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Effect of pasteurization on coagulation properties of bovine milk and the role of major composition traits and protein fractions

This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

*Published Version:*

Franzoi M., Costa A., Vigolo V., Penasa M., De Marchi M. (2022). Effect of pasteurization on coagulation properties of bovine milk and the role of major composition traits and protein fractions. JOURNAL OF FOOD COMPOSITION AND ANALYSIS, 114, 1-9 [10.1016/j.jfca.2022.104808].

*Availability:*

This version is available at: <https://hdl.handle.net/11585/914536> since: 2023-02-10

*Published:*

DOI: <http://doi.org/10.1016/j.jfca.2022.104808>

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**Marco Franzoi, Angela Costa, Vania Vigolo, Mauro Penasa, Massimo De Marchi,**

**Effect of pasteurization on coagulation properties of bovine milk and the role of major composition traits and protein fractions,**

**Journal of Food Composition and Analysis, Volume 114, 2022, 104808, ISSN 0889-1575**

The final published version is available online at:  
<https://doi.org/10.1016/j.jfca.2022.104808>

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1 **Effect of pasteurization on coagulation properties of bovine milk and the role of major**  
2 **composition traits and protein fractions**

3 Marco FRANZOI<sup>a</sup>, Angela COSTA<sup>a\*</sup>, Vania VIGOLO<sup>a</sup>, Mauro PENASA<sup>a</sup>, Massimo DE  
4 MARCHI<sup>a</sup>

5  
6 <sup>a</sup>Department of Agronomy, Food, Natural resources, Animals and Environment (DAFNAE),  
7 University of Padova, Viale dell'Università 16, 35020 Legnaro (PD), Italy

8 \*Corresponding author: Angela Costa, Department of Agronomy, Food, Natural resources, Animals  
9 and Environment (DAFNAE), University of Padova, Viale dell'Università 16, 35020 Legnaro (PD),  
10 Italy; angela.costa@unipd.it

11  
12 e-mail address: marco.franzoi@unipd.it; angela.costa@unipd.it; vania.vilogo@phd.unipd.it;  
13 mauro.penasa@unipd.it ; massimo.demarchi@unipd.it

14

15 **Acknowledgement**

16 The Breeders Association of Veneto region (ARAV) is gratefully acknowledged for the technical  
17 support. Authors thank Dr Selina Sterup Moore (University of Padova) for proof-reading the  
18 manuscript.

19

20 **Declaration of Competing Interest**

21 The authors declare that they have no known competing financial interests or personal relationships  
22 that could have appeared to influence the work reported in this paper.

23

24 **Funding sources**

25 This research was supported by project ‘Innovamilk’ (Innovations in Italian dairy industry for the  
26 enhancement of farm sustainability, milk technological traits and cheese quality) funded by AGER  
27 – Agroalimentare e Ricerca, Grant n. 2017-1153.

28

29    **Highlights**

- 30        •    Coagulation ability of milk after pasteurization depends on raw milk composition
- 31        •    More acidic raw milk shows better cheese-making properties after pasteurization
- 32        •    Raw milk  $\beta$ -lactoglobulin unfavorably affects curd firming time of pasteurized milk
- 33        •    Rennet coagulation time of pasteurized milk can be predicted from raw milk spectra

## 34    **Abstract**

35        Milk coagulation properties (MCP) worsens after heat treatment, however the specific  
36 mechanisms responsible have been scarcely explored. In this study, 100 milk samples were  
37 available to i) identify the raw milk characteristics responsible for unfavorable changes in MCP  
38 after pasteurization and ii) develop infrared prediction models for pasteurized milk MCP using  
39 spectra of raw samples. The loss in coagulation ability due to pasteurization was lower when raw  
40 milk had optimal MCP, higher acidity, greater protein content and lower  $\beta$ -lactoglobulin content.  
41 For the four MCP, the trait measured before pasteurization (raw milk) was the most important  
42 variable influencing the corresponding trait after heating. For example, rennet coagulation time  
43 (RCT),  $\kappa$ -casein, protein, lactose and pH of raw milk significantly affected pasteurized milk RCT  
44 ( $P<0.001$ ). For curd firmness, each unit (mm) corresponded to 58.65 g/100 g  $\kappa$ -casein. In general,  
45 raw milk  $\beta$ -lactoglobulin unfavorably affected pasteurized milk MCP (e.g., the estimate of curd  
46 firming time was 81.39 g/100 g). Results suggested that only the prediction model of RCT  
47 (pasteurized milk) achieved an exploitable coefficient of determination in cross-validation (0.66).  
48 Our outcomes are relevant for dairy plants manufacturing cheese from pasteurized standardized  
49 milk and could support producers' decision-making.

50    *Keywords:* rennet coagulation time; casein; whey protein; heat treatment; cheese; dairy industry

## 51    **1. Introduction**

52        Pasteurization is a common practice in the dairy industry, and is primarily intended for the  
53    reduction of the milk pathogenic bacteria load, which has to be below the admissible level. Heat  
54    treatment translates into an increased shelf-life of milk and limits proliferation and activity of  
55    microorganisms detrimental for cheese processing. Common milk pasteurization techniques  
56    comprise heating at either 63°C for 30 min (low-temperature long-time, LTLT), 72°C for 15 s  
57    (high-temperature short-time, HTST) or any other equivalent thermal treatment (Stumbo, 1973; Liu  
58    et al., 2020). Such temperatures were selected to achieve a 5-log reduction in the presence of the  
59    heat resistant pathogen *Coxiella brunetii* detectable in raw milk (Kelly et al., 2005; FIL-IDF, 2019).  
60    Cheeses commercially available can be produced from either unpasteurized or pasteurized milk.  
61    Some of those entitled with the Protected Designation of Origin label, like Grana Padano and  
62    Parmigiano Reggiano (Mammi et al., 2018; Buonaiuto et al., 2021; Cavallini et al., 2021), are  
63    produced from the former, whereas Cheddar, mozzarella pasta filata and American soft artisanal  
64    cheeses are produced from the latter (Knoll, 2005).

