Food Analytical Methods
https://doi.org/10.1007/s12161-019-01594-5

A New Method to Distinguish the Milk Adulteration with Neutralizers by Detection of Lactic Acid

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Received: 23 April 2019 / Accepted: 10 July 2019

Abstract
A liquid chromatographic method based on determining of the amount of lactic acid was developed to detect milk adulteration with neutralizers. The developed method can be applied to milk with pH values within the regular range of 6.5–6.7 that is suspected of being neutralised. Determination of lactic acid was carried out in milk acidified with lactic acid and neutralised with sodium hydroxide to simulate the adulteration. The validation parameters showed high linearity ($R^2 > 0.99$), good precision (relative standard deviation ≤ 0.123%) and high sensibility (limit of detection 0.1 mg/L and limit of quantification 1 mg/L). The proposed method was applied to bacterially acidified and subsequently neutralised milk and detected a content of lactic acid of approximately 40 mg/100 mL in milk slightly acidified to pH 6.4. The developed method is simple, fast, precise and suitable for detecting the addition of hydroxides in sour milk.

In parallel, the sodium content was determined in the same sodium hydroxide neutralised samples, but the addition of a small amount of this alkali does not affect the natural variation of sodium in milk.

Keywords Milk adulteration · Sodium hydroxide · Lactic acid · Neutralizers

Introduction

In the recent years, the media has highlighted many instances of adulteration of milk and milk products with various kinds of adulterants. In particular, in Italy, public officers recently discovered the adulteration of water buffalo mozzarella with sodium hydroxide. The addition of neutralizers is an illegal practice to prevent the rejection of poor-quality milk. Use of food additives, including sodium hydroxide (E254), in raw and pasteurised milk is forbidden by Regulation (2008).

Despite food legislation, neutralizers such as sodium bicarbonate, sodium carbonate, sodium hydroxide and calcium hydroxide are generally used to mask the pH and acidity values of badly preserved milk, passing it off as fresh milk. These adulterants can be harmful to the consumer; for example, carbonates and bicarbonates can cause disruptions of hormone signalling that regulates development and reproduction (Singuluri and Sukumaran 2014). Carbonates in milk produce gastrointestinal problems including gastric ulcers, diarrhoea, colon ulcers and electrolyte disturbances (Ayub et al. 2007). Sodium hydroxide (caustic soda) contains sodium and acts as a slow poison to those suffering from hypertension and heart ailments. Caustic soda prevents the body from utilizing lysine, an essential amino acid in milk, which is required by growing babies (Maheswara Reddy et al. 2017).

Neutralisation of sour milk is often used in cheese-making factories and it improves renneting properties, probably due to elevated Ca^{2+} activity (Lucey et al. 1996).

The pH of normal healthy cow's milk generally ranges from 6.5 to 6.7, while the pH of water buffalo's milk is between 6.7 and 6.8. Milk with higher pH values should be suspected of either being abnormal milk, such as mastitic milk or neutralised milk. Conversely, the pH values are lower in colostrum (pH 6.0). The capacity of milk to be acidified, which is called the buffering capacity, has been found to be higher for water buffalo's milk than for cow's milk. The pH of water buffalo's milk decreases more slowly than pH of cow's milk during acidification, probably due to the higher casein content in water buffalo's milk compared with cow's milk. The buffering property of milk is also related to its contents of acid and basic compounds. Moreover, inorganic phosphate, which also contributes to the buffering capacity, is also higher in water buffalo's milk than in cow's milk (Ahmad et al. 2008).

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Published online: 26 July 2019

Acidification of milk to pH > 5.5, followed by neutralisation to pH 6.6, hardly reduces buffering (at pH approximately 5.1) because either little colloidal calcium phosphate (CCP) dissolves during acidification or other calcium phosphates are formed during neutralisation. The micellar system is not readily reversible; in fact, once disintegrated by acidification, micelles are not reformed by neutralisation (Lucey et al. 1996).

