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A COMPARISON OF THE DETERMINATION OF THE RENNET COAGULATION PROPERTIES OF BOVINE MILK

Roman Pytel, Květoslava Šustová, Vojtěch Kumbár, Šárka Nedomová

ABSTRACT

The aim of the work was compared of two different methods (the visual method and the nephelo turbidimetry method) for determination of rennet coagulation time. It was observed the effect of heat treatment of milk; types of rennet and addition of different amount of $CaCl_2$ into the pasteurized milk. It was used two different chymosin rennet. For the visual method was milk sample (100 mL) equilibrated at 35 °C, 1 mL of rennet was added into milk and was measured the time required for the first visible flakes (visual method). For the determination rennet coagulation time by nephelo-turbidimetry was removed part of milk with rennet and placed into nephelo-turbidimetry. Milk had a titratable acidity in the range from 6.5 to 7.0 °SH, average pH of milk was 6.68. Dry matter content was in range from 12.351 to 13.142%. The average content of protein by Kjeldahl was 3.14%, fat by Gerber 4.34%, lactose by polarimetry 4.68% and calcium content 1.1%. The pasteurized milk had the worst rennet coagulation time about 32 s compared to the raw milk. The difference coagulation time between milk with addition of 20 µL $CaCl_2$ and 40 µL $CaCl_2$ was in range 21 s to 26 s by visual method. The difference coagulation time between milk with addition of 20 µL $CaCl_2$ and 40 µL $CaCl_2$ was 15 s by nephelo-turbidimetry method. There occurred statistically non-significant differences in most of the measurements, comparing the visual and the nephelo-turbidimetric method. The heat treatment, addition of $CaCl_2$ and using of different rennet had an influence on the curd category. It was obtained, that using nephelo-turbidimetry shown objective results for measuring the rennet coagulation time contrary the subjective visual method. Further, the results obtained by nephelo-turbidimetry are accurate and determined with the lower variation.

Keywords: rennet coagulation time; nephelo-turbidimetry; bovine milk; calcium chloride

INTRODUCTION

Milk coagulation properties are one of the most important technological properties of milk which have influence on the cheese production. The addition of rennet into the sample of milk will be cause of physical and biochemical changes in milk, which include modification of casein micelles. The result of renneting is the change of viscosity and elasticity. Milk coagulation properties is collection of property which are traditionally expressed as the rennet coagulation time, time to curd firmness of 20 mm and curd firmness 30 min after enzyme addition **(Bittante, 2011)**.

The principle of cheese production is the conversion of a viscous liquid (milk) into solid material (curd) which retain casein protein and fat (which was included in milk). If needs a solid material, the whey from curd have to be removed. In the whey is left a major part of water, whey protein and majority of lactose. For this step is important to precipitate the casein from the milk and release the whey from the curds **(Law and Tamime, 2010)**. After the addition of rennet para casein micelles starts to aggregate, thereby increasing the viscosity and elasticity. This transition may also change other physical properties of milk e.g. a light reflectance or a thermal conductivity. Several methods are principally based on the detection of these changes. These methods were developed for measuring and determination of rennet coagulation properties **(Fuquay et al., 2011)**.

The milk coagulation property is influenced by many factors, which have an effect on primary or secondary phase of precipitate of milk or both. The milk composition has direct effect on precipitate of milk and total yield of cheese. Especially important is the content of calcium, particularly the ionic form, which has influence on formation of curd gels consistency **(Roginski et al., 2003)**. Further factors which had an effect on rennet coagulation time are coagulation temperature, pH and concentration of $CaCl_2$. The coagulation temperature had highly significant effect on rennet coagulation time, coagulum firmness, gel firming rate and curd firmness. The effect of pH was highly significant for all parameters too, especially on curd firmness. Concentration of $CaCl_2$ was significant only for rennet coagulation time and coagulum firmness **(Nájera et al., 2003)**. The milk salts have an influence on rennet coagulation of milk and structure of cheese **(Lucey and Fox, 1993)**. The addition of $CaCl_2$ had an effect on the yield of cheese **(Wolfschoon-Pombo, 1997)**. The main salts are Ca and PO_4. Addition of Ca decrease the rennet coagulation time of milk that is due to neutralization of negatively charged residues of casein, which increased the aggregation
of renneted micelles **(Lucey and Fox, 1993)**. **Yuksel**

(2013) confirmed that rennet flocculation and clotting times are decreased with increasing concentration $CaCl_2$.