65        Heating, including the pasteurization process, is known to alter milk composition and impair the  
66    technological traits (Anema et al., 2007; Blecker et al., 2012; Britten & Giroux, 2022; Hyslop,  
67    2003; Lucey, 1995), causing a deterioration of the milk coagulation properties (MCP). Various  
68    MCP have been described in the literature, but rennet coagulation time (RCT, min), curd firmness  
69    ( $a_{30}$ , mm) and curd firming time ( $k_{20}$ , min) are known to be the most important for describing the  
70    milk cheese-ability. The deterioration of MCP observed in heat-treated milk is likely due to the  
71    denaturation of  $\beta$ -lactoglobulin ( $\beta$ -LG) and its subsequent complexation with  $\kappa$ -casein ( $\kappa$ -CN)  
72    through a sulphydril-disulphide interaction (Fox et al., 2017). In this way, rennet enzymes are  
73    sterically prevented from hydrolyzing  $\kappa$ -CN (Dalgleish, 1993; Guinee, 2021). In addition,  $\beta$ -LG  
74    binds to para- $\kappa$ -CN cysteine residues during hydrolysis, reducing the capability of casein micelles  
75    to aggregate (Creamer et al., 2004). Heat treatments are also responsible for the demineralization of  
76    casein micelles, which furthermore minimizes the aggregation capability (Fox, 1981; Touhami et

77 al., 2022). As such, less favorable MCP, e.g. longer RCT and weaker curd, are expected when  
78 dealing with pasteurized rather than raw milk (Yu et al., 2009).

79 The HTST pasteurization has a greater impact on MCP than LTLT. In fact, the extent of the  
80 detrimental effects on both the enzymatic and non-enzymatic phases of rennet-induced coagulation  
81 is determined by the intensity of heating. For instance, even short (15 s) treatments at a temperature  
82 greater than 75°C s cause notable damages to the cheese-making properties of milk (Fox et al.,  
83 2017).

84 The interest to further improve milk MCP has grown in recent years, both within the scientific  
85 community and among manufacturers. Phenomics can be considered a large scale acquisition of  
86 novel phenotypes to be studied and validated for several purposes, including for the definition of  
87 new breeding programs (Cole et al., 2020). However, the validation path requires collection of  
88 reference data for new phenotypes, which can be extremely expensive and cumbersome. Clotting  
89 parameters like RCT,  $a_{30}$  and  $k_{20}$  need to be determined through the reference analysis,  
90 lactodynamography, ideally using milk samples from both bulk tank and individual cows  
91 (Kübarsepp et al., 2005). Lactodynamography consists in the analysis of milk thromboelastography  
92 and provides various descriptors of coagulation speed and curd syneresis. Mid-infrared  
93 spectroscopy (MIRS) has proven very useful in the collection of data of interest in dairy species,  
94 including cattle. Beyond determining milk-related traits, spectral data can be exploited to assess and  
95 monitor the cows' health status. Nowadays, MIRS is the routine technology employed in DHI  
96 programs, as it is fast, easy to implement and relatively inexpensive. In addition, spectral data can  
97 be stored for later retrospective analyses (Gengler et al., 2016). Predictive models have been  
98 proposed in the past for a large scale acquisition of MCP data in cattle, sheep, goat and buffalo; in  
99 some countries, like Italy, such models are used to establish detailed milk payment systems or to  
100 estimate animals' breeding value (Cassandro et al., 2008; De Marchi et al., 2014; El Jabri et al.,  
101 2019). All above-mentioned predictive models rely exclusively on raw milk spectra and reference  
102 MCP data. However, a considerable amount of dairy industries manufacture cheeses from



103 pasteurized milk and knowing the potential coagulative performance of the pasteurized milk in  
104 advance, i.e., before heating (raw), would thus allow them to optimize milk standardization and  
105 processing. The objective of the present study was to understand which components in raw milk  
106 influence the MCP of the pasteurized milk. Particularly, we aim to i) quantitatively determine  
107 compositional traits of raw milk, including the detailed protein profile, that are reported to be  
108 involved in the MCP loss after pasteurization, ii) identify the main variable responsible for the  
109 deterioration of MCP when milk is subjected to heat treatment, and iii) develop MIRS prediction  
110 equations for pasteurized milk MCP using the spectra collected on the untreated (raw) samples.

111

## 112 **2. Material and methods**

### 113 *2.1. Sampling and milk composition analysis*

114 Milk was collected from 100 cows at different lactation stages, i.e. from 5 to 410 days after  
115 calving, by trained personnel during the morning milking. Cows with lactations from 1 to 8 were  
116 represented. Cows belonged to Simmental (40), Jersey (30), Holstein (20) and Rendena (10) breeds  
117 and were reared in 4 single-breed farms located in Northern Italy under intensive or semi-intensive  
118 farming conditions. Sampling took place between July and December 2021.

119 The tubes used for milk collection contained 0.05% (w/w) of preservative (Bronopol; 2-bromo-  
120 2-nitropropan-1,3-diol; Knoll Pharmaceuticals, Nottingham, UK) to prevent microbial spoilage.  
121 After filling, samples were transported (4°C) to the Department of Agronomy, Food, Natural  
122 resources, Animals and Environment of the University of Padua (Legnaro, Italy) within 2 h. For  
123 each sample, various aliquots were obtained: 50 mL was sent to the milk laboratory of Breeders  
124 Association of Veneto Region (ARAV, Padua, Italy) for MIRS spectra collection and gross  
125 composition determination; 10 mL was used to determine raw milk MCP; 10 mL underwent heat  
126 treatment to subsequently assess MCP in the pasteurized matrix; and 0.5 mL was kept for the  
127 determination of raw milk protein fractions via HPLC. The MIRS device MilkoScan FT7 (FOSS  
128 A/S, Hillerød, Denmark) provided information on content of fat, protein, lactose, and casein and

129 pH. According to the ISO 21543:2020 (ISO, 2020), samples were warmed at 37°C and  
130 homogenized by gentle inversion before analysis. The sample intake was set to 5 mL and the time  
131 required for a single analysis was 6 s. The somatic cell count (SCC) was also determined using a  
132 Fossomatic 7 DC (FOSS A/S, Hillerød, Denmark) following the ISO 13366-2:2006 (ISO, 2006)  
133 and was mathematically converted into somatic cell score (SCS) to normalize the distribution of the  
134 data.

## 135 2.2. *Milk coagulation properties*

136 Assessment of MCP at 60 min was performed in parallel for the raw and the pasteurized aliquot  
137 of milk through lactodynamographic analysis (MaPe System, Firenze, Italy). The LTLT  
138 pasteurization was carried out based on Fox et al. (2015), i.e. 63°C for 30 min in a water bath under  
139 mild agitation (Yu et al., 2009). The protocol proposed by Vigolo et al. (2022) was followed for the  
140 lactodynamographic analysis; in brief, milk was dispensed in the wells according to the scheme  
141 depicted in Fig. 1 and the whole plate was thereafter heated (35°C). 200 µL of a commercial calf  
142 rennet solution (Naturen Plus 215, Chr Hansen, Hørsholm, Denmark) diluted in distilled water  
143 (1.2:100 v/v) was added to each well to induce the coagulation. Measurements were taken for 60  
144 min after rennet addition and the traits recorded included RCT,  $a_{30}$ ,  $k_{20}$  and the curd firmness  
145 measured at 2 times the RCT ( $a_{2r}$ ). By definition, RCT is the time between the addition of rennet  
146 and coagulation initiation,  $k_{20}$  is the time necessary to reach a 20 mm firmness of the curd, and  $a_{30}$   
147 measures the consistency of the curd at 30 min of analysis.

