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# The effect of temperature and inoculum concentration on rheological and light scatter properties of milk coagulated by a combination of bacterial fermentation and chymosin. Cottage cheese-type gels

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**Abstract**

The main differences between the processing methods generally used for cottage cheese manufacture are the coagulation temperature and starter concentration. The effects of these factors on cottage cheese gels were investigated. Gels were made with a low rennet concentration. Gel formation was monitored using infrared light backscatter and dynamic small amplitude oscillatory rheology. Light backscatter profile was explained by a complex combination of casein micelle aggregation, curd firming and micelle demineralization. Increasing temperature or inoculum concentration resulted in faster network formation, which resulted in more viscous and less stiff gels. Activation energy of aggregation decreased significantly with increasing starter concentration. Casein aggregation had a higher temperature coefficient than firming and transition from rennet- to acid-type gels mostly occurred during curd firming stage of network formation. The second minimum of the backscatter profile second derivative and the rheological gelation time were highly correlated but not significantly different, suggesting that they both corresponded to the beginning of gel firming.


*Keywords:* Cottage cheese; Milk gels; Light backscatter; Rheology

## 1. Introduction

Milk coagulation is the initial step in the manufacture of many dairy products and can be induced by acidification or by the combined action of acid and enzyme (we will use the term mixed gels to describe gels made by a combination of rennet and acid). Fermented milk products have been increasing in popularity during the last few decades, especially because of their benefits for human health. Even though fermented milk products represent a major segment of the dairy market, the study of acid or mixed milk coagulation has received very little attention when compared with rennet induced coagulation. In fact, our general knowledge about this topic still remains limited and mostly empirical (Lucey & Singh, 2003). Physical–chemical changes induced by acidification, or combination of acidification and rennet action, exert a major influence on the rheological properties of fermented dairy products (Lucey, Tamehana, Singh, & Munro, 2000, 2001; Tranchant, Dalgleish, & Hill, 2001). Horne (1999) considered the lack of detailed information on this topic very surprising since perceived texture in fermented products is one of the most important sensory properties, which helps determine consumer preference and acceptability.

Cottage cheese is generally manufactured by acid coagulation of pasteurized skim milk. Three widely different processing methods, i.e., short-, medium- and long-set, are used industrially to produce cottage cheese

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(Walstra, Geurts, Noomen, Jellema, & van Boekel, 2001). The main differences between them are the coagulation temperature and starter concentration. The incubation times are ~5, ~8 and ~12–16 h for short-, medium- and long-set, respectively. For the short-set method, milk is typically inoculated with ~5% of a non-gas-producing mesophilic culture and incubated at ~32 °C. For the medium-set, the incubation temperature and starter concentration are typically ~27 °C and ~2.75% respectively, while the long-set method uses ~22 °C and ~0.5%. A small concentration of rennet is sometimes added after the starter has been allowed to develop some acidity (i.e., pH ⩾ 5.5). When rennet is used, the gel obtained is predominantly acid but its characteristics are modified to some degree by renneting. Since these manufacturing methods lead to different reaction rates, more information is needed on the biochemical and physical changes taking place in milk systems during this type of mixed coagulation. It was considered that monitoring coagulation by several objective devices under the conditions corresponding to the very different methods used in cottage cheese manufacture would supply useful information for improving the quality of cottage cheese.

Dynamic oscillatory rheometry can provide very useful information about milk gels (Lucey, 2002). It has been widely used to monitor coagulation of rennet (Dejmek, 1987; Niki, Kohyama, Sano, & Nishinari, 1994; Mellema, Walstra, van Opheusden, & van Vliet, 2002) and acid gels (Lucey, Tamehana, Singh, & Munro, 1998). There have been only a few reports on the rheological properties of mixed gels (Lucey et al., 2000, 2001; Tranchant et al., 2001) and none were found on the fundamental rheological properties of cottage cheese gels. Infrared light backscatter has been applied to monitor the cottage cheese fermentation process (Payne, Freels, Nokes, & Gates, 1998). More recently, a kinetic model, based on this technique, has been proposed to monitor gel assembly in goat milk rennet-induced gels (Castillo, Payne, Hicks, Laencina, & López, 2003b). The objectives of this study were to: (a) monitor and analyze the mixed coagulation process using oscillatory rheometry and infrared light (880 nm) backscatter, and (b) study the effect of starter inoculum concentration and incubation temperature on pH, rheological and light backscatter parameters.

## 2. Materials and methods

### 2.1. Experimental design

A randomized factorial design with two factors and three replications was used to study the effect of the different methods of manufacturing cottage cheese on the milk coagulation and properties of the gels. Three levels of starter culture addition (0.5%, 2.75% and 5% (w/w)) and three coagulation temperatures (22, 27 and 32 °C) were investigated using a constant concentration of both calcium chloride ($0.2\,g\,kg^{-1}$) and chymosin ($2\,mg\,kg^{-1}$). A total of 27 tests were conducted with this design. Reconstituted low heat skim milk powder (SMP) from the same batch was used in all the tests to reduce experimental error. Coagulation was monitored using both a backscatter sensor and a rheometer. The pH was monitored until it decreased to 4.8. All the various types of tests were conducted in parallel with time zero being the time that starter was added.

### 2.2. Temperature control and pH measurements

The techniques applied in this experiment required an accurate control of temperature. Heating of samples and control of temperature during testing were performed using water baths having an accuracy of ±0.05 °C. The rheometer was equipped with a precise temperature control system (Peltier TEZ 150P, Physica Messtechnik GmgH, Stuttgart, Germany) having an accuracy of ±0.01 °C. Sample temperatures were measured with a precision thermistor thermometer. All pH measurements were determined using a combination pH electrode (8102BN, Orion Research Inc., Beverly, MA, USA) and an automatic temperature compensation probe (927005, Orion Research Inc., Beverly, MA, USA). Both were connected to the SensorLink pH Measurement System (PCM700, Orion Research Inc., Beverly, MA, USA), which was connected to a personal computer. The pH measurements were recorded every minute during milk acidification. The first derivative of each pH profile was calculated using 41 data points to smooth the first derivative profile. The slope of each data subset was calculated by linear least-squares regression method and assigned to the midpoint of the data subset. The maximum rate of acidification ($R_A$) was defined as the maximum slope (absolute value) determined during the process. Other variables obtained from the pH profiles were $t_{RA}$ or the time to the maximum rate of acidification and pH values at $t_{RA}$, $t_{max}$, $t_{max2}$, $t_{2max}$, $t_{2min}$, $t_{2max2}$, $t_{2min2}$ (see Section 2.7 for definition of light backscatter parameters).

### 2.3. Enzyme

The milk coagulant used was 100% recombinant chymosin (CHY-MAX® extra; EC 3.4.23.4, isozyme B, $600\,IMCU\,mL^{-1}$) supplied by Chr. Hansen's Inc. (Milwaukee, WI, USA). Rennet was added at a concentration of 2 mg per kg of milk, to all the milk samples. Ten minutes before every test, chymosin was diluted in ultra pure water obtained using a Millipore Q purification system (MilliQPlus, Millipore Corporation, Bedford, MA, USA). The dilution was standardized so

that 200 μL of solution would deliver 2 mg of chymosin to 1 kg of reconstituted skim milk sample. Rennet was stored at 4 °C and added to milk 5 min after addition of starter (total stored time of standardized solution ~15 min).

### 2.4. Milk reconstitution and heat treatment

Low heat SMP with 6.51 mg $g^{-1}$ of undenatured whey protein nitrogen (Bradley et al., 1992) was supplied by Dairy America™ (Fresno, CA, USA). A three-liter milk sample was reconstituted the day before gelation experiments by dissolving 10% (w/w) SMP in ultra pure water. The milk was stirred at room temperature (~25 °C) for 3 h in a beaker covered with parafilm to avoid evaporation. The pH of the milk after reconstitution was typically 6.68 ± 0.01. The milk sample was then heat-treated at 63 °C for 30 min in a water bath and cooled by immersion in ice water. A constant proportion of anhydrous calcium chloride (0.2 g $kg^{-1}$) was added to the sample with stirring by adding 3.75 mL of a 1.44 M solution of calcium chloride. The milk sample was stored overnight at 4 °C until used the next morning. No significant change of pH was detected during storage time of heat-treated milk.

### 2.5. Starter culture

A mesophilic lactic culture (Redi-set #82, mixture of *Lactococcus lactis* and *Leuconostoc cremoris,* Chr. Hansen Inc.) was used to make the cottage cheese gels. To reduce variability in the activity of the culture during the experimental period, a mother culture of the starter bacteria was prepared and stored in cryovials. The commercial frozen culture was first grown in skim milk. A 800 g sample of commercial skim milk with ~10% (w/w) total solids was weighed into an autoclavable bottle and 214 mg of $CaCl_2 \cdot 2H_2O$ was added to the milk sample. The milk sample was autoclaved at 121 °C for 15 min. The sterilized milk was cooled to 25 °C, inoculated aseptically (0.4 mL of culture in 800 g milk) and immediately stirred and incubated at 25 °C, for 12.8 h, when the pH was ~5. After incubation, aliquots of ~4 mL were rapidly and aseptically transferred to sterile vials, which were frozen and stored at −80 °C in an ultra-low temperature freezer. Before the gelation experiments, frozen vials were thawed for 20 min at room temperature and a 120 μL aliquot of culture was added aseptically to 240 g of sterilized milk that had been warmed to 25 °C. The inoculated sample was incubated at this temperature and an acidification profile was obtained by monitoring the pH of an aliquot until it was 4.8, which typically required a time period of ~13.5 h. Then, this starter was used to inoculate reconstituted SMP samples at the experimental starter levels.

