Detection of calcium based neutralizers in milk and milk products by AAS

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Abstract Current study was carried out with the intent to standardize detection and estimation method for calcium (Ca) based neutralizers in milk and milk based indigenous products (*khoa* and *paneer*) using atomic absorption spectroscopy (AAS). Neutralized milk, *khoa* and *paneer* samples were prepared using milk with developed acidity to which calculated quantity of neutralizer (Ca based) was added. Rosalic acid test results get masked at times due to developed acidity which neutralizes the alkalinity imparted by neutralizer and hence gives false result with time in neutralizer added samples. Atomic absorption spectroscopy proved to be an accurate estimate which could detect the abnormal rise in mineral concentration even with slight addition of neutralizers in comparison with control milk and milk products. Formalin, which is a commonly used preservative in milk samples for chemical analysis, did not have any significant impact on estimation of calcium in the neutralized milk during storage.

Keywords AAS · Neutralizer · Indigenous products ·
Khoa · *Paneer* · Formalin

Introduction

Milk is a liquid secreted by the mammary gland for the nourishment of newly born containing water, fat, protein, lactose and minerals. Persevering unhygienic conditions, favourable circumstance for microbial growth, poor milk quality, lack of proper facilities to transport milk from production centres to the processing plants soon after milking and non availability of cold storage leads to development of acidity which further reduces the shelf life of milk. Middlemen add neutralizers like alkali bicarbonates, carbonates and hydroxides which improve the shelf life of milk by neutralizing the developed acidity. Addition of neutralizers can cause increased mineral concentration in body fluids and soft organs leading to kidney stone development and commercial preparation of neutralizers might even be contaminated with heavy metals like arsenic, lead, etc. Continuous use of such milk and milk product may cause health hazards (Satoskar et al. 2000). Since the frequency and quantity of milk consumed by infants and children are much more compared to adults, the health risk is more for them (Mohan et al. 2001). Hence, use of neutralizers in milk is prohibited as per Food Laws (FSSAI 2012).

Many methods have been developed for the detection of neutralizers in milk such as rosalic acid test, alkalinity of ash and direct and indirect methods based on principles of colorimetry, ionometry, potentiometry and titrimetry. However, the addition of neutralizers in small quantities and neutralization of added neutralizers by developed acidity hinder their detection using these methods.

In India, various indigenous milk products like *khoa* and *paneer* are widely consumed. *Khoa*, a heat desiccated milk product, is a major intermediate base for a variety of sweets like *burfi*, *peda*, *kalakand*, *gulabjamun*, etc. *Paneer* is a product similar to acid coagulated soft cheese, prepared by coagulating buffalo or blend of buffalo and cow milk and pressing the resulting coagulum (Khan and Pal 2010).

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Atomic absorption spectrophotometry (AAS) is commonly used in many analytical laboratories for estimation of minerals in water and in acid digest of sediment or biological materials (Ballance 1996). Dry ashing (Juarez and Martin-Hernandez 1983; Pollman 1991) or wet digestion (Suddendorf and Cook 1984; Vaessen and Van de Kamp 1990) is used to destroy organic material in dairy products before analysis. In AAS, a fine mist of digested sample solution is aspirated into the flame at a particular wavelength. The decrease in intensity of the radiation from the hollow cathode lamp due to absorption by specific atoms in the flame is measured (Wehr and Frank 2004).

So far, no quantitative method for neutralizers in milk or indigenous dairy products has been established. Therefore, in the present study, AAS was employed to determine addition of neutralizers in milk and milk products, based on the principle that addition of neutralizers would increase the mineral content of products well above the range present in milk. In addition, the results were compared with those of rosolic acid test results which is an approved test by BIS. Formalin being used for the preservation of samples stored for chemical analysis was evaluated for its impact on AAS readings.

Materials and methods

Apparatus and glasswares

The determination of calcium was carried out using a Model AA-7000 atomic absorption spectrophotometer (Shimadzu, Japan). Muffle furnace (Modern Industrial Corporation, Bombay, India) was used for dry digestion, double distillation unit for double distilled water (Bhanu Scientific Instrument company, Bangalore, India). Silica crucible, volumetric flasks (50, 100 and 1,000 ml) and auto pipettes (0.1 to 1.0 ml and 5.0 ml) were used during sample preparation. Calcium was determined using an air-acetylene flame. Acetylene gas of standard commercial 'A' grade (Talwar Gas Agency, Karnal, Haryana, India); B.O.D Incubator (NSW-152, Super Delux Automatic, Narang Scientific Works PVT Ltd., Mayapuri, New Delhi, India), micro processor controlled oven (Metrex Scientific Instruments (P) Ltd., New Delhi, India), electronic balance (Model No. CX65S, Citizen, India), electric heater (Pride hot plates Producers, New Delhi, India), fume hood (Sandeep Instruments and Chemicals, New Delhi, India) were used in the study.

