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Research Article
A Rapid Quality Control Method for the Detection of Adulteration of Milk by Neutralizing Agents

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Abstract

Background and Objective: The adulterated milk could be processed into fluid or other dairy products such as yoghurt and cheese. Milk is not cooled after the milking process or stored for a long period before processing so, the acidity increased and the milk became unfit for heat treatments. So, some raw milk suppliers may neutralize the milk acidity by neutralizing agents to be suitable for processing. So this study aimed to validate a suitable method for detecting the neutralizing substances in milk, yoghurt and soft cheese. **Materials and Methods:** The spectrophotometric method using $FeCl_3$ was used to determine the lactate in sour milk and neutralized milk as well as yoghurt and soft cheese made from neutralized milk. **Results:** Results confirm that the spectrophotometric method could be used for the determination of the lactate in milk even after neutralizing by ammonia, sodium hydroxide or tri-sodium phosphate as there was no significant difference between sour milk (pH 6.2) before neutralization or after neutralization in lactate levels. The yoghurt and soft cheese physiochemical properties did not seem to be impacted by milk neutralization. **Conclusion**: The spectrophotometric method is unable to detect the neutralizing substances in yoghurt made using neutralized milk.

Key words: Neutralizing substances, milk adulteration, spectrophotometric method, tri-sodium phosphate, hypertension, caustic soda, ion-exclusion

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Milk is the main source of proteins, fats, carbohydrates, vitamins and minerals that are all important for bodybuilding and health. It is consumed as a fluid product or converted into a variety of dairy products. For safety, milk flavour, shelf life and consumer satisfaction, milk must be quickly cooled and held at low temperature after milking until pasteurized or processed to dairy products. Keeping the milk without cooling becomes critical especially in warm weather. One of the important criteria of raw milk acceptance in processing plants is its titratable acidity and pH. Acidification in milk is mainly due to lactic acid bacteria which rapidly grow in absence of efficient cooling after milking[1,2]. The anion lactate, which is the final product of lactose fermentation by lactic acid bacteria, is an indicator of milk quality[3]. Therefore, high lactate values in milk indicate inefficient cooling and/or poor farm and supply chain practices[4]. Sometimes, milk producers use a small number of alkaline solutions such as sodium hydroxide, sodium carbonate and sodium phosphate to neutralize acidity in milk so that raw milk does not exceed the acceptable acidity limit[5,6].

The adding of neutralizer substances decrease milk quality and may lead to serious consumers' health issues[7]. Caustic soda (NaOH) contains sodium that acts as a slow poison for hypertension and heart patients[8,9]. It also prevents the body from using an essential amino acid in milk (Lysine), which is needed by growing infants[10]. Adding carbonates in milk causes gastrointestinal problems including gastric ulcer, diarrhoea, colon ulcer and electrolytes disturbance[11,12]. Carbonates and bicarbonates also cause interruptions in growth hormone signaling[13].

The amount of milk and dairy products consumed worldwide makes these products target for potential adulteration with financial benefits for producers[14]. For that reason, it is important to protect the consumer from adulterated milk and dairy products by accurate results using suitable methods for the detection of adulteration. The standard method of Titratable Acidity (TA) is expressed as % lactic acid[15], which is also known as Dornic acidity[16,17]. Also, the Association of Official Analytical Chemists (AOAC) recommend this method for acidity determination in milk[18]. Since milk acidity is due not only to lactic acid but also, the presence of caseins, albumins, CO_2, citrate and phosphates, the results inducted by the TA method always over-estimate acid development[18]. Furthermore, the illegal use of neutralizing substances makes TA a useless method for detecting acid developed after milking. Therefore, the application of lactate detection method that works in case of added neutralizing substances has become very important especially in developing countries.

There were several efficient methods to determine lactic acid as the final product of fermentation such as ion-pair, ion-exclusion and reversed-phase chromatography[19,20]. In addition, oxidation of lactic acid can be oxidized to acetic aldehyde that can be quantified. Also, it is possible to use Atomic absorption spectroscopy which proved to estimate the abnormal rise in minerals concentration due to application to neutralizers[21]. Recently, a spectrophotometric method for lactic acid determination has been developed for the detection of the end product of the reaction between lactate ions and $FeCl_3$[22].