The acidity of milk is composed of natural acidity and developed acidity. Natural acidity is mainly due to phosphate, casein and to a lesser extent, citrate and carbon dioxide. Developed acidity is due to lactic acid produced by the action of bacteria on lactose in milk. The acidity of milk includes the total acidity (natural + developed) or titratable acidity. The titratable acidity of freshly drawn milk ranges between 0.12 and 0.16% (expressed as lactic acid) for cow's as well as water buffalo's milk. The true lactic acid level of freshly drawn milk is approximately 2 mg% (range from 0.4 to 2.8 mg%). As milk ages, milk bacteria proliferate and produce lactic acid from lactose. The predominant bacteria are as follows: *Micrococcus*, *Streptococcus* and nonpathogenic *Corynebacterium* from animal skin (Aaku et al. 2004); *Lactobacillus*, *Coliforms* and *Enterococcus* (from the environment); and psycrotrophic bacteria such as *Pseudomonas*, *Alcaligenes* and *Flavobacterium* from refrigerated tanks (Hantsis-Zacharov and Halpern 2007). The high value of lactic acid can also be due to bad hygienic practices and bad storage and transport conditions. In the latter case, there is the risk that some pathogenic bacteria are present, such as *Mycobacterium tuberculosis* and *bovis*, *Brucella abortus* and *melitensis*, *Streptococcus agalactiae*, *Staphylococcus aureus*, *Salmonella* and *Campylobacter jejuni* (Jayarao and Henning 2001). However, thermal treatment can lower the risk of pathogenic bacteria.

Many methods have been developed to detect neutralizers in milk, such as the rosalic acid test, the alkalinity of ash and pH determination (Sharma et al. 2012). However, these methods are old and have limitations. Rosalic acid is an indicator that shows a change in colour upon addition to alkaline milk. This test will only work when neutralizers are added at excess quantities and milk is alkaline in nature. If sour milk is underneutralised below the normal pH of milk, the rosalic test will fail to detect added neutralizers. The test for the alkalinity of ash is based on the fact that neutralisation of milk invariably increases the ash content and also the total alkalinity of ash from a fixed quantity of milk. However, because of the natural variations in the alkalinity of the ash and its differences in cow's and water buffalo's milk, this test cannot be reliably used to detect and determine neutralizers when added at low concentrations (Sharma et al. 2012). Similarly, determination of the titratable acidity can also be employed for the detection of neutralizers in milk. Milk with low phosphorus, casein and Ca^{2+} contents tends to be low in titratable acidity (Jensen 1995). However, these tests enable the detection of overneutralised milk.

The determination of the true lactic acid/lactate content coupled with a titratable acidity measurement could be a more reliable approach to detect added neutralizers, as the addition of neutralizers to milk will reduce the titratable acidity but does not affect the lactic acid/lactates content. An altered relationship between the lactic acid/lactates content and the titratable acidity can be regarded as an indication of the presence of neutralizers. Procedures based on spectrophotometric analysis to determine lactic acid in milk (Ling 1951), ISO (2005) require time and are laborious. According to (Ling 1951) after precipitation with NaOH-$ZnSO_4$ in the presence of $BaCl_2$, the filtrate is treated with the $FeCl_3$-HCl reagent, and the colour is measured by a spectrophotometer. According to the procedure ISO (2005), fat and proteins are precipitated and then filtered. The filtrate is treated with the following enzymes and biochemical substances, which are added simultaneously but act in sequence: (a) L-lactate dehydrogenase and D-lactate dehydrogenase, in the presence of nicotinamide adenine dinucleotide (NAD) to oxidize lactate to pyruvate and to convert NAD to its reduced form, NADH; (b) glutamate pyruvate transaminase in the presence of L-glutamate to transform pyruvate into L-alanine and to convert L-glutamate to α-ketoglutarate. The amount of NADH produced is determined by spectrophotometric measurement at a wavelength of 340 nm and is proportional to the lactic acid and lactate contents.

The aim of this work was to develop a rapid and precise method to detect adulteration of milk with sodium hydroxide. The chemical reaction when sodium hydroxide is added to sour milk is as follows: lactic acid + sodium hydroxide = sodium lactate + water. Therefore, we tested two methods in neutralised milk: detection the addition of sodium hydroxide by means of determining the Na content and detection of neutralisation via determination of lactic acid (lactate) by high pressure liquid chromatography (HPLC).

Materials and Methods

Materials and Solutions

All of the standards including lactic acid (DL 85%) were purchased from Sigma-Aldrich (St. Louis, MO, US). Acetonitrile, trifluoroacetic acetic acid (TFA), nitric acid, sodium hydroxide and lanthanum chloride were obtained from Merck (Darmstadt, Germany). Deionised water was produced with a Milli-Q water system (Millipore, Billerica, MA, US).