Reagents

All chemicals and reagents used were at least of analytical-reagent grade and procured from Sigma Aldrich (St. Louis,

MO, USA). Glass double distilled water with a resistivity of 10 MΩ cm was used to prepare all samples and standards.

Conc. HCl (35.5 to 36.5 % min assay); conc. HNO₃ (69.71 % min assay); potassium chloride (Qualigen Fine Chemicals, Mumbai, India); sodium chloride of AR grade (Himedia Laboratories, Mumbai, India); calcium carbonate (99.9 % pure); 0.1 N HCl solution; phenolphthalein indicator (1 % w/v); rosolic acid solution (1 % w/v); stock standard solution of calcium (1,000 ppm as Ca); citric acid of AR grade (Shah Scientific, India); chromic acid of AR grade (Qualigens Fine Chemicals, Mumbai, India) were the reagents used.

LaCl₃ solution was prepared by mixing lanthanum chloride (5 g) and 5 ml of concentrated HNO₃, 5 ml double distilled water and the volume was made up to 100 ml with double distilled water.

Neutralizer

Calcium based neutralizer used in the present investigation was calcium hydroxide of AR grade procured from Qualigens Fine Chemicals, Mumbai, India.

Cleaning of glasswares

All glasswares used for AAS analysis were washed properly and soaked in chromic acid solution for 24 h, rinsed with deionised water, dipped in 3 % EDTA solution for 24 h and rinsed with deionised water again before use.

Standard solutions

Stock standard solution

1.269 g calcium carbonate (99.9 %) was mixed with 20 ml water and 20 ml concentrated HNO₃ was added to it and the volume made up to 250 ml.

Standard solutions

Five standards (1, 2, 4, 6 and 8 ppm) were prepared for calibration curve using calcium carbonate stock solution. The stock solutions were diluted according to concentration of standards and lanthanum chloride was added to all standards at 500 µg ml⁻¹.

Calibration

The instrument was calibrated before every analysis and the standard solutions were prepared just before analysis. A blank sample and standard solutions of known concentrations were measured for absorbance.

Sample preparation

Raw fresh milk used throughout the study was collected from cattle yard, National Dairy Research Institute (NDRI), Karnal, India. Samples were standardized (Cow milk- 3.5 % fat, 8.5 % SNF; Buffalo milk-6 % fat, 9 % SNF, Mixed milk- 4.0 % fat, 8.5 % SNF) and after allowing to stand at 37 °C for development of acidity to required level. The samples were neutralized with calculated amount of calcium hydroxide (Ca(OH)_2) to bring down the acidity to 0.14 % L.A.

Khoa was prepared in experimental dairy plant, NDRI, Karnal, India. Control *khoa* samples were prepared by the method of De and Roy (1953) using 5 l of milk. Neutralized *khoa* samples were prepared by using milk (allowed to stand for development of acidity) neutralized using calculated quantity of Ca(OH)_2 .

Paneer was prepared in experimental dairy plant, NDRI, Karnal, India. Control *paneer* samples were prepared by the method of Vishweshwaraiah and Anantakrishnan (1985). Neutralized *paneer* samples were prepared by using milk allowed to stand at 37 °C for development of acidity and neutralized subsequently.

Sample preparation for AAS analysis

Ash was prepared by taking 1 ml of milk samples in silica crucibles followed by heating on hot plate to complete charring. The charred samples were incinerated in a muffle furnace at 550 °C for 3–4 h till white ash was obtained. In case of *khoa* and *paneer*, 1 g of crushed samples were taken and charred followed by ashing in muffle furnace at 550 °C for 6 h.

Dry digestion HNO_3 method (DD HNO_3)

The dishes with samples were cooled in a desiccator to room temperature and 1 ml concentrated nitric acid was added to dissolve the ash, contents were then transferred to volumetric flask and volume was made up to dilution so that the concentration falls within the range of standard solutions concentration.

Dry digestion HCl method (DD HCl)

The dishes were cooled in a desiccator to room temperature and 1 ml concentrated hydrochloric acid was added to dissolve the ash, contents were then transferred to volumetric flask and volume was made up so that the concentration falls within the concentration of standard solutions.

