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Effect of Gel Firmness at Cutting Time, pH, and Temperature on Rennet Coagulation and Syneresis: An in situ ^{1}H NMR Relaxation Study

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The objective of this study was to monitor rennet-induced milk gel formation and mechanically induced gel syneresis in situ by low-field NMR. pH, temperature, and gel firmness at cutting time were varied in a factorial design. The new curve-fitting method Doubleslicing revealed that during coagulation two proton populations with distinct transverse relaxation times ($T_{2,1}$ = 181, $T_{2,2}$ = 465 ms) were present in fractions (f_1 = 98.9%, f_2 = 1.1%). Mechanical cutting of the gel in the NMR tube induced macrosyneresis, which led to the appearance of an additional proton population ($T_{2,3}$ = 1500−2200 ms) identified as whey. On the basis of NMR quantification of whey water the syneresis rate was calculated and found to be significantly dependent on pH and temperature.

KEYWORDS: In situ monitoring; NMR; rennet coagulation; syneresis; casein; slicing

INTRODUCTION

During cheese manufacture rennet is added to milk, where it breaks the bond between the amino acids Phe_{105} and Met_{106} in κ-casein. Subsequently, the casein starts to aggregate and form a gel. This gel retains all of the constituents of the milk including the aqueous phase. An important step in cheese manufacture is the separation of the main part of the water phase from the casein-gel, which is achieved through a process called syneresis (*1*). Syneresis occurs as a result of local stresses in the gel network, leading to rearrangements and local expulsion of whey, a phenomenon termed endogenous syneresis or microsyneresis. Physical separation of whey from the rennet gel (i.e., macrosyneresis) is normally dependent on cutting of the gel (*2*, *3*). Furthermore, the firmness of the gel at the cutting influences the moisture content of the final cheese. If the gel is cut at a too low firmness (cutting too early), the final cheese yield is reduced due to loss of fat and curd fines to the whey. If the gel is cut at a too high firmness (cutting too late), the syneresis is retarded, which results in cheese with high moisture content and undesirable textural properties (*4*, *5*). The process parameters pH and temperature have a major impact on syneresis. Lowering the pH of the gel during syneresis has been found to increase the rate of syneresis (*6*, *7*); increasing the temperature accelerates the rate of syneresis. The kinetics of syneresis is commonly considered to be of first order over the time scale used in cheese production (*2*, *8*). Thus, pH, temperature, and gel firmness at cutting are crucial for the water content and the texture of the final cheese product.

Traditional methodologies for studying syneresis can be divided into physical separation methods and tracer methods. In the physical separation methods the whey and/or gel is weighed to determine the extent of syneresis. The problem with physical separation methods is that the gel expels additional whey when being handled, which results in a biased measurement. The tracer methods measure dilution of an added compound such as Blue Dextran 2000 (*9*, *10*). A difficulty with tracer methods, however, is finding a tracer compound that does not adsorb to or diffuses into the gel (*11*).

Time domain proton (^{1}H) low-field nuclear magnetic resonance (LF-NMR) represents a particularly attractive alternative method for characterizing and quantifying water in food and dynamic food systems such as gel formation and syneresis. The major advantage of LF-NMR in this context is that the method is both nondestructive and noninvasive (*12*). The NMR relaxation of water protons in high water content biological systems is met by different restrictions that increase the rate of relaxation. In this manner different relaxation rates can give selective information about the surrounding environment of different water *pools* or *populations* within biological matter. Restrictions to relaxation are due to water protons being present in different states or sites in the system in a time scale compatible with the NMR experiment: (1) restriction due to chemical exchange between the water protons and the biopolymer protons and (2) restriction of proton relaxation due to physical compartmentalization in the sample. The latter restriction cause is related to diffusion of water protons because water protons will experience fast relaxation in the

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proximity of a physical barrier. Naturally, fast relaxation will only occur if the diffusion time for protons to reach the barrier is shorter than the intrinsic relaxation time of the protons and the time scale of the NMR experiment (*13*).

Several NMR relaxation studies have been reported on milk gel formation (*14*−*17*) and syneresis (*3*, *15*, *17*). The effect of milk gel syneresis on water proton relaxation has so far been studied on undisturbed gels that exhibit only endogenous syneresis (i.e., syneresis caused by pressure being built up by network formation within the gel). This is, however, not representative for actual cheese manufacturing, in which mechanical cutting of the gel into dices is an essential process step. Two inconsistencies exist in the interpretation of the water proton relaxation during to milk gel formation and syneresis: (1) the development (or lack) of the transverse relaxation time constant, T_2, and its corresponding proton population size during gel formation and (2) the number of proton populations necessary to describe water proton relaxation during cheese gel formation. Two studies have found no change in the relaxation time constant, T_2, during milk gel formation (*15*, *17*). On the other hand Hinrichs et al. (*14*) found small changes in T_2 relaxation time during the gel formation. The latter study found (without providing explicit proof) that three populations of water protons were required to describe the relaxation during milk gel formation, whereas the former studies found that one proton population was sufficient to describe the proton relaxation. There is a general agreement that the onset of milk gel syneresis is associated with the appearance of an additional water population with slower relaxation, which are the protons in the whey water (*14*, *15*, *17*). In these studies, however, the syneresis happened spontaneously, and the actual onset of syneresis was not controlled by cutting, which speed up the syneresis.