Instrumentation

An atomic absorption spectrometer, PinAAcle™ 900F, PerkinElmer (MA, USA), was used to determinate sodium content.

Chromatographic analysis was performed using an Agilent high-performance liquid chromatography model 1200 (Agilent, Technologies, CA, USA) equipped with a Rheodyne valve and a 20-μL loop coupled to a diode array detector (DAD). The analytical column was a Spherisorb ODS2 column (250 mm × 4.6 mm, 5 μm; Waters Corporation, Milford, MA, USA). The mobile phases consisted of 0.1% TFA in water (eluent A) and 0.1% TFA in acetonitrile (eluent B); the gradient was 0% of eluent B for the first 10 min, which was then raised to 100% over 2 min. The total run time was 22 min. The flow rate was 0.8 mL/min. Detection of lactic acid was performed at 205 nm.

A Crison pH-meter (Basic 20 Instruments, Barcelona, Spain) was used for pH adjustments. Hettich centrifuge, model D-7200 (Germany), was used to remove interfering substances from milk.

Sample Preparation

Fresh, pasteurised, homogenised whole milk was obtained from three Italian companies located in Fagianeria Piana di Monte Verna (CE), Castrovillari (CS) and Pastorano (CE), coded A, B and C, respectively.

Each of the three samples was divided into thirteen aliquots, leading to a total of 39 aliquots: 3 aliquots without any acidification were used as controls (A, B and C), 18 were chemically acidified over the pH range of 6.4–5.4 (A_{C1}–A_{C6}, B_{C1}–B_{C6}, C_{C1}–C_{C6}) and 18 were bacterially acidified over the same pH range by bacterial growth (A_{N1}–A_{N6}, B_{N1}–B_{N6}, C_{N1}–C_{N6}), as listed in Table 1. Chemical acidification was carried out by the addition of lactic acid under stirring until pH values of 6.4, 6.2, 6.0, 5.8, 5.6 and 5.4 were reached. Natural bacterial acidification was carried out by incubation in oven at 37 °C for 4, 8, 16, 18, 20 and 21 h to allow the natural acidity to occur and reach pH values of 6.4, 6.2, 6.0, 5.8, 5.6 and 5.4, respectively (Table 1). To simulate the illegal practice of neutralisation, both chemically and bacterially acidified samples were immediately neutralised to an initial value of pH 6.7 by addition of 0.25 M NaOH (Table 1).

Table 1 pH and titratable acidity expressed in °SH (mean value ± standard deviation) of milk samples acidified by the addition of lactic acid (A_{C1}–A_{C6}, B_{C1}–B_{C6}, C_{C1}–C_{C6}) and natural bacterial growth (A_{N1}–A_{N6}, B_{N1}–B_{N6}, C_{N1}–C_{N6}); amount of NaOH (mean value ± standard deviation) added to neutralize to the initial pH

