International Journal of Chemical Studies

P-ISSN: 2349–8528
E-ISSN: 2321–4902
IJCS 2017; 5(5): 552-556

Received: 17-07-2017
Accepted: 18-08-2017

Chaudhari Priyankaben Rameshbhai
Former Scholar, Dairy Chemistry Department, SMC College of Dairy Science, Anand Agricultural University, Anand, Gujarat, India

AI Shaikh
Assistant Professor, Dairy Chemistry Department, SMC College of Dairy Science, Anand Agricultural University, Anand, Gujarat, India

SI Patel
Assistant Professor, Dairy Chemistry Department, SMC College of Dairy Science, Anand Agricultural University, Anand, Gujarat, India

Kakade Pooja Vilasrao
Former Scholar, Dairy Chemistry Department, SMC College of Dairy Science, Anand Agricultural University, Anand, Gujarat, India

KD Aparnathi
Professor & Head, Dairy Chemistry Department, SMC College of Dairy Science, Anand Agricultural University, Anand, Gujarat, India

Correspondence
AI Shaikh
Assistant Professor, Dairy Chemistry Department, SMC College of Dairy Science, Anand Agricultural University, Anand, Gujarat, India

Comparative appraisal of qualitative tests for detection of urea in milk

Chaudhari Priyankaben Rameshbhai, AI Shaikh, SI Patel, Kakade Pooja Vilasrao and KD Aparnathi

Abstract
Qualitative tests for the adulterant detection are advantageous because of it being selective in nature. Because of procedural variations in these tests, the efficiency of the test for detection of urea differs. Three reported tests for detection of urea adulteration in milk were compared for their efficiency, ease of performance and rapidity. The suitable methods found for urea detection were BIS (1960) in case of DMAB test, Paradkar *et al*. (2000) in case of urease test and Vishweshwar and Krishnaiah (2005) in case of phenol test. DMAB test was found to be most simple to perform, rapid and had better stability of colour, whereas urease test was found to be most sensitive with limit of detection of 0.06% urea concentration in milk.

Keywords: milk, adulteration, urea, qualitative test

Introduction
Milk plays a crucial role in the diet of humans and is considered as one of the essential food commodities. Milk products have high nutritional value and are consumed all around the world, playing a fundamental role in international commerce and giving milk a great economic importance [1]. However, individuals aiming at higher profits and flouting ethical values renders milk susceptible to several types of adulteration [2]. Recent reports have revealed the use of numerous adulterants in milk intended for either raising the quantity (e.g. water), increasing the compositional values (e.g. urea, sucrose, starch, etc.) or increasing the shelf life (e.g. neutralizers and preservatives)[3].

The most widely practiced approach of adulterating milk is to add water in it and subsequently adding urea to raise solid not fat (SNF). Urea is a normal constituent of milk and amounts to about 55 per cent of the total non-protein-nitrogen in milk [4]. Additionally urea being nitrogenous compound, will give false high level of protein if analyzed by Kjeldahl method [4, 5]. In India, the addition of external urea to the milk is not permitted legally under the FSSAI [4]. However, as per FSSAI 2006, the urea content in milk should not be more than 70 mg/100 ml [6, 7, 8]. Urea is one of the major ingredients of synthetic milk along with caustic soda, detergent, sugar and foreign fats. Adulteration of natural milk with synthetic milk increases the level of urea and it may causes toxicological hazards [8, 9]. An increase in urea concentration causes renal failure such as acute or chronic urinary tract obstruction with shock, burns, dehydration, and gastrointestinal bleeding [10]. Estimation of urea concentration in milk may serve as a tool for checking the menace of adulteration of natural milk with synthetic milk [8]. There are several methods reported for urea detection and estimation in milk such as chemical method, enzymatic methods, infrared (IR) spectroscopy, etc. [4]

The presence of added urea in milk can be detected using qualitative tests like DMAB (Para-dimethylamino benzaldehyde) test [8, 11-17] phenol test [17, 18] and urease [4, 13, 15, 17, 19-24].

