribution of the exchangeable proton to water relaxation was negligible at this concentration.

3.1.2. Characterization of anhydrous milk fat NMR signals

The AMF relaxation decay curves T_2 (Fig. 2A) and T_1 (Fig. 2B) could not be fitted with a single exponential as demonstrated by the MEM method. A complex distribution of relaxation values was observed independently of the temperature. Moreover, because of this complex distribution, a discrepancy between MEM and Marquardt adjustments was observed. The distribution of relaxation times could be explained by the complexity of chemical composition of AMF. Indeed, AMF is a mixture of several triacylglycerols. This complexity originates from the extreme diversity of its fatty acids with different aliphatic chain lengths, different position and number of unsaturations and different branches. All these factors induced multi-exponential behavior [24].

3.1.3. Characterization of fatty protein concentrate NMR signals

The spin–spin relaxation time distribution for the fatty protein concentrate was characterized by a bimodal distribution, whatever the temperature (Fig. 2C). At 20 °C, a first peak with a low population and a T_2 value around 10 ms and a second peak with a higher population and a T_2 value at 45 ms were observed. According to previous NMR studies on cheese [25], the two peaks could be explained by the liquid fat proton relaxation and the water proton relaxation as first approximation.

Indeed, the water proton relaxation T_2 values for fat-free protein concentrate and fatty-protein concentrate were close, 33 and 45 ms, respectively. The difference was explained by the change in water content. Moreover, when the temperature increased, the T_2 variation of the water relaxation was well in agreement with the variation observed for the fat-free protein concentrate, respectively, 25.9% and 26.3% for fatty and fat-free protein concentrate. Below 20 °C, the fat relaxation was shorter than the water relaxation or superimposed with the water relaxation. Above 20 °C, the peak with a mean T_2 value of 140 ms at 30 °C, and 200 ms at 40 °C could be attributed to the fat relaxation.

Nevertheless, it was not possible to separate quantitatively the fat proton and the water proton signal from each other. The spin–spin fat proton relaxation covered a wide range of T_2, which was superimposed with the water proton relaxation, and prevented any discrimination.

