

Comparison of total milk-clotting activity measurement precision using the Berridge clotting time method and a proposed optical method

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ABSTRACT

An objective measurement technique is needed to simplify the determination of total milk-clotting activity (α_t) of rennets and other milk coagulating enzymes. IDF Standard 157: 2007/ISO 11815 is the current standard method for bovine rennets and measures milk-clotting activity by visually determining the Berridge clotting time (i.e., time of appearance of flocks of renneted standard milk substrate on the wall of a rotating test tube). The IDF Standard 157: 2007 method is somewhat subjective because it depends on an operator's skill to consistently identify milk flocculation. An optical method based on changes in infrared light backscatter at 880 nm

during milk coagulation is proposed as an alternative, objective method to Berridge clotting time to eliminate the operator's subjectivity from the IDF Standard 157: 2007. The Berridge clotting time method and a proposed optical method were compared using 29 replications to determine the precision of a_t measurements. The light backscatter profiles collected during the coagulation process were analyzed with five optical time parameters determined for each profile. The a_t , expressed in International Milk-Clotting Units (**IMCU mL⁻¹**), was calculated using Berridge clotting time and each of the five optical time parameters. The calculated milk-clotting activities were compared statistically to determine differences between measurement methods. No significant differences were found between the a_t determined using the Berridge clotting time and the optical time parameters, t_{2min} and t_{2min2} . Preferred optical time parameters t_{max} and t_{2min} were recommended. The results show that the proposed optical method based on measurement of changes in infrared light backscatter is a potential objective method for a_t measurement.

Key words: Berridge, clotting, coagulation, activity, rennet, milk, optical, light backscatter, fiber optic, IMCU, REMCAT, IDF

INTRODUCTION

Milk coagulation properties are of great importance as they significantly influence cheese yield and quality (Kubarsepp et al., 2005). Milk clotting enzymes are one of the most significant cheese making raw materials impacting and regulating milk coagulation properties. Although all milk-clotting enzymes used in cheese manufacture belong to the same group of aspartic proteinases and present, in general terms, similar coagulating properties, they have a large number of important differences that substantially modify their value for cheese making (Law,

1999). Thus, for both economic and processing reasons, it is essential to know the total milk-clotting activity (a_t) of a certain rennet type, which allows the cheese maker to select the adequate enzyme for each type of cheese and adjust the enzyme proportion to control milk coagulation and thus optimize cheese yield and quality. Cheese plants often have to rely on the total milk-clotting activity from the printed label, but enzyme activity might decrease with time leading to inadequate use of the enzyme. On the other hand, the number of rennets and other milk coagulants in the market has steadily risen for the last three decades, which has increased the need for analyzing the different products commercially available.

A number of methods have been proposed and used with that purpose. In the past, Soxhlet's units (**SU**), which is the volume of milk that can be clotted by one unit volume of a rennet within 40 min at 35°C, was widely used to characterize the strength of a rennet solution. While SU is easily understandable by the cheese maker, the measured strength depends substantially on both milk quality and pH. Further, no milk-clotting enzyme reference standards are used and SU should be considered as an indicative measurement of milk-clotting activity. Subsequently, Rennin Units (**RU**) or Berridge units have also been used in the past by the British Standard Institution (**BSI**) but it is no longer used. RU is described as the activity which is able to clot 10 mL of standardized milk in 100 s at 30°C (Law, 1999). Similarly to the SU units, RU units also have some major drawbacks to it. During cheese making, the pH is usually around 6.5-6.7, while the standardized substrate has a pH of 6.3. The pH plays an essential role in the analysis since each enzyme has its own pH-dependency. Not only does the pH cause misleading information, but also the calcium content is abnormally high in standardized milk. This has a major influence on the analysis and may result in measurement errors. In 1997 the International Dairy Federation (**IDF**) developed a standard known as the "relative milk-clotting activity test" or REMCAT (IDF Standard 157A: 1997) for determination of a_t of bovine rennets and fermentation produced chymosin. This standard, which has been recently revised (IDF Standard 157: 2007/ISO 11815) accounts for variations in test conditions which affect the enzyme. Similar methods have been

developed by the IDF for microbial enzymes (IDF Standard 176: 2002) and other coagulants –
ovine and caprine rennets – (e.g., IDF Standard 199: 2006).

The Berridge clotting time method is used as the indicator of milk flocculation in all the IDF
Standards for a_t determination. The Berridge clotting time is determined as the time of visual
appearance of flocks of renneted standard milk on the wall of a rotating test tube. The Berridge
clotting time method is somewhat subjective because it relies on “human eye identification” of
flocculation, which greatly impacts the precision of this observation. The IDF 157: 2007
standard recommends that the repeatability of independent analytical results performed on
identical test material and in a short interval of time by the same operator will in not more than
5% of cases be greater than 1.8% relative to the arithmetic mean of the test results. If two
determinations are obtained under these conditions an absolute difference of 4.9% relative to the
arithmetic mean of the test results should not be exceeded. Recommended absolute difference
between the results of two single measurements performed on identical test material by two
different operators working in different laboratories is doubled to 9.8%.

Several continuous and nondestructive methods based on the measurement of optical properties
such as reflection, absorbance, scatter, and refraction of light have been proposed for monitoring
milk coagulation and determining gelation/clotting time. Korolczuk et al. (1986) found a strong
correlation between the time of inflection point of refractometric curves and the gelation time.
McMahon and Brown (1990) measured changes in light scattering at 600 nm in coagulating milk
and found a correlation between gelation time and the inflection point of turbidity plots. Saputra
(1992) reported a correlation between the first order enzymatic reaction rate constant for κ -casein
hydrolysis and the inflection point of infrared light backscatter profiles ($t_{\max}$). Payne et al. (1993)
reported a relationship between the infrared light backscatter time parameter $t_{\max}$ and curd
cutting time. A number of researchers have also investigated the relationship between clotting
time and optical time parameters (Castillo et al., 2000). These authors reported a strong linear
correlation between $t_{\max}$ derived from infrared light backscatter and the Berridge clotting time.

Lopez et al. (1997) claimed that there were no significant difference between the optical time parameter, which quantifies the time to the point of maximum increase in the near infrared reflectance curve and Berridge clotting time.

These correlations between optically determined parameters and milk clotting time suggest the possibility of developing an optical method for measuring a_t . An optical method for measuring infrared light backscatter has been used in research and commercially since 1990 for monitoring milk coagulation (Payne et al., 1993; Castillo et al., 2003).

The objective of this study was to compare the Berridge clotting time method (IDF Standard 157: 2007) with an optical method for precision of a_t measurement.