The objective of the present study was to validate the spectrophotometric method of lactate determination for the detection of milk adulteration by neutralizing substances. In addition, the effect of the neutralizing substances on the physicochemical and rheological properties as well as the yield of curd produced from acid and rennet coagulation were also assessed.

MATERIALS AND METHODS

Study area: The study was carried out at the Laboratory of Dairy Microorganisms and Cheese Research, Department of Dairy Science and Technology, Faculty of Agriculture, Alexandria University, Egypt from March-October, 2019.

Validation of a spectrophotometric method to detect neutralizing substances

Milk samples preparation: Fresh full-fat cow's milk Table 1, obtained from the Farm of Faculty of Agriculture, Alexandria University was divided into three parts and received the following treatments: (a) control untreated milk (acidity of 0.17%, pH of 6.78), (b) milk was acidified with natural flora to pH 6.4 and (c) milk was acidified with natural flora to pH 6.2. Acidified milk was then divided into three parts and each part was neutralized by one of the following neutralizers: sodium hydroxide 10%, trisodium phosphate 10% or Ammonia 37% to ~ pH 6.7. Milk composition was determined by Miko Scan (FT2, FOSS, Denmark). The pH value of milk was measured using a laboratory pH meter (Milwaukee Mi 150). Titratable acidity was determined as % of lactic acid according to the AOAC[18].

Table 1: Analysis of raw milk

Parameters	Values
Total solids (%)	12.66
pH	6.78
Titratable acidity as lactic acid (%)	0.17

Standard curve of lactate: Serial dilutions of lactic acid were prepared from the stock solution (80 g L^{-1}) using two-fold dilutions according to Borshchevskaya *et al.*[22]. A total of 50 µL of each dilution containing lactic acid were added to 2 mL of a 0.2% $FeCl_3$ and stirred for 5 min and absorbance was then measured at 390 nm (UV/Visible spectrophotometer, Pg T80+, England), against the reference solution (2 mL of a 0.2% $FeCl_3$ solution). Three replicates were carried out.

Determination of lactate in milk samples by the spectrophotometric method: All milk samples (as treated above) were warmed to 40°C and coagulated by rennet in 30 min. After enzymatic coagulation of each milk sample, whey was separated by centrifugation (6000 rpm). In the case of lactate determination in soft cheese (enzymatic coagulation) and yoghurt (acid coagulation), whey after centrifugation was diluted 10 folds.

A total of 50 µL of each sample was added to 2 mL of a 0.2% $FeCl_3$[22]. The concentration of lactate in each sample was calculated using the standard curve.

Studying the effect of neutralizers on the physicochemical properties of acid and rennet curds

Milk preparation: Fresh full fat cow's milk was divided into two batches, batch 1 as control with normal pH (6.78) and batch 2 fermented with yoghurt starter (Lofast 259A, SACCO, Italy) to a pH value of 6.3. Milk at pH 6.3 (batch 2) was then divided into three parts. In part A, the pH was adjusted to 6.8 using Sodium hydroxide (10%), in part B, the pH was adjusted to 6.8 using Trisodium phosphate (10%) and in part C, the pH was adjusted to 6.8 using ammonia solution. Milk from the three parts was used in making yoghurt and soft cheese.

Yoghurt making: All milk samples (control and parts A, B, C) were heated at 90°C for 10 min. After heat treatment, the milk was cooled to the (42°C) followed by thermophilic starter culture (Lofast 259A, SACCO, Italy) addition. When the pH of yoghurt reached 4.7, samples were cooled to 4 ± 2°C[23].