Samples for studying impact of formalin on calcium content of samples

Formalin was added to milk, crushed *khoa* and crushed *paneer* at the rate of 0.4 % and samples were stored at 37 °C in tightly stoppered plastic containers. Samples were withdrawn every 7th day for analysis of milk and every 5th day for analysis of *khoa* and *paneer* samples to study the impact of formalin on calcium concentration.

Sample preparation for rosolic acid test

Rosolic acid test for control and neutralized milk was performed by the procedure of BIS (1961). The total solid content of *khoa* and *paneer* were estimated by the method as described in IS:SP:18 (Part XI, 1981) and the samples for rosolic acid test were prepared by dilution to a total solid content equivalent to milk (~12.5 %) using deionised water.

Statistical analysis

Data reported were expressed as mean values with standard errors. In experiments, carried out with 15 replicates, single way analysis of variance (ANOVA) with a subsequent least significance difference ($P < 0.05$) in the mean values as described by Snedecor and Cochran (1989) was applied.

Results and discussion

Milk

On analyzing by DD HCl and DD HNO_3 method in AAS (Table 1), control cow milk showed a calcium content of 1,164 and 1,186 ppm, respectively which were in alignment with the previous results reported by Fox and Mcsweeney (1998) as 1,000–1,400 ppm calcium content in cow milk. Neutralizer free buffalo milk exhibited a calcium concentration of 1,753 and 1,771 ppm by DD HCl and DD HNO_3 method, respectively. The average calcium content reported by Mathur et al. (1999) in cow milk and buffalo milk was 1,230 and 1,750 ppm, respectively. These findings are in conjunction with the results obtained in the present study for control milk samples. Addition of calculated amount of Ca(OH)_2 to cow milk and buffalo milk i.e., 356, 534, 648 mg/L as calcium increased the overall calcium content proportionately, enabling the neutralizer addition to milk detectable even at minor quantities of less than a gram per litre. Calcium content in control samples (both cow and buffalo milk) using DD HCl and DD HNO_3 were non significantly different ($P < 0.05$), whereas in case of neutralized samples the difference was significant ($P < 0.05$). When neutralizer was added at a lower level (356 mg calcium per litre

milk of buffalo and cow milk), DD HNO₃ method yielded marginally better accuracy i.e., 96.36 and 96.26 % against the recovery of 95.82 and 95.44 % using DD HCl method, respectively. Both the methods differed non significantly when calcium was added at a concentration of 534 mg per litre milk samples. In case of mixed milk, the calcium concentration of control milk was 1,576 and 1,565 ppm which proportionately increased to 2,258 and 2,203 ppm for neutralized milk (with added calcium of 648 mg per litre), respectively using DD HNO₃ and DD HCl methods.

Formalin treated control and neutralized milk samples were analyzed for calcium concentration using AAS by DD HNO₃ method (Table 2). Non significant difference ($P<0.05$) in the calcium content of neutralized samples throughout the storage period of 28 days when analyzed every 7th day was observed. Also similar trend of proportionate increase in the calcium content with Ca(OH)₂ addition was observed which delineated no substantial impact of formalin treatment on the calcium concentration and hence, detection of added neutralizer in the samples during storage preserved with formalin posed no problem.

Rosalic acid test carried out on milk samples treated with different concentration of Ca(OH)₂ (5, 10, 20, 30, 50 mg/100 ml milk) exhibited sensitivity of the test for a concentration of 20 mg

Ca(OH)₂ per 100 ml milk sample onwards when the test was carried out within 20 min of addition of neutralizers but at all concentrations up to 50 mg the test was negative when carried out after 5 h of addition of neutralizer, indicating its neutralization by the developed acidity. These findings were in alignment with previous observations of Gunnery (1979) that the sensitivity of rosolic acid test was altered by developed acidity.

Khoa

Khoa samples when subjected to DD HNO₃ and DD HCl methods demonstrated non significant difference ($P<0.05$) in calcium content. The calcium content (Table 1) of control samples (7,362 ppm by DD HCl method and 7,530 ppm by HNO₃ method) were slightly higher than the previous findings reported by Boghra and Mathur (1991) as value ranging between 6,300 and 7,170 ppm, respectively. The calcium content of neutralized samples increased with addition of neutralizers.

The rosolic acid test when conducted for homogenous mixture of *khoa* samples displayed sensitivity for Ca(OH)₂ above a concentration of 20 mg (added in milk during *khoa* preparation).