MATERIALS AND METHODS

Development of a coagulation measurement apparatus

A coagulation measurement apparatus (Figure 1) was designed and developed to measure near infrared light backscatter during milk coagulation. Two vats were designed into the apparatus to make precise comparisons feasible. Each vat was instrumented to measure near infrared light backscatter, temperature and pH (not used in this study) during coagulation. The sample vats had an inside diameter of 36 mm, height of 115 mm and contained a maximum sample volume of 98 mL. The sample vats were fabricated of stainless steel with the interior surfaces polished for easy cleaning. A plastic cap was used to cover each vat during testing to prevent evaporative cooling. The cap provided access to the vats for mixing the enzyme, inspecting the sample visually, and inserting a pH electrode. The coagulation measurement apparatus had an inlet/outlet connection to a water bath as shown in Figure 1. Linear thermistors (Model OL-710-PP, Omega Engineering, Stamford, CT) were used to measure the temperatures of the milk samples (sample vats #1 and #2) and circulating water bath. A fiber optic unit (Model 5, Reflectronics, Inc.,

Lexington, KY) was used to measure near infrared light backscatter at 880 nm. The fiber optic unit was installed horizontally in each vat as shown in Figure 1. The fiber optic units directed near infrared light from a LED (Model L2791, Hamamatsu Corp., Bridgewater, NJ) into the milk sample and returned the backscattered light to a detector (Model TSL250, TAOS, Plano, TX).

Temperature control

Precise temperature control is essential for accurate comparison of simultaneous enzymatic reactions. The coagulation measurement apparatus was designed to provide identical temperatures in the two milk sample vats by circulating one stream of water from a water bath around both vats. The temperature difference between the water bath and the milk in the vats was minimized by using a plastic vat enclosure (Figure 1) and other plastic parts to minimize heat transfer losses. Plastic hoses (inside diameter of 12.7 mm; length 1 m) connected the vat enclosure to the circulating water bath. A circulating water bath (Lauda RM 220, Brinkman Instruments, Inc., Westbury, NY) having a reported temperature control of $\pm 0.04^{\circ}\text{C}$ was used to circulate water through the vat enclosure at the maximum flow setting provided by the manufacturer and measured to be 10.2 L min^{-1} . Since the internal water volume of the vat enclosure was measured to be 1.6 L, the design provided for a water volume exchange rate of >6 per min.

Precise temperature control was confirmed for the coagulation measurement apparatus. The water bath was filled with water, set to a control temperature of 32.0°C and allowed to stabilize for 8 h with the laboratory environment at 24.2°C . The temperature difference between the milk sample in a vat and water in the water bath were accurately measured using a precision thermistor (model 5831 A, Omega Engineering, Stamford, CT, resolution $\pm 0.01^{\circ}\text{C}$, accuracy $\pm 0.2^{\circ}\text{C}$). The thermistor was inserted in the milk sample, allowed to equilibrate for 1 minute, and 100 temperature measurements were collected on 6 s intervals over a 10 min period. Then, the thermistor was inserted in the water bath and the temperature measurements were likewise

collected. A total of three replications were conducted. The mean and standard deviation of the temperature measurements were $32.02 \pm 0.01^{\circ}\text{C}$ and $32.00 \pm 0.01^{\circ}\text{C}$ for the water bath and sample, respectively. The average temperature difference between the sample and water bath was thus 0.02°C . Therefore the equilibrium sample temperature in the vat is approximately equal to the water bath temperature, and as a result, the temperature control of the vat is the same as the temperature control of the circulating water bath.

Data Acquisition System

A computerized data acquisition system written in Microsoft Visual Basic.net was used to collect, store and analyze the data for determination of optical time parameters during milk coagulation. The analog signals for temperature and light backscatter were digitized using a 16-bit analog to digital board (Model USB-1608FS, Measurement Computing Corp., Norton MA).

Optical time parameters determination

Near infrared light backscatter measurements were collected on 6 s intervals for Vats 1 and 2. The light backscatter ratio profile (Figure 2) was calculated by dividing the voltage output from the detector by the average of the first ten voltage data points collected after enzyme addition according the procedure described by Castillo et al. (2000). An algorithm was developed to calculate the first and second derivatives of the light backscatter profile. Five optical time parameters were defined by the maxima and minima of the derivatives as shown in Figure 2. The times to the first maximum of the first and second derivatives were defined as t_{max} and $t_{2\text{max}}$, respectively, while the time to the second maximum of the second derivative was defined as $t_{2\text{max}2}$. The times to the first and second minima of the second derivative were defined as $t_{2\text{min}}$ and $t_{2\text{min}2}$, respectively.

Experimental Design

A test was designed, according to the IDF Standard 157: 2007, to compare the Berridge clotting time method with the proposed optical method using near infrared light backscatter for precision of measurement of a_t (IMCU mL⁻¹). The optical time parameters ($t_{2\max}$, $t_{\max}$, $t_{2\min}$, $t_{2\max2}$, and $t_{2\min2}$) and the Berridge clotting time (t_{bc}) were determined in parallel. The experimental design used and described in Figure 3 ensured that simultaneous coagulation reactions could be compared (paired tested). A total of 29 total milk-clotting activity determinations for the test rennet were performed over a period of 9 days using a fresh, reconstituted, standard milk sample for each single determination of a_t . Figure 4 shows the set-up of the light backscatter measurement system utilized during testing.

Preparation of standard milk substrate

Standard milk substrate samples were prepared according to the IDF Standard 157: 2007. Figure 3 shows a flow chart of the process used to prepare the milk substrate and perform the tests. Low heat, low-fat, spray dried milk powder purchased from Chr. Hansen (Jernholmen, Denmark) was used. A calcium chloride solution was prepared by diluting 0.662 g of CaCl₂·H₂O into de-ionized water to a volume of 1000 mL (i.e., 0.5 g L⁻¹ CaCl₂ solution). The spray dried milk powder (110 g) was mixed at room temperature to a volume of 1000 mL with calcium chloride solution for 30 min using a magnetic stirrer. The resulting standard milk substrate was then stored at room temperature and in the dark for 30 min. The pH of the standard milk substrate was verified to be ~6.5 upon removal from storage.

Preparation of enzyme solutions

Two coagulating enzyme solutions, a test and a reference standard, were prepared according to the IDF Standard 157: 2007. Acetate buffer solution (pH = 5.5) was used to dilute the enzymes. The acetate buffer solution was prepared by adding 10.0 mL of acetic acid (CH₃COOH) 1 M and

208 10.0 g of sodium acetate trihydrate ($\text{CH}_3\text{COONa} \cdot 3 \text{H}_2\text{O}$) with de-ionized water to make 1000
209 mL.
210

211 The Chymax test solution was prepared using Chymax (100% chymosin; Chr. Hansen's
212 Laboratory Inc., Milwaukee, WI). The Chymax test solution was prepared on a daily basis by
213 diluting 0.25 mL of Chymax (i.e., 0.273 g, m_{chymax}) with buffer solution to make 50 mL
214 (V_{chymax}).
215

