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Colloidal stability of milk: reinterpretation of alcohol test results by digital microscopy

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Abstract

In this research communication we propose a new approach by portable digital microscopy with a 200× objective to improve the visualization of microparticles of pasteurized milk submitted to the alcohol test. Not only did the method reduce the subjectivity of the readings, but also generated high resolution images of the microparticles, which allows the creation of a specific image pattern for each type of final product. In comparison to a control pasteurized milk treatment, the results confirmed the effect and the specificity of added salts (sodium citrate, disodium phosphate or their combination) on the stability of the milk to the alcohol test. Finally, the mixture of stabilizing salts of citrate/phosphate provided the highest degree of stability to pasteurized milk among the treatments studied.

Milk consists of a suspension of micelles of casein in an aqueous phase combined with an emulsion of fat globules, in addition to lactose molecules, whey proteins and minerals distributed in different phases. Besides, there are also other constituents such as vitamins and ions in lesser quantity (Fox and Mcsweeney, 1998) and which may suffer variations in their composition due to several factors such as breed, diet and lactation stage (Walstra *et al*., 2006). The protein nitrogenous compounds in milk represent 95% of all nitrogen-based compounds and of this value 80% are divided among the different forms of casein, with casein stability at high temperatures being the technological basis for the production of several derivatives (Fox and Mcsweeney, 1998; Walstra *et al*., 2006). Casein is in the form of a micelle with a quaternary conformation (Walstra *et al*., 2006).

The thermal treatment of milk is justified by microbiological aspects, however it generates changes in the structure of the milk constituents, with effects on processing characteristics (Ferron *et al*., 1991; da Costa, 2016; Francisquini *et al*., 2018; Francisquini *et al*., 2019; Nunes *et al*., 2019). Heating causes five groups of changes in milk: acid production, calcium phosphate precipitation, changes in casein, Maillard reaction and interaction of sulfhydryl groups (Fox, 1981). The thermal stability of milk (TSM) can be defined as the temperature at which the milk must be heated to promote precipitation in up to two minutes (Miller and Sommer, 1940). Alternatively, Horne and Muir (1990) define that TSM is based on the time required for coagulation when subjected to 140°C. TSM can vary in different ways according to the lactation period (Rose, 1962).

There is a relationship between TSM and stability to alcohol. Several authors estimate that the alcohol test is a good estimate for the verification of TSM (Akkerman *et al*., 2021). Thus, the alcohol test is used to predict TSM in industries and by the Ministry of Agriculture, Livestock and Supply in Brazil. The stability of milk to alcohol can be associated with the minimum concentration of ethanol capable of coagulating the milk when added in the same amount of milk, this coagulation occurs by reducing the dielectric constant of the solution generating a collapse of K-casein. One way to increase TSM in the face of alcohol testing is the addition of stabilizing salts based on phosphates and citrates, as both are calcium chelating agents (Horne and Parker, 1981). Since instability can occur due to the Ca/P ratio (calcium phosphorus), the lower this ratio, the more stable the milk will be in relation to alcohol.

With the lack of precision in the Petri dish technique, which can present a false positive for milk destined for the UHT process, the industry may suffer losses such as discarding a milk that would have suitable conditions for processing or allowing an unsuitable milk that then cloggs the equipment's piping. Another point is that the conventional technique lacks the history of the analyzes performed for previous batches. Hence, the main objective of this work is to compare the current technique with digital microscopy that guarantees a better accuracy for

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the alcohol test, avoiding errors in the measurements, in addition to generating a record of all readings performed in the form of an image, guaranteeing the industry greater reliability and security for UHT processing.

Material and methods

Samples used

For all experiments we used pasteurized standardized whole milk of 3.2% (m v^{-1}) fat, which was supplied by a milk company from Juiz de Fora, Minas Gerais state, Brazil, comprising three groups of samples obtained in different periods of time.

Treatments

From whole pasteurized milk, four treatments were prepared with different mineral environments: milk without added salts (control); pasteurized milk with 0.05% sodium citrate (m v^{-1}); pasteurized milk with the addition of 0.05% disodium phosphate (m v^{-1}); pasteurized milk with the addition of a mixture of citrate and phosphate salts (manufacturer's code LAC8074-7) 0.05% (m v^{-1}), each employing 0.125 g of the salt in 250 ml milk, according to online Supplementary Figure 1S. Weighing employed an analytical balance and salts were add to 250 ml glass pots with mixing at 5°C. All the pots were shaken intermittently for one hour and then shaken with each new intervention. All experiments were done in triplicate.