Soft cheese making: Soft cheese was processed according to Awad *et al.*[24] Milk samples were pasteurized at 63°C for 30 min. Milk was transferred to cheese vats and a commercial starter culture (DVS Lofast 259A, SACCO, Italy) and $CaCl_2$ (0.012%) were added to milk at 40°C. The inoculated milk was held for 1 hand then NaCl was added at a level of 5% (w/w). A suitable amount of chymosin preparation (2% Chymax, Chr. Hansen, Hørsholm, Denmark) was added to coagulate the milk in 90 min. The crud was then transferred to moulds, which were lined with cheesecloth. After 2-3 hrs, a plate and weights were placed to compact the curd. The weights were removed after 4-6 hrs and the cheese mass was divided with a knife into blocks and placed in storage cans.

Physicochemical properties of dairy products (yoghurt and soft cheese): The pH of yoghurt and cheese was measured using a laboratory pH meter model (Milwaukee Mi 150). Moisture was analyzed by the oven method[18], total protein by macro-Kjeldahl[25] and fat content by the Gerber method[18]. While total solid, protein, fat, lactose and freezing point in whey were measured using Miko Scan (FT2, FOSS, Denmark).

Cheese yield calculation: The actual yield of cheese was determined as the quantity of cheese obtained from a given quantity of milk[26].

Measuring the syneresis in yoghurt: Syneresis of yoghurt samples was determined during storage periods using the centrifugation method as described by Saffon *et al.*[27].

Texture analysis: The textural characterization of yoghurt was evaluated by back extrusion (Stable Micro Systems Ltd., Godalming, UK) with a 35 mm disc[28]. The speed was 1 mm s^{-1}, the distance target was 30 mm and the trigger was 10 g. In cheese and curd, the speed was 1 mm s^{-1}, the distance target was 10 mm and the trigger was 5 g. The results were reported as the mean of three measurements.

Statistical analysis: Data reported are the average of 3 replicates and 3 measurements for each trial. The SAS software package[29] was used for one-way ANOVA. Differences were considered significant at $p\leq0.05$.

RESULTS AND DISCUSSION

Validation of the spectrophotometric method to detect neutralizing substances: The result of Fig. 1 shows the standard curve of the absorbance of iron-lactate solution (A) versus the concentration of lactic acid (c). The obtained statistical assurance is approximation 0.9989 as calculated from the standard curve.

Data in Table 2 show that the spectrophotometer method of Borshchevskaya *et al.*[22] using $FeCl_3$ was suitable to determine if milk was neutralized by determining the concentration of lactate. The lactate was significantly increased after fermentation of milk and dropping the pH from 6.78-6.2. The lactate concentration was not affected by

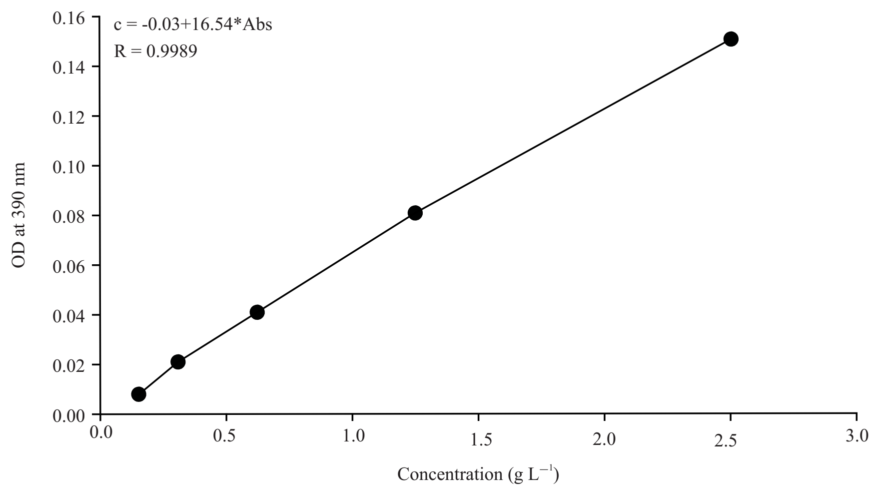

Fig. 1: Standard curve of lactate concentration

Table 2: Lactate concentration (g L^{-1}) in artificial adulterated milk with some neutralizer agents at different pH

