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Effects of pH, temperature, $CaCl_2$ and enzyme concentrations on the rennet-clotting properties of milk: a multifactorial study

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Abstract

The effects of pH, coagulation temperature, $CaCl_2$ and enzyme concentrations on the rennet clotting properties of milk were assessed. Rennet coagulation time, coagulum firmness, curd firmness and gel firming rate were the coagulation parameters measured using a gelograph. A multifactorial design, considering two levels of coagulation temperature (28 and 44 °C), pH (6.0 and 6.8) and concentration of $CaCl_2$ (10 and 18 mM), was applied. pH showed the most important influence on rennet coagulation time and gel firming rate, while coagulation temperature showed the highest contribution to predict the firmness parameters. The effects of the interactions among pH, coagulation temperature and $CaCl_2$ concentration were significant, except for curd firmness. Rennet coagulation time was the parameter most affected by two- and three-factor combinations. The interaction between pH and $CaCl_2$ concentration was the only combination of factors that affected coagulum firmness and gel firming rate. However, curd firmness was affected only by coagulation temperature.

Keywords: Rennet coagulation; Multifactorial study; pH; Temperature; $CaCl_2$ concentration; Enzyme concentration

1. Introduction

Milk clotting by rennet is a normal practice in cheesemaking. The coagulum is usually cut, based on a subjective evaluation of its textural and visual properties. As is well-known, cheese yield and rheological characteristics of the product are strongly affected by the coagulation process (Ikonen et al., 1999; López & Laencina, 1994; van Hooydonk & Walstra, 1987). Such factors as enzyme concentration, temperature, pH and concentration of Ca^{2+} control the rennet clotting of milk (Daviau, Famelart, Pierre, Goudédranche, & Maubois, 2000; Gunasekaran & Ay, 1996; Payne, Hicks, Madangopal, & Shearer, 1993; Picón, Gaya, Medina, & Núñez, 1995). These factors have been widely studied, but few authors have studied the interactions between them and their influence on the rennet clotting properties of milk (Castillo, Payne, Hicks, & López, 2000; Daviau et al., 2000; Noel, 1991).

The rennet coagulation of milk combines an initial enzymic hydrolysis reaction and then a subsequent enzyme-independent protein aggregation reaction (van Hooydonk & Walstra, 1987). Clotting time decreases when enzyme concentration is increased because of a higher level of proteolysis of *k*-casein (Carlson, Hill, & Olson, 1987; Foltmann, 1959; López, Lomholt, & Qvist, 1998; McMahon, Brown, & Ernstrom, 1984). Linear relationships between enzyme concentration and the reciprocal of clotting time or between the reciprocal of the square root of enzyme concentration and the clotting time have been reported (Hyslop, Richardson, & Ryan, 1979; Kopelman, & Cogan, 1976). However, the clotting time of milk by mixtures of enzymes is not linear with the reciprocal of enzyme concentration (Picón et al., 1995). An increase in gel firming has been related to enzyme concentration (Zoon, van Vliet, & Walstra, 1988).

Milk coagulation is strongly dependent on the temperature (Dybowska & Fujio, 1996; Gunasekaran & Ay,

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1996). The velocity of coagulum formation increases progressively from 20 to 40–42 °C, but, at higher temperatures, the coagulation process slows down (Dybowska & Fujio, 1996; Eck, 1990). It has been observed that the temperature of the milk affects protein aggregation rate to a large extent and that increased temperature increases the rate of gel firming (Eck, 1990; Mc Mahon & Brown, 1984; Visser, van Rooyen, & Slangen, 1980). In the literature, most papers focus on the effect of heat-treatment of the milk, before rennet addition, on milk coagulation, most likely because most cheeses are made with pasteurized milk (Balcones, Olano & Calvo, 1996; Daviau et al., 2000; Laporte, Martel & Paguin, 1998; López, Botet, Hellín, Luna & Laencina, 1995; Lucey, Tamehana, Singh & Munro, 2000; Payne, Hicks & Shen, 1993).