In this study was showed that $CaCl_2$ has effects on both – the primary and secondary phase of renneting. $CaCl_2$ reduced the time of coagulation. On the other hand addition of disodium phosphate into homogenized milk increases the time of coagulation **(Maxcy et al., 1955**).

High heat treatment or a low calcium concentration had an effect on prolonged flocculation (slow aggregation rate) with slow firming rate in the samples of reconstituted milk. The coagulation time was negatively correlated with the firming parameters **(Klandar et al., 2007)**.

Several methods were used for measuring and determining the rennet coagulation property of milk. The visual determination of rennet coagulation time is very often used. A nephelo-turbidimetry and the measurement on the formagraph are also used.

The visual method – the coagulation of proteins is often assessed using a visual method that consists of the observation of precipitating milk with naked eye. After the addition of rennet, it was measured the time until the first visible flakes of aggregation casein could be spotted with naked eye. The sample of coagulation milk is observed under continuous gentle mixing, mostly against the light **(Sbodio and Revelli, 2012)**. The result of visual method affects subjective assessment and the experience of the person monitoring the rennetability. For these reasons, it is not regarded as an objective **(Čejna and Přibyla, 2006)**.

The characteristic of rennet was based on the determination of rennet strength. To determine the strength of the rennet, there can be often used these methods: The rennet strength by Soxhlet is defined as the quantity of raw milk, which can be precipitated by 1 mL rennet at 35 °C for 40 min **(Law and Tamime, 2010)**. The method by Berridge – this method is used for determination of the defined strength of rennet to precipitate 10 mL of reconstituted standardized milk (standardized sample of powdered milk reconstituted 0.01 mol/L $CaCl_2$ at pH 6.3 at 30 °C for 100 s **(Fuquay et al., 2011)**. The method of Rolling bottle which is based on giving milk samples in special tubes or bottles. These bottles are heated up to 30 °C, adding 1 mL of diluted chymosin and placed in water bath at an angle of approximately 20 °. Bottles are spinned in this bath at speed of 8 rpm. This test is finished when the film of small casein flocks is formed on the inner wall of the bottle. The coagulation time is determined from number of revolutions from start to the end of this test **(Sommer and Matsen, 1935)**. This method is among nondynamic method. Nondynamic methods are mostly empirical and based on the measurement of rheological or optical properties **(Castillo, 2006)**.

The nephelo-turbidimetry works in the principle of the nephelometry and the turbidimetry. The nephelometry is an optical method, engaged in measuring the intensity of diffusely scattered light to the dispersed particles **(Sojková et al., 2011)**. The optical detector device converts light intensity to an electrical signal. The strain at the output is a function of the intensity of light incident on the optical detector. The milk coagulate reduces the optical signal (turbidimetry) and thereby also a reduction of the measured strain. The resulting recording signal is immediately derived. This result corresponds to the precipitate para-casein and the maximum value of derivate curve **(Chládek and Čejna, 2005)**.

The detection of rennet coagulation properties by formagraph is based on the principle of the movement of small pendulum which is linearly moved in the milk coagulation sample. This movement is recorded on a photographic paper and this curve gives us the dependence of strength to the time. When milk is in liquid form and has a low viscosity, this milk doesn´t have a sufficient strength to move the pendulum, whereas the coagulation milk has a higher viscosity and causes synchronous movement of the pendulum **(McMahon and Brown, 1982)**. Other methods for determination rennet coagulation time are: refractometry, where was measured change in the refractive index during milk coagulation **(Korolczuk et al., 1988)**, Near Infrared spectroscopy (NIR) used a light transmission or reflectance measured in the NIR range for coagulation milk **(O´Callaghan et al., 2000)**, vibration viscometry **(Marshall et al., 1982; O´Callaghan et al., 2000)**, fluorescence spectroscopy where is monitored emission fluorescence spectra of tryptophan residues during coagulation **(Herbert et al., 1999)**, electroacoustic **(Wade and Beattie, 1998)**, ultrasound low frequency used a frequencies in range 50 to 100 kHz **(Nassar et al., 2001)** and reflection photometry where are monitoring coordinates L* and b* which rise during coagulation of milk **(Hardy and Fanni, 1981)**.