One of the reasons for the discrepancy in number of proton components is related to the data analytical methods chosen for studying and deconvoluting the NMR relaxation data. Proton relaxation occurs exponentially with time. If multiple proton populations exist in the samples, the relaxation decay curve is a sum of multiple exponential terms. The major challenge in the analysis of relaxation decay curves of LF-NMR experiments using multiexponential curve fitting is to decide the appropriate number of exponential terms that describe the actual water populations present in a sample. A new exponential curve-fitting method called *Doubleslicing* was introduced by Micklander et al. (*12*) to assist in the determination of water populations. Andrade et al. (*18*) tested the performance of Doubleslicing against existing methods and found that it was accurate in estimating relaxation times and that it outperformed exponential fitting by a factor of 4 with regard to computation time.

The primary objective of this study was to investigate the effect of milk gel formation and in situ mechanically (by cutting) induced gel syneresis by LF-NMR. The secondary objective was to demonstrate Doubleslicing as a method for determining the appropriate number of components in a semiautomated way. For these purposes the effect of milk gel formation and syneresis was studied using an experimental design with three factors: (1) pH, (2) temperature, and (3) gel firmness at cutting time. Time domain LF-NMR measurements were carried out in parallel with rheological measurements. The rheological measurements were done only during gel formation (and not syneresis) to evaluate the gel firmness.

MATERIALS AND METHODS

Experimental Approach. In this study we investigate rennet-induced gel formation of skim milk and the subsequent syneresis process after gel cutting by time domain LF-NMR. Rennet was added to skim milk, and this volume was split into two fractions: one was transferred to an NMR tube with an inner diameter of 17 mm. This tube was immediately inserted in the LF-NMR spectrometer and continuously measured without interruption during milk coagulation, cutting, and syneresis. The other fraction was injected into a rheological instrument that continuously measured gel firmness during gel formation. The role of the rheological measurements was to ensure that the gels formed in the NMR tube for repeated experiments had the same, wanted firmness when being cut. A *knife*, consisting of a thin polycarbonate blade tightly matching the tube diameter, was used to cut the gel manually and straight once over the entire inner diameter of the NMR tube. This was done when the firmness of the gel in the *twin* sample in the rheometer had reached a predefined level. To avoid interruption of the NMR measurements, the gel cutting was done inside the spectrometer in a 4 s delay between consecutive measurements. The delay did not always occur simultaneously with the predefined level of gel firmness, which therefore gave rise to slight variations in gel firmness at cutting.

Experimental Design. During the gel formation and syneresis three experimental factors were investigated on two levels (2^3 factorial design): pH (6.3 and 6.5), temperature (32 and 35 °C), and gel firmness at cutting time (low and high; defined later). The eight combinations of the three design factors were replicated twice, resulting in 16 ($= 2 \times 2^3$) gel and syneresis experiments. A center point with pH 6.4, gel firmness at cutting (middle level), and 35 °C was replicated four times. Unfortunately, a middle temperature level (33.5 °C) could not be tested due to experimental limitations. Overall, this resulted in 20 ($= 16 + 4$) gel and syneresis experiments performed in random order in the NMR spectrometer.

Materials. Arla Foods Ingredients (Denmark) kindly donated low-heat skim milk powder (SMP; Milex 240). The composition of SMP as reported by the manufacturer was 34−39% protein, <1.25% fat, 48−56% lactose, 7−9% ash, and <4% moisture. Chy-Max Plus rennet with 220 international milk clotting units (IMCU) mL^{-1} was obtained from Chr. Hansen A/S (Hørsholm, Denmark). Calcium chloride dihydrate ($CaCl_2 \cdot H_2O$) from Merck A/S (Darmstadt, Germany) and HCl from Acros Organic (Morris Plains, NJ) were used for preparation of the reconstituted milk samples.

Preparation of Milk and Renneting. Reconstituted skim milk samples were prepared by dissolving 100.0 g of SMP in 1000 mL of deionized water. The reconstituted skim milk was left overnight at 5°C to allow the proteins to fully dissolve. The protein concentration of the reconstituted milk was 3.4−3.9% (calculated from manufacturer's data). A 10% $CaCl_2 \cdot H_2O$ stock solution (1.50 mL) was added, resulting in a final concentration of 0.015% (~1.2 mM) $CaCl_2 \cdot H_2O$ in the milk. The pH was adjusted according to the experimental design with weak HCl (0.5 M). The reconstituted milk samples were conditioned to the temperatures of the experimental design for approximately 10 min in a water bath.