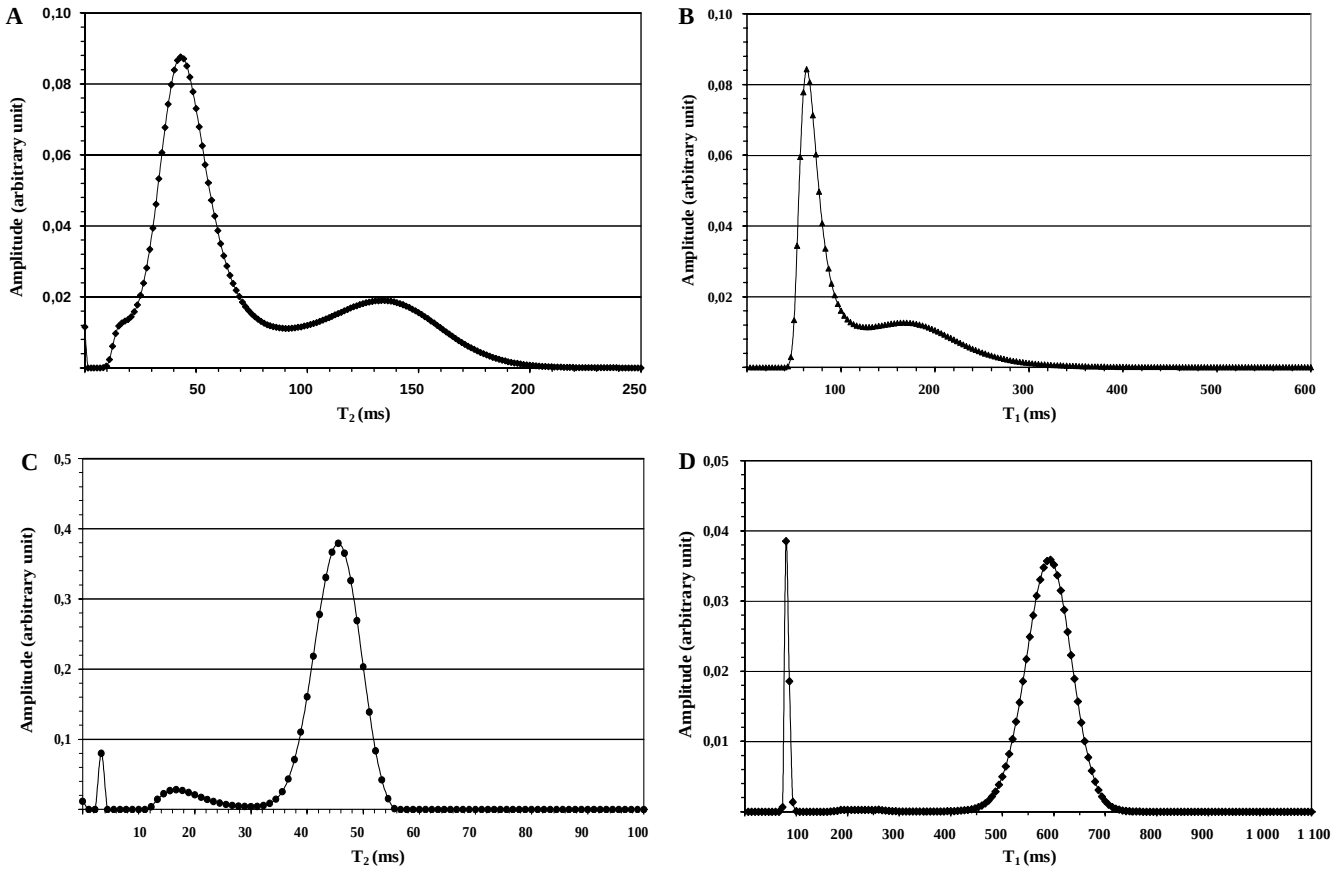

Fig. 2. Spin–spin (A) and spin–lattice (B) relaxation time distribution obtained by MEM for the anhydrous milk fat sample at 20 °C. Spin–spin (C) and spin–lattice (D) relaxation time distribution obtained by MEM for the fatty protein concentrate samples at 20 °C.

Consequently, whatever the value of the echo time in the standard PFG spin-echo sequence, the echo intensity was always dependent on fat and water proteins content. So, water self-diffusion coefficient would not be measured without any perturbations from fat protons.

Because of the larger difference between T_1 relaxations of water in fat-free protein concentrate and of AMF, a better discrimination was possible at any temperature. The T_1 distribution was bimodal (Fig. 2D). The first T_1 value corresponded to the fat relaxation and the second T_1 value corresponded to the water relaxation. As already explained when considering T_2, the T_1 changes for water between fat-free and fatty protein concentrate were explained by the water content variation.

3.2. Diffusion results

3.2.1. Water self-diffusion determination from standard spin-echo sequence for fat-free protein concentrates

The logarithmic plot of the echo attenuation as a function of k is given in Fig. 3 for the fat-free protein concentrate. A straight line was observed whatever the temperature. This demonstrates that the water molecules are not confined or restricted in compartments. So they can diffuse freely over a length, given by the relationship: $\langle r_z^2 \rangle = 6D\Delta$. According to the water self-diffusion coefficients (Table 2), the length in three-dimensional diffusion corresponded to a traveled distance of between ~ 6 and $10\,\mu m$ by the water molecule for a temperature range between 5 and 40 °C. A difference was observed between the pure water self-diffusion and the water self-diffusion in the fat-free protein concentrate. This reduction of the water self-diffusion in fat-free protein concentrate compared to pure water self-diffusion has been already observed in dairy products such as casein dispersions and gels [26] and cheese [8]. The decrease of the water diffusion was explained by the obstruction effect induced by the dairy protein as mainly casein micelle [26].

3.2.2. Water and fat self-diffusion determination from standard spin-echo sequence for fatty protein concentrates

The self-diffusion coefficient in the pure anhydrous milk fat (D_{AMF}) for each temperature is given in Table 3. As expected the fat self-diffusion was very low compared to the water self-diffusion and increased with temperature. The fat self-diffusion coefficient value, we obtained at 30 °C, was of the same order as the one measured for the fat diffusion in bulk milk fat by Callaghan et al. [8], i.e., $\sim 1.1 \times 10^{-11}\,m^2\,s^{-1}$. However, it appeared that the