The influence of pH on clotting time is very strong; the decrease in the pH of milk from 7.0 to 5.2 causes decrease in the clotting time (Eck, 1990; Ernstrom, 1974), the pH optimum for the hydrolysis of *k*-casein being 5.1–5.3 (Humme, 1972; Hyldig, 1993; López et al., 1998; van Hooydonk, Boerrigter & Hagedoorn, 1986). So, the most important effects of lowering the pH of the milk are the solubilization of micellar calcium phosphate (Dalgleish & Law, 1989; Le Gräet & Brul, 1993; Visser et al., 1980), the decrease in the net charge of the casein molecule, and the dissociation of casein from micelles (Dalgleish & Law, 1988; Desobry-Bannon, 1991; Gastaldi, Lagaude, & Tarodo de la Fuente, 1996; van Hooydonk, Hagedoorn & Boerrigter, 1986). It has also been reported that lowering the pH causes an increase in the curd firming rate (Daviau et al., 2000; Ramet, 1980). However, the coagulation of rennetted milk is not very efficient at pH lower than 5.0 (Kowalchyk & Olson, 1977).

Addition of Ca^{2+} decreases the rennet clotting time (Balcones et al., 1996; Montilla, Balcones, Olano & Calvo, 1995; Storry, Grandison, Millard, Owen & Ford, 1986; Tervala, Antila & Syvaejaervi, 1985) but, at high concentrations of $CaCl_2$ ($\geqslant 0.3$ M), the clotting time may be increased (McMahon, Richardson & Brown, 1984; Patel & Reuter, 1986). The addition of $CaCl_2$ also reduces the pH of milk, resulting in an increased protein aggregation rate (Flüeler & Puhan, 1978; Gastaldi, Pellegrini, Lagaude & Tarodo de la Fuente, 1994; Mehaia & Cheryan, 1983; van Hooydonk & van der Berg, 1988). The influence of increasing the concentration of Ca^{2+} on curd firmess has also been reported (Balcones et al., 1996; Patel & Reuter, 1986; Solorza & Bell, 1998). Addition of up to 10 mM Ca^{+2} to milk increased the rennetted gel strength (Lucey & Fox, 1993).

The objective of this work was to study the interrelated effects of pH, coagulation temperature, $CaCl_2$ and enzyme concentrations on the rennet clotting properties of milk. A multifactorial design was applied, considering enzyme concentration as a fixed effect.

2. Materials and methods

2.1. Preparation of the milk substrate

Low-heat spray-dried skim-milk powder (INRA, Poligny, France) was used for the experimental assays. The substrate was prepared according to International Dairy Federation Standard 110A (1987), by stirring 12 ± 0.02 g of the milk powder into 100 ml of aqueous solution of 0.01 M $CaCl_2$ (Panreac, Barcelona, Spain). The substrate was held for 30 min at room temperature in the dark. The pH of the substrate was 6.3 ± 0.1.

2.2. Rennet coagulation

Commercial rennet powder (Naturen, CHR Hansen, Hørsholm, Denmark) consisted of 80% (w/w) bovine chymosin and 20% (w/w) pepsin was used; the rennet strength was 1400 IMCU g^{-1} of coagulant.

The coagulation process was measured using a model NT Gelograph (Gel Instrumente AG, Thalwil, Switzerland) connected to a model DXY-1250 plotter (Roland, Tokyo, Japan). The gelograph is based on the principle of light absorption and scattering in the coagulating milk. Near infra-red light is passed through the milk sample and, depending on its structure, it will be scattered or absorbed to a greater or lesser extent. A photodiode detects the transmitted signal and the relative transmission is evaluated electronically, giving a direct measurement of the structure of the milk sample. Remeuf, Picque, and Corrieu (1993) compared the performance of the gelograph with the formagraph, using skim and whole milk, and found a good correlation between the time to inflection point of the transmission signal and the rennet coagulation time by the formagraph.

Rennet coagulation time, cutting time, coagulum firmness, curd firmness and gel firming rate were the coagulation parameters measured in coagulating milk substrate. Rennet coagulation time (min) was the time from rennet addition to the first appearance of an increase in viscosity of the rennetted milk. Cutting time (min) was established as twice the rennet coagulation time. Coagulum firmness was the firmness of the rennetted milk at the rennet coagulation time. Curd firmness was the firmness of the rennetted milk at the cutting time. The firmness of the rennetted milk was measured as percentage of relative transmission (% RET); so, a higher value of % RET represented a lower firmness. Gel firming rate was the change rate of firmness of rennetted milk at the rennet coagulation time and at the cutting time. Gel firming rate was obtained by dividing the difference in firmness at cutting and rennet coagulation times by the time difference between these two points (% RET min^{-1}).