A diluted rennet solution of 6.60 IMCU mL^{-1} was made within 3 min before initialization of the experiment by mixing 300 μL of Chy-Max Plus (220 IMCU mL^{-1}) with 10 mL of deionized water. Renneting was initialized by adding 1.000 mL of diluted rennet solution to 150 mL of preheated milk placed in a water bath with magnet stirring, resulting in a final concentration of 0.044 IMCU mL^{-1} of milk. Thirty seconds after rennet addition, the stirring was stopped and 1.000 mL of milk was transferred to each of four sample cups in the rheometer. Sixty seconds after rennet addition, 7.000 mL of milk was transferred to a temperature-conditioned NMR tube, and LF-NMR measurements were immediately started.

Rheological Measurements. Firmness of the milk gel at cutting time was regulated by cutting at one of three levels according to the experimental design (low, medium, or high). The viscoelastic properties of the gel were monitored during gel formation by free oscillation rheometry (FOR) by using a ReoRox4 instrument from MediRox (Nyköping, Sweden) using disposable polypropylene sample cups and the accompanying software (ReoRox4 v2.00 and ReoRox Viewer v2.11). In free oscillation rheometry the measurement geometry is released into free oscillations at a frequency of around 10 Hz, and the amplitude and period time are measured by optical sensing. The frequency implies that the method is noninvasive toward the rennet gel system and hence does not disturb the gel during formation (*19*). The gel was cut inside the NMR tube when the elastic modulus G' (10 Hz) reached 90, 125, or 160 Pa, corresponding to the gel firmness defined as low, middle, or high in the experimental design.

This resulted in a cutting-time range for the 20 experiments in the design of 17−61 min. Note that the elastic modulus, G', as well as strain is appreciably higher in FOR than in conventional small-amplitude oscillation rheometry (SAOR) due to the higher oscillation frequency.

NMR Acquisitions. Time domain LF-NMR analysis was done on a benchtop 23.2 MHz Maran pulsed ^{1}H NMR spectrometer (Oxford Instrument, U.K.) equipped with a 17 mm inner diameter variable-temperature probe head. The temperature of the probe was set according to the experimental design. The Carr−Purcell−Meiboom−Gill (CPMG) pulse sequence was used to determine the relaxation behavior. This sequence was chosen because it minimizes the influence of magnetic field inhomogeneities, diffusion, and chemical exchange (*13*). A total of 8100 data points/echo times were acquired, with a 90−180 pulse spacing (τ) value of 500 μs. Only the even-numbered data points were used in the data analysis, resulting in 4050 data acquisition points per measurement. By using every second echo only (even echoes), inaccuracies in the 180° pulse setting are corrected. Prior to the first measurement, the frequency of the instrument was adjusted on a 10 mM $CuSO_4$ standard sample. During gel formation four scans were accumulated with a relaxation delay between consecutive scans of 14 s. Prior to the four scans, each measurement was preceded by two dummy scans, leading to a total measurement time of 2 min and 12 s. Measurements were carried out continuously until a maximum of 100 min after cutting.

NMR Data Analysis by Doubleslicing. Time domain LF-NMR data are most frequently analyzed using multiexponential fitting using, for example, the Levenberg−Marquardt algorithm, which applies nonlinear iterative curve-fitting algorithms to extract and characterize the underlying pure exponentials from random noise in the data (eq 1):

$$M(t) = \sum_{n=1}^{N} M_{0,n} \exp\left(\frac{-t}{T_{2,n}}\right) + e(t) \quad (1)$$

$M(t)$ is the reduced magnetization at time t, $M_{0,n}$ is the concentration or magnitude parameter of the n^{th} exponential, $T_{2,n}$ is the corresponding transverse relaxation time constant, and $e(t)$ is the residual error. After the relaxation curve has been deconvoluted into n exponential components, inspection of residuals can reveal whether the curve has been modeled into too few, too many, or the correct number of components. If the relaxation curve has been resolved into fewer exponential components than actually present, the residuals will show a systematic pattern. If the right number of exponential components is used, the residual will be randomly distributed. If too many exponential components are used, the residuals will also be randomly distributed, but instrumental noise will be incorporated in the fit. With large data sets (e.g., ~800 curves in the present study), this procedure including the inspection of the residual plots can become very time-consuming.

Micklander et al. (*12*) introduced an alternative noniterative and rapid technique for curve resolution called Doubleslicing. The technique utilizes the fact that in every part of a multiexponential decay curve each of the monoexponentials is present, but in different amounts. The technique pseudo-upgrades the single relaxation curve to become trilinear data, by cutting the relaxation curve into slices. By selectively removing parts of the signal curve (slicing) and using the remaining curve, the relaxation curve can be transformed from a one-dimensional signal (a vector $\mathbf{x}$) into two-dimensional data (a matrix X). By repeating this procedure on the matrix, the data are transformed to three-dimensional data (a cube $\underline{X}$). This procedure of converting the relaxation curve into three-way data may appear to be pointless at first glance, but it enables the use of three-way mathematical methods such as direct trilinear decomposition [DTLD (*20*)], which has some very attractive features. Andrade et al. (*18*) tested the performance of Doubleslicing against existing methods and found that it was accurate in estimating relaxation times and that it outperformed exponential fitting by a factor of 4 with regard to computation time. The speed advantage is desirable when large data sets are analyzed. Besides drastic improvement in speed, Doubleslicing also improves modeling and method diagnostic.