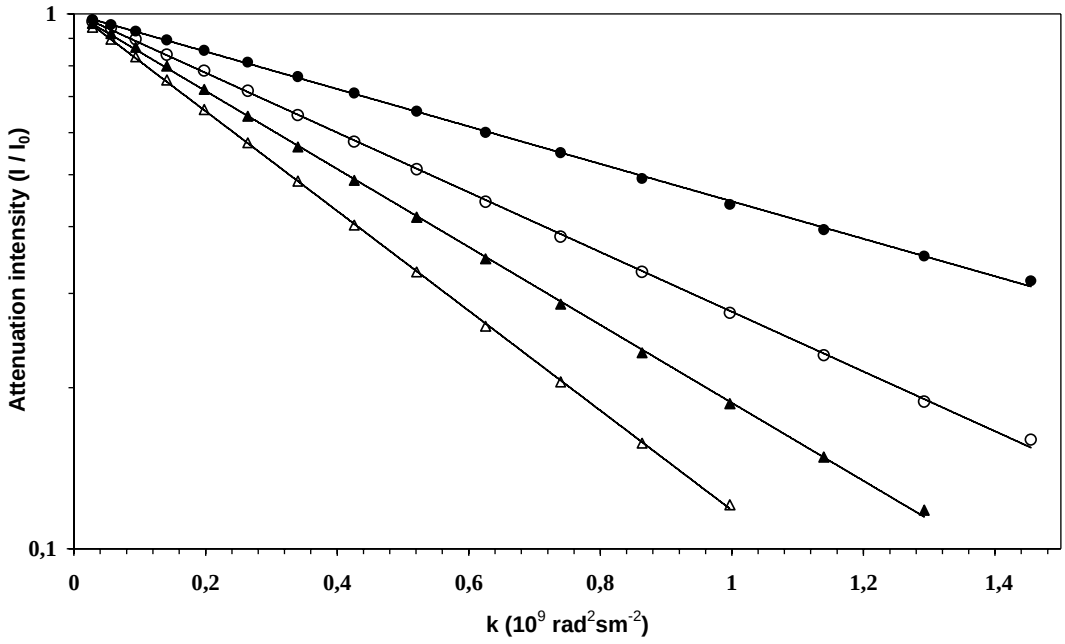

Fig. 3. Experimental echo attenuations versus k ($k = [\gamma^2\delta^2 g^2(\Delta - \delta/3)]$) for water in the fat-free protein concentrate. The lines are the results of the fit of Eq. (2) to the data. Standard errors were less than 1.5% for all the measurements. The NMR experiments were performed at different temperatures: 5 °C (●), 20 °C (○), 30 °C (▲) and 40 °C (△).

Table 3
Fat self-diffusion coefficients obtained at 20 MHz at different temperatures

Temperature (°C)	D_{fat} ($\times 10^{-9}$ m^2 s^{-1}) (in pure AMF)
5	0.008 (±0.003)
20	0.0097 (±0.0003)
30	0.01597 (±0.00009)
40	0.0246 (±0.0005)

Each NMR diffusion value was the mean of three separate acquisitions. Standard errors are given between brackets.

value of the fat self-diffusion coefficient measured experimentally at 5 °C seemed erroneous because no difference can be observed for D_{AMF} between 5 and 20 °C. We obtained a diffusion coefficient value equal to 8×10^{-12} m^2 s^{-1} for fat whereas the correct value estimated from the diffusion activation energy on the temperature range (20–40 °C) is lower and equal to 4.5×10^{-12} m^2 s^{-1}. The difference could be explained by the limits of the apparatus. When the fat was partly crystallized, the self-diffusion was so slow that stronger gradient pulses were required to perform an accurate self-diffusion coefficient measurement.

The experimental echo attenuations versus k for fatty protein concentrate at different temperatures are shown in Fig. 4. A non-linear behavior was observed. The deviation from linearity increased with temperature.

The non-linearity could be explained either by anomalous diffusion when distribution of self-diffusion coefficients is suspected [15,27] or by the effect of fat protons on the echo intensity. Callaghan et al. [8] have observed no curvature for the echo attenuation and concluded that the water molecules could move freely inside a cheese, despite the fat content. So, the effect of fat proton relaxation on the self-diffusion measurements would be the more appropriate explanation of the non-linearity of the echo attenuation.