2.3. Experimental design

A randomized block 2^3 experimental design was used (Hunter, McNuty, & Banks, 1997). Milk substrate was prepared at two pH values (6.0 and 6.8), and two $CaCl_2$ concentrations (10 and 18 mM), and the coagulation process achieved by using 0.02 g l^{-1} (28 IMCU l^{-1}) of commercial rennet at two temperatures (28 and 44 °C). A total of eight tests were conducted under this design for each replication. The experiment was randomly replicated twice. pH adjustment was performed at 25 °C by the gradual addition of 1M HCl or NaOH (Panreac, Barcelona, Spain). $CaCl_2$ concentration was modified by addition of crystals of $CaCl_2{\cdot}2H_2O$ (Panreac, Barcelona, Spain) to the milk substrate. Coagulation temperature was performed in a model Precisterm thermostatted water bath (Selecta, Barcelona, Spain). Milk substrates were held at the coagulation temperature during 15 min before rennet addition.

A 1^5 fixed-effects design was also used to study the changes in the coagulation parameters produced by changing each individual factor within the levels used in the multifactorial study. The effect of the enzyme concentration was also included. Thus, the fixed-effect values in each design were pH = 6.3 ± 0.1, 10 mM $CaCl_2$, coagulation temperature (T) = 32 °C, and 0.02 g l^{-1} (28 IMCU l^{-1}) rennet. The levels of pH were 6.0, 6.2, 6.4, 6.6 and 6.8, those of coagulation temperature were 28, 32, 36, 40 and 44 °C, those of $CaCl_2$ concentration were 10, 11, 12, 14 and 18 mM, and those levels of enzyme concentration were 14, 28, 56, 112 and 168 IMCU l^{-1}. Each individual test was done in triplicate.

The reproducibility of the coagulation parameters, measured by the gelograph, was calculated for six tests made under the same conditions (28 IMCU l^{-1} rennet, T = 32 °C, pH = 6.3 ± 0.1 and 12 mM $CaCl_2$).

2.4. Statistical analysis

The SPSS statistical package, version 9.0 (SPSS Inc., Michigan, USA), was used for the statistical analysis. From the data obtained in the 2^3 experimental design, a three-way analysis of variance (ANOVA) was done to establish the presence or absence of significant differences in the coagulation parameters, considering pH, concentration of $CaCl_2$ and coagulation temperature as factors. The data were also studied using multiple linear regression analysis, in a stepwise manner, considering the factors and the two- and three-factor interactions as independent variables. The levels for each factor were coded from −1 to +1. The stepping algorithm was F; i.e. the entry or removal of the independent variables from the equation was based on F-to-enter ($P \leqslant 0.050$) or F-to-remove ($P > 0.100$) limits. The t-test allowed cancellation of insignificant factors ($P \leqslant 0.010$). So, the final linear equations obtained may be useful for predicting changes in the coagulation parameters produced by changes in pH, coagulation temperature and concentration of $CaCl_2$. Pearson bivariate correlations between the coagulation parameters were also calculated.

From the data obtained in the 1^5 fixed-effects design, simple regression analyses were applied to fit the experimental data for the coagulation parameters with changes in pH, coagulation temperature, $CaCl_2$ and enzyme concentrations.

3. Results and discussion

The results of the reproducibility test showed low variation coefficients for all the coagulation parameters measured using the gelograph: 3.9% for rennet coagulation time (R), 2.2% for coagulum firmness, 0.8% for curd firmness and 3.2% for gel firming rate.

Experimental data from the 2^3 multifactorial design were studied by means of three-way ANOVA and multiple linear stepwise regression analysis. The results of the ANOVA (Table 1) showed that the effect of coagulation temperature (T) was highly significant ($P \leqslant 0.001$) for R, coagulum firmness, curd firmness, and gel firming rate. The efect of pH was also highly significant ($P \leqslant 0.001$) for all the coagulation parameters, except curd firmness. The effect of concentration of $CaCl_2$ (Ca) was significant only for R and coagulum firmness. Significant factor interactions were also observed, depending on the factors and coagulation parameters involved (Table 1). Thus, R was highly affected by all the two-factor interactions and (pH×Ca×T) combination. Coagulum firmness was significantly affected only by (pH×Ca) interaction and curd firmness was not significantly affected by either two- or three-factor interactions. In the case of gel firming rate, (Ca×T) combination was the only non-significant factor interaction (Table 1). These results were partially in agreement with those of Castillo et al. (2000) who reported a significant interaction between pH and coagulation temperature for gel firming rate in goat's milk clotting, although they did not find this interactive effect on