In the present study Doubleslicing was performed similar to the method of Andrade et al. (*18*). To validate that the relaxation curves were resolved into the actual number of exponentials and not under- or overfitting, an extensive range of diagnostic criteria had to be fulfilled. This set of diagnostic criteria enabled the construction of an automated algorithm, which could successively determine the relaxation times and the appropriate number of exponentials in the approximately 800 relaxation curves analyzed in the present study without manual intervention. Doubleslicing with diagnostic criteria was compared with the classical approach discrete exponential fitting using visual inspection of residuals. The implementation of diagnostic criteria and comparison with discrete exponential fitting are described in the Supporting Information.

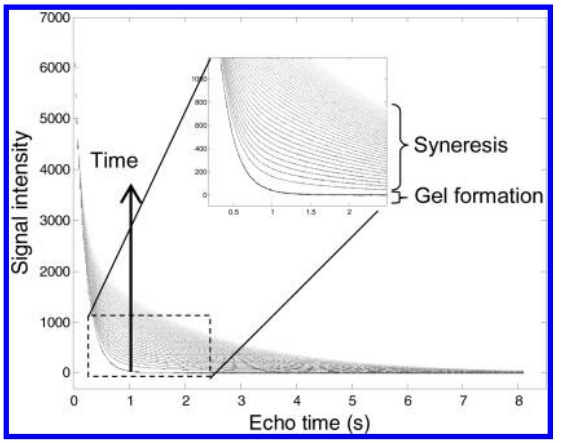

Figure 1. NMR CPMG relaxation curves during gel formation and syneresis in one gel formation and syneresis experiment. Notice that the overall relaxation becomes systematically slower with experiment time.

Syneresis Rate. The expected rate at which water leaves a milk gel when cut (i.e., syneresis) has been found to follow first-order reaction kinetics (*2*), which implies that the rate is dependent on the concentration of water in the gel. First-order reaction kinetics for syneresis can be expressed as (eq 2)

$$W_{\text{gel}} \rightarrow W_{\text{whey}}$$

$$-\frac{\mathrm{d}[W_{\text{gel}}]}{\mathrm{d}t} = k[W_{\text{gel}}] \quad (2)$$

where W_{gel} is the concentration of water in the gel, t is time, and k is the first-order rate constant. Integration and variable separation of eq 2 yields eqs 3 and 4:

$$\ln \frac{[W_{\text{gel}}]}{[W_{\text{gel},0}]} = -kt \quad (3)$$

$$[W_{\text{gel}}] = [W_{\text{gel},0}]\, \mathrm{e}^{-kt} \quad (4)$$

For a first-order reaction, therefore, a plot of natural logarithm versus t is linear and the first-order rate constant is obtained from the slope (*21*). In this study the proton population sizes of the whey water protons are assumed to represent the concentration of water outside the gel.

Statistical Analysis. On the syneresis rate constant k, obtained in the 2^3 factorial design, a three-way analysis of variance (ANOVA) was performed (omitting the partial center point) using the following main-effects model (eq 5)

$$k = \mu + \alpha \times \mathrm{pH} + \beta T + \gamma \times \mathrm{GF} + e \quad (5)$$

where μ is the common mean, α is the coefficient characterizing the pH effect, β is the temperature effect, and γ is the gel firmness effects. Interaction terms were not included, that is, evaluated as insignificant ($p < 0.05$) by iterative testing.

All data analysis steps (data exploration, trilinear modeling, ANOVA, etc.) were performed with Matlab version 7.6 (MathWorks Inc., Natick, MA) and an in-house algorithm.

RESULTS AND DISCUSSION

Development in T_2 Relaxation Time Constants and Population Sizes. Figure 1 shows CPMG relaxation curves recorded in one