Consequently, a bi-exponential function according to Eq. (5) could be used to fit the experimental echo attenuation. To avoid a badly adjusted fitting and unphysical results, conditions were imposed, i.e., $D_{fat} > 0$. In this way, the bi-exponential fit was correct and the water and fat self-diffusion coefficients were estimated (Fig. 4 and Table 4).

If the bi-exponential model seemed mathematically correct to describe the non-linearity of the echo attenuation, it did not provide an accurate estimation of the fat self-diffusion coefficients and large standard errors were deduced (Table 4). The relative intensity contribution for fat and water, P^*_{fat} and P^*_{water} also presented large standard errors.

Each echo attenuation value presented in Fig. 4 corresponded to the mean value of three measurements of echo attenuation obtained from three different NMR tubes. So the error included the error of sampling and the error of diffusion measurement. The fitting process was performed on each of these three experimental curves and the self-diffusion coefficient values and their respective errors were calculated from these three adjustments and then averaged. In this case the error

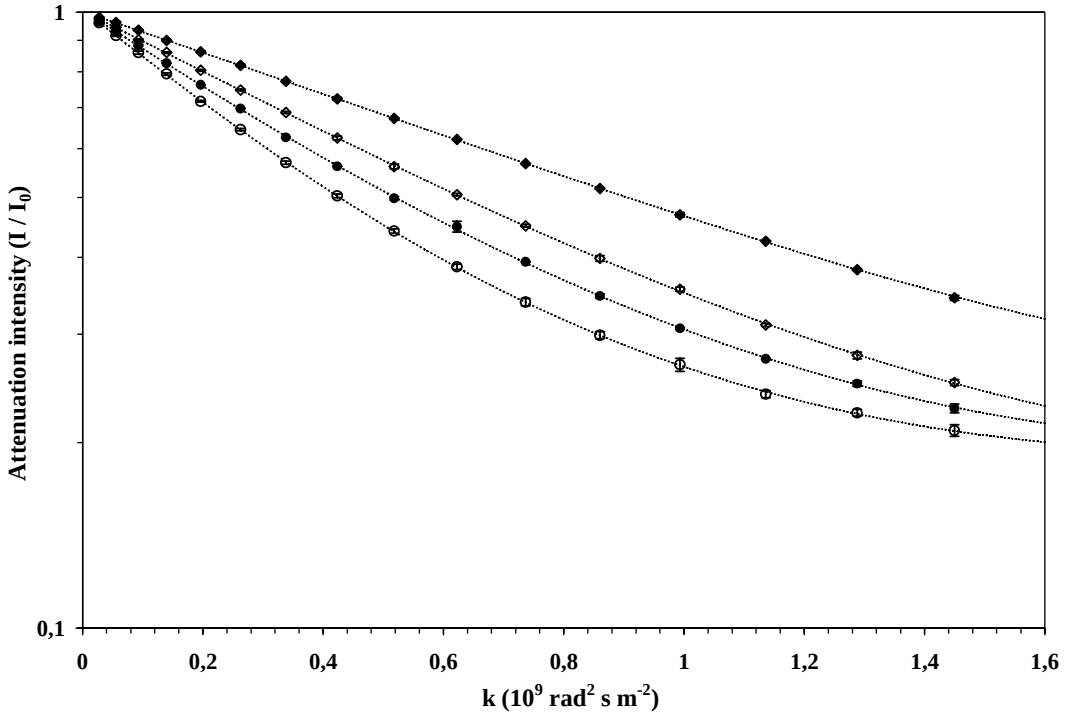

Fig. 4. Experimental logarithm plots of the echo attenuation versus $k(k = [\gamma^2\delta^2 g^2(\Delta - \delta/3)])$ for the fatty protein concentrate samples at different temperatures: 5 °C (◆), 20 °C (◇), 30 °C (●) and 40 °C (○). The lines are the results of the fit of Eq. (5) to the data.