### 2.6. Testing procedures

Each test day, a 3 L SMP sample, was split into two aliquots. Two litres were transferred to the reaction vessel and were used to monitor coagulation by the backscatter sensor. The vessel was stainless steel ($15 \times 13.3 \times 15\,cm^3$) with a removable insulated lid. The remaining litre of milk was kept in a covered flask and used for pH measurements and testing other properties of the gels. Both the flask and the reaction vessel were immersed in a water bath and heated for at least 30 min at the test temperature. When the starter reached pH 4.8, SMP samples were inoculated at the required level (0.5%, 2.75% or 5%). Light backscatter measurements in the reaction vessel were initiated simultaneously with the starter addition to the milk ($t = 0$). Both milk samples were stirred thoroughly with a spatula for 1 min. Rennet was added to the samples after 4 min and stirred for 1 min. The milk contained in the flask was split into several aliquots for the rheometer, and to monitor pH, permeability, syneresis and gel microstructure tests. The coagulation properties are presented in this paper and other properties will be reported in subsequent papers.

### 2.7. Near infrared light backscatter

A light backscatter probe (Model 4A CoAguLite, Reflectronics Inc., Lexington, KY, USA) using near infrared light at 880 nm was used to monitor light backscatter during milk coagulation using the method described by Castillo, Payne, Hicks, and López (2000). Output voltage was zeroed to 1 V. The sensor gain was calibrated to give a 2 V signal response when placed in reconstituted SMP. Response data were collected every 6 s. The initial voltage response ($V_0$) was calculated by averaging the first ten data points after correction for the 1 V zero offset. A light backscatter ratio ($R'$) was calculated by dividing the sensor output voltage (less the 1 V zero output) by $V_0$. The first derivative ($R''$) of the light backscatter ratio profile was calculated by conducting linear least-squares regression on the most recently collected 4 min of data. The calculated slope was assigned to the midpoint of the data subset used. The second derivative ($R''$) was calculated in a similar manner but using 120 data points to smooth the $R''$ profile. The optical parameters derived from the light backscatter profile are defined in Table 1 and were classified as suggested by Castillo et al. (2000) as time-based (units of time, min), response-based (units of light backscatter ratio, i.e., dimensionless) or mixed-based parameters (units of light backscatter ratio divided by units of time, i.e., $min^{-1}$ or $min^{-2}$). Fig. 1 shows a light backscatter ratio profile for mixed milk coagulation with the calculated first and second derivatives.

Table 1
Definition of optical parameters derived from the light backscatter ratio profile[a]

| | |
|---|---|
| $t_{max}$ was the time to the first maximum of $R'$ | $R_{max}$ was the value of $R$ at $t_{max}$ |
| $t^*_{cut}$ was the time to gel cutting[b] | $R^*_{cut}$ was the value of $R$ at $t^*_{cut}$ |
| | $R'_{max}$ was the value of $R'$ at $t_{max}$ |
| $t_{max2}$ was the time to the second maximum of $R'$ | $R'_{max2}$ was the value of $R'$ at $t_{max2}$ |
| $t_{2max}$ was the time to the first maximum of $R''$ | $R''_{max}$ was the value of $R''$ at $t_{2max}$ |
| $t_{2min}$ was the time to the first minimum of $R''$ | $R''_{min}$ was the value of $R''$ at $t_{2min}$ |
| $t_{2max2}$ was the time to the second maximum of $R''$ | $R''_{max2}$ was the value of $R''$ at $t_{2max2}$ |
| $t_{2min2}$ was the time to the second minimum of $R''$ | $R''_{min2}$ was the value of $R''$ at $t_{2min2}$ |

[a] $R$, light backscatter ratio; $R'$, first derivative of $R$ as a function of time; $R''$, second derivative of $R$ as a function of time.
[b] Cutting time was defined as the time required for the gel to reach pH 4.8 (Walstra et al., 2001).

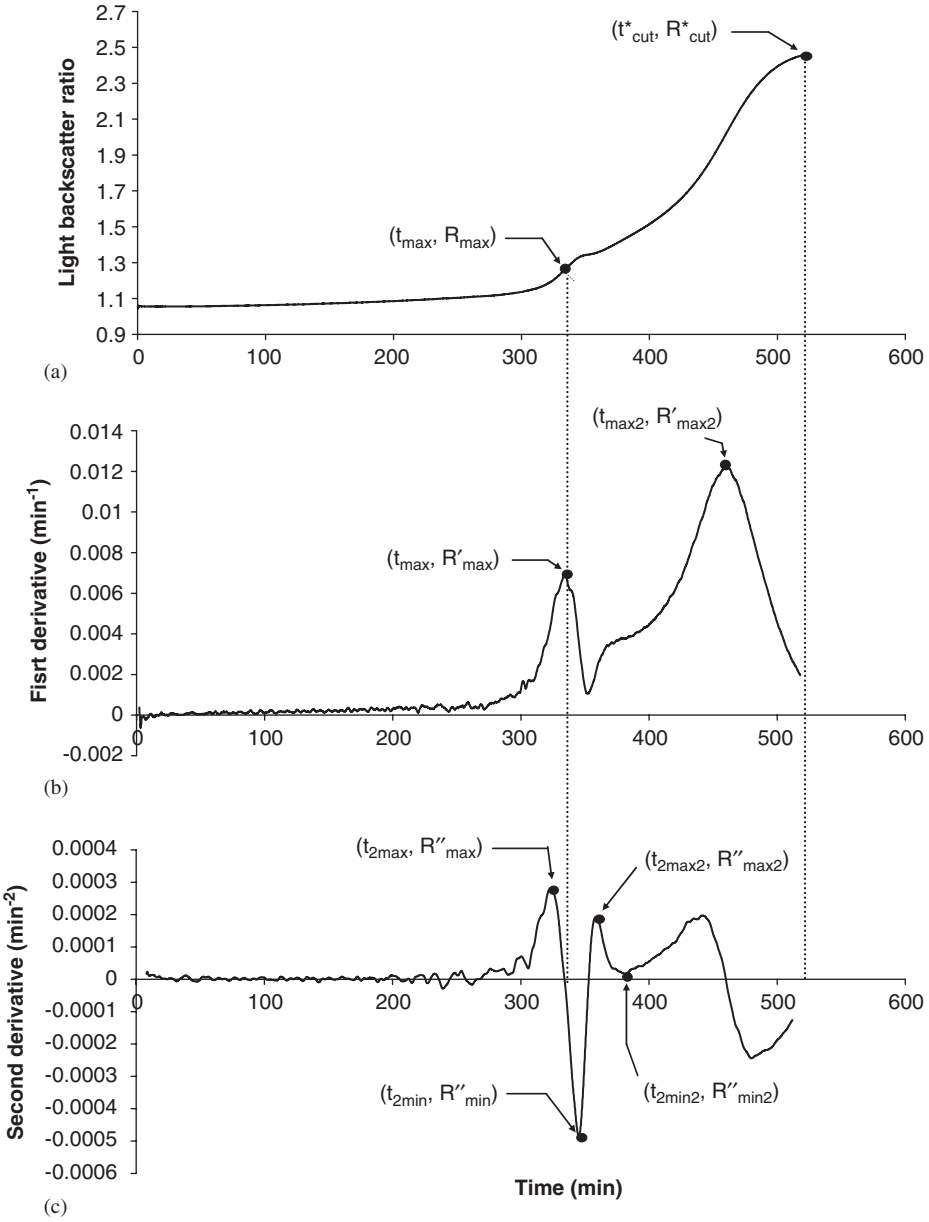


Fig. 1. Light backscatter ratio profile (a) and its characteristic first (b) and second (c) derivatives versus time. Data corresponded to skim milk coagulated at 22 °C using 2 mg kg$^{-1}$ of rennet and 2.75% starter culture. Cutting time was defined as the time when the gel pH reached 4.8.

### 2.8. Rheological assays

Small amplitude oscillatory rheometry (SAOR) was performed using a Universal Dynamic Spectrometer (Paar Physica UDS 200, Physica Messtechnik GmgH, Stuttgart, Germany). A couette measuring geometry (Z3 DIN) was used. Fourteen grams of inoculated milk with rennet added was transferred to the measuring system that was pre-warmed to the assay temperature. Vegetable oil was added on the exposed milk surface to prevent evaporation. The strain applied in the tests was 1%, which is within the region of linear viscoelastic response reported for acid milk gels (van Marle & Zoon, 1995). After temperature equilibration, samples were oscillated at a frequency of 0.1 Hz and measurements were taken every 5 min, until the samples reached pH 4.8. Parameters determined were the elastic or storage modulus ($G'$), the viscous or loss modulus ($G''$) and tan $\delta$ or loss tangent (tan $\delta = G''/G'$). Gelation time ($t_{gel}$) was defined as the time when gels had a $G' \geqslant 1$ Pa (van Marle & Zoon, 1995). When the gel reached pH 4.8, large deformation properties were studied by the method described by Lucey, Teo, Munro, and Singh (1997a). Gels were subjected to a constant shear rate of 0.01 $s^{-1}$ until the gel yielded. Shear stress was plotted as a function of applied strain. The yield point was defined as the point when the shear stress started to decrease. The stress at yield point or yield stress ($\sigma_y$) and the strain at yield point ($\gamma_y$) were measured.