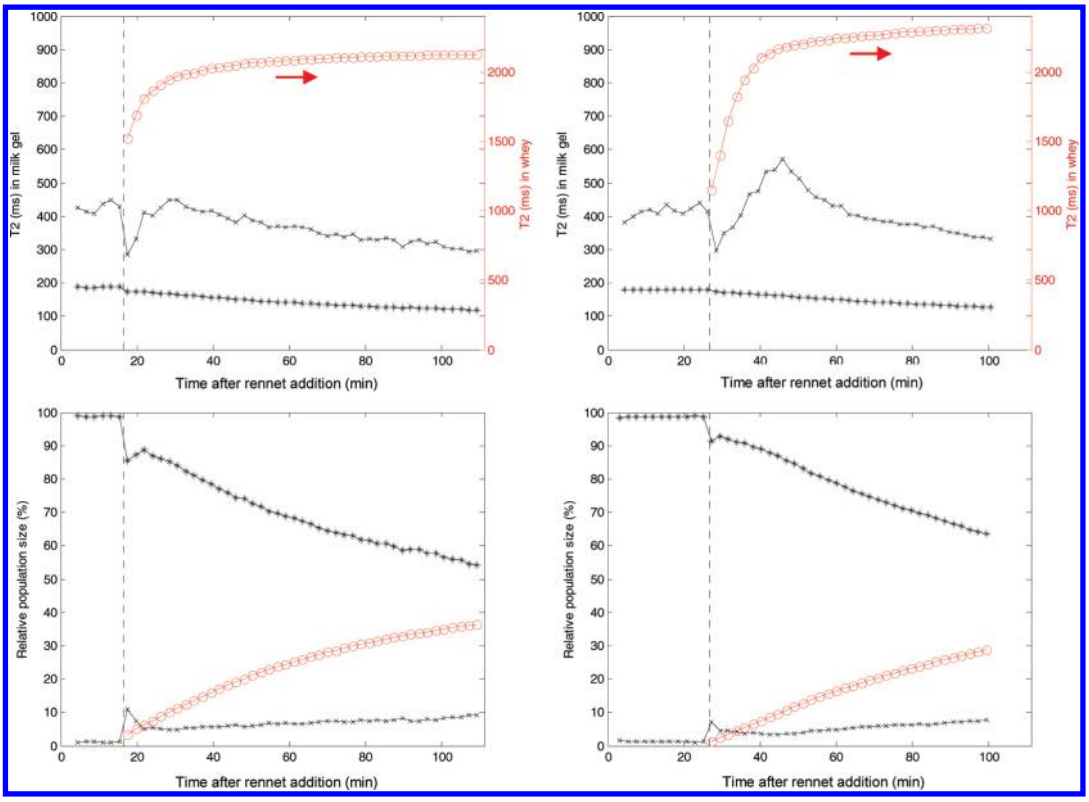

Figure 2. Development in transverse relaxation time constants $T_{2,1}$, $T_{2,2}$, and $T_{2,3}$ (upper row) and the corresponding relative population sizes f_1, f_2, and f_3 (lower row) during gel formation and syneresis of two experiments with different experimental settings. The left-column graphs show the development in an experiment with conditions pH 6.3, 34.9 °C, and low firmness at cutting. The right-column graphs show the development in an experiment with conditions pH 6.5, 34.9 °C, and low firmness at cutting. The vertical broken line indicates the time when the milk gel was cut.

Table 1. Average T_2 and Relative Population Size during Gel Formation and Syneresis for the 20 Experiments

	transverse relaxation time constant (ms)			relative population size (%)		
phase	$T_{2,1}$	$T_{2,2}$	$T_{2,3}$	f_1	f_2	f_3
gel formation	180.7 (5.1)[a]	465.3 (69.0)		98.9 (0.2)	1.1 (0.2)	
gel syneresis	151.1 (5.9)	425.9 (35.1)	1849.2 (125.2)	79.0 (5.7)	5.3 (1.1)	11.8 (5.2)

[a] Standard deviation in parentheses.

batch during gel formation and subsequent syneresis induced by cutting the milk gel at predetermined degrees of gel firmness [i.e., size of the viscous module G'' (10 Hz)]. By visual inspection of the relaxation curves in **Figure 1** it is not possible to observe any changes prior to cutting of the gel. However, overall relaxation becomes systematically slower after cutting of the milk gel, implying that the proton population(s) progressively changes as a result of the syneresis process, when water (i.e., whey) is expelled from the gel network. The trend in **Figure 1** is representative for all experiments in the design.

The development in T_2 and population sizes calculated from the relaxation curves using the correct number of exponential terms for each LF-NMR measurement are shown for two representative experiments (**Figure 2**). Notice that the trend lines through the 50 time points in **Figure 2** are added for interpretational purposes only after DTLD component determination. The abrupt change at the cutting point and the otherwise smooth curves give rise to high confidence in both the modeling approach and the automated method of model rank determination. **Figure 2** displays some key trends that are representative for all 20 batches.

During gel formation and prior to cutting, two components are present, which correspond to two distinguishable populations of protons with characteristic relaxation times $T_{2,1}$ and $T_{2,2}$. Within the gel formation phase the relaxation times $T_{2,1}$ and $T_{2,2}$ and population sizes are relatively constant compared to the syneresis phase (**Figure 2** and **Table 1**). The main part of the (water) protons (relative contribution in total signal of 98.8%, SD = 0.2%) prior to cutting of the gel originates from a population that is characterized by an average $T_{2,1}$ of 180.7 ms (SD = 5.1 ms).

Changes in T_2 and Population Size during Gel Formation. During gel formation and syneresis two proton populations with the characteristic transverse relaxation times $T_{2,1}$ and $T_{2,2}$ are present within the gel (**Figure 2**). The sizes of $T_{2,1}$ and $T_{2,2}$ show that the proton population originates from water associated with different parts/constituents of the gel. Data analysis clearly showed that biexponential behavior characterizes the system during gel formation, prior to cutting. The second component characterized by $T_{2,2}$ has not been previously described in the literature, to the authors' knowledge. The $T_{2,2}$ component represents only ~1% of the water protons during gel formation, but cutting caused it to increase to ~5% of the water. Cutting induced an immediate decrease in $T_{2,2}$ followed by an increase simultaneously with an increase in $T_{2,3}$, suggesting that the events are related. More experiments should be done to further elucidate what $T_{2,2}$ represents, but this is beyond the scope of the present study.