Table 4
Water and fat self-diffusion coefficients at different temperatures for experimental echo attenuation from fatty protein concentrate

Temperature (°C)	Experimental NMR diffusion parameters			
	$D_{water}(\times 10^{-9}\,m^2\,s^{-1})$	$\%P_{water}$	D_{fat} $(\times 10^{-9}\,m^2\,s^{-1})$	$\%P_{fat}$
5	0.83 (±0.03)	98 (±5)	0.00009 (±0.0006)	5 (±5)
20	1.27 (±0.04)	95 (±7)	0.0005 (±0.008)	9 (±6)
30	1.70 (±0.08)	87 (±5)	0.0008 (±0.004)	13 (±8)
40	2.14 (±0.09)	85 (±4)	0.007 (±0.05)	16 (±9)

The echo attenuation plots were fitted with a bi-exponential model. Each experimental value was the mean of three separate acquisitions. Standard errors are given between brackets.

included also the error related to the fitting of the diffusion data in addition to the error of sampling and to the error of NMR measurement. This explained the difference between the error bars so small in Fig. 4 and the errors so large given in Table 4. Moreover, it is well known that the inverse Laplace Transform, required to fit multi-exponential signal, is an ill-conditioned problem: many solutions could be estimated to fit the experimental data [22].

Generally, the fitting methods required a large number of data point with high signal-to-noise ratio. Moreover, the echo attenuation from fat signal in the k-values range studied is very small. For example, at 30 °C, with $D_{fat} = 0.01597 \times 10^{-9}\,m^2\,s^{-1}$ (Table 3), the variation between $k = 0$ and $k = 1.45 \times 10^9\,rad^2\,s\,m^{-2}$ do not exceed 2.5%. Thus, all these reasons explained why so many solutions could be found for fat self-diffusion coefficient with no significant difference in the residuals. So the fat self-diffusion coefficient determination is erroneous.

To conclude, this low accuracy could be explained by both a low signal-to-noise ratio because of the use of a low-field spectrometer, and by the small number of experimental k values with regards with the degrees of freedom of the model.

3.2.3. Water self-diffusion determination from T_1-weighted spin-echo sequence for fatty protein concentrates

The strategic implementation of the T_1-weighted spin-echo sequence resulted from the possible distinction between water and a part of AMF spin–lattice relaxation times and because the fat spin–lattice relaxation was smaller than the water spin–lattice relaxation. For the T_1-weighted sequence, a precise determination of the delay t_i between the 180° and the 90° pulse is required. Generally the T_1 of the fat component was calculated

from $T_1 \ln 2$ [20]. Nevertheless, the T_1 spin–lattice relaxation times distribution of the pure AMF showed a broad T_1 relaxation time distribution (Fig. 2B). Therefore, the estimation of a unique value of T_1 from the adjustment of the spin–lattice decay curve was not possible. Here, this value was estimated experimentally from a T_1-null inversion recovery CPMG sequence performed on anhydrous milk fat at each temperature. The t_i values, given the lowest intensity of CPMG decay curve, were determined. The results are summarized in Table 5.

Table 5
T_i delay experimentally obtained for the T_1-weighted sequence at different temperatures

Temperature (°C)	Experimental t_i values (ms) (by T_1-weighted CPMG sequence)
5	20
20	35
30	50
40	60

The echo intensity attenuation obtained from the T_1-weighted and the spin-echo sequences were compared for each temperature (Figs. 5A–D).

At 5 °C, no difference for the echo attenuation versus k curves was observed between the two sequences. Indeed, at this temperature, the contribution of the fat protons to echo signal was negligible because of the crystallization process. For higher temperatures, the echo attenuation versus k curves became different according to the sequence used. For the T_1-weighted sequence, the semi-logarithmic echo attenuation versus k plots followed a linear behavior up to temperature of 30 °C according to Eq. (7) (Figs. 5A–C).

This behavior was in agreement with the experiments performed on fat-free protein concentrate and also with the high field NMR self-diffusion measurements performed by Callaghan et al. [8].

Moreover, this behavior confirmed that the water molecule in the fatty protein concentrate did not undergo an anomalous diffusion. The water diffusion coefficients, obtained from a linear regression, with this selective sequence and the ones, determined from a