### 2.9. Cutting time determination

The time at which the samples reached pH 4.8 was used as the cutting time ($t^*_{cut}$). This was as recommended by Walstra et al. (2001) for milk acidified with culture and with 2 μL $L^{-1}$ of added rennet.

### 2.10. Statistical analysis

All data obtained were processed and analyzed using the Statistical Analysis System (SAS®, 1999). Pearson correlation coefficients were determined by the correlation (CORR) procedure. The analysis of variance (ANOVA) and regression analysis were performed using the general linear model (GLM) procedure. Least squares means (LSM) and significance of treatments were calculated using type IV sum of squares. LSM were considered to be statistically different when $P<0.05$.

## 3. Results and discussion

Mixed coagulation of milk was monitored by rheological and optical methods, with simultaneous pH measurements. An ANOVA was conducted to determine the main sources of variation in the dependent variables. Starter concentration "$S_0$", temperature "$T$", and the qualitative variable "*Rep*" (to quantify the effect of replication) were selected as main effects in the preliminary ANOVA model. The main interaction "$T \times S_0$" was also included. Replication effect was not significant and was removed from the model. Table 2 shows the ANOVA for dependent variables studied. The ANOVA model was highly significant for all the parameters derived from light backscatter except for $R^*_{cut}$, for the rheological variables, $t_{gel}$ and tan $\delta$ at pH 4.8, and for all the parameters derived from pH measurement. The main effects, temperature and starter concentration, were significant for most variables.

### 3.1. The effect of temperature and inoculum size on coagulation and rheological properties of mixed gels

#### 3.1.1. The effect of temperature

It was found that time parameters ($t_{max}$, $t_{max2}$, $t_{2max}$, $t_{2min}$, $t_{2max2}$, $t_{2min2}$, $t_{gel}$ and $t_{cut}$) decreased significantly ($P<0.0001$) when temperature increased (Table 3). In Table 3, only the LSM for selected parameters were shown to avoid unnecessary redundancy, because the response of some parameters was identical, e.g., $t_{max}$, $t_{max2}$, $t_{2max}$, $t_{2min}$, etc. It is generally accepted that increasing incubation temperature decreases $t_{gel}$ in acid milk gels (Lucey & Singh, 1998; Lucey et al., 1998). Fig. 2 shows how increasing incubation temperature resulted in an increase in the rate of acidification and shortened the gelation time. The inflection point of the backscatter ratio profile, $t_{max}$, appeared before gelation was detected by the rheometer (Fig. 2a) for all samples. A shoulder in the backscatter ratio profile appeared before $t_{gel}$ but after $t_{max}$ (Fig. 2a). Significance of these findings will be discussed later. The interaction between temperature × inoculum level was very significant for all the time parameters studied. Smaller $t_{gel}$ values were observed at high temperatures and inoculum concentrations, but the effect of temperature was more pronounced at low inoculum concentration. Regarding mixed-based parameters, as expected, $R'_{max}$, and $R''_{max}$ increased and $R''_{min}$ decreased, with increasing gelation temperature ($P<0.0001$), reflecting the enhancement of the coagulation rate (Table 3). Castillo, Payne, Hicks, Laencina, and López (2003a) had a similar trend for the effect of temperature on $R'_{max}$ of rennet gels. Concerning the response-based parameters, $R_{max}$ and $R^*_{cut}$, our results also agreed with those of Castillo et al. (2003a). As shown in Table 3, these parameters significantly increased with temperature, except there was little difference between the $R^*_{cut}$ values at 22 and 32 °C. This exception was caused by an unexpected decrease of $R^*_{cut}$ when gelation temperature varied from 27 to 32 °C. As it will be discussed later, we attributed it to a weakening of the protein network at high temperatures when acid

Table 2
Analysis of variance and $F$ statistics for dependent variables[a]

| | Model | | Variation source | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | $T$ | | $S_0$ | | $S_0 \times T$ | |
| | $R^2$ | $F$ | DF | $F$ | DF | $F$ | DF | $F$ |
| $t_{max}$ | 0.993 | $321^{***}$ | 2 | $729^{***}$ | 2 | $539^{***}$ | 4 | $6.81^{**}$ |
| $t_{max2}$ | 0.992 | $241^{***}$ | 2 | $738^{***}$ | 2 | $318^{***}$ | 4 | $13.5^{***}$ |
| $t_{2max}$ | 0.993 | $334^{***}$ | 2 | $740^{***}$ | 2 | $578^{***}$ | 4 | $9.04^{***}$ |
| $t_{2min}$ | 0.992 | $281^{***}$ | 2 | $644^{***}$ | 2 | $467^{***}$ | 4 | $6.75^{**}$ |
| $t_{2max2}$ | 0.994 | $392^{***}$ | 2 | $895^{***}$ | 2 | $646^{***}$ | 4 | $14.2^{***}$ |
| $t_{2min2}$ | 0.994 | $362^{***}$ | 2 | $797^{***}$ | 2 | $630^{***}$ | 4 | $10.2^{***}$ |
| $t_{gel}$ | 0.984 | $140^{***}$ | 2 | $320^{***}$ | 2 | $228^{***}$ | 4 | $6.13^{**}$ |
| $t^*_{cut}$ | 0.992 | $285^{***}$ | 2 | $620^{***}$ | 2 | $492^{***}$ | 4 | $13.1^{***}$ |
| $R'_{max}$ | 0.968 | $68.8^{***}$ | 2 | $270^{***}$ | 2 | $5.44^{*}$ | 4 | $0.04^{ns}$ |
| $R'_{max2}$ | 0.845 | $10.3^{***}$ | 2 | $14.4^{***}$ | 2 | $28.5^{***}$ | 4 | $0.15^{ns}$ |
| $R''_{max}$ | 0.946 | $39.2^{***}$ | 2 | $152^{***}$ | 2 | $3.49^{ns}$ | 4 | $0.81^{ns}$ |
| $R''_{min}$ | 0.965 | $62.9^{***}$ | 2 | $212^{***}$ | 2 | $32.5^{***}$ | 4 | $3.77^{*}$ |
| $R_{max}$ | 0.869 | $14.9^{***}$ | 2 | $48.6^{***}$ | 2 | $2.27^{ns}$ | 4 | $4.43^{*}$ |
| $R^*_{cut}$ | 0.510 | $2.34^{ns}$ | 2 | $6.24^{**}$ | 2 | $1.96^{ns}$ | 4 | $0.58^{ns}$ |
| $G'$ | 0.307 | $1.00^{ns}$ | 2 | $1.27^{ns}$ | 2 | $0.83^{ns}$ | 4 | $0.94^{ns}$ |
| $G''$ | 0.248 | $0.74^{ns}$ | 2 | $0.17^{ns}$ | 2 | $0.41^{ns}$ | 4 | $1.19^{ns}$ |
| tan $\delta$ | 0.855 | $13.3^{***}$ | 2 | $42.7^{***}$ | 2 | $6.50^{**}$ | 4 | $2.00^{ns}$ |
| $\sigma_y$ | 0.421 | $1.64^{ns}$ | 2 | $2.82^{ns}$ | 2 | $1.83^{ns}$ | 4 | $0.95^{ns}$ |
| $\gamma_y$ | 0.375 | $1.35^{ns}$ | 2 | $0.99^{ns}$ | 2 | $2.77^{ns}$ | 4 | $0.82^{ns}$ |
| $R_A$ | 0.600 | $3.38^{*}$ | 2 | $8.27^{**}$ | 2 | $0.82^{ns}$ | 4 | $2.21^{ns}$ |
| pH at $t_{max}$ | 0.676 | $4.69^{**}$ | 2 | $1.05^{ns}$ | 2 | $15.3^{***}$ | 4 | $1.19^{ns}$ |
| pH at $t_{2min2}$ | 0.614 | $3.58^{*}$ | 2 | $2.27^{ns}$ | 2 | $7.23^{**}$ | 4 | $2.41^{ns}$ |

[a] $N = 27$; $T$, temperature; $S_0$, starter concentration; $S_0 \times T$, starter × temperature interaction; $R^2$, determination coefficient; $F$, ANOVA $F$-statistic; DF, degree of freedom; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{ns}$ not significant. For the definition of dependent variables, see the materials and methods section. $G'$, $G''$, tan $\delta$, $\sigma_y$, and $\gamma_y$ were measured at pH 4.8. Cutting time was defined as the time when the gel pH reached 4.8.