Sample	Amount of lactic acid (mg/100 mL) added	pH before neutralisation	°SH before neutralisation	Amount of NaOH (mg/100 mL) added to neutralize to the initial pH	Sample	Incubation time (h) at 37 °C	pH before neutralisation	°SH before neutralisation	Amount of NaOH (mg/100 mL) added to neutralize to the initial pH
A	0	6.70 ± 0.01	7.90 ± 0.01	0		0			0
A_{C1}	45.65	6.42 ± 0.02	8.80 ± 0.03	17.46 ± 0.01	A_{N1}	4	6.42 ± 0.01	8.85 ± 0.01	14.24 ± 0.01
A_{C2}	79.87	6.21 ± 0.01	9.25 ± 0.01	29.10 ± 0.01	A_{N2}	8	6.23 ± 0.01	9.30 ± 0.02	28.60 ± 0.02
A_{C3}	114.06	6.00 ± 0.01	10.45 ± 0.03	39.45 ± 0.02	A_{N3}	16	6.04 ± 0.02	10.40 ± 0.00	34.85 ± 0.01
A_{C4}	159.61	5.82 ± 0.02	12.90 ± 0.01	51.06 ± 0.02	A_{N4}	18	5.85 ± 0.01	13.00 ± 0.00	53.05 ± 0.01
A_{C5}	227.86	5.65 ± 0.03	14.60 ± 0.02	71.06 ± 0.01	A_{N5}	20	5.67 ± 0.02	14.65 ± 0.03	71.93 ± 0.02
A_{C6}	273.31	5.43 ± 0.01	17.80 ± 0.01	86.08 ± 0.02	A_{N6}	21	5.44 ± 0.01	17.90 ± 0.01	87.32 ± 0.01
B	0	6.71 ± 0.01	8.07 ± 0.02	0		0			0
B_{C1}	34.24	6.43 ± 0.03	8.90 ± 0.01	12.61 ± 0.01	B_{N1}	4	6.45 ± 0.01	8.86 ± 0.02	14.23 ± 0.01
B_{C2}	68.46	6.20 ± 0.01	9.80 ± 0.02	22.26 ± 0.02	B_{N2}	8	6.23 ± 0.02	9.31 ± 0.01	28.60 ± 0.01
B_{C3}	114.06	6.01 ± 0.02	10.45 ± 0.03	34.82 ± 0.01	B_{N3}	16	6.05 ± 0.01	10.42 ± 0.01	34.81 ± 0.02
B_{C4}	182.37	5.82 ± 0.01	13.15 ± 0.00	48.54 ± 0.02	B_{N4}	18	5.81 ± 0.03	13.10 ± 0.02	53.01 ± 0.01
B_{C5}	205.12	5.66 ± 0.01	14.75 ± 0.05	65.78 ± 0.01	B_{N5}	20	5.67 ± 0.01	14.70 ± 0.03	71.91 ± 0.01
B_{C6}	273.31	5.45 ± 0.02	17.90 ± 0.01	85.16 ± 0.01	B_{N6}	21	5.43 ± 0.01	17.95 ± 0.02	87.30 ± 0.02
C	0	6.78 ± 0.01	8.00 ± 0.01	0		0			0
C_{C1}	53.59	6.49 ± 0.02	8.80 ± 0.03	17.38 ± 0.01	C_{N1}	4	6.42 ± 0.01	8.86 ± 0.02	14.25 ± 0.01
C_{C2}	80.36	6.23 ± 0.02	9.70 ± 0.02	25.49 ± 0.01	C_{N2}	8	6.20 ± 0.02	9.35 ± 0.02	28.64 ± 0.01
C_{C3}	107.12	6.02 ± 0.01	10.40 ± 0.01	34.89 ± 0.01	C_{N3}	16	6.04 ± 0.01	10.41 ± 0.03	34.85 ± 0.01
C_{C4}	160.60	5.87 ± 0.01	13.00 ± 0.01	49.01 ± 0.01	C_{N4}	18	5.88 ± 0.01	13.06 ± 0.02	53.05 ± 0.02
C_{C5}	205.12	5.66 ± 0.01	14.70 ± 0.04	65.42 ± 0.02	C_{N5}	20	5.66 ± 0.01	14.60 ± 0.02	71.94 ± 0.02
C_{C6}	285.16	5.45 ± 0.01	17.90 ± 0.00	83.86 ± 0.02	C_{N6}	21	5.47 ± 0.01	17.95 ± 0.03	87.33 ± 0.01

Table 2 Sodium content expressed in mg/100 mL (mean value ± standard deviation) of milk samples acidified by the addition of lactic acid (A_{C1}–A_{C6}, B_{C1}–B_{C6}, C_{C1}–C_{C6}) and natural bacterial growth (A_{N1}–A_{N6}, B_{N1}–B_{N6}, C_{N1}–C_{N6}) after neutralisation to the initial pH