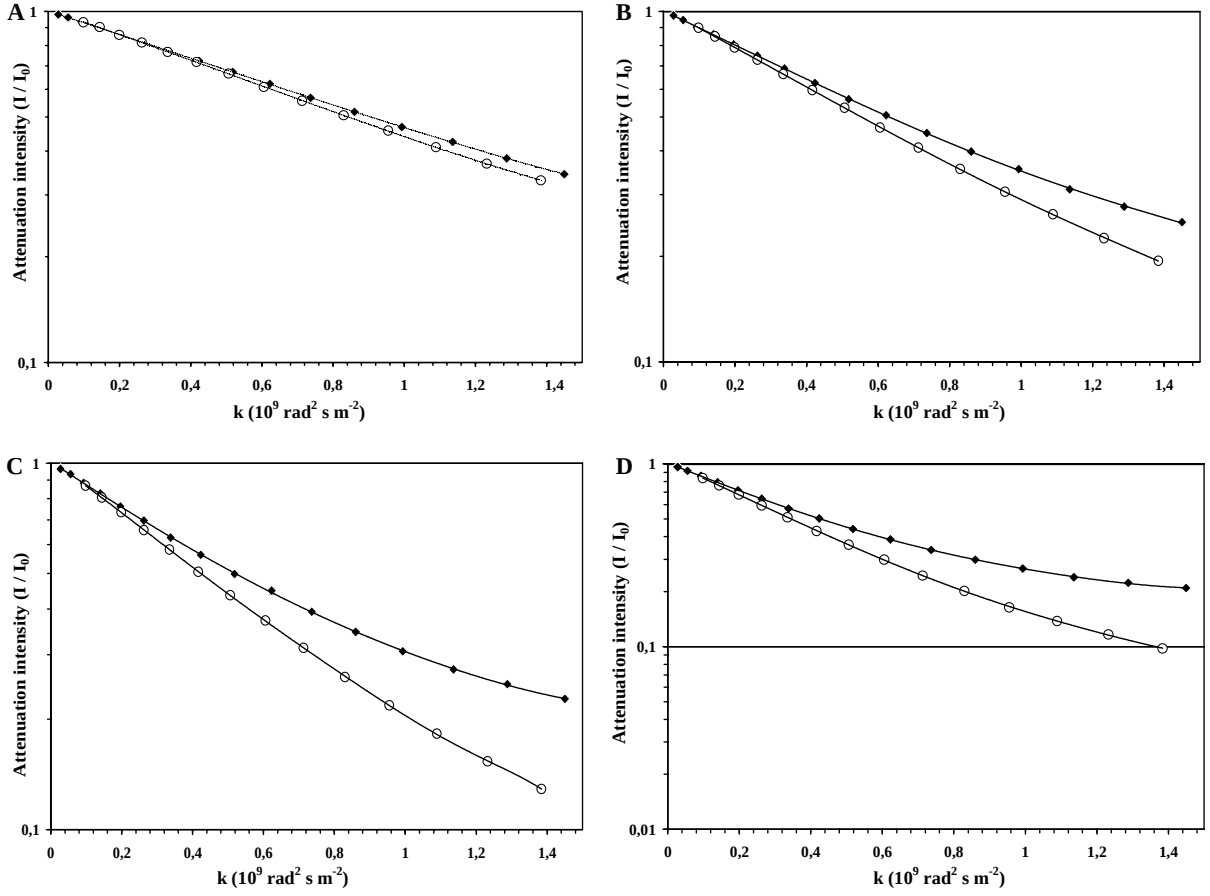

Fig. 5. Experimental echo attenuation versus $k(k = [\gamma^2\delta^2 g^2(\Delta - \delta/3)])$ for the fatty protein concentrate samples obtained from the spin-echo sequence (◆) and the T_1-weighted spin-echo sequence (○) at different temperatures: 5 °C (A), 20 °C (B), 30 °C (C), and 40 °C (D). The echo attenuation plots were fitted with mono- and bi-exponential models.