Table 1
Significance levels (P) of the three-way analysis of variance for the effects of pH, concentration of $CaCl_2$ (Ca) and coagulation temperature (T)

	pH	Ca	T	pH×Ca	pH×T	Ca×T	pH×Ca×T
R	***	***	***	***	***	***	***
CGF	***	**	***	**	NS	NS	NS
CDR	NS	NS	***	NS	NS	NS	NS
GFR	***	NS	***	**	***	NS	**

NS: not significant. R: rennet coagulation time; CGF: coagulum firmness; CDF: curd firmness; GFR: gel firming rate

** $P \leqslant 0.010$.

*** $P \leqslant 0.001$.

rennet coagulation time. Daviau et al. (2000) also found an interactive effect between pH and ionic strength on coagulation parameters.

Table 2 shows the results of the multiple linear stepwise regression analysis for predicting R, coagulum firmness, curd firmness and gel firming rate with changes in pH, T and Ca. According to the significance levels obtained in the three-way ANOVA, the multiple linear regression equation for predicting R showed that significant effects were obtained for pH, T, Ca and all of two- and three-factor combinations. Increases in T or in Ca produced a progressive decrease of R (Figs. 1 and 2, respectively), as has been reported by other authors (Balcones et al., 1996; Dybowska & Fujio, 1996; Gastaldi et al., 1994). R increased progressively as pH increased (Fig. 3) because the pH optimum for the enzymatic hydrolysis phase is below 5 (Hyldig, 1993; López et al., 1998). If the factor loadings in the equation are examined (Table 2), pH showed the highest contribution for predicting R. All the factor interactions showed lower contributions than those of principal effects, except that of (pH×Ca). The three-factor interaction was significant ($P > 0.010$) being its factor loading even higher than that of (T×Ca) interaction (Table 2). Fig. 4 shows the R values calculated from the multiple linear regression equation reported in Table 2. As observed, the longest values of R ($\geqslant$15.46 min) were always obtained at high pH (6.8) and the shortest ($\leqslant$7.26 min) at low pH (6.0), regardless of the other two factors. The contribution of the three factors (pH, T and Ca) was significant to give the longest R (52.00 min). However, the shortest R (2.58 min) was observed at low pH and high T, regardless of Ca. If factor combinations at high pH were considered, the shortest R (15.46 min) was achieved at high values of T (44 °C) and Ca (18 mM), while R was similar when these two factors were combined inversely (Fig. 4). In the case of factor combinations at low pH, the effect of T was the most significant factor to influence changes in R, and the effect of Ca on this coagulation parameter was apparent only at low T (28 °C) (Fig. 4).

The linear regression equation showed that pH, T, Ca and (pH×Ca) had significant effects ($P \leqslant 0.010$) on coagulum firmness (Table 2) which increased progressively

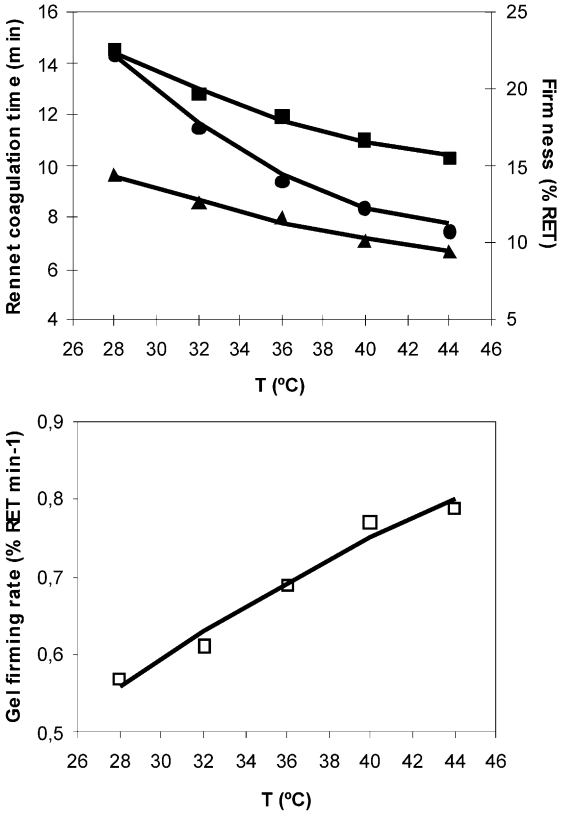

Fig. 1. Changes in rennet coagulation time (●), coagulum firmness (■), curd firmness (▲) and gel firming rate (□) with respect to coagulation temperature (T). Curves were fitted from simple regression equations with explained variance percentages higher than 89%. Points represent the means of three replicates.

