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Effect of protein and temperature on cutting time prediction in goats' milk using an optical reflectance sensor

Manuel Castillo[1]*, Fred A Payne[2], Clair L Hicks[3], José Laencina[1] and María-Belén López[1]

[1] Department of Food Technology, University of Murcia, E-30071, Murcia, Spain
[2] Department of Biosystems and Agricultural Engineering, University of Kentucky, Lexington, KY 40546, USA
[3] Department of Animal Sciences, University of Kentucky, Lexington, KY 40546, USA

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An objective method for determining the coagulum cutting time is needed to improve consistency and processing efficiency of goats' cheese. A fibre optic sensor was used to measure the backscatter of near-infrared radiation at 880 nm during the coagulation of skimmed goats' milk for the purpose of predicting coagulum cutting time. A randomised block design, replicated three times, was used to test the effect of three protein concentrations (3, 5 and 7% (w/w)) on diffuse reflectance parameters for cutting time prediction of milk coagulated at five different temperatures (20, 25, 30, 35 and 40 °C) to assure a wide range of coagulation rates. The inclusion of a protein term in the existing algorithms was essential to reduce the standard error of prediction to under 6·2 min. An algorithm including a time-based parameter and a protein term, $T_{cut}=\beta_0\ T_{2\,min}\ (1+\gamma\%\ Protein)$ was found to predict cutting time with a SEP of 2·42 min and an R^2 of 0·98. γ was considered constant ($\gamma=-0{\cdot}0674$, goats' milk) representing the protein effect on β_0. Algorithms using response-based parameters (such as change in reflectance ratio) and the composition parameter protein required additional regression parameters such as temperature and an intercept term to predict the cutting time with the same precision as algorithms using only time-based parameters. Time-based parameters were found to decrease proportionally with increasing temperature and decreasing protein concentration. Response-based and mixed-based parameters were found to decrease with decreasing temperature. Reflectance ratio at cutting time did not significantly change with protein concentration for skimmed goats' milk. The activation energy of κ-casein hydrolysis was calculated based on changes in reflectance profile parameters and was found to be in the range 63–72 kJ mol^{-1}.

Keywords: Diffuse reflectance, cutting time, protein, temperature, activation energy, goats' milk, rennet.

Traditionally, in cheese plants, the curd is cut after a predetermined time from the enzyme addition or upon the operator's judgement of cutting time based on a simple empirical evaluation of firmness and visual appearance. Cutting the coagulum after a pre-fixed time is questionable, because variations in milk properties and/or processing conditions affect the optimum cutting time. The subjective operator's judgement may be accurate, if an experienced cheesemaker can recognise optimum gel properties for cutting. A number of authors have noted the disadvantages of an inappropriate cutting time selection (Hori, 1985; Payne et al. 1993; Passos et al. 1999). Cutting the curd too early results in a poor yield, while cutting it too late results in poor quality. These reasons suggest the importance of an objective and inline method for predicting the optimum coagulum firmness for cutting that will maximise cheese yield and quality (van Hooydonk & van den Berg, 1988).

Several instruments have been employed for monitoring milk coagulation and/or used to determine optimum firmness for cutting the curd. Most of them expose the gel to stress and deformation or even disrupt the curd during measurements. These instruments are not practical for inline applications where continuous monitoring is required. Other cutting time prediction technologies that have been investigated have based their prediction on measured changes in heat induced convection by a hot wire probe (Hori, 1985), transmission of near infrared radiation (Remeuf et al. 1993), ultrasonic properties (Gunasekaran & Ay, 1994), diffuse reflectance (light backscatter) of near infrared radiation (Payne 1995) and torsional and vibrational properties (Schulz et al. 1997). Optical sensors employing

*For correspondence; e-mail: mcastillo@um.es

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fibres to measure light backscatter have been applied to determine cutting time of cows' milk (Payne et al. 1993) and goats' milk (Castillo et al. 2000). Desirable characteristics of the optical sensor used in this work include its small size, non-destructive measurement, good sanitation characteristics, and their inherent precision. These characteristics make it a potential technology for cutting time prediction. But in addition, it also has been shown that this technology could be useful for some other interesting applications such as clotting time prediction (Castillo et al. 2000).

A cutting time sensor technology must perform over a wide range of milk protein levels to be commercially viable. Van Hooydonk & van den Berg (1988) claimed that due to the effect of protein concentration on the rate of firming, it was necessary to adjust the cutting time selection procedure if pre-concentrated milk was used with the traditional manner of cheese production. Payne et al. (1993) conducted diffuse reflectance tests on cows' milk having protein content of 3·3, 4·3 and 5·3%, and used K_{20} as measured by a Formagraph® for actual cutting time determination. They observed that a protein term was necessary for the cutting time prediction. They tested the equation $K_{20}=\beta_0+\beta_1(T_{max})+\beta_2$ (% Protein). Payne (1995) reported that a sensor employing optical fibres to measure light backscatter at 820 nm was successfully operated inline in a commercial cheese manufacturing plant for over a year. The algorithm used was $T_{cut}=\beta\ T_{max}$, where β was a constant. In practice, β was obtained to match the cheese plant operator's selection of cutting time with the predicted cutting time. Payne (1995) stated that cutting time was accurately predicted using the above algorithm in a cheese plant where protein concentration was relatively constant. O'Callaghan et al. (1999) affirmed that curd firming time was 4–5 times as sensitive to protein changes as to rennet concentration changes. These authors claimed that in order to describe the ratio of curd firming time to gel time, it was necessary to include a protein term in the prediction algorithm if the protein levels varied significantly. As a result the application of optical sensors in cheese plants, where the protein content changes significantly, requires an algorithm that accounts for the effect of protein. Additionally, it is desirable that the algorithm should contain only one calibratable coefficient to simplify in-plant calibration. Thus the goals of this study were to determine:

1. The effect of temperature and protein concentration on the parameters generated from the diffuse reflectance profile of skimmed goats' milk.
2. A cutting time prediction algorithm, containing only one calibration coefficient that predicts cutting time over a wide range of protein concentrations.