216 The calf rennet reference standard solution was prepared using the reference calf rennet standard
217 powder (100% chymosin; Chr., Hansen's Laboratory Inc., Jernholmen, Denmark, ~1000 IMCU
218 mL^{-1} , stored at -18°C). The reference standard solution was prepared by mixing 500 ± 1 mg of
219 reference calf rennet standard powder ($m_{\text{c,ref}}$) with buffer solution to make 10 mL (V_2). The
220 reference standard solution was made on a weekly basis and stored in a refrigerator at 4°C . A
221 reference working solution was prepared from the reference standard solution for each test day
222 by diluting the reference standard solution (0.6 mL; V_1) with buffer solution to make 10 mL
223 (V_3).
224

225 The IDF Standard 157:2007 calls for adding 25 mL of standard milk substrate to a test tube; pre-
226 heating to 32°C ; quickly adding 0.5 mL of the enzyme solution while simultaneously starting a
227 timer; mixing; and placing the test tube on the rotating apparatus. However, in order to ensure
228 proper comparison between Berridge clotting time and the light backscatter parameters, a minor
229 change in the IDF method was made. A standard milk substrate sample (200 ± 0.01 mL) was
230 measured into a container. The sample was placed in the circulating water bath ($32.0 \pm 0.04^\circ\text{C}$)
231 and allowed to come to thermal equilibrium (sample temperature $>31.9^\circ\text{C}$). The enzyme solution
232 (4 mL) was added with the timer simultaneously initiated and the coagulating sample mixed for
233 0.7 min. The coagulating sample (~80 mL) was quickly placed into both Vat 1 and Vat 2; and 25

mL was placed into the test tube. The total time elapsed between adding the enzyme to the standard milk substrate sample and placing the coagulating sample into the two vats and test tube was always < 2 min, which was well before the onset of aggregation.

Total milk-clotting activity calculation

The total milk-clotting activity, a_t (IMCU mL⁻¹), of the Chymax test sample (chymax -100% chymosin), relative to calf rennet reference standard solution, was measured and calculated according to the IDF Standard 157: 2007 as follows:

$$a_t = \left(\frac{c_C a_{tc}}{100} \right) + \left(\frac{c_P a_{ta}}{100} \right) \quad (\text{Eqn. 1})$$

where:

c_C = % of the chymosin content in the Chymax test solution;

c_P = % of the pepsin content in the Chymax test solution;

a_{tc} = total milk-clotting activity of the Chymax test solution relative to the calf rennet reference standard solution (IMCU mL⁻¹);

a_{ta} = total milk-clotting activity of the Chymax test solution relative to the adult bovine reference standard solution (IMCU mL⁻¹).

Since chymax is 100% chymosin ($c_C = 100\%$ and $c_P = 0\%$), there is no need to calculate the milk-clotting activity relative to the adult bovine reference standard solution and Eqn. 1 reduces to the following:

$$a_t = a_{tc} . \quad (\text{Eqn. 2})$$

259

260 The total milk-clotting activity of the Chymax test sample relative to the calf rennet reference
 261 standard solution is determined by the following:

262

$$a_t = a_{tc} = \frac{t_{c,ref} \ m_{c,ref} \ V_1 \ d \ a_{c,ref}}{t_t \ V_2 \ V_3} \quad (\text{Eqn. 3})$$

264

265

266 where:

267 $a_{c,ref}$ = total milk-clotting activity of the calf rennet reference standard solution (IMCU
 268 mL⁻¹);

269 $t_{c,ref}$ = clotting time for the calf rennet reference standard solution (min);

270 t_t = clotting time obtained with the Chymax test solution (min);

271 d = dilution factor of the Chymax test solution (mL g⁻¹);

272 $m_{c,ref}$ = mass of the calf rennet reference standard solution (g);

273 V_1 = volume taken from the first calf rennet reference standard solution (mL);

274 V_2 = final volume of the first calf rennet reference standard solution (mL);

275 V_3 = final volume of the second calf rennet reference standard solution (mL).

276

277 The total dilution factor for the Chymax test solution, d , was determined by dividing the final
 278 volume of the Chymax test solution by the weight of chymax used as follows:

279

$$d = \frac{V_{chymax}}{m_{chymax}} \quad (\text{Eqn. 4})$$

280

281

282 where:

283 V_{chymax} = final volume of the Chymax test solution (mL);

284 m_{chymax} = mass of the chymax (g).

285

286

287 The dilution factor for the second calf rennet reference standard solution was determined as
288 follows:

289

290
$$d_{calf} = \frac{V_2 V_3}{m_{c,ref} V_1}. \quad (\text{Eqn. 5})$$

291

292 Substituting Eqns. 4 and 5 into Eqn. 3 reduces the equation to the following:

293

294
$$a_t = a_{tc} = \left(\frac{t_{c,ref} d}{t_t d_{calf}} \right) a_{c,ref} \quad (\text{Eqn. 6})$$

295

296 The dilution factors using Eqn. 4 and 5 and using the target mass and volume values specified for
297 the above test design gives the following:

298

299
$$d = \frac{50.0 \text{ mL}}{0.273 \text{ g}} = 183 \text{ mL g}^{-1};$$

300

301
$$d_{calf} = \frac{(10.0 \text{ mL})(10.0 \text{ mL})}{(0.500 \text{ g})(0.60 \text{ mL})} = 333 \text{ mL g}^{-1}.$$

302

The total milk-clotting activity calculation for the Chymax test sample using Eqn. 6, the dilution factors calculated above and the average Berridge clotting time of 9 and 8.4 min for calf rennet standard solution and Chymax test solution (presented later), gives the following calculation:

$$a_t = a_{tc} = \frac{(9.00 \text{ min})(183 \text{ mL g}^{-1})}{(8.40 \text{ min})(333 \text{ mL g}^{-1})} (1000 \text{ IMCU mL}^{-1}),$$

which reduces to

$$a_t = a_{tc} = 589 \text{ IMCU mL}^{-1}.$$

Statistical Analysis

An analysis of variance (**ANOVA**) was conducted using the general linear model (**GLM**) procedure of SAS (Statistical Analysis System, SAS[®], 2003) to identify the sources of variation of the collected data for all time parameters and total milk-clotting activities. The actual milk **pH**, and actual coagulation temperature (**T**) were added as covariates to the statistical model to account for differences between replicates. **Enzyme type** and **Day** (since a new calf rennet working solution was prepared for each day) were added as class variables. Student pair wise t-test model procedure of SAS (**TTEST**) was used to determine differences between the optical time parameters and t_{bc} and also the differences between the optically determined and the visually determined total milk-clotting activities. Least squared differences (**LSD**) were also conducted using the GLM procedure to determine the differences between the visually determined and optically determined total milk clotting activities.

The total milk-clotting activities calculated using the optical time parameters and the visually determined parameter, t_{bc} , were tested for repeatability with *Day* as an effect. The Repeatability

Relative Standard Deviation (RSD_r), Within-Laboratory Reproducibility Relative Standard Deviation (RSD_R), Relative Repeatability Value (r_{rel}) and Relative Within-Laboratory Reproducibility Value (R_{rel}) were determined using the International standard ISO 5725-2: 1994 calculation procedure as described in Table 1. The calculation procedure is in compliance with the IDF standard 135B:1991. For this test, the Intracell Standard Deviation was calculated by taking the difference between each of the single measurements conducted each day, y_{bk} , and their daily average, $\bar{y}_b$ (Table 1). The coefficient of variation of reproducibility, which in our study expresses the variability of independent analytical results of different *Days* was also calculated in compliance with the above mentioned standards.