Samples	pH value	Concentration of lactate (g L^{-1})
Control 1	6.78	1.2022 ± 0.01^{B}
Control 2	6.2	1.5744 ± 0.00^{A}
Treatment with NaOH	6.71	1.5488 ± 0.00^{A}
Treatment with Na_3PO_4	6.74	1.5578 ± 0.00^{A}
Treatment with NH_3	6.79	1.5330 ± 0.03^{A}
Control 1	6.78	1.2022 ± 0.01^{B}
Control 3	6.4	1.5248 ± 0.01^{A}
Treatment with NaOH	6.77	1.5248 ± 0.01^{A}
Treatment with Na_3PO_4	6.79	1.5330 ± 0.00^{A}
Treatment with NH_3	6.77	1.5661 ± 0.00^{A}

Control 1: Refer to natural milk without any additives, Control 2: Milk after reducing, pH to 6.2, Control 3: Milk after reducing pH to 6.4, *Same letters indicate no significant differences at the 95% confidence level. Different letters refer to differences among samples. Mean±standard error

the neutralizer addition to milk as there were no significant differences between acidified milk before and after neutralization. Detection of milk adulteration by neutralizing agents would provide useful information to dairy processing plants. The addition of neutralizing agents in milk did not lead to significant changes in physicochemical characteristics of milk, such as freezing point, density, alcohol stability, acidity and pH (Data not shown). Therefore, specific qualitative tests for the detection of these substances are required in the dairy industry to test the quality of receiving milk. Silva *et al.*[30] couldn't detect sodium hydroxide used to neutralize acidity in the milk. The neutralizing agent is a challenge for food safety and human health as they can cause serious health problems[31].

Milagres *et al.*[4] developed and validated the HPLC method to detect lactic acid in sour milk and after neutralization. However, such methods are expensive and HPLC may not be available especially in countries where adulteration is a common practice. Therefore, the spectrophotometric method would be a simple and easy method to detect adulteration and protect consumers with a relatively low running cost.

Effect of neutralizing agents on the properties of yoghurt and soft cheese: Data in Table 3 show that the titratable acidity % of yoghurt decreased by adding the neutralizing substances to the sour milk before yoghurt making when compared to yoghurt made using non-neutralized milk as control. The lactate concentration as measured by the spectrophotometric method, was significantly ($p\leq0.05$) higher in yoghurt samples made from neutralized milk than in the product made from normal milk. The concentration of lactate was very high in treatment 1 (neutralized with NaOH), followed by treatment 2 (neutralized with Na_3PO_4), while treatment 3 (neutralized with NH_3) had the lowest concentration. While there were no significant differences in lactate concentration among sour milk samples before or after neutralization with NaOH, NH_3 or Na_3PO_4 (Table 2), there were differences in lactate concentration in yoghurt and cheese samples made from milk neutralized by NaOH and NH_3. This could be caused by differences in the impact of the neutralizing agents on the metabolic activity of starter cultures. Acidity was significantly higher in the control yoghurt than in the samples made from neutralized milk (Table 3). While the lactate concentration in yoghurt treatment-1(neutralized with NaOH) was three times that in control its acidity was only 10% higher. Whereas the lactate concentration in yoghurt treatment-3 (neutralized with NH_3) was about 40% of that in yoghurt treatment-1, both samples had the same acidity. This may support our hypothesis that there are differences in the impact of the neutralizing agents on the metabolic activity of the starter culture.

Table 3: Physicochemical properties of yoghurt and cheese, which produced from artificial adulterated milk with some neutralizer agents