Alcohol test

Each of these treatments was subjected to the alcohol test. The concentration of ethanol ranged from 72% (v v^{-1}) to 96% (v v^{-1}) with intervals of 2% (v v^{-1}). The concentrations were initially adjusted with a Gay–Lussac alcoholometer; subsequently, the alcoholic grades were checked and readjusted with the aid of a digital densimeter 'Densito 30P' (Mettler Toledo, São Bernardo do Campo 09851-900, Brazil) and for pH 6.8–7.0 with the aid of a Gehaka potentiometer, model PG 1800 (Gehaka, São Paulo 05686-900, Brazil). The alcohol tests were performed, in Petri dishes, with the addition of 2 ml of alcohol over 2 ml of milk (5 ± 2°C) for each alcoholic variation test. The destabilization reaction to alcohol was observed visually. All experiments were done in triplicate.

Observation in digital microscope

After visual observation, a 10 μl portion of the milk/alcohol mixture was transferred to a microscope slide and covered by a coverslip, then examined in the ProScope HR digital microscope (high-resolution hand-held microscope: Bodelin Technologies, Oregon City, OR 97045, USA) with 200× magnification. This equipment consists of a portable, digital optical microscope, with optional kits with up to six interchangeable lenses (10×, 30×, 50×, 100×, 200×, 400×), connected by USB or Wi-Fi cable to a computer or tablet, capable of exploring and capture images and edit short movies. The portable digital microscope reading was obtained directly on a computer screen, using image transfer software 'ProScope HR software'. Each analysis generated a set of 10 to 12 images and 1–2 15-s films totaling 608 images and 60 films. For capturing images and films, the following specifications of the ProScope image program were adopted: Resolution 640 × 480, Frame Rate 15, illumination in the 'Daylight' position, brightness 0, contrast 0, hue 0, saturation 40, sharpness 1, white ratio 4. All experiments were done in triplicate.

Results and discussion

The composition of pasteurized milk used in this study is shown in online Supplementary Table 1S. The addition of calcium-chelating salts has the stabilizing function through the physical modification of milk, forming stable complexes through ionic bonds (Fox and Mcsweeney, 1998). The stabilizing salts increase thermal stability, thus improving the processability conditions of milk (Chavez *et al*., 2004). It can be seen that the addition of salts changed the stability curve of milk due to the reduction of soluble calcium (Horne and Parker, 1981).

The choice of types of salts and their application levels used in the assembly of the four treatments proved to be correct, since the main objective was the comparison of different methods of visualizing the structures formed in the alcohol test. Had the differences been very small and difficult to perceive, other treatments would have been performed. The control treatment (no addition) served as a basis for comparison to assess the increments in the stabilization of the alcohol test of the other treatments. Increasing stability was found in the following order: 0.05% sodium citrate, followed by 0.05% disodium phosphate and finally the combination (0.05% LAC 8074-7).

The phosphate added to milk reduces the soluble phosphate and changes the pH pattern to alkaline values, increasing the time of thermal clotting. In cases where the pH is below the stability range (6.7–6.9), sodium citrate or disodium phosphate are indicated for increased stability (Walstra *et al*., 2006). In the present study, there was a difference in the specificity between citrate and disodium phosphate, however, it was in the mix of stabilizing salts that the synergistic effect between the salts was observed, potentiating the stabilizing effect in relation to the alcohol test. Although the sequestering function is common to the salts used, the alkalinizing property of the phosphates slightly increasing the pH (to 6.94) promoted a better condition of stability to the casein micelles.

There were differences in the behavior of each treatment. Pasteurized milk without the addition of salts showed visible coagulation in alcohol 82% (v v^{-1}), whereas with sodium citrate the visible coagulation occurred in alcohol 88% (v v^{-1}) and with disodium phosphate it was at alcohol 92% (v v^{-1}). Finally, the LAC 8074-7 mixture showed visible coagulation in 96% alcohol (v v^{-1}). Singh (2004) suggests that the alcohol test can be useful in understanding the factors that affect micellar stability and can be used as a method to select types and dosages of stabilizing salts.