MATERIAL AND METHODOLOGY

The bovine milk used for this work was collected in summer 2015. The samples of milk were heated up prior to analyses to 40 °C and then cooled down to 20 °C for better dispersion of the fat globules. After heating and cooling, the samples were immediately analysed. Before measuring the milk samples, there were done some basic laboratory analyses that have an effect on rennet coagulation time. The titratable acidity, pH, the calcium content and the lactose by polarimetry were determined according to Czech state standard No 57 0530. The content of fat was determined by Gerber's method **(ISO 2446:2008)**, the protein content was determined by Kjeldahl's method **(EN ISO 8968-1:2002)**, dry matter content was determined by gravimetry **(ISO 6731:2010)**.

After these analyses, the part of milk for rennet coagulation time was removed. This part of milk was analysed as raw milk. The remaining part of milk was pasteurized at 72 °C for 20 s. This combination is very often used at cheese production. It was premised that heat treatment has an influence on the rennet coagulation time (that the pasteurized milk needs more time for coagulation then raw milk).

Subsequently, the pasteurized milk has been divided into four groups. The first group was only pasteurized. The solution of 36% $CaCl_2$ was added into the next three groups at the quantities of 20 μL, 30 μL and 40 μL 100 $mL^{-1}$of pasteurized milk.

In our work was used a proteolytic enzyme which causes precipitation of proteins. It was used two different chymosin RENNET A (CHY-MAX M200, CHR. HANSEN, Denmark, 190 IMCU/1 mL) and RENNET B (Laktochym CZ 1068 ES, MILCOM a.s.,

Czech Republic, 59.5 IMCU/1 mL). These rennet were diluted so, that the precipitation was in range from 120 s to 240 s.

Each group (raw milk, pasteurized milk, pasteurized milk with 20 μL 100 mL^{-1}, pasteurized milk with 30 μL 100 mL^{-1}, pasteurized milk with 40 μL 100 mL^{-1}) had 6 samples for RENNET A and 6 samples for RENNET B. The measurements were repeated three times.

The measurement of rennet coagulation time of milk was carried out by visual methods and method using the nephelo-turbidimetric sensor for milk coagulation property.

The milk sample (100 mL) was equilibrated at 35 °C, 1 mL of rennet was added into milk and was measured the time required for the first visible flakes (visual method). For the determination rennet coagulation time of coagulation, which was measured by nephelo-turbidimetry.

The milk with adding rennet was placed in a thermostat at 35 °C. Samples of coagulation milk were evaluated according five grade scale (in the Table 1) after one hour in a thermostat.

Statistically, the difference between the two methods using the t-test was evaluated. The results were statistically processed by program STATISTICA 12.

The aim of the work was compare of two difference methods (the visual method and nephelo-turbidimetry method) for determination of rennet coagulation time. It was observed the effect of heat treatment of milk; types of rennet and addition of difference concentration of $CaCl_2$ into the pasteurized milk.

Table 1 Evaluation of rennet curd quality (Kuchtík et al., 2008).

Category	Appearance and firmness of curd and appearance of whey
1	very good and hard curd, keeping its shape after its removal from the container; whey is clear, of yellowgreenish colour
2	good but a little softer curd, not keeping its shape quite perfectly; excretion of whey not perfect; whey is greenish
3	not good, soft curd, partly not keeping its shape; milky white whey
4	very bad curd, not keeping its shape; milky white whey
5	very weak or invisible flocculation of casein

Table 2 The composition of milk and milk properties.

	Average	Standard deviation	Minimum	Maximum	Coefficient of variation (%)
Titratable acidity (°SH)	6.7	0.3	6.5	7.0	3.7
pH	6.68	0.04	6.63	6.71	0.62
Dry matter content (%)	12.740	0.396	12.351	13.142	3.106
Protein content (%)	3.14	0.11	3.04	3.26	3.58
Fat content (%)	4.34	0.31	4.01	4.62	7.11
Lactose content (%)	4.68	0.08	4.61	4.77	1.75

Table 3 The comparison of rennet coagulation time of raw and pasteurized milk by the visual and the nephelo-turbidimetry method (the average value with the standard deviation).