148

## 149 2.3. *Analysis of protein fractions*

150 Quantification of  $\alpha$ -CN s2,  $\alpha$ -CN s1,  $\beta$ -CN,  $\kappa$ -CN,  $\alpha$ -LA and  $\beta$ -LG was performed on a small  
151 representative aliquot of raw milk by using the HPLC station Agilent 1260 Infinity II LC (Agilent  
152 Technologies, Santa Clara, CA) equipped with a quaternary pump (Agilent 1260 Infinity II,  
153 G7111B), a diode array Detector (Agilent 1260 Infinity II, G7115A), a column thermostat (Agilent  
154 1260 Infinity II, G7116A), and an auto-sampler (Agilent 1260 Infinity II, G7129A). A reversed-

155 phase analytical column C8 (Zorbax 300SB-C8 RP, Agilent Technologies) preceded by a pre-  
156 column (300SB-C8 Guard Cartridges  $4.6 \times 12.5$  mm, 4/PK, Agilent Technologies), was used for  
157 separation. Before injection, the samples were prepared as described in Bobe et al. (1998): briefly,  
158 500  $\mu$ L of milk were added to an aqueous solution of guanidine (Gdn) HCl (6 M GdnHCl, 0.1 M  
159 bisTris buffer, 5.37 mM sodium citrate, and 19.5 mM DTT) in a 1:1 ratio (v/v). Each sample was  
160 shaken for 10 s, incubated at room temperature for 1 h, and thereafter centrifuged at 13,000 g for 10  
161 min at room temperature. The aqueous phase was diluted in the proportion 1:3 (v/v) with a solution  
162 containing 4.5 M GdnHCl in water, acetonitrile and trifluoroacetic acid (100:900:1). The  
163 chromatographic conditions were those described by Bonfatti et al. (2008), i.e. gradient elution was  
164 carried out with a mixture of solvent A (0.1% TFA in water) and solvent B (0.1% TFA in  
165 acetonitrile). Separations were performed with the following gradients: linear gradient from 33 to  
166 35% B in 5 min, from 35 to 37% B in 4 min, from 37 to 40% B in 9 min, from 40 to 41% B in 4  
167 min, isocratic elution at 41% B for 5.5 min, linear gradient from 41 to 43% B in 0.5 min, and from  
168 43 to 45% B in 8 min. Before the injection of the subsequent sample, the column was re-  
169 equilibrated at 33% B for 8 min. The flow rate was 0.5 mL/min, the column temperature was kept at  
170 45°C, the detection was made at a wavelength of 214 nm and the injection volume was 5  $\mu$ L  
171 (Bonfatti et al., 2008). Agilent OpenLab 2 CDS software (Agilent Technologies, Santa Clara, SA)  
172 was used for data acquisition and analysis. The identification of single protein fractions was carried  
173 out using external standards of  $\alpha$ -CN,  $\beta$ -CN,  $\kappa$ -CN,  $\alpha$ -LA (Merck, Darmstadt, DE) and  $\beta$ -LG (BOC  
174 Sciences, NY, USA), and the quantification of each chromatographic peak was obtained with 5-  
175 point calibration curves (coefficient of determination  $\geq 0.99$ ).

176

## 177 2.4 Statistical analysis

178 Variables determining MCP of pasteurized milk were identified using the GLMSELECT  
179 procedure of SAS software v. 9.4 (SAS Institute Inc., Cary, NC). The explanatory variables imputed  
180 for RCT,  $a_{30}$ ,  $k_{20}$  and  $a_{2r}$  were: the correspondent MCP measured in the raw milk (both the first and

181 the second power), gross composition traits and all the protein fractions expressed as g/100 g of  
182 total protein. The stepwise selection algorithm was the chosen selection method, with the Akaike  
183 Information Criterion (AIC) used as selection/exclusion criterion; the selection criterion of the final  
184 model was the adjusted coefficient of determination. Selection algorithm was refitted 1,000 times  
185 on 80% of randomly selected samples and the variables selected in the final model were those  
186 included in at least 10% of refitted models. The final output consisted in: intercept, average and SD  
187 of variables regression estimates, first and third quartile of estimates, and variable inclusion rate, i.e.  
188 rate of variables appearing in refitted models. Finally, for each MCP trait, the selected variables  
189 were refitted to the dependent variable (RCT,  $a_{30}$ ,  $k_{20}$  or  $a_{2r}$  of the pasteurized samples) using a  
190 multiple linear regression through the REG procedure of SAS, in order to assess the significance of  
191 each covariate (selected variable), the estimates, and the standard error of estimates.

192

## 193 *2.5 Spectral collection and chemometric analysis*

194 Although milk spectra were collected in the window between 900 and 5,000  $\text{cm}^{-1}$ , every 3.858  
195  $\text{cm}^{-1}$ , prediction models were developed using only part of the whole spectral region. In fact, only  
196 wavelengths belonging to intervals that the manufacturer refers to as “good spectrum” (FOSS A/S,  
197 Hillerød, DK) were kept to exclude regions associated with water-related noise and poor signal-to-  
198 noise ratio. 450 spectral variables in the intervals 964.5 to 1,562.5  $\text{cm}^{-1}$ , 1,720.7 to 2,291.7  $\text{cm}^{-1}$   
199 and 2,415.1 to 2,970.7  $\text{cm}^{-1}$  were available for each sample.

200 In order to improve the linear relationship between the spectra and reference values, statistical  
201 procedures and mathematical pretreatments were applied to the milk spectrum. Prediction equations  
202 were built using a modified partial least squares regression analysis (WinISI III v. 1.60; Foss and  
203 Infracore International LLC, State College, PA) through a 5-fold cross-validation. Several  
204 combinations in terms of both scattering correction (no correction, None; detrend, Det; standard  
205 normal variate, SNV; SNV + Det; and standard multiplicative scatter correction, MSC) and  
206 mathematical treatment (0,0,1,1; 1,4,4,1; 1,8,8,1; 2,5,5,1; and 2,10,10,1) were tested. The 4 digits

207 defining the mathematical treatment indicate: number of the derivative, gap used for derivative  
208 calculation, data points in the first smoothing, and data points in the second smoothing,  
209 respectively. The number of latent variables included in the model were selected according to van  
210 der Voet (1994).