Sample	Detection of Na (mg/100 mL)	Sample	Detection of Na (mg/100 mL)
A	44.40 ± 0.52		
A_{C1}	54.94 ± 1.12	A_{N1}	52.67 ± 3.02
A_{C2}	70.91 ± 5.02	A_{N2}	69.62 ± 0.12
A_{C3}	71.21 ± 4.84	A_{N3}	70.21 ± 0.10
A_{C4}	71.88 ± 2.04	A_{N4}	70.89 ± 0.32
A_{C5}	90.19 ± 0.41	A_{N5}	90.29 ± 1.14
A_{C6}	90.26 ± 0.64	A_{N6}	90.96 ± 1.12
B	53.20 ± 7.23		
B_{C1}	62.63 ± 0.76	B_{N1}	55.67 ± 2.09
B_{C2}	63.20 ± 7.23	B_{N2}	68.92 ± 2.12
B_{C3}	72.63 ± 0.76	B_{N3}	73.19 ± 0.10
B_{C4}	80.41 ± 9.43	B_{N4}	79.07 ± 0.51
B_{C5}	88.74 ± 2.37	B_{N5}	90.29 ± 1.14
B_{C6}	95.61 ± 3.10	B_{N6}	93.05 ± 1.12
C	49.67 ± 9.92		
C_{C1}	53.87 ± 0.18	C_{N1}	51.97 ± 2.02
C_{C2}	94.00 ± 7.11	C_{N2}	66.02 ± 0.12
C_{C3}	95.95 ± 11.99	C_{N3}	69.01 ± 0.10
C_{C4}	98.09 ± 0.88	C_{N4}	70.89 ± 0.32
C_{C5}	107.88 ± 9.22	C_{N5}	80.19 ± 1.14
C_{C6}	110.97 ± 2.45	C_{N6}	89.56 ± 1.12

All samples were prepared in triplicate and submitted to the following analysis.

Titratable Acidity

Titratable acidity was determined by titration with 0.25 N NaOH to a final pH of 8.4, using a Crison pH-meter and is expressed in Soxhlet-Henkel degrees (°SH/100 mL) as described in the Official method of analysis (Cunniff 1995).

Sodium Determination by Atomic Absorption Spectrometry (AAS)

To decompose organic matter, 1.5 g of the 39 samples of milk was placed in an oven. The oven temperature was increased by 50 °C per hour up to 505 °C and maintained at this temperature overnight.

Ashed samples were dissolved in 3 mL of a 0.5 N nitric acid solution. The crucible content was quantitatively transferred into a 25-mL one-mark volumetric flask, and the volume was brought up with water and mixed thoroughly. The solution was diluted 1:100, and a volume fraction of 0.1% of a lanthanum chloride solution (10000 mg/L) was added to suppress phosphate interference and the ionisation of elements in the flame. A blank test was performed using the same procedure. The atomic absorption spectrometer was set at 589.6 nm for Na (Noel et al. 2008). The results are expressed as milligrams of Na/100 mL of milk.

Lactic Acid Determination

Milk samples were prepared as follows: 20 g of each sample of milk was acidified with 2 N HCl until reaching pH 4.6 and was centrifuged at 2000 rpm for 15 min to remove interfering substances, such as caseins and fat. The supernatant fraction was recovered by filtration on Whatman paper, diluted 1:20 with water and filtered again through a 0.22-μm membrane. Twenty microlitres of the recovered supernatant was injected into the HPLC-DAD.

Lactic acid was identified by comparing its retention time with that of the standard lactic acid provided by Sigma-Aldrich. Lactic acid was identified at 4.2 min of elution time. Quantification was performed using an external standard curve at five different concentrations (10, 50, 100, 500 and 1000 mg/L) of lactic acid. The results are expressed as milligrams of lactic acid/100 mL of milk. The linearity range was evaluated by plotting the peak area corresponding to the analyte as a function of the concentration introduced. The repeatability of the method was checked by injecting replicate injections of the five concentrations of lactic acid for 3 days.