Material and Methods

Experimental design and statistical analysis

A complete randomized block design with protein content and coagulation temperature as main factors and with three replications was used to determine protein and temperature effect on the reflectance profile during coagulation process. Different levels of temperature were selected rather than pH or enzyme concentration to obtain reflectance profiles over a range of reaction rates because temperature could be controlled precisely. Protein concentration was varied to determine the effect of substrate (casein) concentration. Skimmed goats' milk was adjusted to three levels of protein (3, 5 and 7%) and coagulated at five different temperatures (20, 25, 30, 35 and 40 °C) using a constant concentration of enzyme (0·06 ml/kg milk) and calcium chloride (0·47 g/kg milk). We observed that homogenization of the samples interfered with the diffuse reflectance signal and a cream on the surface of the samples decreased the accuracy of cutting time determination. For that reason, we skimmed the milk instead of standardizing the fat to a constant level. A total of 15 tests were conducted for each replication. The diffuse reflectance profile and cutting time were collected for each test and the profile parameters were generated.

The data were analysed using the general linear model (GLM) procedure of the Statistical Analysis System (SAS® 1999, SAS Institute Inc., Cary, NC 27513-2414, USA). Protein concentration and temperature were established as the model's main effects. One batch of milk was used for each replication (three replications). The qualitative variable 'Rep' was added, as a main effect, to the statistical model to quantify the confounded effect of the initial milk composition and the replication effect on the dependent variables. The quantitative variable 'actual fat', measured for each sample tested, was included in the statistical model as a co-variable to quantify the effect of the unadjusted fat level on the samples. Calcium activity was also included in the model as a co-variable to account for calcium variability between samples. Calcium activity was removed from the final model because it was not significant for the variables tested.

The least square means (LSM) and significance of each treatment were computed using type IV sum of squares. LSM for each protein level was calculated taking into account all the temperatures (15 observations) while LSM for each temperature was computed considering the three protein levels tested (9 observation in total). Differences between treatment means were considered to be significant when probabilities were $P<0{\cdot}05$. Regression of several different models using the GLM, NLIN and Maximum R^2 procedures of SAS was tested for predicting cutting time, with the independent variables in combination with dependent variables generated from the diffuse reflectance profile.

Enzyme

The coagulation enzyme used was recombinant chymosin (EC 3.4.23.4, isozyme B) supplied by Chr. Hansen Inc. (Milwaukee, WI 53214-4298, USA). An enzyme concentration (E_o) of 0·06 ml/kg milk was used. This resulted in

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cutting times between 10 and 90 mins within the range of temperatures and protein concentrations tested. A constant sample dilution was assured by adding a total volume of 10 ml containing 120 µl (2 °C) of recombinant chymosin and the remainder de-ionised water. The enzyme dilution was added to the 2 kg goats' milk sample after thermal equilibrium between the water bath and sample was reached. The diffuse reflectance measurements were initiated immediately upon adding the enzyme to the sample.

Saturation of the enzyme by the substrate

Several authors found that κ-casein hydrolysis follows the Michaelis-Menten equation (Garnier, 1963; Carlson, 1982; Dalgleish, 1993):

$$\frac{-d[S]}{dt} = \frac{V_{max} \cdot [S]}{K_m + [S]}$$

where, V_{max} is the maximum reaction rate (occurs when the enzyme is saturated by the substrate), K_m the Michaelis-Menten constant, and $[S]$ the substrate concentration at the reaction time t. When $[S] \gg K_m$ the reaction order is approximately zero. Conversely, if $K_m \gg [S]$ the reaction is approximately first order. The condition $[S] \gg K_m$ was viewed as desirable for analysing data, because hydrolysis does not depend on the substrate concentration. Van Hooydonk & Walstra (1987) reviewed the kinetic parameters for the action of chymosin in several systems. K_m values of chymosin on milk were in a wide range 0·001–0·05 mM. Assuming that 73·8% of the total protein in goats' milk is casein and 11·5% of the casein is κ-casein (Quiles & Hevia, 1994), it was estimated that the 3, 5 and 7% protein content samples contained 0·13, 0·22 and 0·31 mM κ-casein/l, respectively. $[S]$ should be ten times greater than K_m to be considered saturated with the substrate. Based on these calculations the saturation condition was not met for 3% protein unless the magnitude of K_m was smaller than 0·013 mM which is unlikely considering the references mentioned previously. Most authors have found that the first order reaction described the enzymatic hydrolysis during coagulation of milk (van Hooydonk et al. 1984; van Hooydonk & Walstra, 1987; Lomholt, 1996; López et al. 1997). The condition $K_m \gg [S]$ is required for this to be true. Assuming that $K_m \gg [S]$ and there is a first order reaction then an increase in protein concentration will result in an increase in the initial hydrolysis rate.

Goats' milk sample preparation

A 44 l batch of goats' milk, from a single goat herd in Eastern Kentucky, was collected on the Monday of each test week, during October and November 1999. Each batch of goats' milk was used to run 15 tests that constituted a replication. The milk was skimmed at 35–40 °C using an electric cream separator (Subitas DE 085, The Coburn Company Inc., Whitewater, WI 53190-0147, USA). An anti-foaming agent (Antifoam 1520-US, Dow Corning Corporation, Midland, MI 48686-0994, USA) was added at a concentration of 10 ppm of active silicone to reduce foaming during the skimming and condensation processes. Skimmed goats' milk was pasteurized at 65 °C for 30 min and then condensed by low temperature evaporation (30–40 °C) in a lab scale vacuum evaporator to reduce the volume to a protein concentration of approximately 10%. After condensation, the milk was stored at 2 °C. The above process was performed the same day that the milk was collected.

For every replication, a salt solution that simulated milk ultrafiltrate (SMUF-skimmed milk ultrafiltrate; Jenness & Koop, 1962) was prepared for diluting the condensed skimmed milk to the desired protein level. SMUF was used instead of de-ionised water to allow casein micelles to equilibrate after pasteurization and evaporation steps. Milkoscan FT 120 (Foss Electric A/S, 3400 Hillerød, Denmark) was calibrated with 12 goats' milk samples supplied by DQCI Services (Mounds View, MN 55112-1400, USA). Condensed skimmed milk from each batch was analysed for protein concentration. Protein concentration was used to calculate mixture proportions of SMUF and condensed skimmed milk to obtain samples of 2060±0·01 g with the target protein concentration (3, 5 and 7%). The calculated amount of condensed skimmed milk and 100 g less than the required amount of SMUF were weighed, mixed and placed into a 2200 ml plastic container. Anhydrous calcium chloride (0·96 g) was added with stirring to each sample by adding 1·5 ml of a 5·77 M dilution of calcium chloride. The determination of the optimum amount of calcium to add presented a perplexing problem because protein crosslinking needs colloidal calcium phosphate for the reaction to take place. Adding increasing protein while maintaining a constant colloidal calcium phosphate concentration could drive to non-valid conclusions. Adding calcium in proportion to the amount of protein could introduce a confounding effect due to the simultaneous change of both factors. For these reasons, the approach selected was to add a constant level of calcium at an excess amount to all three-protein concentration tested. Sufficient calcium chloride for 7% protein was added to assure the availability of enough calcium ions for cross-linking reactions at the three protein levels tested. The concentration of free calcium was expected to vary and thus Ca^{++} ions concentration was measured and placed in the statistical model as a co-variable. After calcium chloride addition, the milk was cooled to 2 °C. A linear regression between ml 1 M NaOH dilution and pH was conducted at 2 °C for milk containing 3, 5 and 7% protein and used to predict the amount of NaOH needed to adjust the pH of milk samples to 6·8. The alkali was added to the milk samples at 2 °C with continuous stirring. The pH-adjusted samples were then stored at this temperature until tested.