(observed as a decrease in % RET) as T and Ca were increased (Figs. 1 and 2, respectively), while pH had the opposite effect (Fig. 3); these results agree with those reported by other authors (Daviau et al., 2000; Lucey & Fox, 1993). pH and T showed the highest contributions for predicting coagulum firmness (Table 2). Fig. 5 shows the coagulum firmness values calculated from the multiple linear regression equation reported in Table 2. As observed, the lowest coagulum firmness values ($\geqslant$22.80% RET) were obtained at high pH (6.8) and low T (28 °C) and the highest values ($\leqslant$12.50% RET) at low pH (6.0) and high T (44 °C), regardless of Ca

Table 2
Effects of pH, concentration of $CaCl_2$ (Ca) and coagulation temperature (T) on rennet coagulation parameters; multiple linear regressions with significant factors at $P \leqslant 0.010$

Equation	R^2	SE
$R = 16.35 + 11.62$ pH-5.40 $T-4.56$ Ca-4.75 (pH×Ca)-3.56 (pH×T)$+2.63$ (T×Ca)$+3.13$ (pH×T×Ca)	1.000	0.47
CGF $= 18.37 + 2.70$ pH-3.10 $T-0.72$ Ca-0.65 (pH×Ca)	0.982	0.69
CDF $= 12.24-2.02$ T	0.995	0.16
GFR $= 0.65-0.21$ pH $+$ 0.07 T $+$ 0.04 (pH×Ca)	0.964	0.05

R: rennet coagulation time; CGF: coagulum firmness; CDF: curd firmness; GFR: gel firming rate; R^2: determination coefficient; SE: standard error of the estimation.

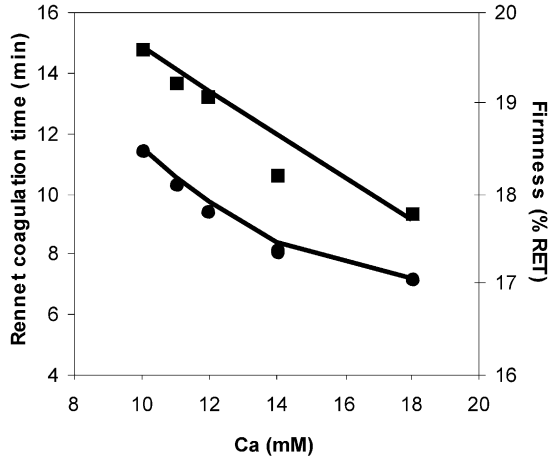

Fig. 2. Changes in rennet coagulation time (●) and coagulum firmness (■) with respect to concentration of $CaCl_2$ (Ca). Curves were fitted from simple regression equations with explained variance percentages higher than 75%. Points represent the means of three replicates.