Reported procedures for these chemical tests differs from author to author making it challenging for the users to select a reliable test and a feasible procedure with respect to sensitivity and precision in results. Therefore, the present study was carried out with the objective to establish a comparative appraisal between different qualitative tests reported for the detection of urea in milk.

Materials and Methods

Milk sample

Raw milk samples were procured from dairy farm of Anand Agricultural University, Anand. Milk Samples were prepared by spiking the raw milk with urea at the suitable level for a particular test.

Chemicals and reagents

DMAB (Loba Chemie Pvt. Ltd.), Trichloroacetic acid (Loba Chemie Pvt. Ltd.), Phenol red (Central Drug House (P) Ltd.), Urease enzyme (Sigma Chemicals Co.), Bromothymol blue (Qualigens Fine Chemicals), Sodium hydroxide (Qualigens Fine Chemicals), Sodium hypochlorite (SD Fine Chemicals Ltd.), Phenol (Spectrochem Pvt. Ltd.) and Urea (SD Fine Chemicals Ltd.).

Test procedures used for detection of adulterants in milk

1. DMAB test

A) Test reported by BIS (2006)[12]

In a test tube 5 ml of the suspected milk sample was taken. To it, 5 ml of the 1.6% w/v DMAB reagent was added and mixed well. In another test tube 5 ml of control milk sample (known to be free from added urea) and 5 ml of DMAB reagent were taken and mixed. The presence of urea was confirmed by appearance of deep yellow colour as compared to control sample, which gave a pale yellow colour due to presence of natural urea content. Same procedure was also reported by various authors[13,14,15,16,17,22].

B) Test reported by BIS (1960)[11] and Sharma *et al.* (2012)[8]

Equal quantity of milk and 24% TCA was taken in a glass stoppered test tube. Contents were mixed and filtered with Whatman No. 42 filter paper. In another test tube 3 ml of filtrate was taken and 3 ml of 1.6% DMAB reagent was added. The occurrence of distinct yellow colour indicated the presence of added urea in milk sample whereas slight yellowish colour was developed in control sample.

2. Urease test

A) Test reported by Paradkar *et al.* (2000) [4]

Paradkar *et al.* (2000) [4] suggested use of crude enzyme extract for detection of urea in milk. Crude enzyme extract was prepared by dissolving 10 g soya flour in 40 ml distilled water. After 10 minutes contents were filtered to get clear solution. In a test tube 5 ml of milk sample was taken and pH was adjusted at 7. In test tube 2 drop of phenol red (0.01 g/ 100 ml) and 1 ml of crude extract enzyme was added. In presence of added urea colour changed from orange to pink within 2 minutes.

B) Test reported by DGHS (2005) [19]

In a glass stoppered test tube 10 ml of suspected milk was taken and small quantity of soybean slurry was added into it. A strip of moistened red litmus paper was inserted into test tube and while adding the contents care was taken that paper did not touch the milk and sides of the test tube. Mouth of test tube was covered with a cork or stopper to make it air tight. The contents were mixed gently. The test tubes were kept undisturbed for 5-10 minutes and colour change in litmus paper was observed. The colour of red litmus paper changed to blue in presence of added urea whereas in control sample no change in colour was observed. NDDB (2009) [15] and Srivastava (2010)[21] reported the same procedure.

C) Test reported by Anon. (2006) [20]

In a test tube 5 ml of milk sample was taken. To it, 0.2 ml urease solution and 0.1 ml Bromothymol Blue (BTB) solution were added. Appearance of blue colour after 10-15 minutes indicates the presence of urea in milk. Normal milk shows faint blue colour due to natural urea present in milk. Many authors have reported the same procedure[13,17,22,23].

D) Test reported by DDDM (2013)[24]

In a test tube 5 ml of suspected milk sample was taken and 20 mg of soybean powder and 2 drops of 0.5 % aqueous solution of bromothymol blue were added to it. Development of blue colour after 10 minutes confirmed the presence of added urea into the sample.