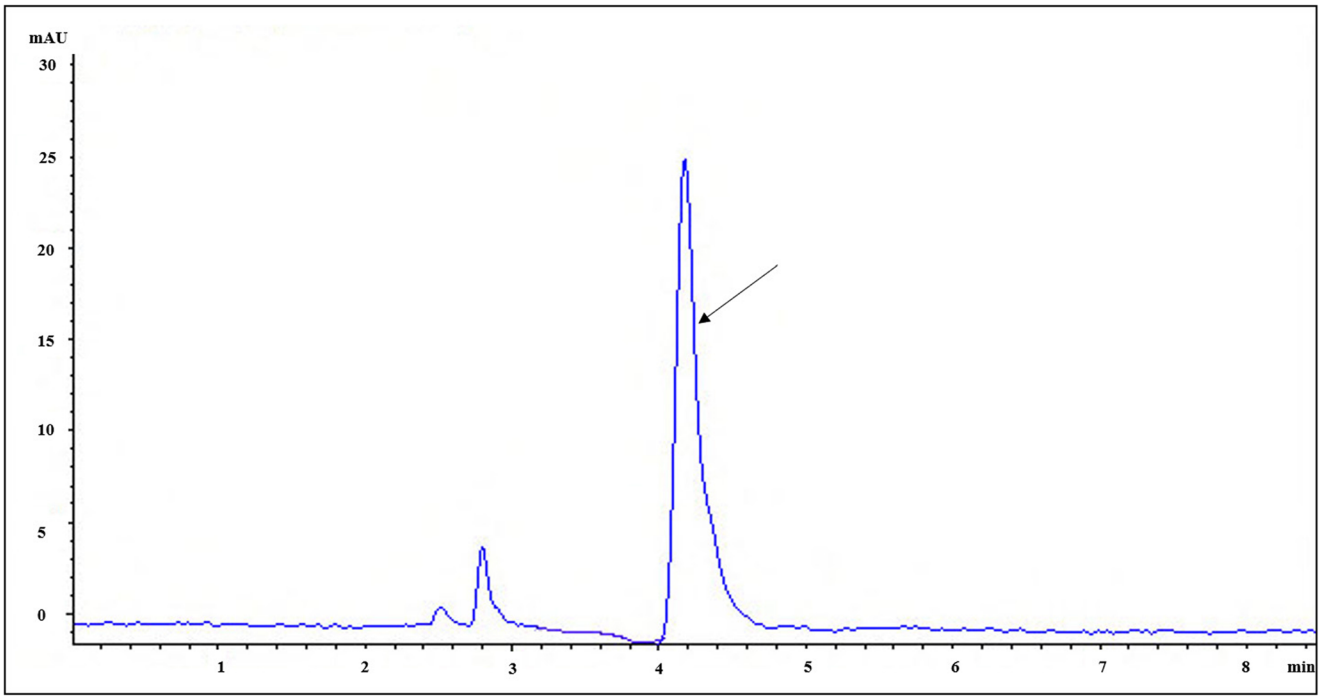

Fig. 1 Typical HPLC chromatogram of lactic acid in milk samples

Statistical Analysis

All determinations were performed in triplicate, and the reported results are the average values of the three repetitions. Differences were considered to be significant when $P \leq 0.05$, and the data were analysed using the software XLSTAT (Addinsoft, New York, NY, USA).

Results and Discussion

The three samples of fresh milk A, B and C used as controls had a pH value of 6.7 and titratable acidities of 7.9, 8.1 and 8.0 °SH, respectively, indicating they were milk samples of good quality (Table 1). The bacterially and chemically acidified samples (A_{N1}–A_{N6}, B_{N1}–B_{N6}, C_{N1}–C_{N6} and A_{C1}–A_{C6}, B_{C1}–B_{C6}, C_{C1}–C_{C6}) were used to simulate milk of poor quality and had values of titratable acidity ranging from 7.9 to 18 °SH before neutralisation (Table 1). Quality parameters such as pH and titratable acidity may have a significant effect on cheese yield (Verdier-Metz et al. 2001).

Bovine fresh milk typically has a pH between 6.5 and 6.7. The pH value of milk is influenced by the phosphate, citrate and protein contents. As milk is a buffer solution, a considerable amount of acid may be present before the pH changes. When milk goes sour, it becomes more acidic because bacteria in milk convert lactose into lactic acid. Values lower than 6.5 denote the presence of bacterial deterioration, and values higher than 6.8 denote mastitic milk. Lowering the pH decreases the colloidal stability of milk. Both enzymatic and aggregations reactions are affected by the pH of milk.

The Soxhlet-Henkel degrees, which represent the titratable acidity, were defined as millilitres of 0.25 M NaOH used to titrate 100 mL of milk in the presence of phenolphthalein as the indicator. Bovine fresh milk typically has 7.0–8.5 °SH of acidity. Casein, mineral matter, traces of organic acids and phosphate contribute to the natural acidity of milk. When lactic acid formation occurs, the value of titratable acidity increases, but this titration measures the buffering capacity of milk and not the true acidity. Titratable acidity plays an important role during all phases of milk coagulation: it affects the reactivity of rennet and the aggregation rate of para-casein micelles. Titratable acidity also determines the suitability of milk for cheese-making and influences the rate of syneresis. Usually, milk with low acidity (hypoacid milk) is considered to not be suitable for cheese-making because of its negative effects on the rheology of the acid-rennet curd and on the textural properties of the cheese paste (De Marchi et al. 2009).