Table 1 Calcium content (ppm) in control and neutralized milk samples estimated by atomic absorption spectrophotometer by using dry digestion methods with HNO₃ and HCl

Samples	Digestion method			
	HNO ₃	% Recovery	HCl	% Recovery
Milk samples				
Control milk samples				
Buffalo milk	1771.3±34.80 ^{aE}		1753.0±50.36 ^{aC}	
Cow milk	1186.6±23.34 ^{aA}		1164.3±12.25 ^{aA}	
Mixed milk	1576.6±50.44 ^{aB}		1565.2±38.12 ^{aB}	
Ca(OH) ₂ added per litre milk				
Buffalo milk+658 mg Ca(OH) ₂	2142.6±17.14 ^{bF}	96.4	2075.3±51.34 ^{aF}	95.8
Cow milk+658 mg Ca(OH) ₂	1602.6±27.28 ^{bC}	96.2	1546.6±27.84 ^{bC}	95.4
Buffalo milk+987 mg Ca(OH) ₂	2443.3±52.06 ^{aF}	96.1	2410.1±45.63 ^{aH}	95.6
Cow milk+987 mg Ca(OH) ₂	1690.2±15.28 ^{aD}	95.4	1718.1±50.76 ^{aE}	94.9
Mixed milk+1,200 mg Ca(OH) ₂	2258.3±26.03 ^{bG}	95.8	2203.3±22.19 ^{aG}	97.3
Khoa samples				
Control	7,530±175 ^{aA}		7,362±112 ^{aA}	
Ca(OH) ₂ added per 5 kgs milk				
<i>Khoa</i> +1,644 mg Ca(OH) ₂	8,242±39 ^{aB}	95.4	8103.7±66 ^{aB}	94.4
<i>Khoa</i> +3,288 mg Ca(OH) ₂	9,241±34 ^{aC}	96.3	9,129±49 ^{aC}	92.8
<i>Khoa</i> +4,932 mg Ca(OH) ₂	10,165±58 ^{aD}	94.1	10132.6±58 ^{aD}	93.4
<i>Khoa</i> +6,572 mg Ca(OH) ₂	11,045±74 ^{aE}	93.5	10972.3±107 ^{aE}	92.1
Paneer samples				
Control	4,975±34 ^{bA}		4,867±73 ^{aA}	
Ca(OH) ₂ added per 2 kgs milk				
<i>Paneer</i> +656 mg Ca(OH) ₂	5,029±87 ^{bB}	94.3	4,953±61 ^{aB}	94.8
<i>Paneer</i> +987 mg Ca(OH) ₂	5,312±53 ^{aC}	96.4	5,261±91 ^{aC}	97.4

HNO₃ nitric acid; HCl
Hydrochloric acid

Means within rows (a, b) and columns (A, B, C) with different superscript letters are significantly different ($P<0.05$) ($n=15$)

Table 2 Calcium content (ppm) in formalin treated control and neutralized milk samples stored at room temperature (37 °C) estimated by atomic absorption spectrophotometer

Formalin treated samples	Storage period (in days)				
	0	7th	14th	21st	28th
Cow milk	1,132±23 ^{bA}	1,152±42 ^{aA}	1,155±44 ^{aA}	1,143±25 ^{aA}	1,169±23 ^{aA}
Cow milk+Ca(OH) ₂ 50 mg	1,213±35 ^{aB}	1,198±37 ^{aB}	1,201±26 ^{aB}	1,176±33 ^{aB}	1,197±39 ^{aB}
Cow milk+Ca(OH) ₂ 100 mg	1,259±31 ^{aC}	1,271±29 ^{aC}	1,253±38 ^{aC}	1,265±36 ^{aC}	1,276±27 ^{aC}
Buffalo milk	1,680±31 ^{aA}	1,723±57 ^{bcA}	1,746±44 ^{cA}	1,689±51 ^{abA}	1,759±38 ^{cA}
Buffalo milk+Ca(OH) ₂ 50 mg	1,780±53 ^{aB}	1,802±60 ^{aB}	1,811±39 ^{aB}	1,798±55 ^{aB}	1,792±47 ^{aB}
Buffalo milk+Ca(OH) ₂ 100 mg	1,842±41 ^{aC}	1,856±42 ^{aC}	1,848±42 ^{aC}	1,862±46 ^{aC}	1,870±50 ^{aC}

Calcium based neutralizer, Ca(OH)₂, added per 100 ml of sample

Means within rows (a, b) and columns (A, B, C) with different superscript letters are significantly different ($P<0.05$) ($n=15$)

Khoa samples when treated with formalin (Table 3) and analyzed for calcium concentration displayed a slight but statistically significant increase ($P<0.05$) with storage period in case of control sample and the concentration was not influenced with storage period in Ca(OH)₂ added samples. In addition, a proportionate increase with increase in the concentration of Ca(OH)₂ addition was also observed, tracing the presence of neutralizers in samples.