Groups of milk	Rennet	Visual method (s)	Nephelo-turbidimetry (s)	Significance
Raw	A	165 ±4	148 ±6	*
	B	135 ±15	130 ±16	NS
Pasteurized	A	184 ±23	162 ±23	*
	B	159 ±27	142 ±15	NS

Note: * – significant difference at $p < 0.05$; NS – nonsignificant difference at $p > 0.05$.

by nephelo-turbidimetry was removed part of milk with rennet and placed into nephelo-turbidimetry. The result of nephelo-turbidimetry measurement of coagulation of milk is a curve with an inflection point representing the milk coagulation time. From each group, there was calculated an average compared to the time

RESULTS AND DISCUSSION

Milk, which has been used for comparing of rennet coagulation time, had a titratable acidity in the range from 6.5 to 7.0 °SH, average pH of milk was 6.68. Dry matter content was in range from 12.351 to 13.142%. The average content of protein by Kjeldahl was 3.14%, fat by Gerber

Groups of milk	Rennet	Visual method (s)	Nephelo-turbidimetry (s)	Significance
Raw	A	165 ±4	148 ±6	*
	B	135 ±15	130 ±16	NS
Pasteurized	A	184 ±23	162 ±23	*
	B	159 ±27	142 ±15	NS

4.34%, lactose by polarimetry 4.68% and calcium content 1.1%. The detail results of milk composition and properties are shown in Table 2.

It was compared visual and nephelo-turbidimetry method for determination of rennet coagulation time in raw and pasteurized milk. The rennet coagulation time determined by two different methods is shown in the Table 3.

It was used two different rennet for determination of coagulation time in this work. The RENNET A was added in the raw milk. The coagulation time determinated by visual method was 165 s and the coagulation time determined by nephelo-turbidimetry was 148 s. According **Bujko et al., (2011)** this rennet coagulation time is evaluated as "less good" because coagulation time is in range 140 – 200 s. The difference between the visual and the nephelo-turbidimetry method was 17 s. For raw milk, there is statistically significant difference between these methods.

The same samples of milk were obscured by RENNET B. The coagulation time determinated by visual method was 135 s and the coagulation time determined by nephelo-turbidimetry was 130 s. According **Bujko et al., (2011)** this rennet coagulation time is evaluated as "good" because coagulation time is in range 110 – 140 s. The difference between the visual and the nephelo-turbidimetry method was 5 s. This difference is not statistically significant.

The pasteurized milk had the worst rennet coagulation properties because heat treatment caused changes in solubility of calcium ions. The rennet coagulation time was increased by about 32 s compared to the raw milk. The pasteurized milk with RENNET A gave a statistically significant difference between both methods. However comparing these methods with RENNET B, it was found that there is no significant difference.

It was observed an influence of different amount $CaCl_2$ to rennet coagulation time. The results of rennet coagulation time are shown in the Table 4.

The rennet clotting time for pasteurized milk with 20 µL by visual method (RENNET A) was 150 s. The rennet clotting time by nephelo-turbidimetry was 134 s. Difference between these methods was 16 s with a statistically significant difference.

The rennet clotting time for pasteurized milk with 20 µL by visual method (RENNET B) was 129 s. The rennet clotting time by nephelo-turbidimetry was 124 s. Difference between these methods was 5 s. This difference between these methods is a statistically not significant.

This statistically not significant difference is due to the fact that RENNET B with $CaCl_2$ forms larger flakes which are better visibly detected by naked eye.

The higher addition than 20 µL of calcium chloride into the milk caused that the difference between the visual and the nephelo-turbidimetry methods is not statistically significant. With the increasing levels of calcium chloride begin to form the larger flakes without influence to type of rennet. The rennet coagulation time are decreasing with increasing the addition of $CaCl_2$. The difference

Table 4 The comparison of rennet coagulation time of pasteurized milk with the addition of different amount of $CaCl_2$ by the visual and the nephelo-turbidimetry methods (average value with standard deviation).

Groups of milk	Rennet	Visual method (s)	Nephelo-turbidimetry (s)	Significance
Pasteurized with 20 µL	A	150 ±20	134 ±11	*
	B	129 ±22	124 ±18	NS
Pasteurized with 30 µL	A	136 ±20	124 ±13	NS
	B	121 ±17	111 ± 9	NS
Pasteurized with 40 µL	A	124 ±19	119 ±15	NS
	B	108 ±17	109 ±15	NS

Note: * – significant difference at p <0.05; NS – nonsignificant difference at p >0.05.