3. Phenol test

A) Test reported by Vishweshwar and Krishnaiah (2005) [18]

In a conical flask 5 ml milk sample was taken followed by 5 ml of sodium acetate buffer or trichloroacetic acid solution (24%). The flask containing sample was heated for 3 minutes in boiling water bath (no heating if trichloroacetic acid is used). Filtering is done through Whatman no. 42 filter paper and 1 ml filtrate is collected in a test tube. One ml of sodium hydroxide solution (2%) was added to the filtrate, followed by 0.5 ml of sodium hypochlorite solution (2%) and finally 0.5 ml of 5% (w/v) phenol solution was added and contents were mixed well. Appearance of blue or bluish green colour indicates presence of added urea whereas pure milk remains colourless.

B) Test reported by Kamthania *et al.* (2014) [18]

Five ml of suspected milk sample was treated with equal quantity of trichloroacetic acid (24% TCA) to precipitate fat and proteins of milk and filtrate was collected. One ml filtrate was taken into a test tube and 0.5 ml sodium hypochlorite (2% w/v), 0.5 ml sodium hydroxide (2% w/v) and 0.5 ml phenol solution (5% w/v) were added to test tube containing filtrate. Contents were mixed well and colour change was observed. A characteristic blue or bluish green colour develops in presence of added urea whereas pure milk remains colourless.

Results and Discussion

The qualitative and/or quantitative variations are reported in DMAB test, urease test and phenol test used for detection of urea in milk. Therefore, variations within a particular test were evaluated to check their effect on the test results.

1. Evaluation of variations in DMAB test

The use of DMAB test for detection of urea in milk has been reported by BIS (1960)[11], BIS (2006) [12], Dairyforall (2006) [13], Draaiyer *et al.* (2009) [14], NDDB, (2009) [15], FSSAI (2012) [16], Sharma *et al.* (2012) [8] and Kamthania *et al.* (2014) [17]. The sample of milk was prepared by addition of 0.2 g of urea per 100 ml of milk. The sample of milk was subjected to detection of urea by the DMAB test along with the qualitative and quantitative variations and the results obtained are presented in this Table 1.

Table 1: Comparative appraisal of DMAB test for detection of urea in milk

Test	Variation in test	Observation
A	5 ml **milk** + 5 ml 1.6 % DMAB reagent [8,12,13,14,15,16,17,22]	• Ease in perform • Quick method • Colour clearly distinguished
B	Equal quantity of milk and 24% TCA + Filter + 3 ml **milk filtrate** + 3 ml 1.6 % DMAB Reagent[8,11]	• Coagulation and filtration required • Time needed to perform test

Reports depicts that there are two variation in DMAB test. In first variation DMAB test was directly performed in milk whereas another test was performed in filtrate which is obtained after treating the milk with TCA. After performing both the tests, positive result was obtained in both the variation and sensitivity of tests was found similar. However, test B required coagulation and filtration as well as more time consuming whereas test A was found to be relatively rapid. Hence, as per the obtained results and observations test A was found suitable among the two variation.

2. Evaluation of variations in urease test

Enzyme based urease test for detection of urea in milk has been reported by Paradkar *et al.* (2000) [4], DGHS (2005) [19], Anon. (2006) [20], Dairyforall (2006) [13], NDDB (2009) [15], Srivastava (2010) [21], Dixit (2012) [22], Singh *et al*. (2012) [23], DDDM (2013) [24] and Kamthania *et al*. (2014) [17]. The sample of milk was prepared by addition of 0.1 g of urea per 100 ml of milk. Qualitative and quantitative variations in urease test were studied for the milk sample and the results obtained are presented in Table 2.