Two identical and optically monitored coagulation tests were conducted during each coagulation measurement using Vat 1 and Vat 2 of the coagulation measurement apparatus. The instrument precision for the optical time parameters obtained was determined by calculating the RSD_r and the r_{rel} as described in Table 1. In this case the measurements for Vat 1 (y_{bk1}) and Vat 2 (y_{bk2}) were considered to be two single measurements and the repeatability based on their difference, $(y_{bk1} - y_{bk2})$.

RESULTS AND DISCUSSION

Light backscatter parameters as milk-clotting indicators

Light backscatter time parameters obtained and evaluated in this study were selected as potential milk-clotting indicators as they have been previously reported to be correlated to different chemical reactions taking place during milk coagulation (Saputra, 1992; Castillo et al., 2003; Castillo et al., 2006).

The light backscatter ratio profiles for the Chymax test solution were averaged ($N = 29$) and the average presented in Figure 2 with the calculated first and second derivatives. The average light backscatter ratio increase observed during the milk coagulation process was $\sim 30\%$. Figure 2 also shows the average location of the Berridge clotting time (t_{bc}) and the optical time parameters (t_{2max} , t_{max} , t_{2min} , t_{2max2} , and t_{2min2}). Table 2 shows the mean and standard deviation for t_{bc} and the optical time parameters for both the test and reference standard solutions.

ANOVA and the F-statistic are shown in Table 3 for t_{bc} and the optical time parameters t_{max} and t_{2min2} . The statistics for t_{2max} , t_{2min} , and t_{2max2} were not included in the table because they were similar to those for t_{max} . As expected, the covariates T and pH were found to be insignificant for all optical time parameters and t_{bc} . These results show that the milk coagulation apparatus and test procedure were consistent for T and pH . *Enzyme type* and *Day* were found to be significant for all optical time parameters except for t_{2min2} (Table 3). Thus it appears that *Day* was significant because a new calf rennet reference working solution (V_3) was mixed each day possibly changing the dilution factor. *Enzyme type* was expected to differ and the t_{bc} means (Table 2) were 8.42 and 9.03 min for the chymax test and calf rennet reference standard solutions, respectively. The difference between the two means was 0.6 min (36 s). This difference was within ± 40 s as prescribed by the IDF Standard 157: 2007. The lack of significance of *Day* for t_{2min2} resulted from the increased variability ($\sim 50\%$) in the measurement for this optical time parameter as shown in Table 2. This effect will be discussed later.

Comparison between Berridge clotting time and optical time parameters

The difference between the Berridge clotting time and the optical time parameters was studied using the student pair wise t-test and the results shown in Table 4. Optical time parameters were found to be significantly different from t_{bc} . Berridge clotting time and t_{2min} had the smallest mean difference (0.33 min). The optical time parameters, t_{max} and t_{2max2} had a mean difference of 1.32 and 1.37 min, respectively.

Klandar et al. (2007) used NIR transmission spectra analysis to compare visual flocculation time (i.e., t_{bc}) with several optical time parameters and found that t_{1max} (time to the first maximum in the first derivative of the transmission profile) and visual flocculation time were not significantly different. In view of the fact that visual observation of clots requires production of sufficient amount of aggregated nuclei material (McMahon et al., 1984), the discrepancy between our results and those obtained by Klandar et al. (2007) must be attributed to different stages of micelle aggregation at the inflection point of the two optical profiles compared (i.e., light backscatter and transmission profiles). Results from Klandar et al. (2007) strongly suggest that the inflection point of the transmission profile, t_{1max} , requires a similar amount of aggregated material than t_{bc} , while our previous studies (Castillo et al., 2003) concluded that the light backscatter generated inflection point is very closely located to the onset of aggregation (degree of hydrolysis at $t_{max} \sim 78\%$). Note that casein micelles cannot appreciably aggregate until the degree of hydrolysis reaches 60-80% (Carlson et al., 1987; Dalglish, 1993). This hypothesis was confirmed by the relationship encountered in our study and in previous studies between t_{max} and t_{bc} . The optical time parameter, t_{max} was calculated to be $\sim 84\%$ of t_{bc} for both enzymes used in our study (chymax and calf rennet; table 2) and $\sim 89\%$ of t_{bc} by Castillo et al. (2000). Results from Green et al. (1978) who reported that aggregation was microscopically observed at 70-90% of t_{bc} clearly pointed in the same direction.

Then, it was not surprising that the light backscatter coagulation measurement apparatus was able to generate three optical time parameters (t_{2max} , t_{max} , and t_{2min}) well before (~ 2.3 , 1.3 and 0.3 min, respectively) milk flocculation was visually observed (t_{bc}). These results were in agreement with previous studies by Payne and Castillo (2007). It should also be noted that the time parameters (t_{2max} , t_{max} , and t_{2min}) occur before the storage modulus, G' , becomes measurable (Castillo et al. 2006).

Total milk-clotting activity calculation

405 The Berridge clotting time, t_{bc} , and optical time parameters were used to calculate the total milk-
406 clotting activity, a_t , as discussed in the materials and methods section. Table 5 shows the
407 repeatability and within-laboratory reproducibility of the total milk-clotting activity, $a_t(t_{bc})$,
408 calculated using the Berridge clotting time measurement and the total milk-clotting activity
409 $a_t(t_{2max})$, $a_t(t_{max})$, $a_t(t_{2min})$, $a_t(t_{2max2})$, and $a_t(t_{2min2})$ calculated using optical time parameters
410 t_{2max} , t_{max} , t_{2min} , t_{2max2} and t_{2min2} , respectively. The relative repeatability value, r_{rel} , for the
411 optically determined a_t was less than that calculated using the Berridge clotting time except for
412 $a_t(t_{2min2})$. This suggests that the optical method using t_{2max} , t_{max} , t_{2min} , and t_{2max2} is as good as or
413 better than the t_{bc} method.

414 The visual and optically determined a_t were compared using the student pair wise t-test (Table
415 5). All optically determined a_t were found to be significantly different from the visually
416 determined $a_t(t_{bc})$ except for t_{2min} and t_{2min2} . However, the mean for $a_t(t_{2max})$, $a_t(t_{max})$, $a_t(t_{2min})$,
417 $a_t(t_{2max2})$, and $a_t(t_{2min2})$ differed from the mean for $a_t(t_{bc})$ by less than 1.4%. Thus even though
418 they are significantly different, the difference is small reflecting the precision of measurement.
419 All a_t were grouped according to their time parameter and compared using LSD. The $a_t(t_{bc})$ was
420 found to be significantly different from $a_t(t_{2max2})$, and $a_t(t_{2min2})$.