Sodium Determination

The concentration range of sodium salt in cow's milk is from 39 to 64 mg/100 mL and is considered to be relatively constant, but slight variations can be observed in some cases. The concentration of minerals varies with the time of the lactation period and during mastitis; in particular, the concentrations of sodium and chloride ions are strongly increased during mastitis. Some differences in the milk salt

Table 3 Lactic acid content expressed in mg/100 mL (mean value ± standard deviation) determined by HPLC in milk samples acidified by the addition of lactic acid (A_{C1}–A_{C6}, B_{C1}–B_{C6}, C_{C1}–C_{C6}) and natural bacterial growth (A_{N1}–A_{N6}, B_{N1}–B_{N6}, C_{N1}–C_{N6}) after neutralisation to the initial pH

Sample	Added amount of lactic acid (mg/100 mL)	pH before neutralisation	Detection of lactic acid (mg/100 mL)	RSD (%) for accuracy	Sample	pH before neutralisation	Detection of lactic acid (mg/100 mL)
A	0.00	6.70 ± 0.01	n.d.				
A_{C1}	45.65	6.42 ± 0.02	44.81 ± 0.60	1.84	A_{N1}	6.42 ± 0.01	39.96 ± 0.14
A_{C2}	79.87	6.21 ± 0.01	78.41 ± 0.36	1.83	A_{N2}	6.23 ± 0.01	69.82 ± 0.33
A_{C3}	114.06	6.00 ± 0.01	112.28 ± 0.14	1.56	A_{N3}	6.04 ± 0.02	107.45 ± 1.45
A_{C4}	159.61	5.82 ± 0.02	157.66 ± 0.30	1.22	A_{N4}	5.85 ± 0.01	144.44 ± 0.19
A_{C5}	227.86	5.65 ± 0.03	226.44 ± 0.66	0.62	A_{N5}	5.67 ± 0.02	220.61 ± 0.08
A_{C6}	273.31	5.43 ± 0.01	271.51 ± 0.11	0.66	A_{N6}	5.44 ± 0.01	270.11 ± 0.14
B	0.00	6.71 ± 0.01	n.d.				
B_{C1}	34.24	6.43 ± 0.03	33.83 ± 0.68	1.20	B_{N1}	6.45 ± 0.01	40.03 ± 0.14
B_{C2}	68.46	6.20 ± 0.01	67.82 ± 0.14	0.93	B_{N2}	6.23 ± 0.02	68.09 ± 0.30
B_{C3}	114.06	6.01 ± 0.02	113.30 ± 0.82	0.67	B_{N3}	6.05 ± 0.01	106.71 ± 1.48
B_{C4}	182.37	5.82 ± 0.01	180.76 ± 0.16	0.88	B_{N4}	5.81 ± 0.03	145.77 ± 0.22
B_{C5}	205.12	5.66 ± 0.01	202.94 ± 0.22	1.06	B_{N5}	5.67 ± 0.01	221.01 ± 0.12
B_{C6}	273.31	5.45 ± 0.02	272.19 ± 0.25	0.41	B_{N6}	5.43 ± 0.01	271.00 ± 0.15
C	0.00	6.78 ± 0.01	n.d.				
C_{C1}	53.59	6.49 ± 0.02	52.91 ± 0.30	1.27	C_{N1}	6.42 ± 0.01	39.04 ± 0.15
C_{C2}	80.36	6.23 ± 0.02	79.81 ± 0.41	0.68	C_{N2}	6.20 ± 0.02	70.02 ± 0.41
C_{C3}	107.12	6.02 ± 0.01	106.07 ± 0.71	0.98	C_{N3}	6.04 ± 0.01	106.95 ± 1.60
C_{C4}	160.60	5.87 ± 0.01	158.29 ± 0.05	1.44	C_{N4}	5.88 ± 0.01	144.02 ± 0.25
C_{C5}	205.12	5.66 ± 0.01	203.35 ± 0.46	0.86	C_{N5}	5.66 ± 0.01	220.98 ± 0.15
C_{C6}	285.16	5.45 ± 0.01	280.54 ± 0.85	1.62	C_{N6}	5.47 ± 0.01	270.99 ± 0.14

n.d. not detected

concentration can be observed during changes in season and diet (Gaucheron 2005).