211 Before each regression, spectral data points were evaluated for global Mahalanobis distance  
212 (GH) and those with  $GH > 3$  were excluded. Hereafter, potential outliers were removed using the T-  
213 outlier test (Soyeurt et al., 2012) available in the WinISI software (Foss, Hillerød, Denmark), by  
214 setting the critical value to 3. Both the modified partial least squares regression and the outlier  
215 determination were iterated three times and the best prediction equation was chosen based on the  
216 standard error of cross-validation ( $SE_{CV}$ ). The standard error ( $SE_C$ ) and the coefficient of  
217 determination in calibration ( $R^2_C$ ) as well as the coefficient of determination ( $R^2$ ) in cross-validation  
218 ( $R^2_{CV}$ ) were reported to evaluate the model performance.

219

### 220 **3. Results & discussion**

#### 221 *3.1. Overview of milk traits*

222 Descriptive statistics of all milk parameters available are summarized in Table 1. Overall, the  
223 average and SD of gross composition traits and pH were in line with multi-breed studies carried out  
224 in Italy (Gottardo et al., 2017; Benedet et al., 2020) and other countries (Visentin et al., 2017;  
225 Frizzarin et al., 2021). The SCS averaged 2.38 and was characterized by a large coefficient of  
226 variation (81.11 %), in accordance with previous studies (Costa et al., 2019; Franzoi et al., 2020).  
227 The minimum, median and maximum SCC were 5 000, 55 500 and 6 077 000 cells/ $\mu$ L,  
228 respectively, suggesting that collected samples were representative of different udder health  
229 conditions (Gill et al., 1990; Franzoi et al., 2020). Casein fractions and concentration of whey  
230 proteins (g/100 g of total protein) revealed that the total protein content was mostly given by two  
231 fractions, the  $\alpha$ -CN s1 and the  $\beta$ -CN fractions (Holt et al., 2013). These were also characterized by  
232 the lowest variability compared to the other fractions, with a CV of 6.13 and 10.65%, respectively.

233 Such low phenotypic variability is in agreement with Sanchez et al. (2019) who investigated protein  
234 fractions predicted via MIRS. In that study, MIRS models used for protein fractions were  
235 characterized by a moderate to good accuracy, with  $R^2_v$  ranging from 0.59 to 0.92. The contribution  
236 of  $\alpha$ -CN s1 (33%) and  $\beta$ -CN (31%) to total protein content of Sanchez et al. (2019) is similar to the  
237 contribution seen in the present study: 27% and 30%, respectively. The same can be said for the  
238 contribution (15.5%) of the two whey proteins, which was equal to 16.5% in Sanchez et al. (2019).

239 Considering protein titers expressed in relation to volume (mg/mL of milk), average values  
240 reported in Table 1 are similar to those of Niero et al. (2016) who investigated casein fractions of  
241 114 cows belonging to Holstein, Brown Swiss and Jersey breed. The amount of whey proteins,  
242 however, was slightly higher compared to previous studies; indeed,  $\beta$ -LG and  $\alpha$ -LA averaged 3.71  
243 and 1.30 mg/mL in Simmental cows (De Marchi et al., 2009) and 2.7 and 1.1 mg/mL in Jersey cows  
244 (Eskildsen et al., 2016). Nevertheless, results in Table 1 are similar to those reported by Frizzarin et  
245 al. (2021) for Irish cows:  $\alpha$ -CN s2 (3.67 g/L, CV = 26%),  $\alpha$ -CN s1 (14.09 g/L, CV = 17%),  $\beta$ -CN  
246 (12.80 g/L, CV = 17%),  $\kappa$ -CN (5.77 g/L, CV = 25%),  $\alpha$ -LA (1.12 g/L, CV = 27%) and  $\beta$ -LG variant  
247 A (2.49 g/L, CV = 47%) and variant B (2.45 g/L, CV = 69%). In that study, the authors assessed the  
248 milk protein profile of cows belonging to various breeds via HPLC and determined MCP using the  
249 Formagraph (FOSS A/S, Hillerød, Denmark). As regards the MCP, Frizzarin et al. (2021) obtained  
250 descriptive statistics similar to the current study, with a mean equal to 20.81 min, 5.82 min and  
251 32.24 mm for RCT,  $k_{20}$  and  $a_{30}$ , respectively. Moreover, Costa et al. (2019b) and Niero et al. (2021)  
252 reported similar statistics for MIRS-predicted traits related to coagulation ability. For instance, in  
253 Niero et al. (2021) the RCT averaged 21.64 min using multi-breed data. In the paper of Costa et al.  
254 (2019b), RCT and  $k_{20}$  of Holstein cows averaged 23.25 and 6.07 min, respectively. In that case, CV  
255 of MCP were smaller than those observed in the present study, which may likely be due to the  
256 larger sample size (>120,000 records) and to the presence of just a single breed. On the other hand,  
257  $a_{30}$  differed from previous results obtained by Costa et al. (2019a), De Marchi et al (2007) and  
258 Niero et al. (2021) using traits predicted via MIRS, but were instead highly similar to results

259 reported by Frizzarin et al. (2021) and Zendri et al. (2017) for traits measured with the reference  
260 instrument. Using a database of samples from Holstein, Brown Swiss, Simmental, Rendena and  
261 Alpine Grey breeds reared in 15 farms located in Italian mountain areas, Zendri et al. (2017)  
262 reported an average  $a_{30}$  of 33.6 mm. Overall, slight discrepancies between the present study and  
263 literature are attributable to the limited amount of data (100 samples) and to the relative  
264 contribution of each breed to the database. Milk  $a_{2r}$  has often been measured with the Formoptic, a  
265 device that provides the measurement in Firmness Index (FI); El Jabri et al. (2019) found values  
266 ranging from 13.35 to 29.04 FI, with a mean of 22.86 FI (CV = 11.74%). In the paper of Sanchez et  
267 al. (2019), the  $a_{2r}$  was predicted through an equation developed using Formoptic measurements as  
268 reference data ( $R^2_v = 0.69$ ) and obtained an  $a_{2r}$  average of 18.9 FI with a SD of 1.80.