The final pH of each sample was adjusted when necessary to 6·8 just prior to coagulation by adding either 1·0 M HCl or 1·0 M NaOH to the sample at 2 °C.

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A constant sample dilution was maintained by using de-ionised water as needed to bring the total volume added (calcium dilution, NaOH, HCL, and/or de-ionised water) to 100 ml. Two 20 ml aliquots from each sample were taken while continuously stirring to verify sample protein and fat concentration. Another aliquot of 20 ml was collected to measure calcium activity. The 2000 g samples were then heated to the desired temperature and transferred to a 2 l vat for coagulation.

Ca^{+2} and pH measurements

Measurements of pH were performed using a combination glass pH electrode (BN 81-04, Thermo Orion, Beverly, MA 01915-6199, USA) connected to an expandable ion analyser (EA 940, Thermo Orion). The pH meter had a sensitivity of ±0·01 pH units and was calibrated at 2 °C using a two-point calibration method (Buffer salt 7·00 and 4·01; Thermo Orion). Calcium measurements were made using a calcium-selective electrode (93-20, Thermo Orion) and a single junction reference electrode (90-01, Thermo Orion) filled with the reference electrode filling solution both connected to the expandable ion analyser. The calcium-selective electrode had a reproducibility of ±4% and it was calibrated using a two-point calibration method by direct measurements. Solutions of 10^{-4} and 2×10^{-3} M were prepared using a 0·1 M calcium standard (Thermo Orion). An ionic strength adjustor (Calcium ISA, Thermo Orion) was added to all the calcium standards and samples (ratio of solution to ISA, 50 : 1) so that the background ionic strength was high and constant relative to variable concentrations of calcium ion. The calcium-selective electrode was calibrated at 2 °C and all sample measurements were made at this temperature.

Monitoring diffuse reflectance profiles

The reaction vessel used to monitor sample coagulation was stainless steel (15 cm × 13·3 cm × 15 cm) with a removable insulated lid (to reduce evaporative cooling). Sample temperature was controlled using a circulating water bath (Lauda RM 20, Brinkmann Instrument Inc., Westbury, NY 11590-0207, USA) with a control accuracy of ±0·01 °C. Milk temperature was measured with a precision thermistor thermometer (5831 A, Omega Engineering Inc., Stamford, CT 06907-0047, USA) with resolution of ±0·01 °C and accuracy of ±0·2 °C. Samples (2000 g) were warmed using a microwave oven to 0·1 °C above the target test temperature and transferred to the reaction vessel. The samples were allowed to thermally equilibrate for approximately 15 min. Both the water bath and milk temperatures were measured during each test to insure that they were in thermal equilibrium. A 10 ml dilution (2 °C) containing 120 µl enzyme was added to the milk and stirred thoroughly with a spatula for 1 min. Reflectance measurements were taken simultaneously with enzyme addition to the milk. The diffuse reflectance of coagulating goats' milk was monitored using a fibre optic sensor (Model 4A CoAguLite, Reflectronics Inc., Lexington, KY 40502, USA) consisting of one 600 µm diameter fibre for light emission into the milk and one 600 µm diameter fibre for transmitting backscattered light from the milk to the photodetector. The fibres were spaced 1200 µm apart. An infrared LED light (880 nm) was used as the light source and a Texas Instruments TSL 245 detector (Texas Instrument Incorporated, Dallas, TX 75243-4136, USA) was used to measure reflected light. The sensor had a 4-20 mA output that was dropped across a 250 Ω resistor to produce an output voltage between 1 and 5 V. The data acquisition portion of the system consisted of a personal computer (486, 33 MHz) equipped with a DAS 802 analog to digital board (Keithley Instrument Inc., Cleveland, OH 44139, USA) and programmed for data acquisition using Visual Basic 4.0 (Microsoft Corporation, Redmond, WA 98052-6399, USA) and VTX 1.1 (Keithley Instrument Inc.).

The sensor output voltage was zeroed to 1 V by placing a black rubber stopper over the distal tip of the probe (zero light reflectance). The sensor gain was calibrated to give a 2 V signal response when placed in goats' milk. The reflectance voltage was collected every two seconds with an average recorded every six seconds. The first ten data points (1 min of data) were corrected for the 1 volt zero offset and averaged to describe the initial voltage response (V_0). A reflectance 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 reflectance ratio profile was calculated by using 4 min of the most recently collected data (41 data points) by linear least-squares regression. The calculated slope was assigned to the midpoint of the data set used. The second derivative (R'') was calculated in a similar manner. The following reflectance parameters were derived from the reflectance ratio profile: T_{max}, the time to the maximum slope of the reflectance ratio profile; $T_{2\,max}$, the time to the maximum second derivative of the reflectance ratio profile; $T_{2\,min}$, the time to the minimum second derivative of the reflectance ratio profile; D_{max}, the maximum value of dR/dt; R_{max}, the reflectance ratio at T_{max}; and R_{cut}, the reflectance ratio at the actual cutting time.

Visual cutting time determination

A glass rod (8 mm diameter and 30 cm in length) with rounded ends was used to assist in the determination of cutting time (T_{cut}). As the curd approached T_{cut}, the glass rod was positioned vertically without support in the 12 cm of milk in the reaction vat. When the glass rod maintained the vertical position for a period of 3 s, three consecutive times, then a manual determination of T_{cut} was initiated. T_{cut} was determined with a knife when the edges of the cuts were clean and smooth (Castillo et al. 2000).