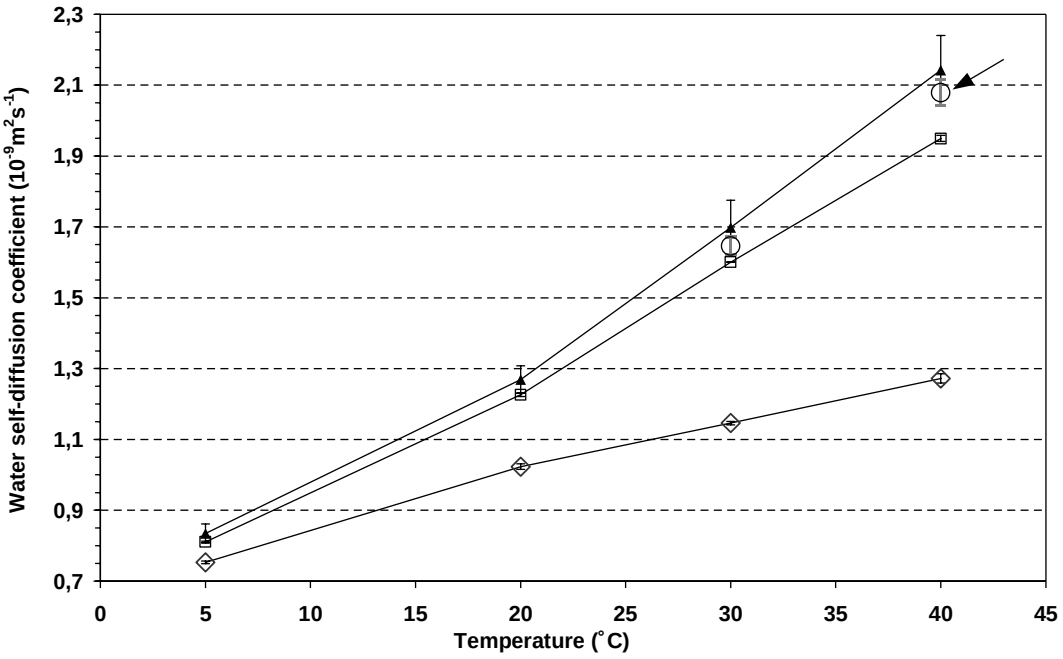

Fig. 6. Water self-diffusion coefficients versus the temperature for fatty protein concentrate samples measured with the two diffusion sequences. The diffusion data are fitted with a mono-exponential for the classical spin-echo sequence (◇) and T_1-weighted spin-echo sequence (□) and with a bi-exponential model for the classical spin-echo sequence (▲) and for the data at 30 and 40 °C obtained with the T_1-weighted spin-echo sequence (○).

bi-exponential fitting, with the basic sequence were not significantly different (Fig. 6). In comparison to these diffusion values, previously determined, a mono-exponential adjustment for diffusion data obtained with the classical sequence have given lower values of water self-diffusion coefficient. This could be explained by the interference of the fat proton contribution on the estimation of the water diffusion coefficient. The advantage of this T_1-weighted spin-echo sequence is that the accuracy of the water diffusion coefficient can be improved significantly. Indeed, the diffusion coefficient accuracy can be increased up to a factor of 10 without changing the water diffusion coefficient values between the basic sequence and the T_1-weighted spin-echo sequence (Fig. 6). So, this latter one allows a more confident interpretation of the self-diffusion, and avoids confusion with anomalous diffusion behavior. Moreover, the effect of the diffusion time Δ on the water self-diffusion could be studied without any perturbations from the echo-intensity from the fat protons. Nevertheless, the T_1-weighted spin-echo sequence required a preliminary determination of the T_1 or the t_i and thus increased the experimental time. Moreover, when the T_1 relaxation does not behave as a single mono-exponential, the fat proton relaxation could not be totally suppressed with single t_i value. Residual signal from fat protons could be detected for higher k values whereas the echo attenuation became non-linear for k values above 1×10^9 rad^2 m s^{-2} as observed at 30 and 40 °C (Figs. 5C and D). In that latter case, a mono-exponential was not enough accurate and a bi-exponential fitting should be used as the classical spin-echo sequence (Fig. 6). Consequently, the use of the T_1-weighted spin-echo sequence for suppression of the fat signal was limited to samples with small amounts of liquid fat or with fat characterized by a small distribution of spin–lattice relaxation times, at any temperature.

4. Conclusion

In this paper, we explored the application of the PFG-NMR ^{1}H to the water phase of a complex reconstituted fatty product. The results clearly show that the characterization of the water self-diffusion coefficient in a complex dairy product is possible using a bi-exponential fitting adjustment. However, we had to pay attention to the accuracy and precision of the values. Indeed, this required constraining the measurement time as well as taking a large number of measurements and a high ratio signal/noise for a precise estimation of the water self-diffusion coefficient D_{water}.

Therefore, the use of the T_1-weighted spin-echo sequence is one solution for improving the precision of the results and to make it possible to demonstrate the existence of particular diffusion behavior. Moreover, compared to the use of high field NMR spectrometers, for which the generally expected errors are about 5%; we obtained standard errors lower than 0.5%. This clearly shows the interest of this type of low-field bench top NMR equipment for self-diffusion studies on food products.

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