Online Supplementary Figure 2S shows the milk microstructures before starting the alcohol test. A homogeneous background with a rough appearance is seen, typical as a reference of milk microstructures visualized by digital microscopy at a magnitude of 200×. Figure 1 shows, for the control treatment, the comparative results of the visual observation and digital microscopy. While direct optical visualization found coagulation to the alcohol test at graduation 82% (v v^{-1}), digital microscopy anticipated the visualization of the formation of the first clots to 76% alcoholic strength (v v^{-1}). In the digital microscopy images, there is an increase in the roughness of the background of the

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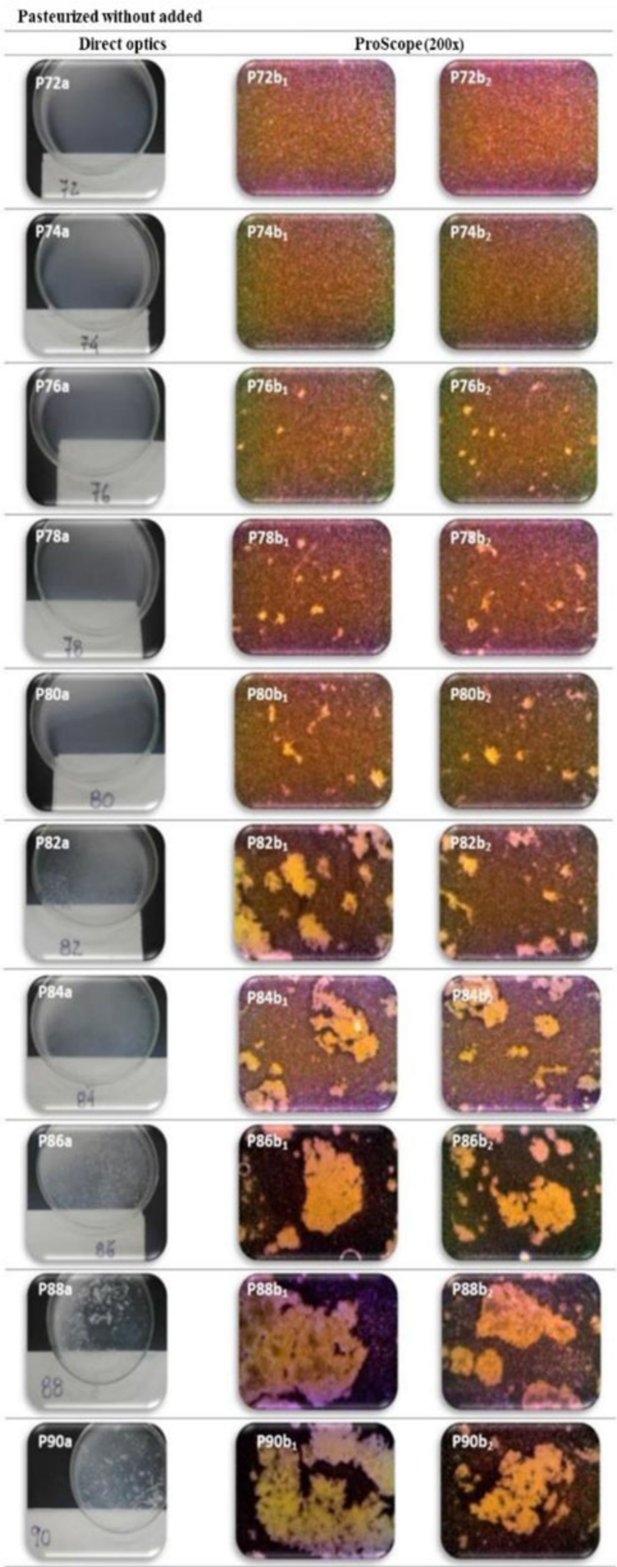

Fig. 1. Comparative images of the alcohol test visualization for pasteurized milk without addition of salts (P) in alcohol concentrations ranging from 72% (v v^{-1}) to 90% (v v^{-1}) (P72 through to P90). Direct optical visualization (a), and duplicate portable digital microscope 200× fields (b1 and b2).

images and the appearance of small yellowish white clusters that appear from 76% (v v^{-1}) alcohol and grow as the alcohol content increases.