Results and Discussion

A model system was used to monitor the coagulation of goats' milk to determine which variables would accurately

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Table 1. Protein (and fat) content of goats' milk batches[a], condensed skimmed milk[a], and mixed test samples[b]

Values are means ± SD for $n=2^a$ or 10^b

	Raw milk		Condensed skimmed milk	Protein content of mixed test samples, % Target protein level		
Batch	Protein, %	Fat, %	Protein, %	3	5	7
1	5·01 ± 0·01	4·14 ± 0·02	9·71 ± 0·02	3·06 ± 0·01	5·06 ± 0·02	7·08 ± 0·05
2	4·38 ± 0·03	4·89 ± 0·01	9·80 ± 0·03	3·05 ± 0·01	5·03 ± 0·01	7·04 ± 0·01
3	5·17 ± 0·01	5·61 ± 0·02	9·74 ± 0·02	3·23 ± 0·03	5·27 ± 0·05	7·37 ± 0·04

Table 2. Analysis of variance and *F* statistic for dependent variables (fat as a co-variable): T_{max}, time to the maximum first derivative of the reflectance ratio profile; T_{cut}, actual cut time; $T_{2\,max}$, time to the second derivative maximum; $T_{2\,min}$, time to the second derivative minimum; D_{max}, value of the slope at T_{max}; R_{max}, reflectance ratio at T_{max}; R_{cut}, reflectance ratio at actual cut time, and V_0, initial voltage of coagulating goats' milk

		F value							
Source	*DF*	T_{max}	T_{cut}	$T_{2\,max}$	$T_{2\,min}$	R_{max}	R_{cut}	D_{max}	V_0
Protein	2	87·0***	16·7***	87·5***	81·6***	67·6***	$0·2^{ns}$	$1·3^{ns}$	961·3***
Temperature	4	71·3***	63·5***	74·2***	70·5***	113·1***	122·6***	52·9***	78·1***
Rep	2	$2·2^{ns}$	5·1*	$2·1^{ns}$	$2·4^{ns}$	$3·3^{ns}$	$1·1^{ns}$	5·9**	13·3***
Protein * Temperature	8	5·8***	$1·4^{ns}$	5·1***	5·3***	$0·4^{ns}$	$1·6^{ns}$	$0·5^{ns}$	$2·3^{ns}$
Fat (co-variable)	1	11·7**	4·2*	12·7**	11·1**	9·1**	4·5*	$1·8^{ns}$	96·2***

DF, Degrees of freedom; *F* value, ANOVA *F*-statistic
* $P<0·05$; ** $P<0·01$; *** $P<0·001$; ^{ns} not significant

describe the coagulation process and ultimately be useful in developing mathematical models for prediction of an accurate cut point. A new batch of goats' milk was used for each experimental replication. Table 1 shows the chemical composition of the raw goats' milk, the condensed skimmed milk and the samples adjusted for protein for the three different batches (replications) of milk used in these coagulation experiments. Fat concentration in the milk varied between batches from 4·14 to 5·61%, which affected the fat concentration in the condensed skimmed milk and subsequently the final fat concentration in the mixed test samples where protein was standardized. Therefore, most of the modelling calculations were adjusted for replication or 'Rep' and for the actual fat concentration in the final mixed test sample.

Dependent variables tested were classified as time-based (T_{max}, $T_{2\,max}$, $T_{2\,min}$, T_{cut}), response-based (R_{max}, R_{cut}, V_0) or mixed-based (D_{max}) parameters as a function of their units (Castillo et al. 2000). An analysis of variance (ANOVA) was conducted to determine the main sources of variation in the dependent variables. Analysis of variance and *F* statistics for dependent variables are shown in Table 2. Temperature was found to have a statistically significant effect on all dependent variables tested. Protein had a statistically significant effect on T_{max}, T_{cut}, $T_{2\,max}$, $T_{2\,min}$, R_{max} and V_0 but not on R_{cut} and D_{max}. 'Rep' was significant for T_{cut}, D_{max} and V_0. The co-variable fat was found to be statistically significant for each variable except D_{max}. The interaction Protein × Temperature was significant for T_{max}, $T_{2\,max}$ and $T_{2\,min}$. Least square means of the studied parameters for protein and temperature main treatments are presented in Table 3.

Effect of protein concentration on time-based parameters

The protein concentration effect on the four time-based parameters was statistically significant ($P<0·0001$) except for least square means of T_{cut} between 3 and 5% protein as shown in Table 3. Temperature × Protein interaction was significant for T_{max}, $T_{2\,max}$ and $T_{2\,min}$. The time-based parameters increased with increasing protein concentration while the relative time distance between T_{max} and T_{cut}, measured as $(T_{cut}-T_{max})/T_{max}$, decreased significantly ($P<0·0001$; Fig. 1). An initial increase in the hydrolysis rate with increasing protein was expected for our experimental conditions but this data does not suggest that it occurred. Although some authors found a decrease rather than an increase in clotting time, when protein concentration increased by ultrafiltration (Reuter et al. 1981; Lucisano et al. 1985; Mehaia & El-khadragy, 1998; O'Callaghan et al. 1999), the results of this study are consistent with the work of others. Dalgleish (1980) claimed that increasing the casein concentration by ultrafiltration or addition of a milk ultrafiltrate brought about an increase in final curd firming while clotting time was not affected or just slightly increased. Similarly, Culioli & Sherman (1978), Schmutz & Puhan (1978) and Garnot et al. (1982) found that clotting time increased with increasing protein content. In addition, several authors found that the κ-casein hydrolysis rate constant did not increase with

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Table 3. Effect of temperature and protein on the time to the maximum first derivative of the reflectance ratio profile (T_{max}), actual cut time (T_{cut}), time to the second derivative maximum ($T_{2\,max}$), time to the second derivative minimum ($T_{2\,min}$), value of the slope at T_{max} (D_{max}), reflectance ratio at T_{max} (R_{max}), reflectance ratio at actual cut time (R_{cut}), and initial voltage (V_0) of coagulating goat's milk