421 According to Payne and Castillo (2007), t_{max} was a good estimator of t_{bc} and compares to the
422 repeatability requirements by the IDF standards 110A:198 and 157A:1997 for t_{bc} and a_t
423 determination. The RSD_r of a_t calculated using optical time parameters, t_{max} , t_{2max} , t_{2min} and
424 t_{2max2} were 1.80, 1.80, 1.82, and 1.49, respectively (Table 5). All would meet the precision
425 requirement of the IDF Standard 157: 2007 which is 1.8%. Thus, the optical time parameters
426 t_{max} , t_{2max} , t_{2min} , and t_{2max2} are considered acceptable for calculating a_t . The measurement of a_t
427 were also found to be reproducible between *Days* for all optical time parameters ($RSD_R < 3.5\%$)
428 except t_{2min2} ($RSD_R = 3.82\%$). However, it is generally felt by the authors that it would be more
429 practical to use the first derivative parameter, t_{max} , because it has been shown a number of times
430 during previous research to be more reliable than other time parameters and is obtained by a

more direct calculation than a second derivative parameter. The optical time parameter t_{2min} occurs closest to t_{bc} and would be equally acceptable.

Instrument precision of the coagulation measurement apparatus

The instrument precision of the light backscatter coagulation measurement apparatus was determined by comparing the optical time parameter measurements between vat 1 and vat 2. The measurements for vat 1 (y_{bk1}) and vat 2 (y_{bk2}) were considered to be two single measurements and the repeatability based on their difference, ($y_{bk1} - y_{bk2}$). Thus, the light backscatter profile for both vats yielded two measurements of optical time parameters for each of the 29 tests conducted, which allows the relative repeatability standard deviation and the relative repeatability value to be calculated according to the procedure described in Table 1. Table 6 shows the instrument precision for the optical time parameters using the Chymax test solution. The instrument precision for parameters measured using calf rennet reference standard solution was nearly identical and is not reported. Repeatability relative standard deviation, RSD_r , for t_{2min} , t_{max} , t_{2max} , and t_{2max2} , and t_{2min2} was less than 1.8%, which compares well with repeatability required by the IDF Standard 157:2007 for a_t determination. The repeatability for a_t determination using optical time parameters would depend on the instrument precision.

CONCLUSIONS

Berridge clotting time method (IDF Standard 157: 2007) was compared with an optical method using infrared light backscatter for precision of total milk-clotting activity measurement. Five optical time parameters t_{2max} , t_{max} , t_{2min} , t_{2max2} , and t_{2min2} were generated from each of 29 coagulation tests, while Berridge clotting time (t_{bc}) was measured in parallel. The optical time parameters were found to be significantly different from the Berridge clotting time with optical time parameter t_{2min} occurring within 0.33 min of t_{bc} .

The optical time parameters and the Berridge clotting time were used to calculate the total milk-clotting activity and the mean difference was determined to be less than 1.4%. The repeatability relative standard deviation for the optically determined total milk-clotting activities suggests that the optical method using $t_{2\max}$, $t_{\max}$, $t_{2\min}$, and $t_{2\max2}$ is as good or better than the Berridge clotting time method. The instrument precision of the light backscatter coagulation measurement apparatus was less than or equal to 1.8%, which compares well with repeatability required by the IDF Standard 157:2007 for total milk-clotting activity determination. These results show that the optical time parameters could be used as an objective indicator of clotting time for determining the total milk-clotting activity. The first derivative parameter, $t_{\max}$, is recommended as the preferred optical parameter for calculating total milk-clotting activity however, a second derivative calculation $t_{2\min}$ that occurs closest to t_{bc} , would be equally acceptable. It is concluded that the infrared light backscatter method represents a viable, repeatable and alternative method for measuring total milk-clotting activity of rennets and milk coagulating enzymes.

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REFERENCES

Carlson, A., Hill, C. G. y Olson, N. F. (1987). Kinetics of milk coagulation: I. The kinetics of kappa casein hydrolysis in the presence of enzyme deactivation. *Biotechnology and Bioengineering* 29: 582-589.

483 Castillo, M., Payne, F.A., Hicks, C.L., and Lopez, M.B. 2000. Predicting cutting and clotting
484 time of coagulating goat's milk using diffuse reflectance: effect of pH, temperature and
485 enzyme concentration. *Int. Dairy J.*, 10:551-562.

486 Castillo, M., Lucey, J.A., and Payne, F.A. 2006. The effect of temperature and inoculum
487 concentration on rheological and light scatter properties of milk coagulated by a
488 combination of bacterial fermentation and chymosin. *Cottage cheese-type gels. Int. Dairy*
489 *J.*, 16:131-146.

490 Castillo, M.; Payne, F.A.; Hicks, C.L.; Laencina, J.S.; Lopez, M.B. 2003. Modeling casein
491 aggregation and curd firming in goats' milk from backscatter of infrared light. *J. Dairy*
492 *Res.*, 70, 335–348.

493 Dalglish, D. G. 1993. The enzymatic coagulation of milk. In *cheese: Chemistry, Physics and*
494 *Microbiology*, 1, 69-100. (Ed. Pf Fox). London: Chapman and Hall.

495 Green, M. L., Hobbs, D.G., Morant, S.Y., and Hill, V.A. 1978. Intermicellar relationships in
496 rennet-treated separated milk II. Process of gel assembly. *J. Dairy Res.*, 45, 413-422.

497 IDF 1991. Precision characteristics of analytical methods – outline of collaborative study
498 procedure. Brussels, Belgium: International IDF Standard 135B.

499 ISO 1994. Accuracy (trueness and precision) of measurement methods and results. Part 2: Basic
500 method for the determination of repeatability and reproducibility of a standard
501 measurement method. Switzerland: International Standard, ISO 5725-2.

502 IDF 1997. Determination of Total Milk Clotting Activity. Brussels, Belgium: International IDF
503 Standard 157A.

504 IDF 2002. Milk and milk products. Microbial coagulants. Determination of total milk-clotting
505 activity. Geneva, Switzerland: IDF International Standard 176.

506 IDF 2006. Milk and milk products – Ovine and caprine rennets – Determination of total
507 milk clotting activity. Brussels, Belgium: International IDF Standard 199.

508 IDF 2007. Determination of the total milk clotting activity of bovine rennets. Brussels,
509 Belgium: International IDF Standard 157.

510 Klandar, A.H., Lagaude, A., and Chevelier-Lucia, D. 2007. Assessment of the rennet coagulation
511 of skim milk: A comparison of methods. *International Dairy Journal*, 17:1151-1160.

512 Korolczuk, J., Maubois, J.-L. and Loheac, J. 1986. Suivi de la coagulation-présure du lait à l'aide
513 d'un nouveau capteur réfractométrique. *Le Lait*, 66: 327-339.