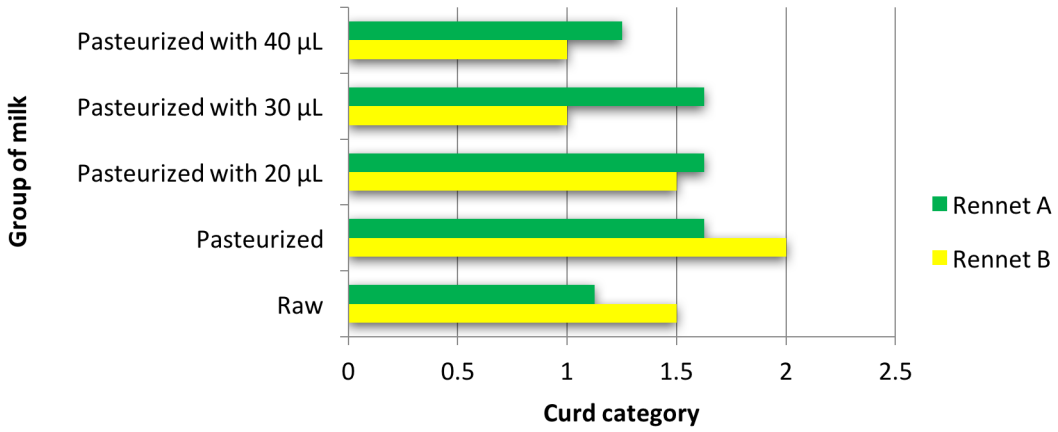

Figure 1 Curd category evaluation of milk group.

coagulation time between milk with addition of 20 µL $CaCl_2$ and 40 µL $CaCl_2$ was in range 21 s to 26 s by visual method. The identical results present **Erdem (1997)**, which studied the effect of $CaCl_2$ concentration on the clotting time. **Fox et al. (2004)** report, that the best rennet coagulation time had pasteurized milk with added calcium chloride. This finding was confirmed by our results – rennet coagulation time for milk with addition of 40 µL was 124 s while the rennet coagulation time for pasteurized milk was 184 s (RENNET A).

According to the study by **Čejna and Přibyla (2006)** the good milk coagulation property means that milk needs a short time to precipitate the milk by adding rennet. The results of this study confirmed possibility to reduce rennet coagulation time by addition of $CaCl_2$.

When was compared the curd quality, it was confirmed the premise that the heat treatment had an effect to curd quality. These curd were soft but the curd harder released a whey. The released whey was a slightly turbid. The curd quality was also affect by rennet which was used (Figure 1). At the higher amount $CaCl_2$ is better used a RENNET B. This curd was solid, keep the shape and whey was yellowgreen clear colour. At amount of 30 or 40 µL $CaCl_2$ was formed a first category of curd. At amount of 20 µL $CaCl_2$ was formed a worse curd category. The curd was a less solid and releasing of whey was more difficult. RENNET A formed a better curd category without addition of $CaCl_2$ or with an addition of 20 µL $CaCl_2$.

CONCLUSION

The visual method for determining rennet coagulation time is a subjective method, which is influenced by the experience of person monitoring rennet coagulation time, by the type of rennet and its concentration. It was obtained, that using nephelo-turbidimetry shown objective results for measuring the rennet coagulation time contrary the subjective visual method. There were found statistically non-significant differences in most of the measurements, comparing these methods. Using nephelo-turbidimetry was obtained the objective results for measuring the rennet coagulation time. Further, the results obtained by nephelo-turbidimetry are accurate and there are determined with the lower deviation. The determination rennet coagulation time by nephelo-turbidimetry is more expensive, but is unaffected by the experience of person monitoring these properties. Thus, this method can replace the subjective visual method.

The heat treatment, addition of $CaCl_2$ and using of different rennet had an influence on the curd category. For milk where is used a less amount of $CaCl_2$ or without $CaCl_2$ is better used RENNET A, which formed a better curd category. The combination of higher amount $CaCl_2$ with RENNET A is not suitable for formed good curd category. RENNET B is better used with higher amount of $CaCl_2$ for formed good curd category.

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Contact address:

Ing. Roman Pytel, Mendel University in Brno, Faculty of AgriSciences, Department of Food Technology, Zemědělská 1, 613 00 Brno, Czech Republic, E-mail: roman.pytel@mendelu.cz.

prof. Ing. Květoslava Šustová, Ph.D., Mendel University in Brno, Faculty of AgriSciences, Department of Food Technology, Zemědělská 1, 613 00 Brno, Czech Republic, E-mail: sustova@mendelu.cz.

Ing. Vojtěch Kumbár, Ph.D., Mendel University in Brno, Faculty of AgriSciences, Department of Technology and Automobile Transport, Zemědělská 1, 613 00 Brno, Czech Republic, E-mail: vojtech.kumbar@mendelu.cz.

doc. Ing. Šárka Nedomová, Ph.D., Mendel University in Brno, Faculty of AgriSciences, Department of Food Technology, Zemědělská 1, 613 00 Brno, Czech Republic, E-mail: snedomov@mendelu.cz.