Table 3
Influence of main effects (temperature and starter concentration) on light backscatter parameters, rheological properties, and pH measurements[a,b]

| | Main effects | | | | | |
|---|---|---|---|---|---|---|
| | Temperature[c] (°C) | | | Starter concentration[d] (%) | | |
| | 22 | 27 | 32 | 0.5 | 2.75 | 5 |
| $t_{max}$ (min) | 354.2a | 242.8b | 191.4c | 342.5a | 242.0b | 204.0c |
| $t_{2min2}$ (min) | 404.9a | 279.0b | 223.4c | 394.2a | 279.4b | 233.8c |
| $t_{gel}$ (min) | 401.5a | 283.4b | 215.6c | 388.0a | 279.0b | 233.4c |
| $t^*_{cut}$ (min) | 536.4a | 380.7b | 322.4c | 523.5a | 382.2b | 333.8c |
| $R'_{max}$ ($min^{-1} \times 10^{-4}$) | 71.72a | 136.1b | 201.6c | 126.3a | 138.8b | 144.3b |
| $R''_{max}$ ($min^{-2} \times 10^{-4}$) | 3.237a | 8.090b | 11.61c | 7.026a | 7.609ab | 82.99b |
| $R''_{min}$ ($min^{-2} \times 10^{-4}$) | −5.048a | −9.631b | −12.78c | −7.474a | −9.532b | −10.45b |
| $R_{max}$ | 1.202a | 1.251b | 1.307c | 1.250a | 1.244a | 1.267a |
| $R^*_{cut}$ | 2.346a | 2.480b | 2.387a | 2.446a | 2.399a | 2.369a |
| $G'$ (Pa) | 80.3a | 76.4a | 64.4a | 81.4a | 70.7a | 69.0a |
| $G''$ (Pa) | 30.9a | 30.8a | 29.1a | 32.1a | 29.3a | 29.3a |
| tan $\delta$ | 0.386a | 0.408b | 0.456c | 0.401a | 0.420b | 0.428b |
| $\sigma_y$ (Pa) | 61ab | 89a | 52b | 51a | 82a | 70a |
| $\gamma_y$ | 1.19a | 1.38a | 1.12a | 0.98a | 1.39b | 1.32ab |
| $R_A$ ($min^{-1} \times 10^{-3}$) | 7.14a | 8.48ab | 9.77b | 7.99a | 8.63a | 8.77a |
| pH at $t_{max}$ | 5.82a | 5.87a | 5.87a | 5.97a | 5.83b | 5.76b |
| pH at $t_{2min2}$ | 5.57a | 5.61ab | 5.64b | 5.92a | 5.78b | 5.72b |

[a] LSM with same letters were not significantly different ($P<0.05$); number of replications = 3; number of observations, $N = 27$.
[b] For the definition of dependent variables, see the materials and methods section. $G'$, $G''$, tan $\delta$, $\sigma_y$, and $\gamma_y$ were measured at pH 4.8. Cutting time was defined as the time when the gel pH reached 4.8.
[c] LSM for each temperature was based on average of nine trials over a range of three starter concentration levels.
[d] LSM for each starter concentration was based on average of nine trials over a range of three temperature levels.

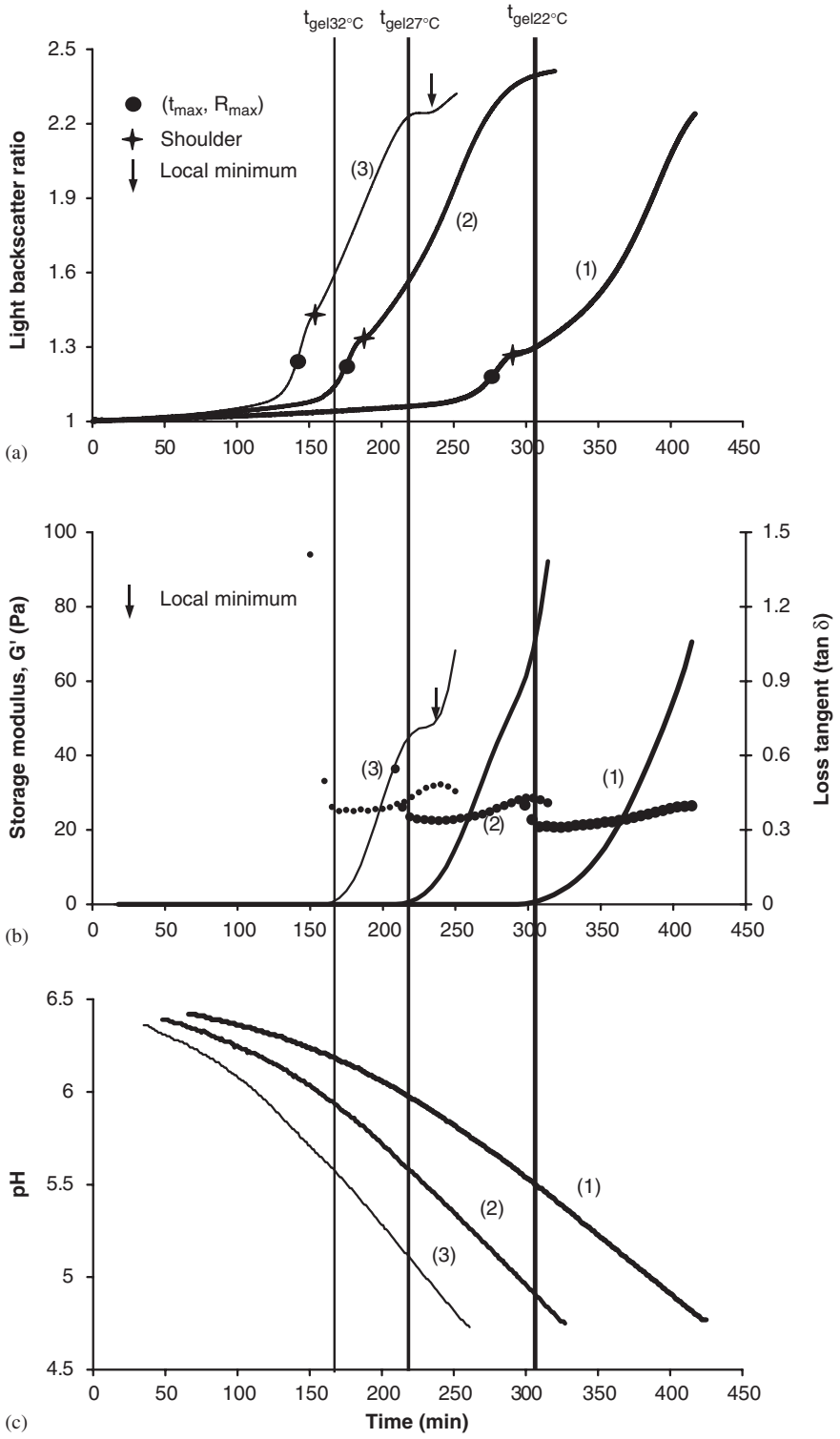


Fig. 2. Effect of temperature on (a) light backscatter profiles, (b) rheological properties (solid line, storage modulus; (●) loss tangent) and (c) acidification profiles: (1) 22 °C, (2) 27 °C, (3) 32 °C. Data corresponded to skim milk coagulated using 2 mg $kg^{-1}$ of rennet and 5% of starter culture. Vertical lines are for comparison between the different signals at the rheologically derived gelation time.

coagulation took place in the presence of a small amount of rennet.

The rheological parameters $G'$, $G''$, tan $\delta$, yield stress ($\sigma_y$) and yield strain ($\gamma_y$) were evaluated when the gel reached pH 4.8. The $G'$ and $G''$ values of gels decreased with increasing gelation temperature (Table 3). Gels made at 27 °C had the highest $\sigma_y$ and $\gamma_y$ values but differences between samples were not significant due to

variability in the data for this large deformation test. There was a significant increase in tan $\delta$ with increasing gelation temperature (Table 3). In agreement with our findings, Walstra et al. (2001) claimed that the lag time between the inoculation of the milk and the reaching of a determined pH value was smaller as temperature increases, but the gels obtained were less firm. Our results agree with the results of Lucey, van Vliet, Grolle, Geurts, and Walstra (1997c) who reported that at higher temperature, acid gelation proceeds faster but that large junctions do not form and, the weaker junctions have fewer protein–protein bonds per junction. According to Roefs and van Vliet (1990) and Lucey, van Vliet, Grolle, Geurts, and Walstra (1997b), increasing temperature enhances hydrophobic forces primarily inside each casein micelle, leading to shrinkage of the particle, resulting in smaller contact zones between casein micelles.

### 3.1.2. Effect of inoculum size

The time parameters significantly decreased with increasing inoculum level (Table 3; $P<0.0001$), reflecting a faster coagulation process. As for the increase in temperature, there was a significant increase in $R'_{max}$ and $R''_{max}$ when starter concentration increased while $R''_{min}$ decreased (Table 3). Effect of glucono-$\delta$-lactone (GDL) concentration on coagulation rate of acid milk gels has been widely reported. Lucey et al. (1997b) and Horne (2003) found that higher GDL concentrations resulted in shorter gelation times. But no reports were found regarding the effect of starter concentration on mixed coagulation. No significant differences were observed in either $R_{max}$ or $R^*_{cut}$ as a function of inoculum concentration (Table 3). Despite the time needed for the starter culture to reach pH 4.8 being shorter at higher starter concentrations, the value of light backscatter ratio at $t_{max}$ or at pH 4.8 (defined in the current study as cutting time) remained constant and was not dependent on the inoculum concentration. Castillo (2001) working with the enzymatic coagulation of goat milk did not find any significant differences in $R_{cut}$ as a function of coagulation factors such as pH, protein concentration, calcium addition and enzyme concentration. The effect of increasing inoculum concentration on $G'$, $G''$, tan $\delta$, $\sigma_y$ and $\gamma_y$ measured at pH 4.8 (Table 3) was similar to trends observed with increasing incubation temperature. There was a significant increase in tan $\delta$ when inoculum concentration increased. This may reveal a weakening of the casein structure (van Vliet, van Dijk, Zoon, & Walstra, 1991) that may be related to a faster rate of solubilization of colloidal calcium phosphate (CCP). The $G'$ values of the gels decreased with increasing inoculum concentration. Gels made with 2.75% starter had the highest $\sigma_y$ and $\gamma_y$ values.