Le Dean et al. (*22*) studied the origin of proton populations in milk and milk protein mixtures using a factorial design of various

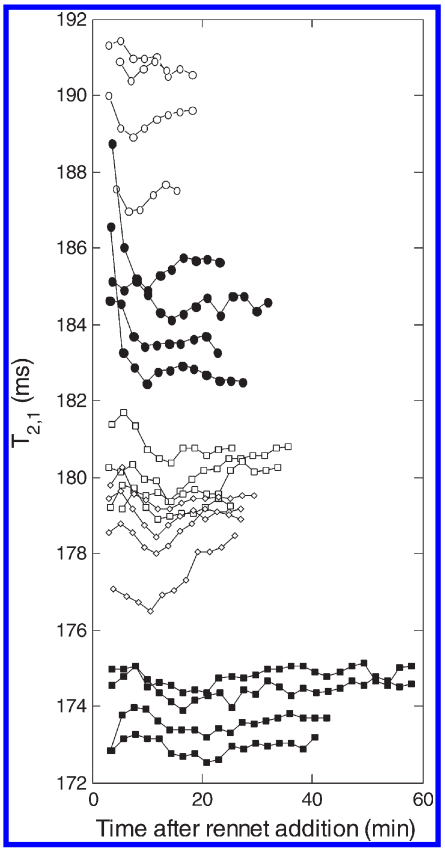

Figure 3. Development of $T_{2,1}$ with time after rennet addition, before cutting, in 20 coagulation experiments: (circles) pH 6.3; (diamonds) pH 6.4; (squares) pH 6.5; (open symbols) 32 °C; (solid symbols) 35 °C. Notice the V-like shape present in the graphs, that is, an initial decrease followed by an increase.

levels of milk components (i.e., caseinates, whey protein, $CaCl_2$, and lactose). They found that a bulk proton component having a T_2 = 163−205 ms was present in both milk and milk protein mixtures and that caseinate concentration explained most of the variation in relaxation time T_2. This finding was a confirmation of previously observed variations in T_2 due to casein concentration (*23*). It has also been shown that addition of lactose and whey protein slightly decreased the relaxation time T_2 of milk protein mixtures (*22*). According to Le Dean et al. (*22*) and Davenel et al. (*23*), the bulk proton component ($T_{2,1}$ = 180.7 ms, **Table 1**) found in the present study could correspond to water protons strongly associated with casein. During gel formation and prior to cutting, the bulk water protons represented by $T_{2,1}$ make up ∼99% of the water protons (**Figure 2**). **Figure 3** shows the development in $T_{2,1}$ before cutting for all 20 experiments in the factorial design. The effect of pH on $T_{2,1}$ is that a reduction in pH from 6.5 to 6.3 slightly increases $T_{2,1}$ (**Figure 3**). Assuming that $T_{2,1}$ represents a proton fraction strongly associated with casein, an increase in $T_{2,1}$ can be interpreted as an enrichment of water to the protein hydration layer. This effect of pH is consistent with the previous NMR findings (*22*, *24*), but in contrast with other studies concluding that lowering the pH reduces hydration of casein micelles (*25*, *26*). In the present study the effect of temperature on $T_{2,1}$ during gel formation is small but significant. $T_{2,1}$ in gels formed at 32 °C (**Figure 3**, open symbols) is lower than $T_{2,1}$ in gels formed at 35 °C (**Figure 3**, solid symbols). T_2 of protons is in general sensitive to temperature due to differences in molecular diffusion and the Boltzmann distribution of protons, which could explain the $T_{2,1}$ variation due to temperature.

Close inspection of $T_{2,1}$ development (**Figure 3**) shows a small trend over the experiments. The trend in the majority of the 20 experimental runs is that $T_{2,1}$ initially decreases and then increases in a V-like shape. This shape development could be some sort of NMR artifact or coincidental. Further studies should be done to validate the V-like shape. Assuming it is a real sample phenomenon, it could be interesting to determine if it is related to proteolysis of κ-casein and aggregation steps taking place during coagulation.

Changes in T_2 and Population Size during Syneresis. The most noticeable trend during syneresis is the rise of a new population of slowly relaxing water protons just after cutting, which is clearly the whey phase. Moreover, we observe that the average $T_{2,3}$ of the water protons in the whey grew asymptotically toward a near steady state, showing that the whey water is being progressively diluted until a certain limit. That the initial water leaving the gel has a lower $T_{2,3}$ than the water leaving later (**Figure 2**, upper row) suggests that the initial water contains more substances (i.e., whey, lactose, and minerals) that restrict relaxation. The population size of the whey water protons increase simultaneously (**Figure 2**, lower row), also toward an expected steady state, which is, however, not reached in the time span of the experiment.