	Reflectance profile parameters							
	T_{max}, (min)	T_{cut}, (min)	$T_{2\,max}$, (min)	$T_{2\,min}$, (min)	D_{max}, (min^{-1})	R_{max}	R_{cut}	V_o, (v)
Temperature[1], °C								
20	$39{\cdot}4^{a}$	$61{\cdot}7^{a}$	$32{\cdot}4^{a}$	$45{\cdot}1^{a}$	$0{\cdot}016^{a}$	$1{\cdot}172^{a}$	$1{\cdot}345^{a}$	$1{\cdot}242^{a}$
25	$26{\cdot}8^{b}$	$40{\cdot}5^{b}$	$22{\cdot}8^{b}$	$30{\cdot}2^{b}$	$0{\cdot}027^{a}$	$1{\cdot}173^{a}$	$1{\cdot}371^{a}$	$1{\cdot}283^{b}$
30	$19{\cdot}5^{c}$	$29{\cdot}5^{c}$	$16{\cdot}4^{c}$	$22{\cdot}1^{c}$	$0{\cdot}041^{b}$	$1{\cdot}196^{b}$	$1{\cdot}443^{b}$	$1{\cdot}325^{c}$
35	$16{\cdot}3^{cd}$	$25{\cdot}7^{c}$	$13{\cdot}0^{d}$	$18{\cdot}8^{cd}$	$0{\cdot}059^{c}$	$1{\cdot}255^{c}$	$1{\cdot}601^{c}$	$1{\cdot}359^{d}$
40	$14{\cdot}1^{d}$	$24{\cdot}5^{c}$	$10{\cdot}8^{d}$	$17{\cdot}3^{d}$	$0{\cdot}093^{d}$	$1{\cdot}339^{d}$	$1{\cdot}800^{d}$	$1{\cdot}395^{e}$
Protein[2], %								
3	$13{\cdot}3^{f}$	$30{\cdot}7^{f}$	$10{\cdot}7^{f}$	$16{\cdot}0^{f}$	$0{\cdot}050^{f}$	$1{\cdot}173^{f}$	$1{\cdot}504^{f}$	$1{\cdot}104^{f}$
5	$22{\cdot}0^{g}$	$33{\cdot}8^{f}$	$18{\cdot}2^{g}$	$25{\cdot}2^{g}$	$0{\cdot}050^{f}$	$1{\cdot}233^{g}$	$1{\cdot}513^{f}$	$1{\cdot}373^{g}$
7	$34{\cdot}5^{h}$	$44{\cdot}7^{g}$	$28{\cdot}4^{h}$	$38{\cdot}9^{h}$	$0{\cdot}042^{f}$	$1{\cdot}275^{h}$	$1{\cdot}519^{f}$	$1{\cdot}486^{h}$

[a–h] Means within the same column with same superscript were not significantly different ($P<0{\cdot}05$)
Number of replications = 3; Number of observations = 45; [1], LSM for each temperature is based on average of 9 trials over a range of 3 protein levels; [2], LSM for each protein level is based on average of 15 trials over a range of 5 temperatures

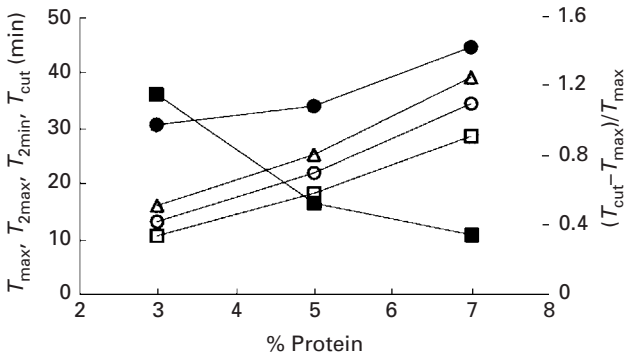


Fig. 1. Effect of protein concentration on ○, T_{max}; □, $T_{2\,max}$; △, $T_{2\,min}$; ●, T_{cut} and ■ $(T_{cut}-T_{max})/T_{max}$.

increasing substrate concentration, as the Michaelis-Menten equation and first order kinetics predicted. Rather it decreased with increasing substrate concentration of milk (van Hooydonk et al. 1984; Saputra, 1992; Hylding, 1993; Lomholt, 1996). This unexpected drop in the hydrolysis rate constant might result from the retardation of the effective diffusion rate of the enzyme due to increasing amount of substrate and might explain why T_{max}, $T_{2\,max}$, $T_{2\,min}$ and T_{cut} increased when protein concentration increased. This is in agreement with the findings of Muhr & Blanshard (1982), who observed that the diffusion rate of a solute in a polymer solution is dependent on the polymer concentration. Likewise, Van Hooydonk et al. (1984) proposed a diffusion model to describe the enzymatic hydrolysis of κ-casein in milk, in which the diffusion rate of enzymes to the casein micelle surface controls the reaction rate. Saputra (1992) analysed the enzymatic hydrolysis of milk by measuring diffuse reflectance of near infrared light radiation. He assumed that enzymatic hydrolysis followed first order kinetics as described by the above mentioned diffusion model and found that T_{max} increased with increasing protein concentration at all wavelengths, temperatures and enzyme concentrations tested. The results of our study confirmed his work.

From another point of view, it is well known that para-κ-casein produced in the micelles by renneting can only aggregate when the whole micelle is capable of aggregating, thereby causing the lag stage that can be observed before aggregation. A casein micelle is able to aggregate only if a critical percentage of its κ-casein has been hydrolysed (Dalgleish, 1993). Therefore if the enzyme is saturated by the substrate, an increase in the concentration of casein micelles, at a constant enzyme concentration, should bring about an increase in the lag time for the required percentage of hydrolysis to be achieved. The enzyme may have been saturated by the substrate under the experimental conditions of this study, though this is unlikely, as discussed in Materials and Methods. This might explain the observed increase in the four time-based parameters studied. On the other hand, it was expected that an increase in protein concentration would increase the aggregation rate because of the increased number of collisions between the casein micelles. Several authors have found that the rate of curd firming increased with increasing protein concentration (van Hooydonk & van den Berg, 1988; Guinee et al. 1997). Even with an increased aggregation rate as a result of increased protein concentration, the delay in the beginning of aggregation could lead to a larger T_{cut}. This could explain why T_{cut} increased when protein increased and at the same time the relative time distance between T_{max} and T_{cut} decreased when protein concentration increased.

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Effect of protein concentration on response and mixed-based parameters

Three response-based parameters (R_{max}, R_{cut}, V_0) and one mixed-based parameter (D_{max}) generated from the diffuse reflectance profile were determined for each sample. Least square means of these variables are presented in Table 3. V_0 and R_{max} increased with increasing protein ($P<0{\cdot}0001$) while no significant effect of protein was found on R_{cut} and D_{max}. The observed behaviour for V_0 agreed with other authors. Payne et al. (1993) found that the sensor output voltage (V) increased with increasing protein and Lochte-Watson et al. (1998) claimed that diffuse reflectance of milk increased linearly with an increase in protein concentration. On the other hand, diffuse reflectance ratio at cutting time (R_{cut}) was found to be the same at different protein concentrations. It appears that for skimmed milk, protein concentration does not affect the final diffuse reflectance ratio but only the rate at which it is reached. In addition, the observed time decrease between T_{max} and T_{cut} when protein concentration increases qualitatively agrees with the conclusions found by Garnot et al. (1982). They observed a decrease in the time period between clotting time and cutting time as protein concentration increased for curd having firmness equivalent to that typically attained in the manufacture of cheddar cheese.