### 3.1.3. Relationships between light backscatter, rheological and pH parameters

The Pearson's correlation coefficients were determined to evaluate relationships between dependent variables. It was observed (Table 4) that a slow fermentation rate, due to low temperature or starter concentration, affected the gel formation rate, which was supported by: (a) the rate of acidification, $R_A$ was positively correlated with $R'_{max}$ and $R''_{max}$ and negatively correlated with $R''_{min}$ and time parameters; (b) time parameters were negatively correlated with $R'_{max}$ and $R''_{max}$ and positively correlated with $R''_{min}$; (c) $R_A$ was positively correlated with incubation temperature ($r = 0.61$, $P<0.0008$). Fig. 3 shows the increase in time parameters and $R''_{min}$, and the decrease in $R'_{max}$ and $R''_{max}$ caused by decreasing temperature. These results are consistent with those published by Ustunol, Hicks, Payne, and Milton (1993) and Castillo et al. (2000). Due to the simultaneous effect of enzyme and continuous acidification on casein micelles, the faster network formation at higher incubation temperatures was

Table 4
Pearson correlation coefficients between dependent variables obtained by light backscatter, rheological, and pH measurements[a]

| | $t_{max}$ | $t_{2min2}$ | $t_{gel}$ | $t^*_{cut}$ | $R'_{max}$ | $R''_{max}$ | $R''_{min}$ | $R^*_{cut}$ | tan $\delta$ |
|---|---|---|---|---|---|---|---|---|---|
| $t_{2min2}$ | $0.999^{***}$ | — | — | — | — | — | — | — | — |
| $t_{gel}$ | $0.993^{***}$ | $0.992^{***}$ | — | — | — | — | — | — | — |
| $t^*_{cut}$ | $0.993^{***}$ | $0.993^{***}$ | $0.990^{***}$ | — | — | — | — | — | — |
| $R'_{max}$ | $-0.805^{***}$ | $-0.794^{***}$ | $-0.807^{***}$ | $-0.777^{***}$ | — | — | — | — | — |
| $R''_{max}$ | $-0.796^{***}$ | $-0.786^{***}$ | $-0.795^{***}$ | $-0.770^{***}$ | $0.978^{***}$ | — | — | — | — |
| $R''_{min}$ | $0.904^{***}$ | $0.898^{***}$ | $0.889^{***}$ | $0.886^{***}$ | $-0.940^{***}$ | $-0.936^{***}$ | — | — | — |
| $R^*_{cut}$ | $0.005^{ns}$ | $0.002^{ns}$ | $0.051^{ns}$ | $0.014^{ns}$ | $0.188^{ns}$ | $0.234^{ns}$ | $-0.195^{ns}$ | — | — |
| tan $\delta$ | $-0.753^{***}$ | $-0.741^{***}$ | $-0.751^{***}$ | $-0.744^{***}$ | $0.816^{***}$ | $0.781^{***}$ | $-0.814^{***}$ | $-0.054^{ns}$ | — |
| $R_A$ | $-0.525^{**}$ | $-0.524^{**}$ | $-0.516^{**}$ | $-0.505^{**}$ | $0.580^{**}$ | $0.566^{**}$ | $-0.536^{**}$ | $0.124^{ns}$ | $0.668^{***}$ |

$^{***}P<0.001$, $^{**}P<0.01$, $^{*}P<0.05$, $^{ns}$ not significant. For the definition of dependent variables, see the materials and methods section. Cutting time was defined as the time when the gel pH reached 4.8.

[a] $N = 27$.

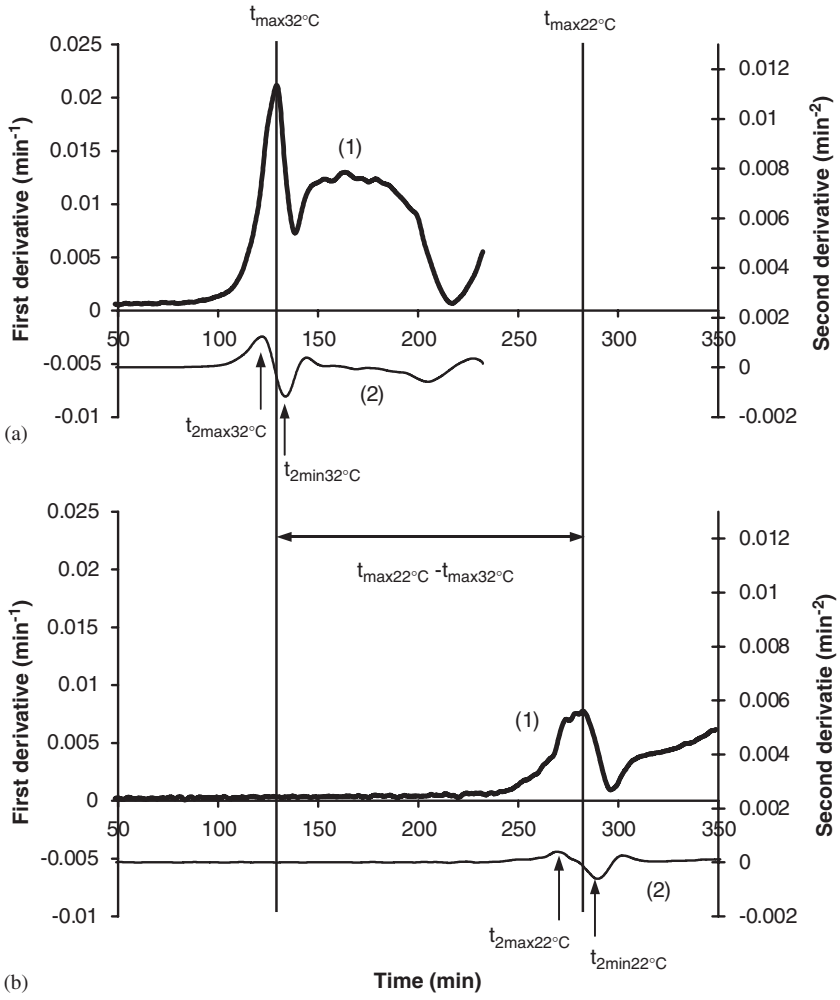


Fig. 3. Effect of temperature on the combined acid–rennet skim milk coagulation during the formation of cottage cheese gels. Curves represented the first (1) and second (2) derivatives of the light backscatter time profiles. Data corresponded to skim milk coagulated using both 2 mg kg$^{-1}$ of rennet and 5% starter culture: (a) incubation temperature 32 °C; and (b) incubation temperature 22 °C. Note that derivative values at the times $t_{max}$, $t_{2max}$ and $t_{2min}$ corresponded to $R'_{max}$, $R''_{max}$ and $R''_{min}$, respectively. Variables were defined in materials and methods section.

attributed to a complex combination of effects. Temperature affects the enzymatic hydrolysis of κ-casein, which in turn may affect milk gelation properties. On the other hand, increasing the incubation temperature by 10 °C raised $R_A$ by 37%. Thus, the combined effect of increasing temperature on hydrolysis, acidification rate, micelle aggregation and curd firming processes probably caused the decrease in the time parameters (this will be discussed further later). The significant correlation coefficients found between tan δ and time parameters (negative correlation), and tan δ and light backscatter response-based parameters (positive correlation: $R'_{max}$, $R''_{max}$; negative correlation: $R''_{min}$) (Table 4) clearly showed that when coagulation rate increased the gel had a more viscous-like character. That behavior agreed with previous studies (Lucey et al., 1997b,c; Lucey & Singh, 1998) on acid-induced gelation using GDL.

### 3.1.4. Relationship between time parameters

A number of Student's *t*-tests were conducted between the means of $t_{gel}$ and light backscatter parameters. Table 5 shows that the time parameters investigated were very significantly different from $t_{gel}$ except for $t_{2min2}$. The correlation between $t_{2min2}$ and $t_{gel}$ was very strong ($r = 0.992$, $P < 0.0001$; Table 4) and the pH values at these two times were not significantly different (Table 5). The parameter $t_{2min2}$ was first defined by Castillo et al. (2003b) as the second minimum of the second derivative of the light backscatter profile versus time. These authors estimated the kinetic constant for the curd firming reaction assuming a first-order reaction for the disappearance of crosslinking sites and using as a start time for the curd firming reaction $t_{2min2}$ (in curves with two maxima in the first derivative, as was observed in our profiles). This result strongly suggested that the rheological parameter, $t_{gel}$, and the light backscatter

Table 5
Student's $t$-test for the difference between means for light backscatter parameters and $t_{gel}$[a]

| $H_0$ | $N$ | Mean (min) | SD | $t$-value | $P$ |
|---|---|---|---|---|---|
| $t_{gel}-t_{2max}=0$ | 27 | 45.52 | 3.21 | 14.17 | 0.0001 |
| $t_{gel}-t_{max}=0$ | 27 | 37.32 | 3.22 | 11.59 | 0.0001 |
| $t_{gel}-t_{2min}=0$ | 27 | 29.53 | 2.75 | 10.75 | 0.0001 |
| $t_{gel}-t_{2max2}=0$ | 27 | 16.62 | 2.63 | 6.32 | 0.0001 |
| $t_{gel}-t_{2min2}=0$ | 27 | −2.32 | 2.48 | −0.94 | 0.36 |
| $t_{gel}-t_{2max2}=0$ | 27 | −47.3 | 6.65 | −7.12 | 0.0001 |
| $pH(t_{gel})-pH(t_{2min2})=0$ | 27 | −0.02 | 0.02 | −1.30 | 0.21 |

[a]$H_0$, null hypothesis; $N$, number of observations; Mean, mean of the differences between every two paired data; SD, standard deviation of the differences; $t$-value, $t$-Student statistics; $P$, probability that the $t$-test statistic will exceed its observed value. For the definition of variables, see the materials and methods section.