514 Kubarsepp, I., Henno, M., Kart, O., and Tupasela, T. 2005. A comparison of the methods for
515 determination of the rennet coagulation properties of milk. *Acta Agriculturae Scand*
516 *Section A*, 55:145-148.

517 Law, Barry A., 1999. *Technology in Cheesemaking*. Brooktondale, N.Y.: F.V. Kosikowski and
518 Associates.

519 Lopez, M.B., Jordan, M.J., Luna, A., Banon, S., Garrido, M.D., and Laencina, J. 1997. Diffuse
520 reflectance profiles of goat's milk during coagulation by various enzymes. *Food Science*
521 *and Technology International*, 3:299-301.

522 McMahon, D.J., Richardson, G.H., and Brown, R.J. 1984. Enzymic milk coagulation: Role of
523 equations involving coagulation time and curd firmness in describing coagulation.
524 *Journal of Dairy Science*, 67:1185-1193.

525 McMahon, D.J., and Brown, R.J. 1990. Development of surface functionality of casein particles
526 as the controlling parameter of enzymic milk coagulation. *Colloids and Surfaces*, 44:263-
527 279.

528 Payne, F.A., Hicks, C.L., Shen, and Pao-Sheng 1993. Predicting optimal cutting time of
529 coagulating milk using diffuse reflectance. *J. Dairy Science*, 76:48-61.

530 Payne, F.A. and Castillo, M. 2007. Light backscatter sensor applications in milk coagulation.
531 *Encyclopedia of Agriculture, Food, and Biological Engineering*, 1:1, 1-5.

532 Saputra, D. 1992. Analysis of enzymatic hydrolysis of milk using diffuse reflectance of near
533 infrared radiation. PhD diss. Lexington, KY.: University of Kentucky, USA.

534 SAS® User's Guide: Statistics, Version 9.1 Edition. 2003. SAS Inst., Inc., Cary, NC.

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Table 1: Repeatability and reproducibility calculations for milk-clotting activity according to ISO Standard 5725-2: 1994 and IDF Standard 135B: 1991.^a

Intracell Mean	$\bar{y}_b = \frac{1}{n_b} \sum_{k=1}^{n_b} y_{bk}$
Intracell standard deviation	$s_b = \sqrt{\frac{1}{n_b - 1} \sum_{k=1}^{n_b} (y_{bk} - \bar{y}_b)^2}$
Repeatability variance	$s_r^2 = \frac{\sum_{b=1}^t (n_b - 1) s_b^2}{\sum_{b=1}^t (n_b - 1)}$
	$\bar{y} = \frac{\sum_{b=1}^t n_b \bar{y}_b}{\sum_{b=1}^t n_b}$
	$\bar{n} = \frac{1}{t-1} \left[\sum_{b=1}^t n_b - \frac{\sum_{b=1}^t n_b^2}{\sum_{b=1}^t n_b} \right]$
	$s_R^2 = \frac{1}{t-1} \sum_{b=1}^t n_b (\bar{y}_b - \bar{y})^2$
Between-Day variance	$s_D^2 = \frac{s_R^2 - s_r^2}{2}$
Reproducibility variance	$s_R^2 = s_r^2 + s_D^2$
Repeatability Relative Standard Deviation (C.V.%)	$RSD_r = \frac{100 * s_r}{\bar{y}}$
Relative Repeatability Value (95% Confidence Interval, %)	$r_{rel} = 2RSD_r$
Within-Laboratory Reproducibility Relative Standard Deviation (C.V. %)	$RSD_R = \frac{100 * s_R}{\bar{y}}$
Relative Within-Laboratory Reproducibility Value (95% Confidence Interval, %)	$R_{rel} = 2RSD_R$
^a b = days; b = 1 to t; k = observations; k = 1 to n _b .	

Table 2: Mean and standard deviation for t_{bc} and all optical time parameters using both chymax test and calf rennet reference standard solutions^a

Parameter	Chymax test solution		Calf rennet reference solution	
	Mean (min)	SD (min)	Mean (min)	SD (min)
t_{bc}	8.42	0.22	9.03	0.18
t_{2max}	6.07	0.21	6.57	0.16
t_{max}	7.07	0.23	7.62	0.16
t_{2min}	8.06	0.23	8.66	0.17
t_{2max2}	9.76	0.25	10.4	0.21
t_{2min2}	12.4	0.46	13.0	0.39

^aNumber of experiments, N=29; SD, standard deviation.

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Table 3: Analysis of variance and F-statistic for Berridge clotting time, $t_{\max}$, and $t_{2\min2}$ (Day and enzyme type as a class)^{ab}

Source	DF	F value		
		t_{bc}	$t_{\max}$	$t_{2\min2}$
Day	8	2.20*	3.38**	1.19 ^{ns}
Enzyme type	1	145.33**	144.04**	28.06**
T	1	1.28 ^{ns}	0.93 ^{ns}	0.73 ^{ns}
pH	1	2.65 ^{ns}	0.26 ^{ns}	0.98 ^{ns}

^aNumber of experiments = 29; DF, degrees of freedom; F-value, ANOVA F statistic. *Significant at 95%, **Significant at 99%, ^{ns}Not significant at the 0.05 level.

^bThe statistics for $t_{2\max}$, $t_{2\min}$, and $t_{2\max2}$ were not included in the table because they were similar to those for $t_{\max}$.

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Table 4. Student t-test for the difference between means for t_{bc} and optical time parameters^a

H_0	N	Mean (min)	SD (min)	t-value	P
$t_{bc}-t_{2max}$	29	2.33	0.07	147	<0.0001
$t_{bc}-t_{max}$	29	1.32	0.07	80.2	<0.0001
$t_{bc}-t_{2min}$	29	0.33	0.09	20.7	<0.0001
$t_{bc}-t_{2max2}$	29	-1.37	0.10	-71.2	<0.0001
$t_{bc}-t_{2min2}$	29	-4.09	0.37	-57.3	<0.0001

^a H_0 , null hypothesis; N, number of observations; Mean, mean of the differences between every two paired data; SD, standard deviation of the difference; t-value, t-student statistics; P, probability that the t-test statistic will exceed its observed value. Key for parameters, see the text and Figure 2.

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Table 5: Repeatability and reproducibility of total milk-clotting activity (a_t) measurement using t_{bc} and optical time parameters as milk clotting indicators and Day as an effect^a

a_t (time parameter)	$\bar{x}$ (IMCU mL ⁻¹)	RSD_r (%)	r_{rel} (%)	RSD_R (%)	R_{rel} (%)
$a_t(t_{bc})$	581	2.17	4.35	2.74	5.48
$a_t(t_{2max})$	589**	1.80	3.59	2.68	5.36
$a_t(t_{max})$	584*	1.80	3.59	2.51	5.03
$a_t(t_{2min})$	581 ^{ns}	1.82	3.64	2.60	5.21
$a_t(t_{2max2})$	578*	1.49	2.99	2.46	4.92
$a_t(t_{2min2})$	580 ^{ns}	3.57	7.14	3.82	7.63

^a $\bar{x}$, mean; RSD_r , repeatability relative standard deviation; r_{rel} , relative repeatability value; RSD_R , within-laboratory reproducibility relative standard deviation; R_{rel} , within-laboratory relative reproducibility value; * Significant at 95%; ** significant at 99%. ^{ns} not significant at the 0.05 level.