269 The MCP of pasteurized milk were less favorable compared to those of raw milk (Table 1; Fig.  
270 2). However, for some samples it was not possible to determine all MCP within the time frame (60  
271 min) of the analysis. The deterioration observed could therefore somehow have been  
272 underestimated. In particular, some samples did not reach a curd firmness of 30 mm within the 60  
273 min resulting in missing data points for the  $a_{30}$ . On average, the absolute difference between raw and  
274 pasteurized samples was 7.19 min and 6.54 min for RCT and  $k_{20}$ , respectively. Moreover,  
275 pasteurization had an adverse effect on firmness-related traits, with a decrease of 13.02 mm and  
276 10.31 mm for  $a_{30}$  and  $a_{2r}$ , respectively. Casiraghi et al. (1989) reported longer RCT and slower  
277 coagulation rate for pasteurized milk and retentates derived from ultrafiltration, compared to their  
278 raw counterpart. Most of the studies that demonstrated a deterioration of MCP after heat treatment  
279 were conducted using higher temperatures and/or longer durations compared to conventional and  
280 commercial pasteurization treatments used in the field. Consequently, their results are not suitable  
281 for a direct comparison with findings reported in the present study (Ustunol & Brown, 1985;  
282 Anema et al., 2007). Blecker et al. (2012) demonstrated that milk heated to 60°C for 20 min had  
283 longer gelation time (0.7 min and 1.6 min at 30 and 40°C gelation temperature, respectively)

284 compared to raw milk. Moreover, compared to raw milk, the maximum firming rate of heated milk  
285 was 13 and 47% lower at 30°C and 40°C gelation temperature, respectively (Blecker et al., 2012).

286

### 287 3.2. Raw milk variables selected

288 The most informative variables selected by the algorithm to explain the pasteurized milk MCP  
289 are reported in Table 2, Table 3, Table 4 and Table 5. For all the MCP, the specific trait itself  
290 determined in raw milk was selected by the algorithm as being greatly relevant for determining its  
291 corresponding value in the pasteurized samples (Fig. 2).

292

#### 293 3.2.1 RCT

294 The RCT of pasteurized milk was primarily influenced by the RCT measured in raw milk. The  
295 proposed regression model resulted in a Lin's concordance correlation coefficient (CCC) of 0.87  
296 (Lin, 1989) with an  $R^2$  of 0.88. The Lin's CCC provides information about the concordance  
297 between a predicted and a reference (gold standard) trait. In order of importance, the other  
298 influencing variables were lactose content, protein content, pH and  $\kappa$ -CN, with the latter being  
299 selected in only 17.8% of the resampling iterations (Table 2). In fact, the effect of  $\kappa$ -CN was not  
300 significant in the subsequent fitted regression ( $P = 0.188$ ). This confirmed findings of previous  
301 publications by Marziali & Ng-Kwai-Hang (1986) and Politis & Ng-Kwai-Hang (1988) who  
302 demonstrated that  $\kappa$ -CN concentration does not have a significant effect on RCT in raw milk.  
303 Nevertheless, literature has shown that that  $\kappa$ -CN and  $\beta$ -LG interact during heating, causing a  
304 reduction in the ability of the milk to coagulate. The presence of  $\kappa$ -CN in just 17.8% of the variable  
305 selection iterations together with the not significant P-value found in regression ( $P=0.188$ ; Table 2)  
306 may suggest that future studies would benefit from a larger sample size to increase statistical power  
307 of the study. This would allow a better understanding of the behavior of  $\kappa$ -CN in pasteurized milk  
308 and thus disclose the relationship between  $\kappa$ -CN and RCT.



309 All the other selected variables showed a significant *P*-value in the multiple linear regression for  
310 RCT. The desired values are those in the negative direction, thus raw milk with higher protein  
311 content and lower lactose content had shorter/better RCT after pasteurization. This was also  
312 demonstrated in Guinee et al. (1996) and is important for cheese producers who rely on pasteurized  
313 milk. In fact, findings indicate that proper standardization adjustments of protein content at tank  
314 level (raw milk) could compensate the inevitably longer RCT. By studying the effect of processing  
315 procedures like heat treatment and mechanical stress on MCP, Casiraghi et al. (1989) observed that  
316 pasteurization was the main milk treatment responsible for longer RCT. The lactose content shows  
317 low variability in bovine milk, especially in standardized conditions, like bulk tank in industrial  
318 dairy plants. Nevertheless, in this study the raw milk lactose content was one of the main  
319 determinants of the RCT of pasteurized milk, with greater concentrations being related to  
320 longer/worse RCT. In cows, both greater lactose content and better MCP were observed in milk  
321 secreted by healthy mammary glands (Costa et al., 2019a, 2019b) which is in contrast with results  
322 observed in this study. Although the mechanisms that make lactose relevant in RCT after heating  
323 deserve a more thorough investigation, two potential explanations may be considered: i) the  
324 outcome is an artefact due to the small sample size and the low variability of lactose content, ii)  
325 during heat treatment, the isomerization of lactose -coupled with its interaction with certain milk  
326 components- could make the starting raw milk lactose concentration particularly relevant for RCT  
327 of heat-treated milk. The latter seems a reliable hypothesis, as specific lactose-derived compounds  
328 such as lactulose and furosine are detectable exclusively in heat-treated milk. Lactulose is the  
329 product of lactose isomerization, while furosine represents the first stable product of the Maillard  
330 reaction (Mendoza et al., 2005; van den Oever and Mayer, 2021). Lactulose, for example, can be  
331 used as an indicator of the level of heat treatment to which the milk was subjected (Olano et al.,  
332 1989). In addition, the concentration of both lactose and minerals in the starting raw milk directly  
333 determines the amount of lactulose produced during the thermal treatment (Olano et al., 1989; van  
334 den Oever and Mayer, 2021). Based on Fox et al. (2015), treatments at temperatures greater than

100°C result in lactose degradation along with the liberation of its organic compounds, followed by a decrease in pH. Further investigations may reveal the exact dynamics that link raw milk lactose and pasteurized milk RCT.

Finally, the pH of the raw milk had an effect on the RCT after pasteurization (Table 2). In particular, more acidic raw milk samples were those presenting a shorter RCT after heat treatment. A greater milk acidity is generally in favor of MCP due to the concentration of desirable salts, like  $\text{Ca}^{2+}$  (Fox et al., 2015) and the intrinsic ability of low pH to increase the heat stability of milk (Miller & Sommer, 1940; Rose, 1962). This is in agreement with Ménard et al. (2005) who demonstrated that raw milk pH is one of the factors responsible for differences in RCT before and after pasteurization of reconstituted milk. In particular, the difference in RCT between raw and pasteurized milk was smaller if the starting milk pH was lower, i.e. more acidic. Having a lower pH at pasteurization stabilizes the  $\kappa$ -CN interaction with casein micelles, reducing migration of  $\kappa$ -CN to the whey phase (Ménard et al., 2005).