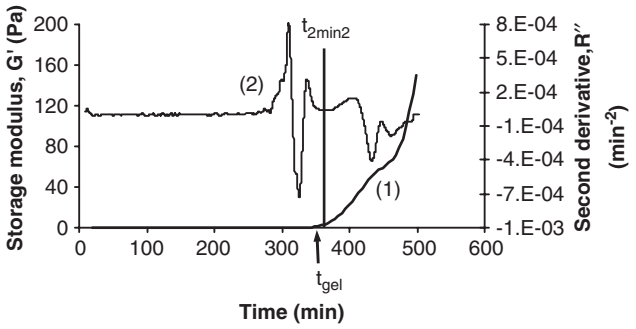


Fig. 4. Relationship between the rheologically defined gelation time obtained from the storage modulus profile (1) and the light backscatter parameter $t_{2min2}$, which corresponds to the second minimum of the second derivative light backscatter ratio profile (2). Rheological and light backscatter data corresponded to skim milk coagulated at 27 °C using both 2 mg kg$^{-1}$ rennet and 0.5% starter culture.

parameter, $t_{2min2}$, corresponded the beginning of the gel firming process (Fig. 4).

### 3.2. Light backscatter properties of mixed gels induced by bacterial fermentation and chymosin

#### 3.2.1. CCP solubilization in mixed gels

Profile 3 in Fig. 2a showed a local minimum after the initial increase in the light backscatter ratio. This local minimum was not observed in gels made at 22 °C, but it was sometimes found at 27 °C and it always occurred at 32 °C. Since the local minimum was found especially at 32 °C, this was probably the reason for the decrease in $R^*_{cut}$ when temperature was raised from 27 to 32 °C as mentioned in Section 3.1.1. The local minimum occurred at pH ~5.0. This relative minimum occurred concomitantly with a decrease or flattening of the $G'$ profile and an increase in tan $\delta$ (Figs. 2a and b). In acid-induced gels made from heated milk, a flattening of $G'$ occurs concomitantly with an increase in tan $\delta$ (e.g., Lucey & Singh, 1998). Lucey et al. (2000) reported the same phenomenon during the acidification of mixed gels with GDL and the minimum increased with increasing rennet. They did not observe this decrease in gels formed with rennet or acid alone. Tranchant et al. (2001) observed a similar shoulder in $G'$ at pH ~5.2 in mixed gels using starter culture to acidify milk. The shoulder was also accentuated by increasing the concentration of enzyme. A high tan $\delta$ indicates an increased susceptibility of bonds and strands in the gel to break or relax, thus facilitating more rearrangements of the gel (van Vliet et al., 1991). The maximum in tan $\delta$ may be a consequence of a partial loosening of the weak initial gel network due to the solubilization of CCP, while at lower pH values there would be increased protein–protein attractions between casein particles as the net charge decreases with the approach of the isoelectric point (Lucey & Singh, 2003). According to Lucey et al. (2000, 2001), a tan $\delta$ maximum should be observed in acid gels that have a high gelation pH, e.g., gels made from heated milk or unheated milk to which some rennet is added. Thus, it is suggested that the local minimum observed in the light backscatter ratio during mixed coagulation was caused by the progressive micelle demineralization induced by ongoing acidification of gelling or gelled casein particles. Note that at pH 5.25 all of the inorganic micellar phosphate is probably solubilized, whereas ~10% of the micellar calcium remains in the micelles (Walstra et al., 2001). As mentioned above, the minimum was not observed at 22 °C. At low temperatures, enzymatic action at the concentration used (2 mg kg$^{-1}$) was not able to induce a primary network until there was a significant pH drop because aggregation might become rate limiting at low temperatures. If the minimum was caused by CCP demineralization in the presence of rennet at high temperature, it should not occur in the absence of rennet.

This hypothesis was tested in triplicate at 32 °C and 0.5% starter concentration under the same conditions described in materials and methods but without adding rennet—it was anticipated that this conditions would exhibit the most distinct minimum in the light backscatter profile. Fig. 5 compares the typical profiles obtained with and without rennet. The pH profile was

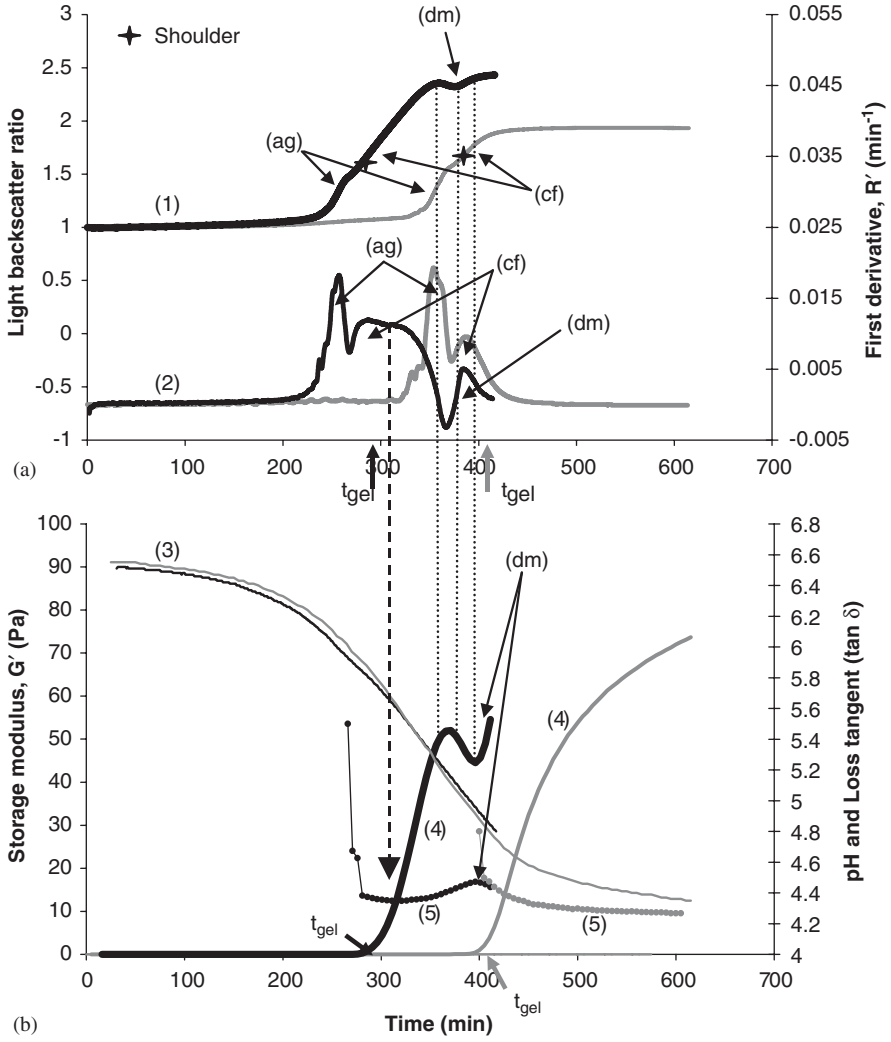


Fig. 5. Effect of rennet on formation of cottage cheese gels. Data corresponded to skim milk coagulated at 32 °C using 0.5% starter culture. Black line, gels made with 2 mg kg$^{-1}$ of rennet; Gray line, gels made without rennet: (a) light backscatter profiles (1) and their first derivatives (2); (b) pH profiles (3), storage modulus (4), and loss tangent values (5). Loss tangent values were incremented in 4 units to adjust their magnitude to the pH scale. (ag) aggregation domain; (cf), curd firming domain; (dm), CCP demineralization; $t_{gel}$, rheologically derived gelation time. Vertical lines are for comparison between the different signals.

almost identical for both gel types (Fig. 5b, 3). However, as expected, light backscatter profiles of mixed gels always had a local minimum (Fig. 5a, 1, dm) that was never observed in gels made without enzyme. The minimum had an effect on the first derivative. A third maximum was observed only in mixed gels (Fig. 5a, 2, dm). This third peak has never been previously observed by the authors in rennet-induced gelation (Castillo, 2001; Castillo et al., 2000, 2003a,b). As expected, the local minimum in the light backscatter ratio was always accompanied by a minimum in $G'$ (Fig. 5b, 4, dm) and a maximum in tan $\delta$ (Fig. 5b, 5, dm) that were absent in strictly acid gels. The results demonstrated that light backscatter measurement detects the partial loosening of bonds caused by CCP solubilization, which occurred at low pH within the casein particles in mixed gel networks. Roefs, Walstra, Dalgleish, and Horne (1985) studied the change in casein micelles with pH and reported a slight minimum in both hydrodynamic diameter and light scatter (90°) intensity at pH 5.2 and 8 °C. The authors related these phenomena to the swelling of casein micelles and some degree of casein dissociation caused by solubilization of CCP (almost complete at this pH). Dalgleish and Horne (1991) measured the change in scattered light intensity and apparent particle size during mixed coagulation of skim milk using diffusing wave spectroscopy (DWS), a dynamic light scattering technique. When coagulation of cultured milk was dominated by the effect of rennet, after an initial increase of both scattered light intensity and apparent particle radius and at pH ~5.3, they observed a decrease in both parameters. In contrast to

our results, the decrease in scattered light intensity was not followed by a subsequent increase. An increase should be expected since electrostatic attraction between and within casein micelles should begin to dominate the effect of CCP solubilization as negative $\zeta$-potential of casein decreases with acidification below pH 5.0.