Effect of temperature on time-based parameters

It is well known that temperature affects both the enzymatic hydrolysis and the aggregation reaction of milk coagulation (McMahon & Brown, 1984; van Hooydonk & van den Berg, 1988). These authors observed that the aggregation phase is more sensitive to temperature, which has far less effect on the enzymatic hydrolysis than on the aggregation reaction. Ustunol et al. (1993) and Castillo et al. (2000) respectively monitored diffuse reflectance of coagulating cows' and goats' milk using an optical probe and observed that T_{max} and T_{cut} decreased as temperature increased. Results confirm these previously reported outcomes. Least square means of the four time-based parameters determined (T_{max}, $T_{2\,max}$, $T_{2\,min}$ and T_{cut}) are shown in Table 3. The rate of change in these parameters decreases with increasing temperature. This observation could be attributed to the non-linear relationship between temperature and the four time-based parameters. Most authors found a non-linear effect of temperature on clotting time (Ramet & Weber, 1980; Zoon et al. 1988) (Fig. 2). Zoon et al. (1988) claimed that at higher temperatures, the renneting reaction is faster, resulting in a measurable value of the moduli sooner after rennet addition (especially between 20 and 30 °C). Mehaia & Cheryan (1983) calculated the Q_{10} values for the whole milk coagulation process (primary plus secondary phases) between 20–30 °C and 30–40 °C. They found a higher value of Q_{10} between 20 and 30 °C than between 30 and 40 °C, which may be related to the observed non-linear

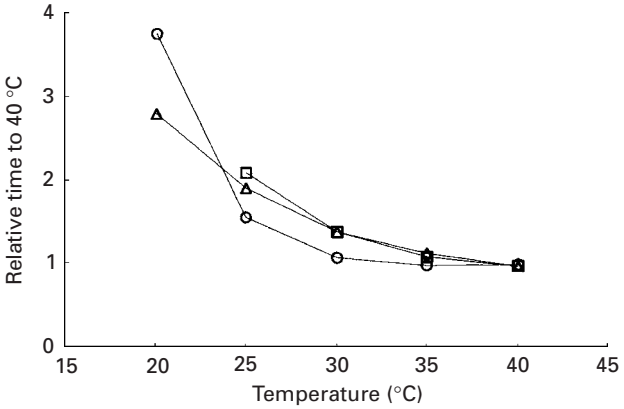


Fig. 2. Effect of temperature on time-based parameters. ○, Zoon et al. (1988) (Clotting time); □, Ramet and Weber (1980) (Clotting time); △, Castillo, et al. (2000) (T_{max}).

effect of temperature on clotting time. This behaviour could be explained by heat inactivation of chymosin with increasing temperatures.

Effect of temperature on response and mixed-based parameters

Table 3 shows the least square means of the response-based parameters (V_0, R_{max}, R_{cut}) and the mixed-based parameter (D_{max}). V_0 significantly increased with increasing temperature. R_{max}, R_{cut} and D_{max} also increased significantly when temperature increased except for the values obtained between 20 and 25 °C, in which the increase was not significant. These results agree with Castillo et al. (2000) who found that R_{max}, R_{cut} and D_{max} increased significantly with temperature. The effect of temperature on these parameters was also found to be non-linear.

Effect of temperature on the κ-casein hydrolysis rate

Saputra (1992) found that diffuse reflectance response for infrared wavelengths of 820, 940, 1250 and 1650 nm have the potential to measure enzymatic hydrolysis of milk. D_{max}, which is the maximum slope of the diffuse reflectance profile, occurs a short time before the Berridge clotting time (Castillo et al. 2000). López et al. (1998) found that the maximum rate of change in G' (rheological elastic modulus) versus time was linearly related to the hydrolysis rate constant at pH values of 6·74, 6·50 and 6·25. Assuming that D_{max} is correlated with the rate of κ-casein hydrolysis, the following Arrhenius-type equation can be written to model the effect of temperature on κ-casein hydrolysis:

$$\ln D_{max} = \ln A - \left(\frac{E_a}{RT}\right)$$

where

A = Estimate of the Arrhenius frequency factor,
R = Universal gas constant, 8·314 J . mol^{-1} . K^{-1},

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Table 4. Algorithms for cutting time prediction

Model number	Model	Coefficients	SE	R^2	SEP (min)
I	$T_{cut}=\beta_0+\beta_1\ T_{2\,min}$	$\beta_0=6{\cdot}76$	1·85	0·888	6·19
		$\beta_1=1{\cdot}11$	0·0600		
II	$T_{cut}=\beta_0+\beta_1\ T_{2\,min}+\beta_2$ Protein T_{max}	$\beta_0=-0{\cdot}845$	0·886	0·983	2·45
		$\beta_1=2{\cdot}21$	0·0759		
		$\beta_2=-0{\cdot}166$	0·0109		
III	$T_{cut}=\beta_0+\beta_1\ T_{2\,min}+\beta_2$ Protein $T_{max}+\beta_3$ Protein	$\beta_0=4{\cdot}94$	2·56	0·985	2·32
		$\beta_1=2{\cdot}01$	0·111		
		$\beta_2=-0{\cdot}127$	0·0193		
		$\beta_3=-1{\cdot}07$	0·448		
IV	$T_{cut}=\beta_0\ T_{max}$	$\beta_0=1{\cdot}48$	0·0424	0·823	7·71
V	$T_{cut}=\beta_0\ T_{2\,min}$	$\beta_0=1{\cdot}30$	0·0339	0·854	7·01
VI	$T_{cut}=\beta_0\ T_{max}+\beta_1$ Protein T_{max}	$\beta_0=2{\cdot}61$	0·0708	0·976	2·89
		$\beta_1=-0{\cdot}187$	0·0113		
VII	$T_{cut}=\beta_0\ T_{2\,min}+\beta_1$ Protein T_{max}	$\beta_0=2{\cdot}15$	0·0494	0·982	2·45
		$\beta_1=-0{\cdot}160$	0·0090		
VIII	$T_{cut}=\beta_0\ T_{2\,min}\ (1+\gamma$ Protein)	$\beta_0=2{\cdot}19$	0·0508	0·983	2·42
		$\gamma=-0{\cdot}0674$	0·0022		
IX	$R_{cut}=\beta_0+\beta_1 R_{max}+\beta_2$ Protein $R_{max}+\beta_3$ Temp	$\beta_0=-1{\cdot}30$	0·123	0·970	0·0341[a]
		$\beta_1=2{\cdot}39$	0·135		
		$\beta_2=-0{\cdot}0412$	0·0032		
		$\beta_3=0{\cdot}0047$	0·0013		

The above regressions were conducted over a data set having 45 observations for each parameter; [a] Dimensionless
SE, Standard error of estimate for coefficients; R^2, Coefficient of determination (sum of squares corrected for the means); SEP, Standard error of prediction

E_a = Estimate of the activation energy of κ-casein hydrolysis.