During syneresis the bulk proton population characterized by $T_{2,1}$ steadily decreases to a level of ∼50−70% of the water protons after 100 min. If we assume this proton population is primarily associated with casein micelles as previously suggested (*22*), then the decrease in population size initiated by cutting suggests that the casein micelles are being steadily dehydrated. The simultaneous increase in proton populations characterized by $T_{2,2}$ and $T_{2,3}$ (primarily $T_{2,3}$, the whey) indicates that during syneresis the protons are transferred from the population characterized by $T_{2,1}$ to the populations characterized by $T_{2,2}$ and $T_{2,3}$. A steady decrease is also observed in $T_{2,1}$ during syneresis (**Figure 2**), which presumably is related to the up-concentration of solutes in the gel after expulsion of whey. A more concentrated solution of, for example, lactose and minerals in this water population would generally cause the water protons to relax more quickly. Another possible explanation for the steady decrease in $T_{2,1}$ during syneresis is that the simultaneous loss of water from the gel means that protons have less space for self-diffusion, which in turn will affect relaxation time.

Experimental Repeatability. To investigate the reproducibility of the cheese-making process as well as the data analytical approach, examples of repeated run are superimposed in **Figure 4**. The process in **Figure 4A** shows excellent repeatability, but the process in **Figure 4B** displays reduced repeatability. Especially the syneresis process is sensitive, as evidenced by **Figure 4B** showing the development in relative population sizes, where one of the gels exhibits faster syneresis. Because pH, temperature, and gel firmness are controlled by the design, a possible source for the reduced repeatability could lie in differences in the action of cutting. Indeed, Mateo et al. (*27*) studied the effect of cutting intensity on syneresis and found that gel moisture significantly depends on cutting intensity. It is thus possible that the not fully standardized, manual cutting intensity used in the present study may explain (modest) duplicate differences in syneresis rates.

Effect of Temperature, pH, and Gel Firmness at Cutting on Syneresis Rate. The relative population sizes determined during modeling quantitatively show how much water (protons) with different T_2 values is present at a given time during syneresis. The primary development can be summarized as follows: fast-relaxing water ($T_{2,1}$ ∼ 180 ms) within the gel is mainly converted to slow-relaxing water ($T_{2,3}$ ∼ 2000−2200 ms) situated outside the gel. A small fraction of the fast-relaxing water protons is seemingly converted into water present within the gel with an intermediate relaxation rate ($T_{2,2}$ ∼ 400−500 ms). The rate by

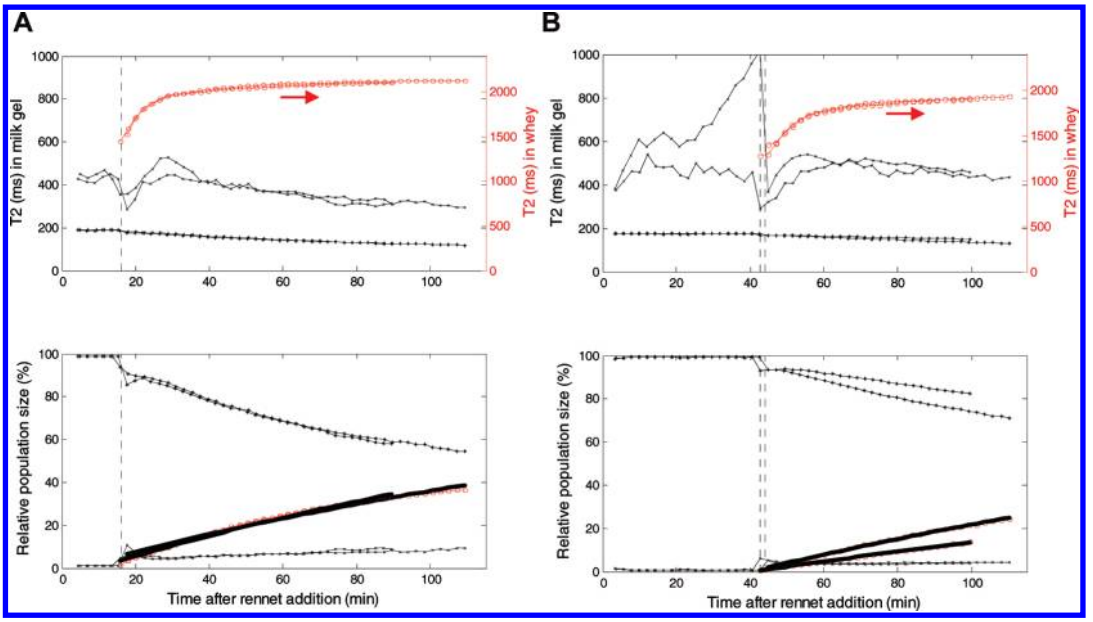

Figure 4. Development in two duplicated experiments of $T_{2,1}$, $T_{2,2}$, and $T_{2,3}$ (upper row) and the corresponding relative population sizes f_1, f_2, and f_3 (lower row): (**A**) duplicates at pH 6.3, 35 °C, and low firmness at cut; (**B**) duplicates at pH 6.5, 32 °C, and low firmness at cut. Broken lines show when the gel was cut. The thick lines are trend lines based on first-order reaction rate constants calculated from natural logarithmic transformed population size values.