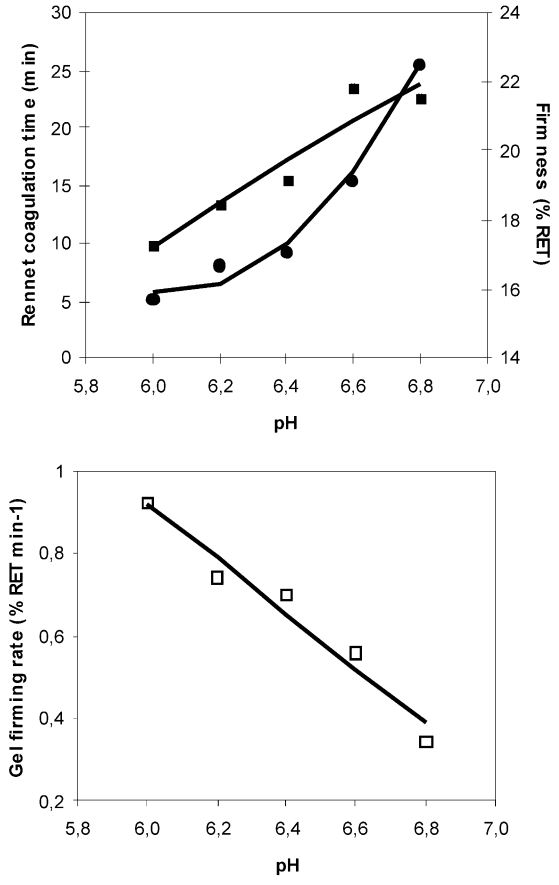

Fig. 3. Changes in rennet coagulation time (●), coagulum firmness (■) and gel firming rate (□) with respect to pH. Curves were fitted from simple regression equations with explained variance percentages higher than 88%. Points represent the means of three replicates.

factor. On the other hand, the effect of Ca on coagulum firmness was not significant at low pH (6.0) while at high pH (6.8) the highest level of Ca yielded a higher coagulum firmness. Also, it was noticeable that similar

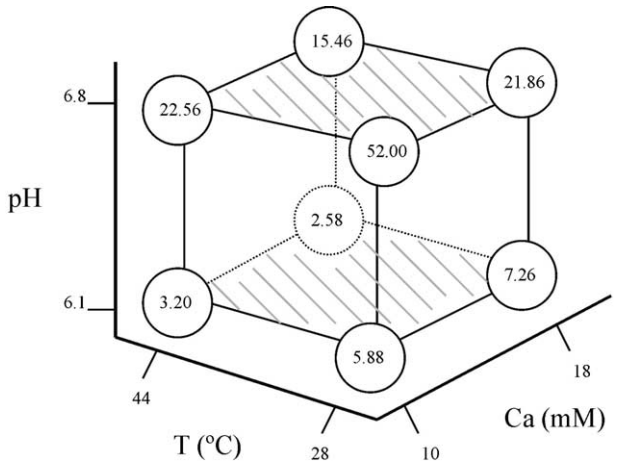

Fig. 4. Values of rennet coagulation time (min) calculated from the multiple linear regression equation reported in Table 2, considering all possible combinations of the factors pH, coagulation temperature (T) and $CaCl_2$ concentration (Ca).

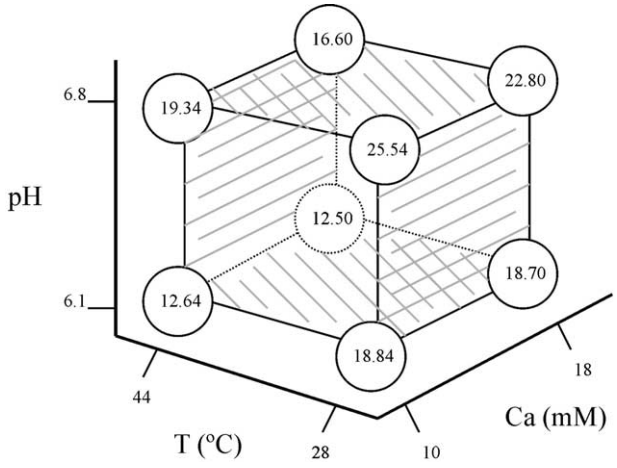

Fig. 5. Values of coagulum firmness (% RET) calculated from the multiple linear regression equation reported in Table 2, considering all possible combinations of the factors pH, coagulation temperature (T) and $CaCl_2$ concentration (Ca).

coagulum firmness values were obtained at low pH and T, regardless of Ca, and at high pH and T at low Ca (10 mM) (Fig. 5).

T was the only significant factor affecting curd firmness (Table 2). Curd firmness increased progressively (observed as a decrease in % RET) as T increased (Fig. 1). As was expected, no significant changes ($P \leqslant 0.050$) were obtained in curd firmness with pH and Ca (mean values of 12.71 ± 0.24 and 12.81 ± 0.29% RET, respectively). The curd firmness values calculated from the linear equation at low (28 °C) and high T (44 °C) were 14.26 and 10.22% RET, respectively (Table 2).