Samples	Concentration of lactate (g L^{-1})	Acidity (%)	Total solids (%)	pH	Syneresis (%)
Yoghurt analysis					
Control	2.9253 ± 0.04^{D}	0.771 ± 0.00^{A}	15.35 ± 0.08^{AB}	4.88	44.13 ± 0.03^{A}
Treatment Y_1	8.7143 ± 0.14^{A}	0.698 ± 0.00^{B}	15.44 ± 0.20^{B}	4.8	41.33 ± 0.16^{B}
Treatment Y_2	7.9700 ± 0.00^{B}	0.664 ± 0.01^{C}	16.23 ± 0.51^{A}	4.89	37.28 ± 0.32^{C}
Treatment Y_3	3.9177 ± 0.04^{C}	0.702 ± 0.01^{B}	16.14 ± 0.36^{AB}	4.88	37.12 ± 0.07^{C}
Cheese analysis					
Control	1.6848 ± 0.00^{C}	0.380 ± 0.01^{C}	35.68 ± 60.13^{A}	6.46	ND
Treatment CH_1	3.6696 ± 0.19^{A}	0.615 ± 0.02^{A}	35.39 ± 0.63^{A}	5.99	ND
Treatment CH_2	2.6772 ± 0.16^{B}	0.52 ± 0.03^{B}	35.08 ± 0.56^{A}	6.24	ND
Treatment CH_3	2.2637 ± 0.14^{BC}	0.24 ± 0.001^{D}	34.58 ± 0.07^{A}	6.67	ND

Treatment Y_1: Yogurt made from adulterated milk with NaOH, Treatment Y_2: Yogurt made from adulterated milk with Na_3PO_4, Treatment Y_3: Yogurt made from adulterated milk with NH_3, Treatment CH_1: Soft cheese made from adulterated milk with NaOH, Treatment CH_2: Soft cheese made from adulterated milk with Na_3PO_4, Treatment CH_3: Soft cheese made from adulterated milk with NH_3. ND: Not determined. *Same letters indicate no significant differences at the 95% confidence level. Different letters refer to differences among samples. Mean±standard error

Table 4: Yield of cheese produced from adulterated milk with some neutralizer agents

Samples	Yield (%)
Control	21.09 ± 0.0^{B}
Treatment CH_1	20.49 ± 0.0^{C}
Treatment CH_2	20.55 ± 0.0^{C}
Treatment CH_3	23.18 ± 0.0^{A}

Treatment CH_1: Soft cheese made from adulterated milk with NaOH, Treatment CH_2: Soft cheese made from adulterated milk with Na_3PO_4, Treatment CH_3: Soft cheese made from adulterated milk with NH_3. *Same letters indicate no significant differences at the 95% confidence level. Different letters refer to differences among samples. Mean±standard error

The lactate concentration in soft cheese treatment-1 (neutralized with NaOH) was about two times higher than that in the control sample but the lactate concentration was only 1.6 and 1.3-times higher in treatments 2 and 3, respectively than in the control sample. This finding is similar to what we found in yoghurt but the titratable acidity in soft cheese is different from that in yoghurt. The acidity in treatment-1 (neutralized with NaOH) is significantly higher than that in control by about 1.6 times while the acidity in treatment-3 (neutralized with NH_3) is significantly lower than in control. Some differences between yoghurt and soft cheese include salt addition at 5% and concentration of proteins and fat by drainage. The addition of salt to cheese milk could influence the metabolic activities of the starter in soft cheese. The obtained results confirmed that the determination of lactate by the spectrophotometric method is not suitable to detect the neutralization of milk used in yoghurt or cheese making. While the titratable acidity in yoghurt was not influenced by milk neutralization it increased in soft cheese made from milk neutralized by NaOH and decreased in cheese made from milk neutralized by NH_3.

The result of Table 3 shows that making yoghurt from neutralized milk has little effect on moisture and total solids contents. Syneresis (%) was lower decreased in yoghurt made from neutralized sour milk than normal milk. Using ammonia as a neutralizing agent produced the lowest syneresis (37.12%) in yoghurt among the other treatments. At the same conditions, data in Table 3 indicated that there were non-significant differences among treatments and control cheese in moisture content. Kamthania *et al.*[32], Ahirwar[33] reported that the physicochemical properties of milk did not change by adulteration of milk with neutralizing substances.