### 3.2.2 Other MCP

According to Table 3, the  $a_{30}$  of pasteurized milk can be predicted from various raw milk traits, namely  $a_{30}$  (squared),  $\kappa$ -CN,  $\alpha$ -CN s1,  $\beta$ -CN, whey proteins, lactose, SCS and pH. The model of  $a_{30}$  was characterized by a Lin's CCC of 0.84 and an  $R^2$  of 0.72. Although the most important variables in terms of inclusion rate were  $a_{30}$  (squared) and whey proteins, the multiple linear regression revealed that only the former was a significant covariate factor for the targeted trait. Similarly, only a few variables among the total selected showed a significant effect on the  $k_{20}$  in regression (Table 4). In order of inclusion rate, the  $k_{20}$ -related variables were protein content,  $\alpha$ -CN s2,  $\alpha$ -LA and  $\beta$ -LG (Lin's CCC = 0.73,  $R^2$  = 0.58). Similarly, raw milk  $\beta$ -LG had a significant and negative effect also on the  $a_{2r}$  (Table 5) and the other significant covariate found for this MCP was the squared raw milk  $a_{2r}$  (Lin's CCC = 0.83,  $R^2$  = 0.70). The undesired effect of raw milk  $\beta$ -LG on the coagulation ability of pasteurized milk has previously been discussed by Kannan & Jennes (1961) and current

361 results support the hypothesis that  $\beta$ -LG exerts its detrimental effect by binding to para- $\kappa$ -CN  
362 cysteine residues, reducing casein micelles aggregation rate (Creamer et al., 2004). Overall, whey  
363 proteins measured in raw milk were important for all the MCP, suggesting that high or low  
364 concentrations determine a difference in the coagulation ability. As an example, elevated  
365 concentrations of  $\alpha$ -LA in the raw matrix were associated with a more desirable  $a_{30}$  (Table 3) and  
366  $k_{20}$  (Table 4) after pasteurization. As regards the  $\beta$ -LG, a lower concentration in the raw sample  
367 resulted in a better  $a_{2r}$  (Table 5) with an inclusion rate of 91.4%; concurrently,  $\beta$ -LG was also  
368 selected as an explaining variable for  $a_{30}$  (Table 3) and  $k_{20}$  (Table 4) in the negative direction.

369 In general, for all MCP, the selected variables highlight the importance of supplying dairy  
370 factories with raw milk of good technological aptitude.

371

### 372 3.3.MIRS prediction

373 Table 6 shows the prediction performance of MIRS for MCP using spectra collected on the raw  
374 matrix, and the scatter plots of reference and the predicted values for both raw and pasteurized milk  
375 are reported in Fig. 3. Based on the outcomes, the coagulation ability of pasteurized milk can be  
376 predicted with an accuracy sufficient for screening purposes (Grelet et al., 2020). The only  
377 exception is given by  $k_{20}$ , which was not predictable using the raw milk spectral data, having an  
378  $R^2_{CV}$  of 0.26. In both milks, RCT was the trait with the best  $R^2_{CV}$ , equaling to 0.64 and 0.66 prior  
379 and post pasteurization, respectively. Overall, the MIRS predictive ability for pasteurized milk  
380 MCP mirrors the predictive ability of the raw milk (Table 6). On a routine basis, MCP prediction  
381 equations are used for raw milk in Italy and their accuracy only allows for a rough screening  
382 (Visentin et al., 2016). In Visentin et al. (2016), lower accuracies were achieved:  $R^2_{CV}$  of 0.55, 0.56  
383 and 0.59 for  $k_{20}$ ,  $a_{30}$  and RCT, respectively. On the other hand, by using lactodynamography as the  
384 gold standard, De Marchi et al. (2013) reported greater  $R^2_{CV}$  compared to the present study: 0.72  
385 ( $k_{20}$ ), 0.70 ( $a_{30}$ ) and 0.76 (RCT).

386        Given the accuracies presented in Table 6, predicted and reference values were expected to show  
387        a diverging distribution. As reported in previous research (e.g., Costa et al., 2021), an MIRS-  
388        predicted trait can be scarcely correlated with its reference at the phenotypic level. This is  
389        particularly true for difficult-to-measure phenotypes like MCP, whose prediction accuracy tends to  
390        be moderate to low.

391

#### 392    **4. Conclusion**

393        Regression coupled with prior variable selection allowed for the identification of raw milk traits  
394        responsible for the reduction in pasteurized milk coagulation ability. Findings revealed that raw  
395        milk technological properties, pH, total protein content and detailed protein fractions were  
396        important factors to consider when assessing the detrimental effect of heating on MCP. The raw  
397        milk delivered to the dairy factory by farmers must be of high quality to preserve the technological  
398        ability and maintain favorable MCP after pasteurization. Out of the four MCP traits, only RCT had  
399        reliable prediction accuracy (coefficient of determination in cross-validation = 0.66) and can thus be  
400        predicted in advance using the raw milk spectra. This study provides new insights into the  
401        deterioration of cheese-making properties of milk following heat treatment. Such novel insights are  
402        of great interest for dairy plants manufacturing cheese from pasteurized milk as having knowledge  
403        of the MCP of pasteurized milk in advance is useful for defining proper strategies and to support  
404        decision-making about the incoming raw milk. In perspective, efforts should be made to develop  
405        more robust prediction models using bulk milk data ,considering both HTST and LTLT  
406        pasteurization protocols available.

407

#### 408    **Acknowledgement**

409        This research was supported by project ‘Innovamilk’ (Innovations in Italian dairy industry for the  
410        enhancement of farm sustainability, milk technological traits and cheese quality) funded by AGER  
411        – Agroalimentare e Ricerca, Grant n. 2017-1153. The Breeders Association of Veneto region

412 (ARAV, Vicenza, Italy) is gratefully acknowledged for the technical support. Authors thank Dr.  
413 Selina Sterup Moore (University of Padua) for proof-reading the manuscript.

414

#### 415 **Declaration of Competing Interest**

416 The authors declare that they have no known competing financial interests or personal relationships  
417 that could have influenced the work reported in this study.

418

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**Table 1**Descriptive statistics<sup>a</sup> of milk composition traits, protein profile, and coagulation properties.