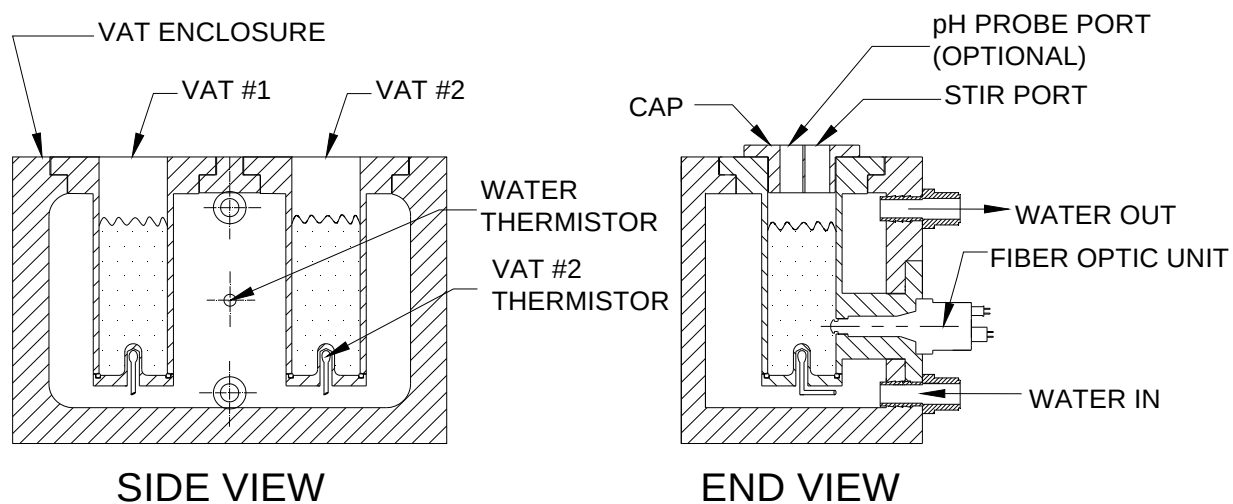
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Table 6: Instrument precision between the milk coagulation apparatus vats for measurement of optical time parameters using chymax^a

Optical time Parameter	$\bar{x}$ (min)	s_r (min)	RSD_r (%)	r_{rel} (%)
t_{2max}	6.58	0.03	0.44	0.89
t_{max}	7.62	0.02	0.32	0.64
t_{2min}	8.64	0.03	0.31	0.62
t_{2max2}	10.39	0.02	0.16	0.33
t_{2min2}	13.04	0.17	1.31	2.62

^a $\bar{x}$, mean; s_r , repeatability standard deviation; RSD_r , repeatability relative standard deviation; r_{rel} , relative repeatability value.

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Figure 1. Schematic of the coagulation measurement apparatus used to measure near infrared light backscatter during milk coagulation.