Effect of neutralizing substances on cheese yield and whey properties: There were significant differences in cheese yield among treatments and between them and the control Table 4. Using ammonia as a neutralizing agent caused increased cheese yield, while sodium hydroxide and tri-sodium phosphate decreased it as compared with control. This means that the milk adulterated with sodium hydroxide or tri-sodium phosphate produced a negative effect on cheese yield unlike adulteration with ammonia. There were no significant differences among the yield of cheeses made from milk neutralized by sodium hydroxide (treatment CH_2) and tri-sodium phosphate. Whey of cheese made using milk neutralized by tri-sodium phosphate contained higher fat and protein than that of cheese made using milk neutralized by sodium hydroxide or ammonia (Table 5).

However, using sodium hydroxide or ammonia as neutralizing substances decreased the fat and protein in acid and sweet whey than those in control (cheese made from natural milk). The freezing point of acid and sweet whey was not affected by neutralizing substances as there were no differences between treatments and control (Table 5). The low cheese yield and high losses of fat and protein in whey of cheese made using milk neutralized by tri-sodium phosphate are related to the solubilization of colloidal calcium phosphate in casein. Neutralization with tri-sodium phosphate occurs more slowly than with sodium hydroxide. Simultaneously to

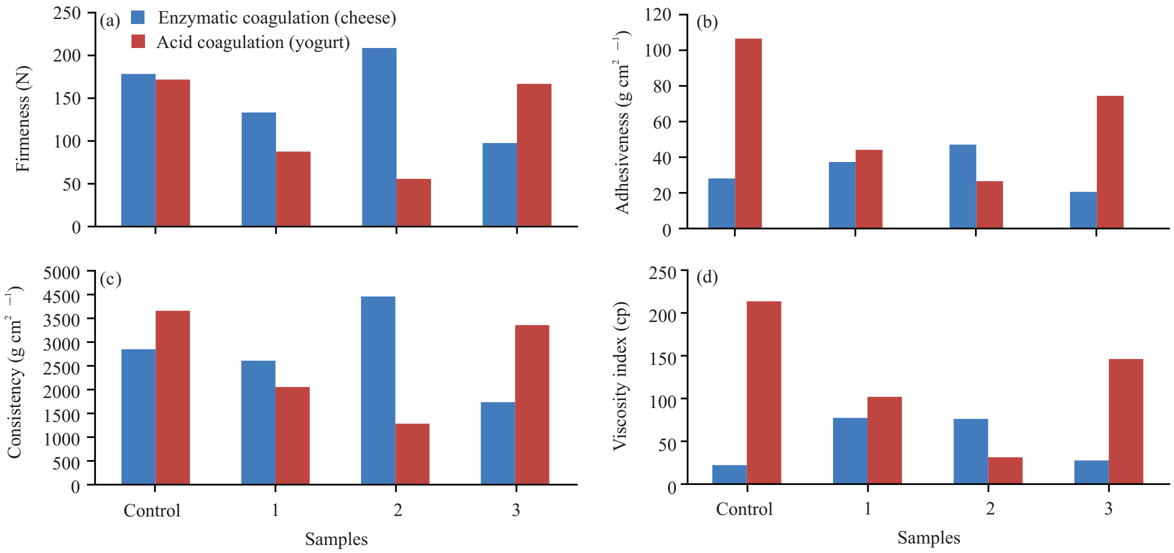

Fig. 2(a-d): Rheological properties of curd producing from acid coagulation (yoghurt) and enzymatic coagulation (cheese)
1: Curd neutralized with sodium hydroxide, 2: Curd neutralized with trisodium phosphate and 3: Curd neutralized with ammonia