### 3.2.2. Weakening of the mixed gels structure with increasing temperature

As commented before, increasing gelation temperature resulted in a less stiff gel (lower $G'$) at pH 4.8, an increase in network propensity to rearrange (higher $\tan\delta$), and an increase in light backscatter ($R^*_{cut}$ significantly increased from 22 to 27 °C; Table 3). In contrast, Figs. 2 and 5 likewise show a decrease of $G'$ and an increase in $\tan\delta$, but a decrease in light backscatter ratio. This local minimum in the gel stiffness took place at pH ~5.0 and was observed only in gels formed at high temperatures. The weakening of the network at pH 4.8 induced by increasing coagulation temperature was attributed to the shrinkage of casein micelles, which could have caused the observed increase in light backscatter. Light backscatter intensity depends, among other properties, on the material of the particle (i.e., the complex index of refraction) and its relative size (i.e., size parameter $= \pi d/\lambda$, where $d$ is the diameter of the particle and $\lambda$ is the wavelength). A complex combination of effects due to changes in the size parameter and in the index of refraction induced by the shrinkage of casein micelles are considered responsible for the observed increase in light backscatter. By contrast, it is proposed that the release of CCP from a gelling structure as pH decreased during milk acidification, in the presence of rennet, at high temperatures and at pH ~5.0, causes both a weakening of the existing network and a decrease of light backscatter. The decrease of light scatter may be related to modification of the refraction index of the micelle associated with the release of CCP.

### 3.2.3. The effect of rennet addition on gelation time of mixed gels

As it could be observed in Fig. 5, mixed gels coagulated faster and at higher pH than strictly acid gels (see pH values at $t_{max}$, $t_{max2}$, and $t_{gel}$). At 32 °C incubation temperature and 0.5% starter concentration, the average values for $t_{max}$, with and without rennet, were 4.3 and 6.0 h, respectively, while for $t_{gel}$ they were 4.8 and 7.0 h. In mixed and acid gels, the average pH values at $t_{max}$ were 6.0 and 5.3 and at $t_{gel}$ were 5.8 and 4.8, respectively. These results agreed with those reported by Lucey et al. (2001) for unheated milk. They found that mixed gels had much shorter $t_{gel}$ and higher pH values at gelation than acid gels. This could be expected since a continuous decrease of pH and enzymatic hydrolysis of $\kappa$-casein have a synergistic

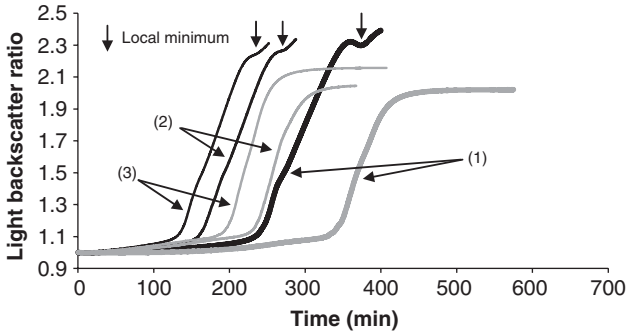


Fig. 6. Effect of inoculum size on CCP solubilization. Data corresponded to skim milk coagulated at 32 °C using different starter culture concentrations. Black lines, gels made with 2 mg kg$^{-1}$ of rennet; Gray lines, gels made without rennet: (1) 0.5% of starter culture; (2) 2.75% of starter culture; and (3) 5% of starter culture.

action on destabilize the casein macropeptide (CMP) "hairy layer" of casein micelles (Tranchant et al., 2001). Note that for acid gels, gelation occurred at pH 4.8, close to the isoelectric point of casein (pH = 4.6). Lucey and Singh (1998) reported that acid gelation of unheated milk generally occurs at pH 4.8.

At pH 4.8 (i.e., at cutting time as defined in this work), 32 °C and 0.5% starter concentration, light backscatter ratio and $G'$ values were higher for mixed than for acid gels (Fig. 5). Fig. 6 shows the same phenomenon but at different starter concentrations. It is observed that the light backscatter minimum due to demineralization of mixed gels was similar at 2.75% and 5% starter concentration but more pronounced at 0.5% starter concentration. That was attributed to a faster rennet–acid gel transition in gels having higher starter/rennet ratio.

### 3.2.4. Casein micelle aggregation of mixed gels

The shape of the light backscatter ratio and its first derivative was also distinctly affected by rennet action even before the demineralization minimum took place. Prior to any indication of coagulation by the rheometer ($t_{gel}$, Fig. 5b, 4) in either acid or mixed gels, the light backscatter profiles showed a small shoulder after $t_{max}$ (Figs. 2a and 5a, 1). This shoulder was indicated by the presence of two maxima in the first derivative (Fig. 5a, 2) corresponding to $t_{max}$ and $t_{max2}$. Castillo et al. (2003b) claimed that the appearance of a shoulder in the light backscatter profile after $t_{max}$ showed that gel formation during enzymatic goat milk coagulation consisted of two distinct and overlapping processes caused by the micelle aggregation and the protein network firmness development, respectively. In agreement with these authors, the first derivative peak (i.e., surroundings of $t_{max}$) was considered dominated by aggregation kinetics (Fig. 5a, ag) and the second derivative peak (surroundings of $t_{max2}$) by the curd firming kinetics (Fig. 5a, cf). According to Lucey and Singh (1998), it is possible that

the increase of $G'$ after $t_{gel}$ in acid gels reflects the inclusion of additional casein aggregates into the network as well as fusion and rearrangement of bonds between micelles in the network. This argues in favor of the existence of two stages during gel formation in mixed gels. It seems reasonable to conclude that gel development of mixed gels consists of two different stages, aggregation and firming, both of which contribute to the light backscatter profile.

Typically, there were not noteworthy differences in the shape of the first peak (i.e., consistently sharp) between gels made with or without enzyme. This seemed to suggest the absence of substantial differences in the initial aggregation mechanisms of those two different types of gels. According to Lucey and Singh (1998), milk gels are examples of 'particle gels' and have been described as fractal in nature. Fractal aggregates implies, among other characteristics, that the structure of the aggregates is scale-invariant (e.g., observation at different scales basically shows the same image). The number of particles in a cluster of radius, $R$, is proportional to $R^D$, where $D$ is a constant $<3$ called the fractal dimensionality. Lucey and Singh (1998) claimed that for either rennet- or acid-induced casein gels $D \approx 2.3$. Vétier, Desobry-Banon, Ould-Eleya, and Hardy (1997) claimed that $D$ for casein aggregates obtained at different acidification rates was constant and equal to 2.38. Since acid and enzymatic gels made under the same conditions, even at different aggregation rates, have similar $D$, it is not surprising that the change of light backscatter ratio during the aggregation step of acid gel formation remains the same if some rennet is added. Nevertheless, the structure of a fractal gel does not only depend on $D$ but also on $a$, and $\varphi$, where $a$ is the radius of primary particle and $\varphi$ is the volume fraction of particles in the whole system. In gels with the same $D$, gel properties can vary to a large extent depending on both parameters $a$ and $\varphi$. Thus, the important microstructural and rheological differences observed between acid and mixed gels should be attributed to changes on these two latest parameters rather than on $D$.

On the other hand, Castillo et al. (2003b) located the onset of aggregation in rennet-induced coagulation close to $t_{max}$. These authors estimated the kinetic rate constant for the aggregation reaction by fitting the observed light backscatter data points between $t_{max}$ and $t_{2min}$ to a second-order equation. It was assumed that $R'_{max}$ was correlated to the rate of casein aggregation during the primary network growth. Thus, the following Arrhenius-type relationship could be established:

$$\ln R'_{max} = \ln A - (E_a/RT), \quad (1)$$

where $A$ is an estimate of the Arrhenius factor, $R$ is the universal gas constant ($8.314\,J\,mol^{-1}\,K^{-1}$), $E_a$ an estimate of the activation energy for primary network formation, and $T$ the absolute temperature. A linear relationship was observed between $\ln R'_{max}$ and $1/T$ at each starter concentration ($R^2$ values were 0.984, 0.978 and 0.974 for 0.5, 2.75 and 5% starter concentration, respectively). The observed activation energy values were 83.1, 77.2 and $72.6\,kJ\,mol^{-1}$ for 0.5%, 2.75% and 5% starter, respectively. These results were consistent with those obtained by Kim and Kinsella (1989) for GDL-induced acid gels and tested using a rigidity scanning apparatus. From their data, the energy of activation for network formation during the acidification of preheated milk with GDL was $52\,kJ\,mol^{-1}$. As observed in Fig. 7, the apparent activation energy for the primary network growth decreased with increasing inoculum concentration. The decrease was statistically significant ($P<0.05$) between the two extreme starter concentrations. It may be related to a diminished energy barrier against aggregation at the beginning of the aggregation process caused by a lower pH at $t_{max}$. As shown in Table 3, the pH value at $t_{max}$ decreased with increasing starter concentration.