Trait <sup>b</sup>	N	Mean	SD	CV, %	Minimum	Maximum
Raw milk						
Gross composition						
Fat (%)	100	4.65	1.51	32.52	2.24	11.23
Protein (%)	100	3.63	0.44	12.26	2.78	5.08
Lactose (%)	100	4.73	0.21	4.45	4.03	5.23
SCS	100	2.38	1.93	81.11	-1.32	8.93
pH	100	6.57	0.08	1.26	6.24	6.79
Protein fractions (mg/mL)						
$\alpha$ -CN <sub>s2</sub>	100	5.76	1.41	24.48	3.25	11.37
$\alpha$ -CN <sub>s1</sub>	100	11.82	1.98	16.78	8.30	19.04
$\beta$ -CN	100	13.25	2.80	21.10	1.61	23.25
$\kappa$ -CN	100	6.25	1.33	21.24	3.19	10.61
$\alpha$ -LA	100	1.71	0.26	15.32	1.23	2.50
$\beta$ -LG	100	5.01	1.09	21.67	2.00	7.62
Protein fractions (g/100g)						
$\alpha$ -CN <sub>s2</sub>	100	13.18	2.72	20.60	7.49	30.31
$\alpha$ -CN <sub>s1</sub>	100	27.00	1.65	6.13	23.67	35.83
$\beta$ -CN	100	30.11	3.21	10.65	4.28	35.03
$\kappa$ -CN	100	14.25	1.85	12.96	7.41	18.29
$\alpha$ -LA	100	3.95	0.62	15.76	2.62	5.72
$\beta$ -LG	100	11.55	2.31	19.97	4.47	16.98
Coagulation properties						
RCT (min)	94	20.46	8.53	41.68	6.75	50.63
k <sub>20</sub> (min)	87	6.41	3.88	60.52	1.75	21.50
a <sub>30</sub> (mm)	83	30.86	13.22	42.83	1.00	51.90
a <sub>2r</sub> (mm)	78	37.65	7.32	19.43	17.64	51.00
Pasteurized milk						
Coagulation properties						
RCT (min)	92	27.65	12.38	44.75	8.50	59.00
k <sub>20</sub> (min)	65	12.95	7.83	60.47	2.13	36.00
a <sub>30</sub> (mm)	62	17.84	11.31	63.38	1.00	47.00
a <sub>2r</sub> (mm)	61	27.34	8.30	30.37	12.70	43.10

<sup>a</sup> N: number of samples; SD: standard deviation; CV: coefficient of variation. <sup>b</sup> SCS: somatic cell score,  $\alpha$ -CN<sub>s1</sub>:  $\alpha$ -casein s1;  $\alpha$ -CN<sub>s2</sub>:  $\alpha$ -casein s2;  $\beta$ -CN:  $\beta$ -casein;  $\kappa$ -CN:  $\kappa$ -casein;  $\alpha$ -LA:  $\alpha$ -lactalbumin;  $\beta$ -LG:  $\beta$ -lactoglobulin; RCT: rennet coagulation time; k<sub>20</sub>: curd-firming rate; a<sub>30</sub>: curd firmness; a<sub>2r</sub>: curd firmness at two times the rennet coagulation time.

**Table 2**

Variables selected by the stepwise algorithm explaining variability of rennet coagulation time of pasteurized milk and multiple linear regression output.

Trait <sup>a</sup>	Stepwise variable selection						r <sup>c</sup>	Multiple linear regression		
	Estimate <sup>b</sup>					Effect inclusion rate (%)		Estimate	SE <sup>d</sup>	P-value
	Average	SD	q1	median	q3					
Intercept	143.80	21.36	131.89	144.01	156.49	100.0		143.69	39.75	<0.001
Raw milk										
RCT (min)	1.38	0.04	1.36	1.38	1.41	100.0	0.91*	1.39	0.06	<0.001
κ-CN (g/100g)	-34.18	12.70	-42.28	-33.69	-25.43	17.8	0.02	-34.31	25.86	0.188
Protein (%)	-4.06	0.50	-4.36	-4.06	-3.74	99.9	-0.06	-4.07	1.09	<0.001
Lactose (%)	10.58	1.64	9.52	10.53	11.68	100.0	-0.03	10.54	2.66	<0.001
pH	-26.58	3.64	-28.95	-26.68	-24.41	99.9	-0.22*	-26.52	6.32	<0.001

<sup>a</sup> RCT: rennet coagulation time; κ-CN: κ-casein. <sup>b</sup> SD: standard deviation; q1: first quartile; q3: third quartile. <sup>c</sup> r: Pearson correlation (\* $P < 0.05$ ) with pasteurized milk RCT. <sup>d</sup> SE: standard error of the estimate.

**Table 3**

Variables selected by stepwise algorithm explaining the variability of curd firmness of pasteurized milk and multiple linear regression output.

Trait <sup>a</sup>	Stepwise variable selection						r <sup>c</sup>	Multiple linear regression		
	Estimate <sup>b</sup>					Effect inclusion rate (%)		Estimate	SE <sup>d</sup>	P-value
	Average	SD	q1	median	q3					
Intercept	-115.72	49.06	-147.43	-115.17	-85.81	100.0		-114.33	89.73	0.208
Raw milk										
a <sub>30</sub> * a <sub>30</sub> (mm <sup>2</sup> )	0.01	0.00	0.01	0.01	0.01	99.5	0.80*	0.01	0.00	<0.001
κ-CN (g/100g)	60.21	33.33	39.83	59.98	79.90	25.4	-0.09	58.65	54.94	0.291
α-CN <sub>s1</sub> (g/100g)	44.53	37.81	19.14	42.16	69.05	21.1	0.19	43.89	71.75	0.543
β-CN (g/100g)	-39.43	29.36	-57.73	-38.74	-20.24	12.9	0.09	-38.93	61.09	0.527
α-LA (g/100g)	282.24	131.36	210.44	284.40	363.79	50.5	-0.02	280.77	181.19	0.127
β-LG (g/100g)	-85.60	36.70	-108.84	-84.16	-62.13	47.8	-0.22	-84.41	58.21	0.153
Lactose (%)	-7.95	3.11	-9.92	-7.79	-5.84	11.4	0.02	-8.01	5.85	0.177
SCS	-0.37	0.29	-0.52	-0.33	-0.17	16.4	-0.09	-0.38	0.47	0.426
pH	21.58	6.91	16.72	21.66	26.41	30.6	0.08	21.45	14.43	0.143

<sup>a</sup> a<sub>30</sub>: curd firmness; α-CN<sub>s1</sub>: α-casein s1; β-CN: β-casein; α-LA: α-lactalbumin; β-LG: β-lactoglobulin; SCS: somatic cell score. <sup>b</sup> SD: standard deviation; q1: first quartile; q3: third quartile. <sup>c</sup> r: Pearson correlation (\*P<0.05) with pasteurized milk a<sub>30</sub>; SE: standard error of the estimate.