Table 2: Comparative appraisal of urease test for detection of urea in milk

Test	Variation in test	Observation
A	5 ml milk + 2 drop phenol red + 1 ml soyabean crude extract enzyme [4]	▪ Ease in detection ▪ Quick result
B	10 ml milk + Add soybean slurry + Insert red litmus paper + Cover the test tube [15, 19, 21]	▪ Time consuming ▪ Fresh soyabean slurry needed
C	5 ml milk + 0.2 ml urease enzyme + 0.1 ml bromothymol blue solution [13, 17, 20, 22, 23]	▪ Faint blue colour was found ▪ Less sensitive
D	5 ml milk + 20 mg of soybean powder + 2 drops of bromothymol blue solution [24]	▪ Ease in detection ▪ Less sensitive

Based on the source of urease enzyme and procedural modifications, four variations have been reported for the detection of urea by urease test. Tests were performed and all the variations were studied for its sensitivity, rapidness and easefulness. From the above table, it can be concluded that test A is simple, sensitive as well as rapid. Test B also was found to be sensitive but in addition, was time consuming due to the requirement of slurry preparation and method was not fully standardized. Moreover, test C and D were simple to perform but less sensitive (LoD 0.1%) than the test A and B. In test C faint blue colour appeared after 15 min; which was improved if amount of urease enzyme added was increased to 1 ml. Considering the above observations for all the tests, it can be concluded that test A gives better confirmation of added urea in milk.

3. Evaluation of variations in Phenol test

Phenol test for the detection of urea in milk is reported by several authors and as a consequence, methodical variations results. As shown in Table 3, two variations in phenol test have been reported by Vishweshwar and Krishnaiah, (2005) [18] and Kamthania *et al*. (2014)[17]. The sample of milk was prepared by addition of 0.1 g of urea per 100 ml of milk and the qualitative and quantitative variations in the phenol test were studied using the prepared sample.

Table 3: Comparative appraisal of phenol test for detection of urea in milk

Test	Variation in test	Observation
A	Equal volume of milk and sodium acetate buffer + Boiling for 3 minutes + filter + 1 ml filtrate + 0.5 ml NaOCl + 0.5 ml NaOH + 0.5 ml Phenol solution : appearance of blue colour[18]	• Clarity in differentiation • Sensitive
B	5 ml milk + 5 ml 24% TCA + filter + 1 ml filtrate + 0.5 ml of 2% NaOCl + 0.5 ml of 2% NaOH + 0.5 ml of 5% Phenol solution : appearance of blue colour[17]	• Difficult to differentiate control and sample

In first variation DMAB test was directly performed in milk whereas another test was performed in filtrate which is obtained after treating the milk with TCA. After performing both the tests, positive result was obtained in both the variation and sensitivity of tests was found similar. However, test B required coagulation and filtration and hence time consuming whereas test A could be rapidly performed. Hence, as per the obtained results and observations test A was found suitable among the two variation.

4. Qualitative tests for detection of urea

The best suitable variation within the test was selected for the comparative evaluation of three available chemical test for the detection of urea in milk. For comparison of different qualitative tests, the milk samples were prepared by addition of urea at the rate of 0 (control), 0.01, 0.02, 0.04, 0.06, 0.08, 0.10, 0.15 and 0.20 (g per 100 ml milk) and were subjected to DMAB test, urease test and phenol test. The observations made on other relevant aspect of the test are summarised in Table 4.

Table 4: Comparative appraisal of qualitative tests for detection of urea in milk

Sr. No.	Test	Observation	LoD (g/100 ml)
1	DMAB	• Easy detection • Quick • Colour formation clearly distinguished • Stable colour • Lower sensitivity	0.20
2	Urease	• Simple to perform • Requires less time • Better sensitivity • Source of urease enzyme may affect result	0.06
3	Phenol	• Coagulation and Filtration required - time consuming • Hypochlorite reagent is unstable	0.06

The LoDs of DMAB test, urease test and phenol test were found to be 0.20, 0.06 and 0.06 g/100 ml respectively.

Therefore, among the different qualitative tests performed for detection of urea in milk, minimum level of detection was found in the urease test and phenol test.