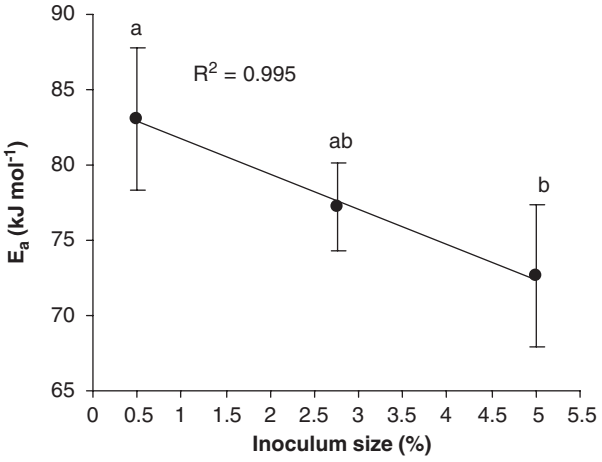


Fig. 7. Effect of inoculum level on activation energy of aggregation estimated by light backscatter. (●) Average value of replicates ($N = 3$). Error bars corresponded to $\pm 1$ SD interval of the average value. Averages with the same letters were not significantly different ($P<0.05$).

### 3.2.5. *Curd firming of mixed gels*

In Fig. 5a (2, ag), it is observed that the duration of the first peak of the first derivative is similar for mixed and pure acid gels. By contrast, the duration of the second peak was typically longer (i.e., more similar to a plateau than to a maximum) with rennet than without rennet (Fig. 5a, 2, cf) suggesting differences during the firming process between acid and mixed gels. The long duration of the second peak is related to a steady increase of the light backscatter ratio in mixed gels. Roefs, van Vliet, van den Bijgaart, de Groot-Mostert, and Walstra (1990) reported that mixed gels formed at pH > 5.15 had rheological properties essentially similar

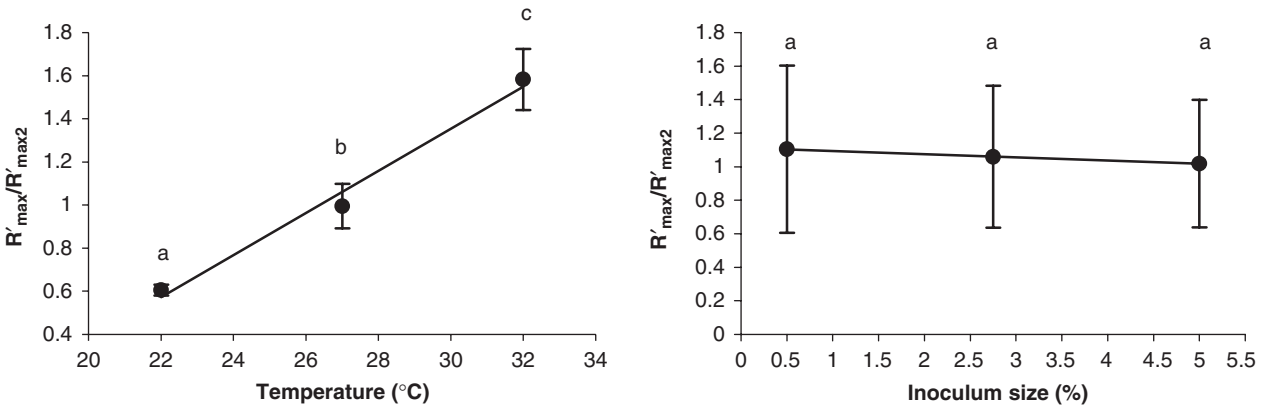


Fig. 8. Effect of temperature and inoculum size on the ratio between the first ($R'_{max}$) and the second ($R'_{max2}$) maxima of the light backscatter profile first derivative. (●) Average value of replicates ($N = 3$). Error bars corresponded to ±1 SD interval of the average value. Averages with the same letters were not significantly different ($P < 0.05$).

to rennet gels, while at pH < 5.15 the properties were similar to those of acid gels. The local minimum in $G'$ and 'consistency' curves of mixed gels observed by Tranchant et al. (2001) was interpreted as an intermediate stage of gel softening caused by CCP demineralization, reflecting the gradual transition from gels with rennet to acid character. According to these authors, this transition typically occurred within the pH range 5.5–5.0. As it was commented above, and shown in Fig. 5, we consider that the phenomenon described by Tranchant et al. (2001) is identical to that revealed by the minimum in the light backscatter profile at pH ~5, and thus must corresponds to the stage of CCP demineralization. Thus, the differences in the second peak of the light backscatter first derivative should reveal preliminary network changes, very likely related to initial CCP demineralization of casein micelles at very low modulus values. It should be noted, that this initial stage of rennet–acid gel transition was not accurately detected by the rheometer (Fig. 5, discontinuous lines) until modulus increased measurably (i.e., when the third peak of the light backscatter first derivative appeared). According to van Hooydonk, Hagedoorn, and Boerrigter (1986), in the pH region from 6.0 to 5.6, the casein micelles start swelling due to dominating effect of CCP solubilization. In Fig. 5, no sign of coagulation was detected by light backscatter above pH ~6.2 and $t_{max2}$ occurred at pH ~5.8. As pH decreased CCP was dissolved from the micelles (van Hooydonk et al., 1986), the charge on casein micelles was substantially altered, and the ionic strength of the solution increased. As a result, in mixed gels, the forces responsible for the integrity of the modified casein micelles constituting the initial network structure should be altered at pH values less than 6. This might affect the first derivative of light scatter profile, but in contrast, sufficient gel strength was required for the effects of demineralization to cause a measurable reduction of $G'$. Our results suggested that the main differences between acid and mixed gels are related to curd firming development.

### 3.2.6. *Relative effect of temperature and inoculum size on gel assembly of mixed gels*

Castillo et al. (2003b) proposed a method to estimate the kinetic rate constants for aggregation, $k_2$, and curd firming, $k_1$, reactions from the light backscatter profile in rennet-induced coagulation. The estimation of those kinetic rate constants was not attempted here because CCP demineralization substantially interfered with the light backscatter ratio profile. However, the relative effect of temperature and inoculum size on aggregation and curd firming was evaluated through the analysis of the rate ratio, $RR = R'_{max}/R'_{max2}$. Fig. 8 shows that inoculum size did not significantly affect RR, but RR did vary significantly with temperature. We consider that this reflected a greater temperature coefficient ($Q_{10}$) for aggregation as compared with curd firming. Castillo et al. (2003b) observed that $Q_{10}$ for aggregation was larger than for curd firming. Tranchant et al. (2001) claimed that $Q_{10}$ for aggregation was larger than for hydrolysis or growth of lactic acid bacteria. Then, it should be not surprising that at low temperatures the aggregation reaction becomes rate limiting. Tranchant et al. (2001) claimed that in mixed gels the aggregation reaction becomes rate limiting at temperatures below ~30 °C. This could be one of the reasons for the constant presence of the shoulder in the light backscatter profile after $t_{max}$ and before $t_{gel}$ (Figs. 1, 2 and 5).

## 4. Conclusions

Three commonly used procedures for making cottage cheese, i.e., short-, medium- and long-set, were

investigated by a randomized factorial design. Increasing the coagulation rate by raising either the incubation temperature or inoculum concentration resulted in a significant increase in the loss tangent value at cutting time, which suggested the gel was more flexible and susceptible to rearrangements. The storage modulus and the light backscatter ratio were higher in cottage cheese gels made with rennet than for strictly acid cottage cheese gels. Comparison of the parameters derived from rheological tests and infrared light backscatter at 880 nm showed that light backscatter could represent an alternative and potential useful method to study the coagulation of cottage cheese. Gel assembly of mixed gels consisted of two different stages, aggregation and firming, both of which contribute to the light backscatter profile. Aggregation had a higher $Q_{10}$ than curd firming in cottage cheese gels. The energy of activation of primary network formation was estimated by analysing the light backscatter parameters and was observed to decrease significantly from 83.1 to 72.6 kJ mol$^{-1}$ when the level of added starter increased from 0.5% to 5%, which could be related to a reduction in the energy barrier against aggregation caused by enhanced acidification rate. No significant difference was found between the rheologically determined gelation time and the light backscatter parameter, $t_{2\min 2}$, which suggested that $t_{2\min 2}$ could correspond to the initial formation of the gel network. A weakening of the gel (decrease of $G'$ and light backscatter ratio) was observed during coagulation at higher incubation temperatures in the presence of rennet and was attributed to CCP demineralization. Solubilization of CCP during continuous acidification in the presence of small amount of rennet marked a transition from a rennet-type of gel to an acid-type of gel, especially at higher temperatures. This transition was detected earlier by the optical measurement than by the rheological parameters.

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