The linear regression equation showed that significant effects ($P \leqslant 0.010$) on gel firming rate were obtained for pH, T and (pH×Ca) (Table 2). Gel firming rate increased progressively as T increased (Fig. 1); these

results agree with those found by other authors, who reported that coagulation temperature markedly affected the rate of protein aggregation (Dybowska & Fujio, 1996). Considering that low pH values slightly promote the coagulation of rennetted milk (Kowalchyk & Olson, 1977; Ramet, 1980; Daviau et al., 2000), a progressive decrease of gel firming rate was observed as pH increased (Fig. 3). On the other hand, gel firming rate did not change significantly as Ca increased (mean value of $0.65 \pm 0.03\%$ RET min^{-1}). Other authors (Balcones et al., 1996; Patel & Reuter, 1986) have reported an increase in milk gel firming rate as the concentration of $CaCl_2$ is increased, but in a range of $CaCl_2$ concentrations lower than 10 mM. Also, addition of higher concentrations of $CaCl_2$ may enhance aggregation while inhibiting gelation (Patel & Reuter, 1986). According to the factor loadings in the multiple linear regression equation (Table 2), pH showed the largest contribution for predicting curd firmness. Fig. 6 shows curd firmness values, calculated from the multiple linear regression equation reported in Table 2. As observed, the fastest gel firming rate ($\geqslant 0.75\%$ RET min^{-1}) was always obtained at low pH (6.0) and the slowest rate ($\leqslant 0.55\%$ RET min^{-1}) at high pH (6.8), regardless of T and Ca. As expected, regardless of pH, high T (44 °C) produced faster gel firming rate than low T (28 °C). However, Ca had opposite effects depending on pH, that is, when the milk contained 18 mM of $CaCl_2$ an increase in gel firming rate at high pH (6.8) was observed, but the opposite ocurred at low pH (6.0) (Fig. 6). These results confirmed that the effect of Ca on gel firming rate was strongly dependent on the pH and agree with results found by other authors (Daviau et al., 2000) indicating that electrostatic interactions, together with steric repulsions, play complementary roles in the destabilisation of the casein micelle system.

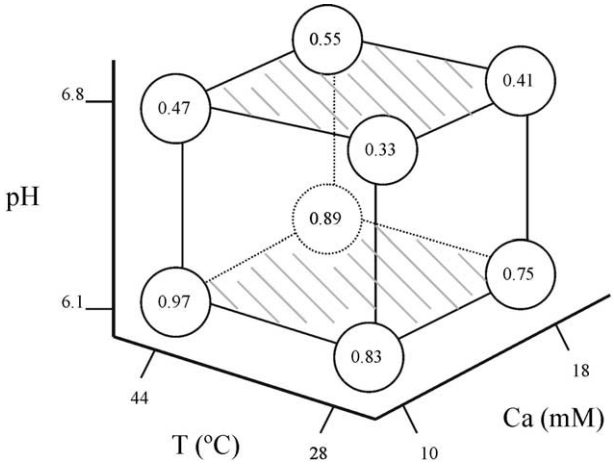

Fig. 6. Values of gel firming rate (% RET min^{-1}) calculated from the multiple linear regression equation reported in Table 2, considering all possible combinations of the factors pH, coagulation temperature (T) and $CaCl_2$ concentration (Ca).

The effect of concentration of enzyme on coagulation parameters was also studied but considering pH, T and Ca as fixed-effects. Fig. 7 shows the changes in the coagulation parameters with the concentration of enzyme. R decreased as concentration of enzyme increased and the decrease was most pronounced from 14 to 56 IMCU l^{-1}. From 56 to 168 IMCU l^{-1} the R tended towards a constant value. These results agree with those reported by other authors (Carlson et al., 1987; López et al., 1995). Coagulum firmness increased progressively as the concentration of enzyme increased. However, an increase in the concentration of enzyme produced a slight decrease in curd firmness, particularly when the enzyme concentration increased from 14 to 56 IMCU l^{-1} (Fig. 7). This effect could be due to the strong decrease in R as enzyme concentration increased, yielding excessively short cutting times ($2 \times R$). As a consequence, the time for aggregating the protein was excessively brief to get a more consistent rennet gel. This observation agrees with mathematical equations established to