The second treatment submitted to the alcohol test was pasteurized milk added with 0.05% sodium citrate (m v^{-1}). Online Supplementary Figure 3S shows the result of the two methods. While the direct optical visualization found the coagulation of the treatment with sodium citrate to the alcohol test at 88% (v v^{-1}), the digital microscopy anticipated the visualization of the formation of the first clots for the alcoholic graduation of 86% (v v^{-1}), thus demonstrating that the addition of this salt increased the thermal stability of the milk. The third treatment was pasteurized milk with 0.05% disodium phosphate (m v^{-1}). Online Supplementary Figure 4S below shows the result of the two qualitative methods. In direct optical visualization, the coagulation in treatment with disodium phosphate was found to be at 92% (v v^{-1}), whilst digital microscopy anticipated the visualization of the formation of the first clots at 88% (v v^{-1}). Again, an increase in the thermal stability of the milk is noticeable, which was greater than that of the treatment with added sodium citrate. Stability in this case is increased because the salt decreases the concentration of soluble phosphate and raises the pH of the milk. The last treatment submitted to the alcohol test was pasteurized milk with a mixture of LAC salts 8074-7 at 0.05% (m v^{-1}). Online Supplementary Figure 5S shows the results. While the direct optical visualization found coagulation with LAC 8074-7 salt mix to the alcohol test at 96% (v v^{-1}), digital microscopy anticipated the visualization of the formation of the first clots at 94 alcoholic graduation % (v v^{-1}).

We compared the results obtained using the two methodologies (Table 1). The control treatment showed structural changes at lower alcoholic concentrations (78% optical direct and 76% under the microscope) indicating a lower milk stability. With the addition of salts, the stability of the milk was increased.

Among the three products that underwent the addition of salts, the one with the lowest stability was the one that underwent addition of sodium citrate, where structural changes were perceived in 88% for optical direct and in 86% by the microscope. Then, the treatment with the addition of disodium phosphate showed instability at alcoholic concentrations of 92% for optical direct and of 88% for the microscope. The treatment added with LAC 8074-7 salt showed the highest stability in the presence of alcohol, alcoholic solutions were used at a concentration of 96% to observe modifications by direct optical and 94% by microscope. The addition of these salts can help in the production of UHT milk, as their addition increases the thermal stability of the products, thus avoiding loss before processing and also prevents equipment damage caused by line clogging. Finally, an important conclusion is that the use of the digital microscope showed a better efficiency when compared to the conventional alcohol test, a fact that can be proven by the reduction in the perception of coagulation in all treatments performed, sometimes reducing the perception by more than two alcoholic degrees.

In conclusion, the portable digital microscope is a useful equipment for the UHT milk industry in order to improve the visualization of the microparticles of pasteurized milk submitted to the alcohol test. The data made it possible to differentiate between the two methods evaluated as well as to visualize the effect of adding salts on the increased alcohol stability, with the highest stability achieved using a mixture of sodium citrate and disodium phosphate (LAC 8074-7). Not only did the digital method reduce the subjectivity of the readings, but it also generated high-resolution images of the microparticles, which will allow the creation of a specific image pattern for each type of final product.

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Table 1. Data recording of the alcohol test applied to the four treatments

Alcoholic concentration	Treatments evaluated in pasteurized milk							
	Control (no salt addition)		Addition of sodium citrate 0.05% (m v^{-1})		Addition of disodium phosphate 0.05% (m v^{-1})		Addition of LAC 8074-7 0.05% (m v^{-1})	
	Optical direct	PDM 200×	Optical direct	PDM 200×	Optical direct	PDM 200×	Optical direct	PDM 200×
Control	–	–	–	–	–	–	–	–
72	–	–	–	–	–	–	–	–
74	–	–	–	–	–	–	–	–
76	–	1	–	–	–	–	–	–
78	1–	1	–	–	–	–	–	–
80	1–	1	–	–	–	–	–	–
82	1	1 +	–	–	–	–	–	–
84	1 +	1 +	–	–	–	–	–	–
86	1 +	1 + +	–	1	–	–	–	–
88	1 + +	1 + + +	1	1 +	–	1	–	–
90	1 + + +	1 + + +	1 + +	1 + +	–	1	–	–
92	X	X	1 + + +	1 + + +	1	1 + +	–	–
94	X	X	X	X	1 +	1 + + +	–	1
96	X	X	X	X	X	X	1	1 +
99	X	X	X	X	X	X	1 + +	1 + +

Where: (X) analysis not performed, (–) performed without apparent destabilization, (1–) possible destabilization, (1) apparent coagulation and (1 + , 1 + +, 1 + + +) increasing and confirming coagulation visualizations.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029922000176

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