According to the literature LoD for DMAB test is 0.20 per cent as reported by FSSAI (2012) [16] and Sharma *et al*. (2012) [8]. For urease test LoD reported by Paradkar *et al.* (2000) [4] is 0.06 per cent. The LoD for phenol test as reported by Vishweshwar and Krishnaiah (2005)[18] is 0.1 per cent. Thus LoDs for DMAB test and urease test found in present study are in accordance with reported LoDs in literature. However in case of phenol test LoD observed in present study was lower than the reported LoD in literature. The variation in LoD found in present study may be attributed to variations in composition of milk and variation in judgement of colour from person to person and other aspects such as chemical composition.

Amongst three qualitative tests attempted to detect urea in milk, greater sensitivity (lowest LoD) was observed in case of urease test followed by phenol test. DMAB test was found to be least sensitive but it has advantages of simplicity of performance, rapidity and better stability of colour. Urease test was simple to perform, better sensitivity and ease in distinction between the positive and negative result. However, the results may be affected by the type and quality associated to source of urease enzyme *i.e*. soybean or jack bean. Furthermore, the phenol test is more time consuming test as it requires coagulation and subsequent filtration. It has disadvantage in that, it uses hypochlorite reagent which is an unstable reagent and hence necessitates the use of fresh preparation very time. Based on the sensitivity of the test, stability of colour, convenience in performance and other relevant parameters of the test, DMAB test was found to be most suitable test for the detection of urea in milk.

Summary and Conclusion

In DMAB test two variations have been reported for the detection of urea in milk. In one of the procedure the test is to be performed directly in milk, whereas, in the second procedure the test is performed using serum obtained by coagulating the milk with TCA followed by filtration. The sensitivity of the test was found similar in both the cases. However, the test performed in milk was quick and simple to perform compared to that performed using milk filtrate.

Amongst the four variations reported for the detection of urea in milk by urease test, various procedures involves the use of phenol red and soy aqueous extract, red litmus paper and soybean slurry, bromothymol blue and jack bean meal and bromothymol blue and soy powder. After performing these all four tests, it was observed that the test involving use of phenol red and soy enzyme extract was found the most simple, sensitive, easy in detection and confirmative compared to the other tests.

Procedural differences in phenol test has been reported by authors where different reagents such as sodium acetate buffer and TCA are employed for the coagulation of milk. In the present study, these variations were compared and obtained results indicated that that phenol test performed using sodium acetate buffer was more accurate and sensitive compared to that using TCA.

Following the evaluation of variations in the tests for detection of urea in milk, suitable variation within the three tests was selected and the tests were compared for its best suitability for the detection of urea in milk. Among these tests DMAB test was found to be most simple to perform, rapid and had better stability of colour. Urease test was found to be most sensitive, however, wide variation in result has been encountered depending on the sources of enzyme. In addition, phenol test being simple to perform and had better sensitivity has limitation of consuming longer time to perform as filtration is involved in the method.