Table 5: Chemical analysis of yoghurt and cheese whey

Samples	Protein	Fat	TS	SNF	Freezing point	Lactose
Yoghurt whey analysis						
Control	0.74±0.03^B	0.44±0.01^A	6.47±0.01^A	6.03±0.01^{AB}	-0.33±0.0^A	4.08±0.01^A
Treatment wY_1	0.68±0.01^{BC}	0.14±0.01^C	6.10±0.06^B	5.96±0.05^B	-0.33±0.0^A	4.05±0.03^A
Treatment wY_2	0.94±0.01^A	0.23±0.01^B	6.35±0.01^A	6.12±0.01^A	-0.32±0.0^A	3.30±0.01^C
Treatment wY_3	0.61±0.01^C	0.14±0.00^C	5.80±0.01^C	5.66±0.01^C	-0.31±0.0^A	3.79±0.01^B
Cheese whey analysis						
Control	0.94±0.01^B	0.20±0.01^B	6.90±0.01^A	6.78±0.01^A	-0.40±0.01^A	4.60±0.01^A
Treatment wCH_1	0.77±0.01^C	0.10±0.00^C	6.45±0.01^B	6.35±0.01^B	-0.36±0.01^A	4.48±0.01^B
Treatment wCH_2	1.04±0.01^A	0.96±0.01^A	6.92±0.01^A	5.96±0.01^C	-0.36±0.01^A	4.39±0.01^B
Treatment wCH_3	0.76±0.01^C	0.10±0.00^C	6.10±0.01^C	6.03±0.03^C	-0.32±0.01^A	4.05±0.01^C

Treatment wY_1: Yogurt whey produced from adulterated milk with NaOH, Treatment wY_2: Yogurt whey produced from adulterated milk with Na_3PO_4, Treatment wY_3: Yogurt whey produced from adulterated milk with NH_3, Treatment wCH_1: Soft cheese whey made from adulterated milk with NaOH, Treatment wCH_2: Soft cheese whey made from adulterated milk with Na_3PO_4, Treatment wCH_3: Soft cheese whey made from adulterated milk with NH_3. *Same letters indicate no significant differences at the 95% confidence level. Different letters refer to differences among samples. Mean±standard error

increasing the net negative charge with sodium hydroxide, calcium bridging would occur between individual proteins within the colloidal particles, keeping them intact and preventing further disintegration of the supramolecular structure[34]. Casein micelles in milk neutralized with ammonia would then be expected to have a more compact structure due to cross-linking by numerous calcium bridges predominantly occurring via phosphoserine in caseins.

Effect of neutralizing substances on the texture properties of yoghurt and soft cheese: Milk neutralization impacted the rheological properties of yoghurt and soft cheese (Fig. 2a-d). The firmness is lower in yoghurt made from treated milk than in control. Milk Neutralization with ammonia produced higher firmness than other neutralizing substances (Fig. 2a). In cheese curd, adding trisodium phosphate increased firmness compared to control. Adhesiveness was significantly lower in yoghurt made from neutralized milk than in control. In contrast, adhesiveness was higher in cheese curd made from milk neutralized by sodium hydroxide and tri-sodium phosphate (Fig. 2b). Adding neutralizing substances to sour milk reduced the consistency in yoghurt (acid coagulation). Cheese curd made using milk neutralized with tri-sodium phosphate has the highest consistency value (Fig. 2c). All neutralizing substances caused a decrease in the viscosity value of yoghurt (Fig. 2d).

Trisodium phosphate (food grade) is allowed as an acidity regulator in milk powder but not ammonia or sodium hydroxide. However, there is no guarantee that adulterants would use expensive food-grade substances to neutralize milk which can cause health problems. Yoghurt made from milk neutralized with ammonia has acceptable physicochemical properties. Moreover, yoghurt made from milk neutralized with ammonia had better syneresis and good texture properties than control. The soft cheese made from milk neutralized with ammonia had a better yield and textural properties than the control. Because it is not possible to detect neutralization in yoghurt or cheese milk by testing the finished product with spectrophotometric, raw milk should be tested before processing.

CONCLUSION

The spectrophotometric method using $FeCl_3$ was able to detect the neutralizers in milk. This is a simple, reliable and cheap method that can be used by quality control to detect milk adulteration by chemicals. The texture and physiochemical properties of yoghurt and soft cheese were not impacted by milk neutralization. However, since this method could not detect adulteration in yoghurt and cheese, it would be recommended for only raw milk testing before processing.

SIGNIFICANCE STATEMENT

This study discovers a new reliable and fast method to detect the adulterated milk by neutralizing agents that can be beneficial for the dairy industry to reject the adulterated milk with neutralizing substances. This study will help the authorities in food safety foundations to uncover the critical areas of the adulterate milk with neutralizing agents that researchers were not able to detect this substance in a short time.

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