T = Absolute temperature.

As expected a linear relationship was found between ln D_{max} and $1/T$ at each protein concentration (for each protein concentration, every D_{max} value at each temperature was the average of three replications). R^2 values were 0·999, 0·996 and 0·995 for 3, 5 and 7% protein respectively. Calculated E_a values for each protein concentration gave 63·5, 68·1 and 71·7 kJ mol^{-1} for 3, 5 and 7% protein, respectively. The E_a values increased proportionally with increasing protein concentration, even though this effect was not significant. This result is in agreement with Saputra (1992), who observed that the hydrolysis rate constant was inversely related to protein concentration. Other authors have found this inverse relation between substrate concentration and hydrolysis rate constant in milk (van Hooydonk et al. 1984; Hyldig, 1993; Lomholt, 1996).

Algorithm development for cutting time prediction

Castillo et al. (2000) tested several multiple linear regression models for predicting T_{cut} in goats' milk. They found an algorithm that predicted T_{cut} with a standard error of 84·5 s using a single parameter (T_{max}) derived from the diffuse reflectance profile. This model ($T_{cut}=\beta*T_{max}$) had the advantage that only one regression coefficient (β) was needed to predict the operator's judgement of T_{cut}. β was determined in the cheese plant by dividing the operator's selection of T_{cut} by T_{max}. An analysis of variance was conducted to determine the effect of protein concentration on β. The results showed that β decreased with increasing protein ($P<0{\cdot}0001$). This suggests that including a protein term in the cutting time prediction equation would improve the prediction accuracy of the algorithm.

Dependent and independent variables as well as three calculated dependent variables (Protein$*T_{max}$), (Protein$*R_{max}$) and (($T_{2\,min}-T_{2\,max}$)$*(T_{max})$) were used in regression models for predicting T_{cut} and R_{cut}. SAS® (1999) procedures NLIN, GLM and Maximum R^2 were used in the regression analysis. The Maximum R^2 procedure was used to select the best one-variable, two-variable and three-variable models for predicting T_{cut} and R_{cut}.

The parameters $T_{2\,min}$, (Protein$*T_{max}$) and Protein were found to be the best descriptors of T_{cut} throughout the various linear regression models tested as shown in Table 4. According to the SEP and R^2, Model I was the best one-parameter algorithm and contained the parameter $T_{2\,min}$. The SEP was viewed as too large for an accurate prediction of T_{cut}. Model II was the best two-variable algorithm and contained parameters $T_{2\,min}$ and (Protein$*T_{max}$). The best three-parameter model (Model III) included the terms (Protein$*T_{max}$), Protein and $T_{2\,min}$. Even though Models II and III had smaller SEP they were not considered practical for plant application because they would require three and four regression coefficients respectively to be determined in the cheese plant. Model IV was $T_{cut}=\beta*T_{max}$ and was tested for comparison with the tests described by Castillo et al. (2000). To simplify in-plant calibration, Model V was developed from Model I and contained only $T_{2\,min}$ without an intercept. Model V was found to have a lower SEP than

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Model IV. The relatively large SEP observed in Models IV and V prove that the simple models are inadequate for industrial applications where protein content varies. Models VI and VII added term (Protein$*T_{max}$) in an effort to improve a description of the effect of protein on cutting time. The introduction of the protein term reduced the SEP values 64% compared with Models IV and V. Unfortunately, Models VI and VII are two parameter models and would require considerable effort for in-plant calibration. Considering the strong linear regression between $T_{2\,min}$ and T_{max} (R^2=0·997), Model VIII was developed for ease of calibration in a dairy plant and contained parameters $T_{2\,min}$ and Protein, a constant γ used to correct β_0 for protein and one coefficient β_0 requiring in-plant calibration. Model VIII had the highest R^2 and the lowest SEP of all models tested except for Model III. However, Model VIII has the advantage that only one parameter, β_0, would be required in-plant calibration, assuming that γ is a constant.

Model IX, a response-based model for predicting R_{cut}, had an R^2 of 0·970 and a SEP of 0·034. Fig. 3 compares the cutting time predictions between Model VIII (time-based) and Model IX (response-based). The comparison was made in term of R^2 and SEP for the regression, with an intercept set to zero, between observed T_{cut} and the predicted T_{cut}. For Model VIII, R^2 was 0·983 and the SEP was 2·38 min. Model IX had a smaller R^2 value (0·947) and a larger SEP (4·32 min). However, when the two outlying points marked in the Fig. 3 were removed from the regression, the SEP and R^2 values were 2·19 min and 0·984 for Model IX and 2·25 min and 0·984 for Model VIII. This result shows that for skimmed milk, response-base parameters can be used to predict T_{cut}, when adjusted for protein and temperature, with accuracy equal to time-based parameters. A conclusion from this comparison is that time-based parameters are better predictors of cutting time because they do not require adjustment for coagulation temperature. A comparison of the effect of protein content on time-based and response-based prediction models is shown in Fig. 4. The first set of figures (a, b) shows that a single parameter algorithm for either time-based or response-based model is inadequate for predicting cutting time when protein varies in skimmed milk. The second set of figures (c, d) shows that a one-parameter time-based and a one-parameter response-based algorithm with an intercept can predict cutting time in skimmed milk when protein is constant. Finally, the third set of figures (e, f) shows a comparison between Model VI and Model IX. The conclusion from this analysis is that both response-based and time-based parameters can be used to predict T_{cut} with equal accuracy for skimmed milk; however, the response-based algorithm must include temperature and an intercept.

These data show the advantage of time-based parameters for predicting cutting time, and that when protein concentration varies, a protein term is required in either a response-based or time-based cutting time prediction model to account for the effect of protein. This result is in agreement with Payne et al. (1993) and with O'Callaghan et al. (1999). Models containing $T_{2\,min}$ instead of T_{max} showed a lower SEP and higher R^2 value. It should be noted that T_{max} and $T_{2\,min}$ have basically the same behaviour and are highly correlated.