Reference

1. Astrid Hilding-Ohlsson, Jonathan A Fauerbach, Natalia J Sacco, Celina Bonetto M, Eduardo Cortón. Voltamperometric Discrimination of Urea and Melamine Adulterated Skimmed Milk Powder. Sensors (Basel), 2012; 12(9):12220-12234.
2. Souza SS, Cruz AG, Walter EHM, Faria JAF, Celeghini RMS, Ferreira MMC *et al*. Monitoring the authenticity of Brazilian UHT milk: A chemometric approach. Food Chemistry. 2011; 124:692-695.
3. Sukumaran MK, Hemanth Singuluri. Milk Adulteration in Hyderabad, India - A Comparative Study on the Levels of Different Adulterants Present in Milk. Indian Journal of Dairy Science. 2015; 68(2):190-193.
4. Paradkar MM, Singhal RS, Kulkarni PR. An approach to the detection of synthetic milk in dairy milk: 1. Detection of urea. *Int. J. Dairy Technol*., 2000; 53:87-91.
5. Sadat A, Mustajab P, Khan IA. Determining the adulteration of Natural milk with synthetic milk using ac conductance measurement. J Food Eng. 2006; 77(3):472–477.
6. Trivedi UB, Lakshminarayana D, Kothari IL, Patel NG, Kapse HN, Makhija KK *et al*. Potentiometric biosensor for urea determination in milk. Sensor Actuat B-Chem. 2009; 40(1):260-266.
7. Mishra GK, Mishra RK, Bhand S. Flow injection analysis biosensor for urea analysis in adulterated milk using enzyme thermistor. *Biosens Bioelectron*, 2010; 26(4):1560-1564.
8. Sharma R, Rajput YS, Barui AK, Laxmana NN. Detection of Adulterants in Milk - A Laboratory Manual, Edition-1. NDRI Publication No: 88/2012. National Dairy research Institute, Karnal-132001, 2012.
9. Shaikh AI, Parmar SC, Raj D. Validation of methods for detection of adulterants, neutralizers and preservatives in khoa and burfi. Ind. J. Dairy Sci. 2011; 64(5):400-405.
10. Tiwari A, Aryal S, Pilla S, Gonga S. An amperometric urea biosensor based on covalently immobilized urease on an electrode made of hyperbranched polyester functionalized gold nanoparticles. *Talanta.,* 2009; 78:1401-1407.
11. BIS. Methods of test for dairy industry, Part- I: Rapid examination of milk, Bureau of Indian standard, ManakBhavan, 9 Bahadur Shah Zafar Marg, New Delhi - 110002, 1960.
12. BIS. Quick methods for detection of adulterants/contaminants in common food products. Indian Standards: 15642 (Parts 1 and 2): 2006. Bureau of Indian Standards, ManakBhavan, 9 Bahadur Shah Zafar Marg, New Delhi-110002., 2006.
13. Dairyforall. Tests to detect adulteration in milk, 2006. Cited from http://www.dairyforall.com/milk-adulteration.php (05-10-2014).
14. Draaiyer J, Dugdill B, Bennett A, Mounsey J. Milk Testing and Payment Systems Resource Book - a practical guide to assist milk producer groups. FAO. 2009, 38-43.
15. NDDB. Methods for detection of common adulterants in milk and milk products. National Dairy Development

Board. 2009; 83:1-36. http://www.dairyknowledge.in/sites/default/files/technews_83.pdf cited on 24-11-2015.
16. FSSAI. Manual of methods of analysis of foods (milk and milk products) - A Laboratory Manual. Food safety and standards authority of India, Ministry of health and family welfare, Government of India, New Delhi, 2012,
17. Kamthania M, Saxena J, Saxena K, Sharma DK. Milk adulteration: Methods of detection &remedial measures. Int. J. Engg. Tech. Res. 2014; 1:15-20.
18. Vishweshwar K, Krishnaiah N. Quality control of milk and processing. State Institute of Vocational Education, Director of Intermediate Education Government of Andhra Pradesh, 2005.
19. DGHS. Manual of methods of analysis of foods (milk and milk products): Laboratory Manual-1. Directorate General of Health Services, Ministry of Health and Family Welfare, Government of India, New Delhi, 2005.
20. Anonymous. Test to detect adulteration in milk. Posted in Dairyforall, 2006. http://www.dairyforall.com/milk-adulteration.php (05-10-2014).
21. Srivastava MK. Adulteration and detection of adulterants in milk. In Handbook on analysis of milk – Chemical and microbiological analysis of liquid milk". IBDC publishers, Lucknow, India. 2010, 95-155.
22. Dixit S. Test yourself – detect adulteration in milk. Cited from, 2012. http://face-cii.in/sites/default/files/dr_sitaram_dixit_-1.pdf (18-10-2014).
23. Singh A, Chandra G, Aggarwal A, Kumar P. Adulteration detection in milk. *Res. New For U.*, 2012; 5:52.
24. DDBM. Quality control. Dairy development department, Maharashtra state. 2013. http://dairy.maharashtra.gov.in/index.php?option=com_content&view=article&id=23:quality-control&catid=2:uncategorised&Itemid=137&lang=en (22-10-2014).