Milk samples A, B and C had 44.40, 53.20 and 49.67 mg/100 mL of sodium, respectively, all of which are within the natural range for milk (Table 2).

The content of sodium, as detected by atomic absorption spectroscopy, ranged from 53.87 to 110.97 mg/100 mL in the chemically acidified samples (A_{C1}–A_{C6}, B_{C1}–B_{C6}, C_{C1}–C_{C6}) and from 51.97 to 93.05 in the bacterially acidified samples (A_{N1}–A_{N6}, B_{N1}–B_{N6}, C_{N1}–C_{N6}), as shown in Table 2. These results show that the addition of a small amount of sodium hydroxide to samples A_{C1}, B_{C1}, B_{C2}, C_{C1}, A_{N1}, B_{N1} and C_{N1} cannot be detected because the sodium content is within the natural range of milk. Therefore, it is difficult to detect illegal additions of sodium hydroxide in weakly acidic milk of poor quality.

Lactic Acid Determination and Method Validation

Good separation of the lactic acid peak was obtained using the previously described conditions, and the retention time was found to be 4.2 min (Fig. 1). The validation parameters consisted of the linearity range, precision and limits of detection and quantification (Ribani et al. 2004). A high linearity ($R^2 > 0.999$) was obtained, and the relative standard deviation (RSD) of the peak's area was found to be 0.092% for intraday and 0.123% for interday, indicating good precision. The limit of detection (LOD) and limit of quantification (LOQ) were calculated to be 0.1 and 1 mg/L, respectively, indicating the high sensitivity of the system. The LOD and LOQ are defined as the minimal concentrations of the analyte that produce a peak height three times and ten times the noise baseline, respectively.

In all untreated samples (A, B and C) submitted to HPLC analysis, lactic acid was not detected (Table 3). Generally, in fresh milk, the amount of lactic acid ranges from 0.4 to a maximum of 3 mg/100 mL (Alais 2000), (Ling 1951).

Chemically acidified samples after neutralisation (A_{C1}–A_{C6}, B_{C1}–B_{C6}, C_{C1}–C_{C6}) showed a lactic acid content ranging from 33.82 to 280.54 mg/100 mL. Accuracy, which is a measure of the degree of closeness of a measured or calculated value to its actual value, always showed values of less than 2% (Table 3). The developed method, therefore, was precise and accurate.

To verify the performance of the proposed method in bacterially acidified milk after neutralisation, the samples A_{N1}–A_{N6}, B_{N1}–B_{N6}, C_{N1}–C_{N6} were submitted to HPLC analysis (Table 3). In samples slightly acidified to pH 6.4 (A_{N1}, B_{N1},

C_{N1}), the lactic acid content varied from 39 to 40 mg/100 mL. Considering that the maximum content of lactic acid in milk is 3 mg/100 mL, the developed method is able to detect the precise concentration of the developed lactic acid, which leads to a slight lowering of the pH of milk.

Conclusion

Sodium determination to detect the addition of sodium hydroxide in weakly acidified milk is not adequate because the addition of a small amount of sodium hydroxide does not affect the natural variation of sodium in milk. Sodium determination can only be used to detect overneutralised milk.

Conversely, determination of lactic acid by HPLC also allows the detection of added hydroxides in slightly acidic milk with pH values around 6.4, which showed a lactic acid content of approximately 40 mg/100 mL in our sample. This new method allows to detect the neutralisation via determination of lactic acid produced from lactose by milk bacteria. The validation parameters showed high linearity ($R^2 > 0.99$), good precision (RSD $\leq 0.123\%$) and high sensitivity (LOD 0.1 mg/L and LOQ 1 mg/L). The developed method is simple and fast, and sample preparation just requires acidification, dilution and filtration before injection.

Compliance with Ethical Standards

Conflict of Interest Alessandra Aiello declares that she has no conflict of interest. Fabiana Pizzolongo declares that she has no conflict of interest. Nadia Manzo declares that she has no conflict of interest. Raffaele Romano declares that he has no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent Not applicable.

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