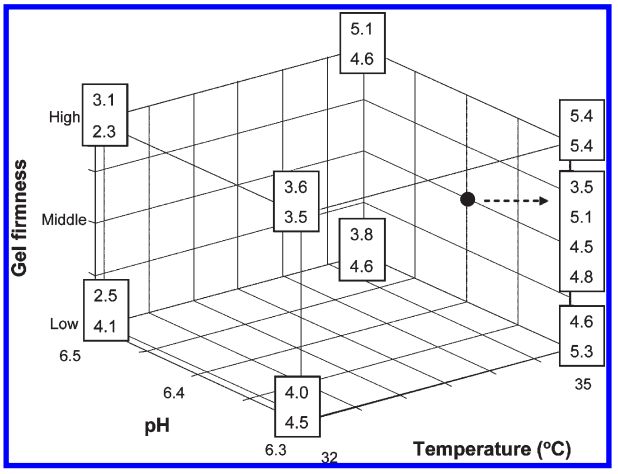

Figure 5. 2^3 factorial design with partial centerpoint used in the present milk coagulation and syneresis study. In the squares are given the first-order reaction rate constants k ($\times$ 10^{-3}) of whey syneresis estimated using LF-NMR. k is given for all design combinations and replicates.

which the water leaves a cut milk gel (i.e., syneresis) can be described as a reaction of first order, meaning that the rate of water expulsion is dependent on the concentration of water present at a given time (*2*). The evolution in population size of the whey water in **Figure 4** indicates first-order reaction behavior, seen as an initial rapid increase that asymptotically flattens out. The model of a first-order reaction was fitted to the evolution in population size of the whey water, and a nice fit with small randomly distributed residuals confirmed that whey water expulsion in the present study followed first-order reaction kinetics.

Figure 5 shows the first-order reaction rate constant k from eqs 2 and 3 for how water is arriving to the (water) proton population, making up the whey water for all 20 gel formation and syneresis experiments inside the factorial design. **Table 2** shows the corresponding results from an analysis of variance (ANOVA) evaluation on whether or not the first-order reaction rate constant k significantly depends on the design factors temperature, pH, and/or gel firmness at cutting.

Table 2. Analysis of Variance: Effects Temperature, pH and Gel Firmness at Cutting on the First-Order Water Proton Syneresis Rates k during Milk Gel Syneresis

	k ($\times$ 10^{-3})	
	P value	av
temperature (°C)		
32	< 0.001	3.4 (0.5)[a]
35		4.8 (0.3)
pH		
6.3	0.036	4.4 (0.3)
6.5		3.8 (0.5)
gel firmness at cutting		
low	0.65	4.2 (0.5)
high		4.0 (0.3)

[a] Parentheses show standard deviation.

A noticeable trend is that k is higher at pH 6.3 than at pH 6.5, which is consistent with previous findings showing that lowering the pH increases the rate of syneresis (*6*, *7*). Another trend is that k is highly temperature dependent; thus, the rate of syneresis accelerates as temperature increases, which is known from previous studies as well (*2*, *8*).

No significant dependence of the syneresis rate constants on gel firmness at cutting was found within the experimental design (**Figure 5**; **Table 2**), which is consistent with previous findings (*28*, *29*). This result presents a paradox because one could expect that a firmer gel with high endogenous pressure should expel whey more quickly than a less firm gel. Within the experimental design the temperature showed a major influence on the syneresis rate constant and is thus of great importance for process control.

To summarize, LF-NMR was used to characterize skim milk gel formation and syneresis qualitatively and quantitatively. A new automated algorithm based on Doubleslicing proved to be precise in finding the appropriate number of underlying exponential components (i.e., proton populations) in single relaxation curves measured during gel formation and syneresis. All 20 batches showed the same tendency that two underlying components of water protons were present during gel formation,

whereas the syneresis initiated by cutting produced an extra proton population with slow relaxation, identified as whey water. Quantitatively, we demonstrated that LF-NMR could monitor the relative amount of water present in each of the populations by the signal magnitude. In this way the first-order syneresis rates of whey being separated from the gel was derived. ANOVA showed that the syneresis rate constant is dependent on pH in the range from 6.3 to 6.5 and on temperature in the range from 32 to 35 °C; gel firmness at cutting did not show any significant effect on syneresis rate. The present approach enables the quantification of macrosyneresis on a rational basis and could be useful in, among other things, studying the relationship between the kinetics of rennet coagulation and syneresis.

Supporting Information Available: NMR data analysis by Doubleslicing, solution diagnostics, exemplification of solution diagnostics, and comparison with discrete exponential fitting. This material is available free of charge via the Internet at http://pubs.acs.org.

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