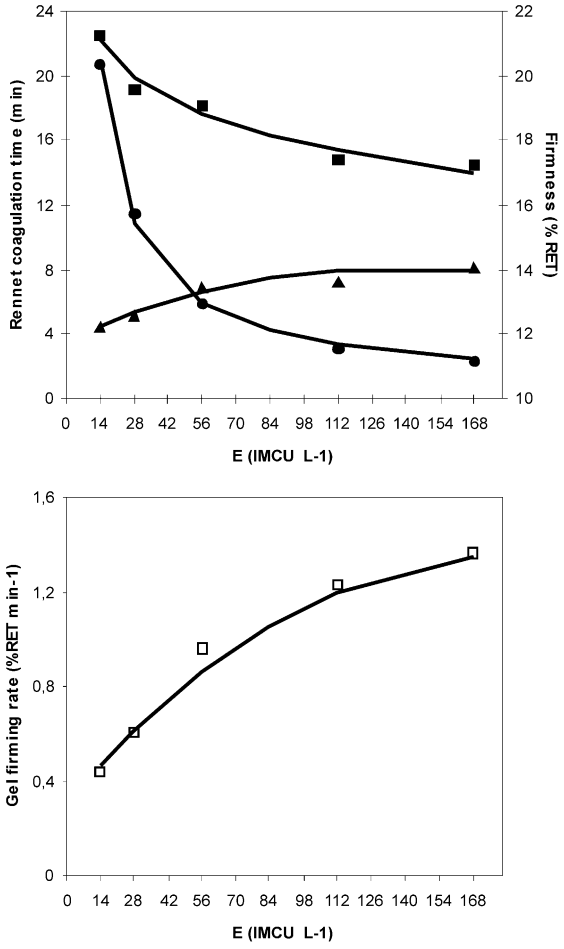

Fig. 7. Changes in rennet coagulation time (●), coagulum firmness (■), curd firmness (▲) and gel firming rate (□) with respect to concentration of enzyme (E). Curves were fitted from simple regression equations with explained variance percentages higher than 85%. Points represent the means of three replicates.

Table 3
Pearson bivariate correlations among rennet coagulation time (R), coagulum firmness (CGF), curd firmness (CDF) and gel firming rate (GFR)

	R	CGF	CDF
R	[–]	[–]	[–]
CGF	0.854[a]	[–]	[–]
CDF	0.365[b]	0.737[a]	[–]
GFR	−0.861[a]	−0.813[a]	−0.312[b]

[a] $P \leqslant 0.001$.
[b] Non-significant correlation ($P \leqslant 0.050$).

describe coagulation processes (Scott Blair & Burnett, 1963; Scott Blair & Oosthuizen, 1961). According to other authors, a progressive increase in gel firming rate was produced by increase in the concentration of enzyme (Castillo et al., 2000).

Table 3 shows the Pearson bivariate correlations between coagulation parameters. High bilateral correlations ($\geqslant 0.850$) between R and coagulum firmness and R and gel firming rate were observed; i.e. a longer R, a lower coagulum firmness (measured as higher % RET) and lower gel firming rate. In this last case, this result was because the cutting time was arbitrarily established (as $2\times R$) in this study. Nevertheless, R and curd firmness were not significantly correlated ($P \geqslant 0.05$); for example, R values of 52 or 7 min, values obtained at 44 °C under different conditions of pH and Ca, gave similar curd firmness (around 14% RET). These experimental results were due to the strong effect of T on curd firmness, regardless of the other coagulation factors (Table 2). Bilateral correlation between coagulum firmness and curd firmness was also found; i.e. a higher coagulum firmness, correlated with a higher curd firmness. Gel firming rate and coagulum firmness were highly correlated ($\geqslant 0.810$); thus, a faster gel firming rate gave a higher coagulum firmness. However, gel firming rate and curd firmness were not significantly correlated ($P > 0.05$); in other words, regardless of the differences in gel firming rate produced by different factor combinations (pH, Ca and T), the curd firmness was only dependent on T (around 10% RET at 28 °C and 14% RET at 44 °C).

4. Conclusions

Rennet coagulation time (R), coagulum firmness, curd firmness and gel firming rate were strongly affected by coagulation temperature, pH, $CaCl_2$ and enzyme concentrations. The multifactorial study revealed that pH showed the most important influence on R and gel firming rate, while T showed the highest contribution to predict the firmness parameters. Except for curd firmness, the coagulation parameters were also considerably affected by interactions among pH, T and Ca. R was the variable most affected by two- and three-factor combinations. The interaction (pH×Ca) was the only factor combination that affected coagulum firmness and gel firming rate. However, curd firmness was only strongly affected by T, but not by the other factors. On the other hand, the coagulation parameters were considerably affected by changes in enzyme concentration although interactions between enzyme concentration and pH, T or Ca were not studied.

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