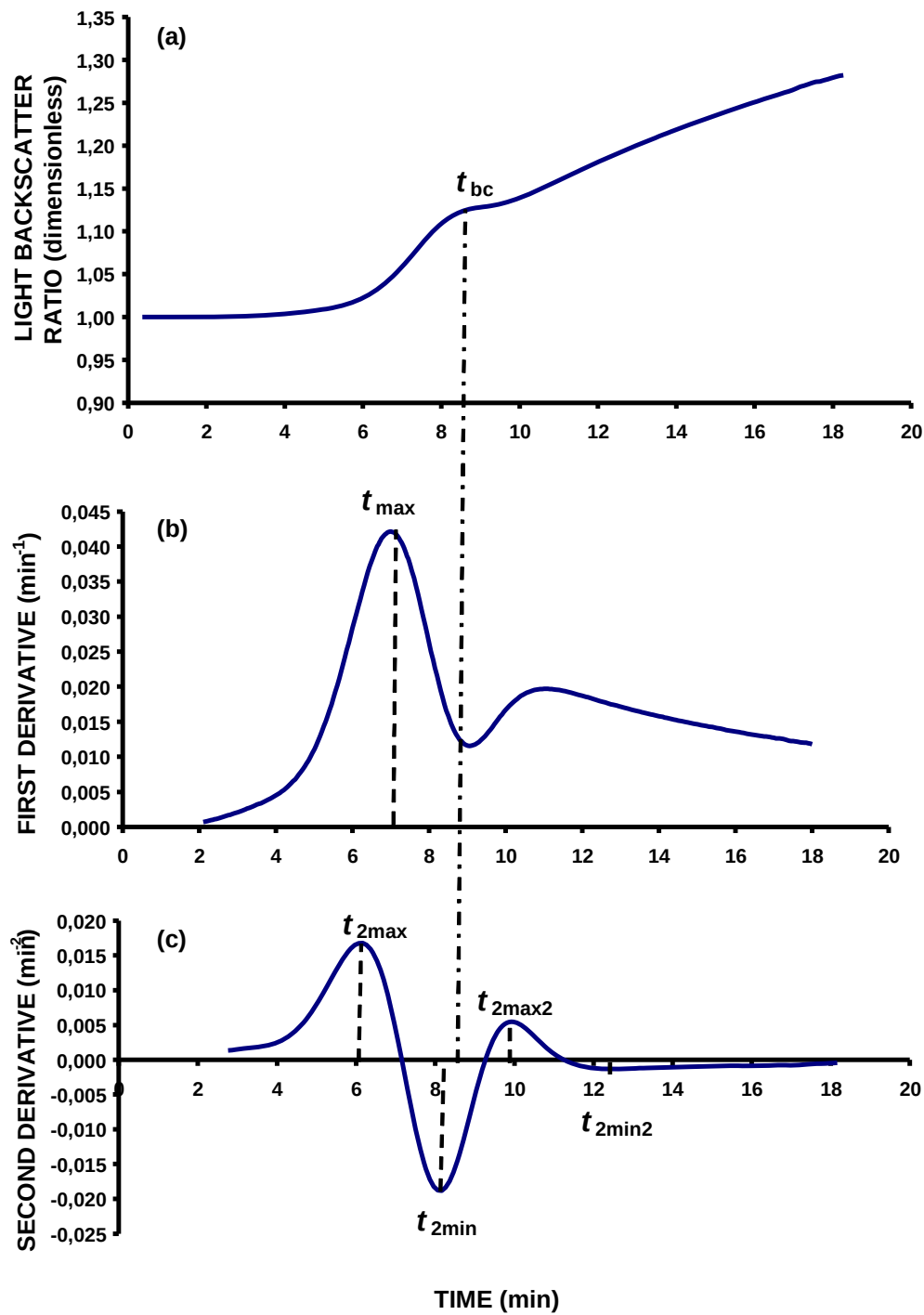
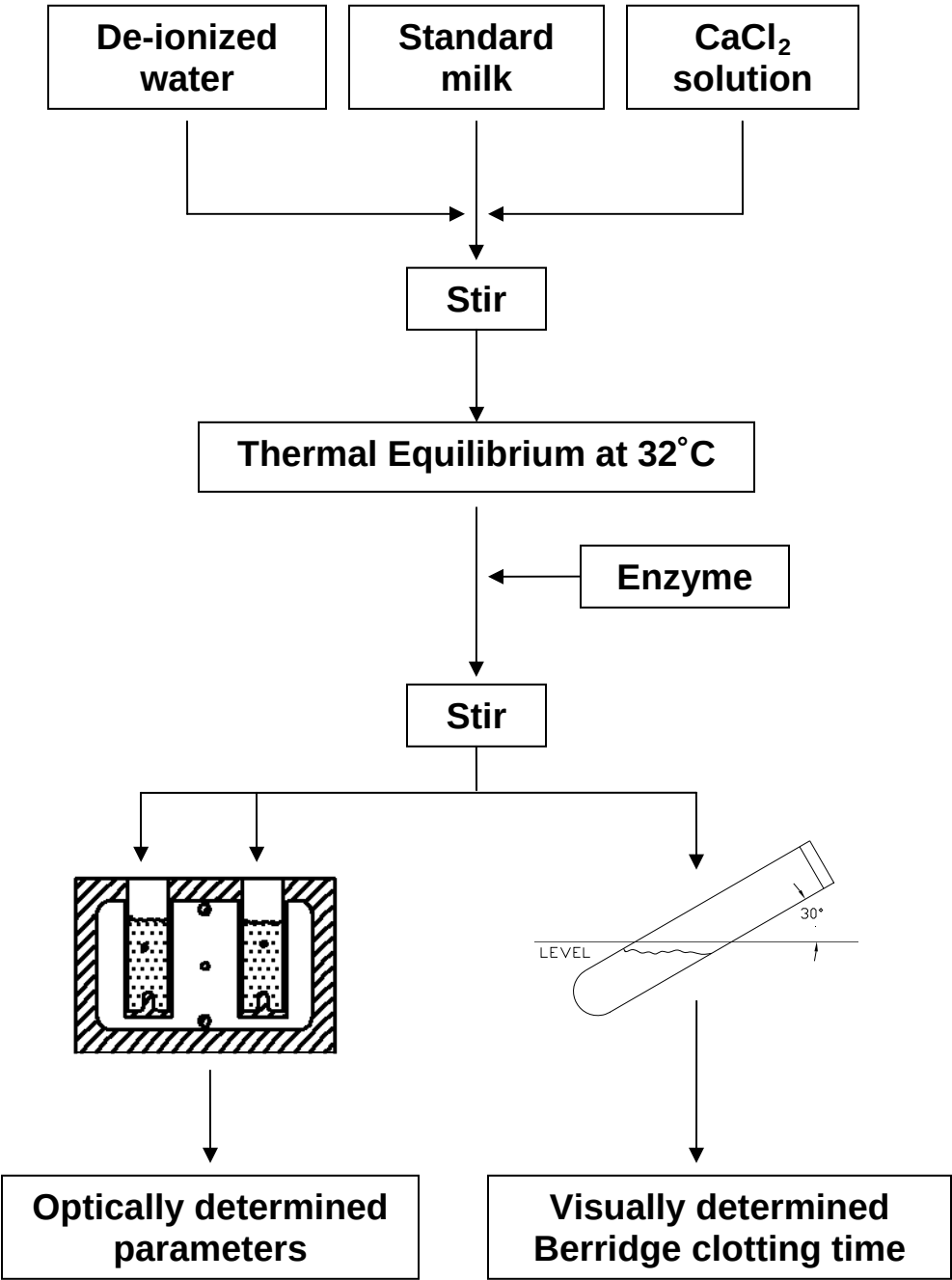


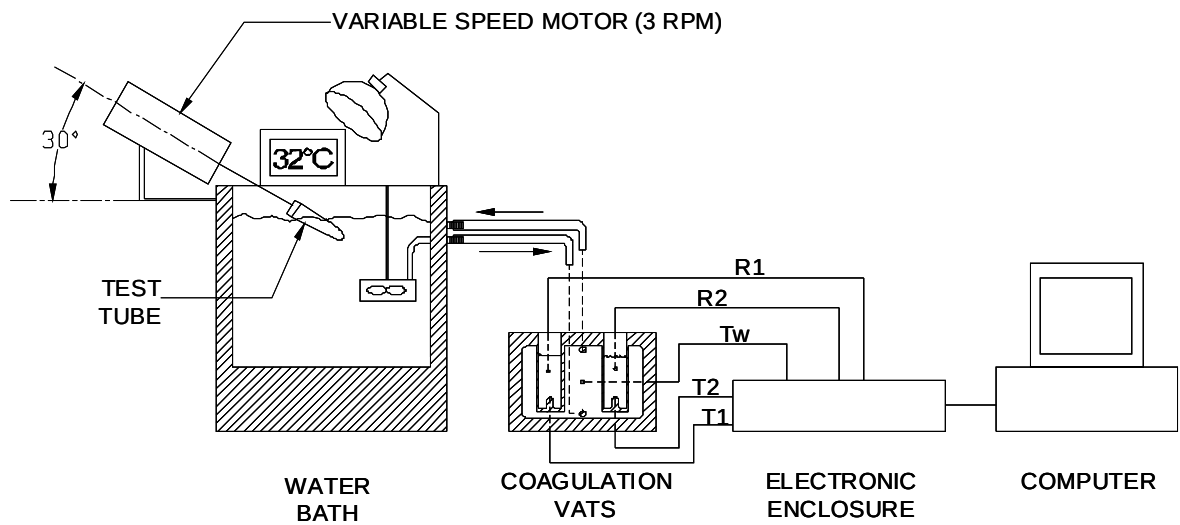
Figure 2. An average light backscatter ratio profile (a) and its calculated first derivative (b) and second derivative (c) obtained by averaging the measured infrared light backscatter ratio profiles for the 29 tests using the Chymax test solution. The average Berridge clotting time, t_{bc} , and optical time parameters (t_{2max} , t_{max} , t_{2min} , t_{2max2} , and t_{2min2}) are indicated with dotted lines. See material and methods section for definition of optical time parameters.



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Figure 3. Schematic of testing procedure for comparing the Berridge clotting time method with the proposed optical method using near infrared light backscatter for determining measurement precision of total milk-clotting activity.

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576 Figure 4. A schematic of the measurement system used to determine Berridge clotting time and
577 optical time parameters. Instrument connections: R1 = light backscatter sensor for Vat 1; R2 =
578 light backscatter sensor for Vat 2; T1 = thermistor for Vat 1; T2 = thermistor for Vat 2; and Tw =
579 thermistor for circulating water inside coagulation vat enclosure.
580