**Table 4**

Variables selected by stepwise algorithm explaining the variability of curd firming time of pasteurized milk and multiple linear regression output.

Trait <sup>a</sup>	Stepwise variable selection						r <sup>c</sup>	Multiple linear regression		
	Estimate <sup>b</sup>					Effect inclusion rate (%)		Estimate	SE <sup>d</sup>	P-value
Average	SD	q1	median	q3						
Intercept	13.55	11.16	7.12	14.83	20.66	100.0		14.90	14.25	0.300
Raw milk										
k <sub>20</sub> (min)	2.50	1.06	1.88	2.47	3.14	60.8	0.63*	2.37	1.92	0.221
k <sub>20</sub> *k <sub>20</sub> (min <sup>2</sup> )	-0.03	0.09	-0.07	-0.03	0.03	39.3	0.63*	-0.02	0.17	0.906
α-CN <sub>s2</sub> (g/100g)	101.18	23.33	87.07	101.74	117.06	77.5	0.13	99.20	39.66	0.015
α-LA (g/100g)	-337.48	81.24	-391.87	-341.73	-282.12	73.0	0.05	-341.68	151.27	0.028
β-LG (g/100g)	80.69	19.63	68.55	80.66	93.25	63.6	0.14	81.39	34.34	0.021
Fat (%)	0.47	0.40	0.19	0.39	0.74	17.3	0.12	0.42	0.54	0.435
Protein (%)	-6.49	1.49	-7.52	-6.85	-5.78	93.7	-0.54*	-6.60	1.86	<0.001

<sup>a</sup>k<sub>20</sub>: curd firming rate; α-CN<sub>s2</sub>: α-casein s2; α-LA: α-lactalbumin; β-LG: β-lactoglobulin. <sup>b</sup>SD: standard deviation; q1: first quartile; q3: third quartile. <sup>c</sup>r: Pearson correlation (\*P<0.05) with pasteurized milk k<sub>20</sub>. <sup>d</sup>SE: standard error of the estimate.

**Table 5**

Variables selected by stepwise algorithm explaining the variability of curd firmness at two times the rennet coagulation time of pasteurized milk and multiple linear regression output.

Trait <sup>a</sup>	Stepwise variable selection						r <sup>c</sup>	Multiple linear regression		
	Estimate <sup>b</sup>					Effect inclusion rate (%)		Estimate	SE <sup>d</sup>	P-value
	Average	SD	q1	median	q3					
Intercept	27.76	3.91	25.14	27.31	30.24	100.0		27.45	7.38	<0.001
Raw milk										
a2r <sup>*</sup> a2r (mm <sup>2</sup> )	0.01	0.00	0.01	0.01	0.01	95.5	0.81*	0.01	<0.01	<0.001
α-CN <sub>s2</sub> (g/100g)	-41.27	24.05	-55.56	-40.06	-24.89	14.8	-0.11	-40.39	36.65	0.275
β-LG (g/100g)	-87.48	15.18	-97.07	-87.33	-77.38	91.4	-0.21	-87.37	32.49	0.001
Fat (%)	-0.68	0.27	-0.85	-0.66	-0.50	34.4	-0.20	-0.65	0.47	0.168

<sup>a</sup> a2r: curd firmness at two times the rennet coagulation time;  $\alpha$ -CN<sub>s2</sub>:  $\alpha$ -casein s2;  $\beta$ -LG:  $\beta$ -lactoglobulin. <sup>b</sup> SD: standard deviation; q1: first quartile; q3: third quartile. <sup>c</sup> r: Pearson correlation (\* $P$ <0.05) with pasteurized milk a2r. <sup>d</sup> SE: standard error of the estimate.

**Table 6**

Spectral treatments and fitting statistics<sup>a</sup> of the Fourier-Transformed mid-infrared spectroscopy calibrations developed for the four coagulation properties.

Trait <sup>b</sup>	Correction	Math treatment <sup>c</sup>	N	SE <sub>C</sub>	R <sup>2</sup> <sub>C</sub>	SE <sub>CV</sub>	R <sup>2</sup> <sub>CV</sub>
Raw milk							
RCT (min)	SNV	2,5,5,1	83	3.43	0.79	4.53	0.64
k20 (min)	None	2,5,5,1	69	1.20	0.73	1.55	0.54
a30 (mm)	MSC	2,5,5,1	76	6.85	0.74	9.02	0.54
a2r (mm)	D	0,0,1,1	66	3.39	0.76	3.74	0.70
Pasteurized milk							
RCT (min)	None	2,5,5,1	79	5.41	0.79	6.89	0.66
k20 (min)	D	1,8,8,1	52	4.02	0.51	4.89	0.26
a30 (mm)	D	1,4,4,1	49	6.09	0.69	8.04	0.45
a2r (mm)	SNV	1,4,4,1	56	3.64	0.80	4.80	0.65

<sup>a</sup> SNV: standard normal variate; MSC: multiplicative scatter correction; D: detrending; N: number of samples; SE<sub>C</sub>: standard error in calibration; R<sup>2</sup><sub>C</sub>: coefficient of determination in calibration; SE<sub>CV</sub>: standard error in cross-validation; R<sup>2</sup><sub>CV</sub>: coefficient of determination in cross-validation. <sup>b</sup> RCT: rennet coagulation time, k20: curd firming rate, a30: curd firmness, a2r: curd firmness at two times the rennet coagulation time. <sup>c</sup> Digits indicate number of the derivative, gap used for derivative calculation, data points in the first smoothing, and data points in the second smoothing, respectively.

13 **Figures captions**

14 **Fig. 1.** Representation of the plate used for lactodynamographic analysis of raw (R) and pasteurized  
15 (P) milk samples (n = 5 at each run).

16

17 **Fig. 2.** Diagrams obtained from the lactodynamographic analysis of three bovine milk samples A)  
18 after and B) before pasteurization.

19

20 **Fig. 3.** Scatter plot of measured and predicted A) rennet coagulation time (RCT), B) curd firming  
21 time ( $k_{20}$ ), C) curd firmness ( $a_{30}$ ), and D) curd firmness at 2 times RCT ( $a_{2r}$ ) in raw (●) and  
22 pasteurized (■) milk.