Paneer

Paneer samples studied for calcium concentration by DD HNO₃ and DD HCl method (Table 1) revealed a calcium concentration of 4,975 ppm and 4,867 ppm, respectively which was similar to the results reported by Boghra and Mathur (1991) as 4,120 ppm to 4,750 ppm in control *paneer*.

The pro rata rise in calcium concentration ($P<0.05$) with the incorporation of Ca(OH)₂ ascertained the precision of the method.

Rosalic acid test carried out for crushed *paneer* samples was negative for control as well as neutralizer added samples as the pH of the product commands the test results and pH of *paneer* sample is around 5.3 due to acid addition during preparation which suppresses any increase in pH due to neutralizer addition.

Formalin treated control *paneer* samples as depicted in Table 3 showed non significant ($P<0.05$) difference in calcium concentration throughout the storage period. Addition of formalin to neutralized samples did not impact the calcium concentration and thereby did not hinder the proportionate increase in calcium concentration with addition of Ca(OH)₂ addition.

Table 3 Calcium content (ppm) in formalin treated control and neutralized samples (stored at 37 °C) of *Khoa* and *Paneer* estimated by atomic absorption spectrophotometer

Samples	Storage period (days)				
	0th	5th	10th	15th	20th
<i>Khoa</i>					
Control	7,580±101 ^{aA}	7,800±113 ^{cA}	7,620±101 ^{abA}	7,760±107 ^{cA}	7,714±95 ^{bcA}
<i>Khoa</i> +Ca(OH) ₂ 1,644 mg	8,318±104 ^{abB}	8,264±96 ^{aB}	8,426±89 ^{bB}	8,341±118 ^{abB}	8,289±107 ^{aB}
<i>Khoa</i> +Ca(OH) ₂ 3,288 mg	9,231±110 ^{abC}	9,186±126 ^{aC}	9,314±111 ^{bC}	9,451±107 ^{cC}	9,302±114 ^{bC}
<i>Khoa</i> +Ca(OH) ₂ 4,932 mg	10,121±125 ^{aD}	10,211±114 ^{aD}	10,341±129 ^{cD}	10,187±120 ^{abC}	10,297±121 ^{bcD}
<i>Khoa</i> +Ca(OH) ₂ 6,572 mg	10,985±108 ^{aE}	11,062±109 ^{aE}	11,131±112 ^{bE}	10,964±103 ^{aE}	10,943±119 ^{aE}
<i>Paneer</i>					
Control	4,932±221 ^{aA}	4,945±212 ^{aA}	4,953±196 ^{aA}	4,938±201 ^{aA}	4,923±200 ^{aA}
<i>Paneer</i> +Ca(OH) ₂ 656 mg	5,106±208 ^{aB}	5,121±231 ^{aB}	5,116±207 ^{aB}	5,127±177 ^{aB}	5,112±212 ^{aB}
<i>Paneer</i> +Ca(OH) ₂ 987 mg	5,467±205 ^{aC}	5,453±218 ^{aC}	5,475±225 ^{aC}	5,462±229 ^{aC}	5,458±198 ^{aC}

Calcium based neutralizer, Ca(OH)₂, added per 5 kg of milk from which *khoa* and *paneer* samples were prepared

Means within rows (a, b) and columns (A, B, C) with different superscript letters are significantly different ($P<0.05$) ($n=15$)

Conclusion

Atomic absorption spectroscopy carried out using DD HNO_3 as well as DD HCl method showed similar calcium concentration in milk when the calcium level was 534 mg/L or above. Also the addition of formalin at a rate of 0.4 % (v/v or v/w) did not impact the results. Hence, AAS can be considered as a reliable method for detection of calcium based neutralizers in *paneer*, where rosolic acid fails to detect the added neutralizer. In case of milk and *khoa* AAS is better than existing detection method viz. rosolic acid test in terms of sensitivity and accuracy for the detection of calcium based neutralizers added even in lower quantities or in samples stored for longer time.

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