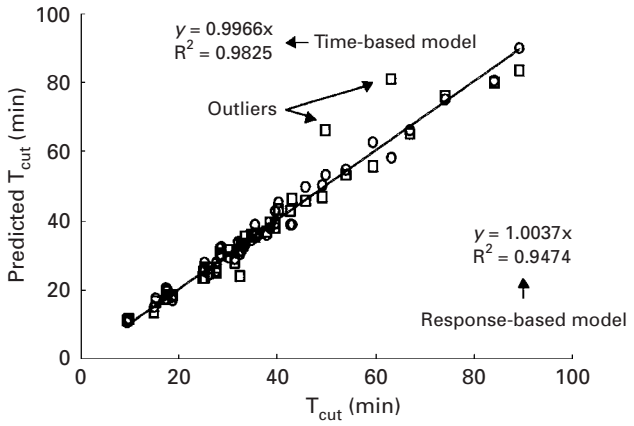


Fig. 3. Comparison between cutting time prediction: time-based (Model VIII) or response-based (Model IX) models. ○, Time-based; □, Response-based.

As a resume, our results confirm that prediction of cutting time by backscatter of near infrared radiation is accurate when protein concentration is kept constant. But in addition, it has been shown that when protein concentration varies in cheese production (ultrafiltration, adding casein or skimmed milk powders) a protein term is required to accurately predict cutting time. Models for cutting time prediction containing time-based parameters are more advantageous than those including response-based parameters because the former do not need the inclusion in the algorithm of temperature and an intercept.

Conclusions

Several multiple regression models for predicting the cutting time were tested with protein concentration varying from 3 to 7% in a wide range of coagulation rates attained by using temperatures between 20 and 40 °C. Models containing $T_{2\,min}$ predicted cutting time with less standard error of prediction than models using T_{max}. Models, which did not include a protein term, had SEP greater than 6·2 min. An algorithm including $T_{2\,min}$ and a protein term, $T_{cut}=\beta_0\ T_{2\,min}\ (1+\gamma$ Protein), was found to predict cutting time with a SEP of 2·42 min. γ was considered a constant of −0·0674 for goats' milk and described the protein effect on β_0. Cutting time prediction based on response parameters needed the addition of other terms, such as temperature, to achieve accuracy similar to time-based models. Time-based parameters decreased when temperature increased or protein decreased while response-based parameters and mixed-based parameters increased with

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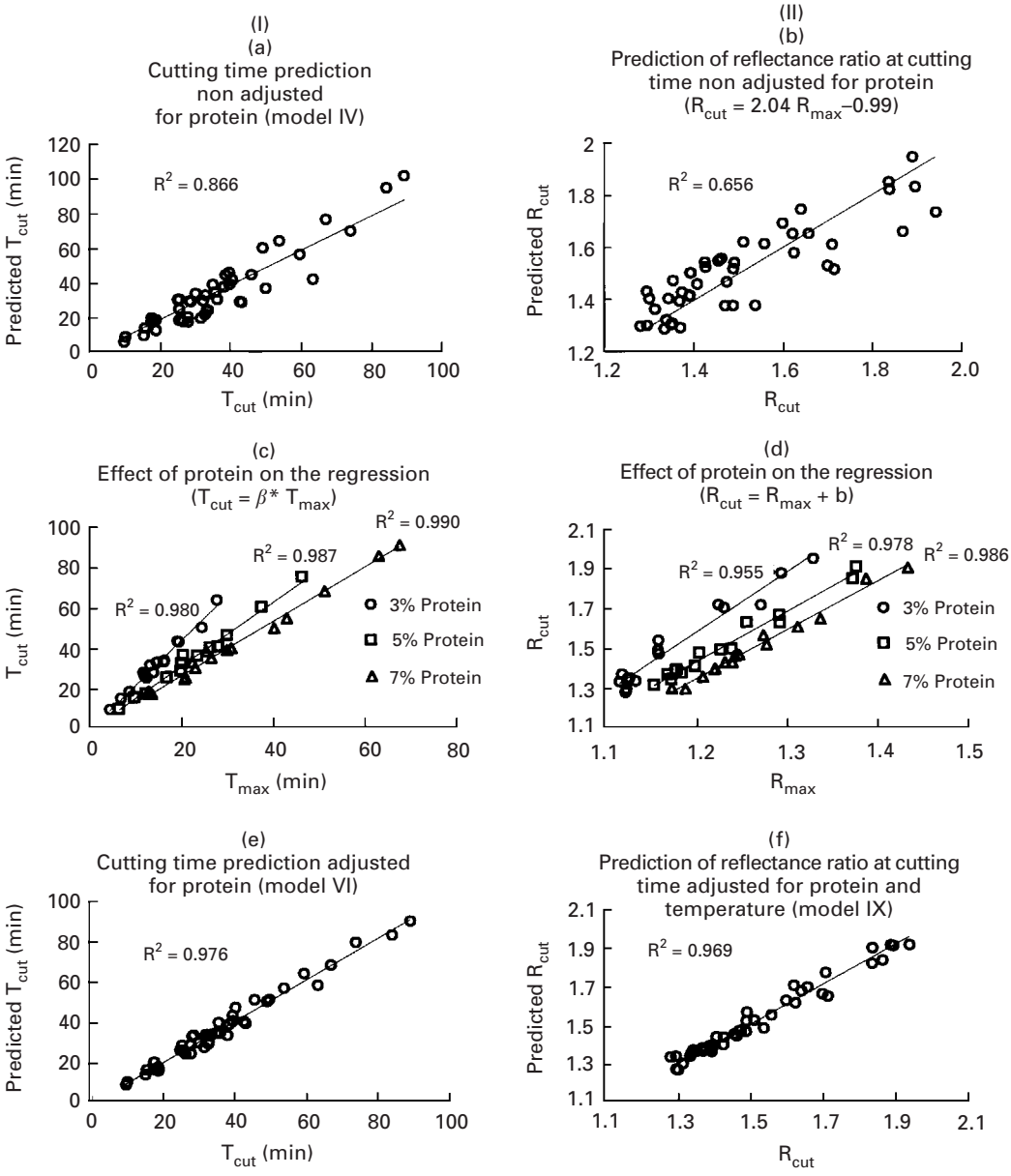


Fig. 4. Comparison of the effect of protein content for cutting time prediction using time-based (I) and response-based (II) prediction models.

increasing temperatures. Values of activation energy for the enzymatic hydrolysis of goats' milk coagulation were found to be between 63 and 72 kJ mol^{-1} using a calculation method based on diffuse reflectance.

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