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# Assessment of Biogenic Amines in Milk and Selected Milk Products

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## ABSTRACT

**Background:** Biogenic amines (BAs) are low molecular weight organic bases with biological activity that are formed in foods by microbial decarboxylation of the corresponding amino acids or by transamination of aldehydes and ketones by amino acid transaminases. As these BAs are toxic and act as a spoilage indicator, their presence determines the quality of food.

**Methods:** Milk and selected milk products were assessed for the presence of selected BAs such as histamine, tyramine, tryptamine, spermine, spermidine, cadaverine and putrescine using HPTLC.

**Result:** It was observed that histamine, spermidine and tryptamine were present in the raw as well as pasteurized milk of cow and buffalo. Pasteurised milk found to show low concentration of tryptamine than the raw milk. Cheese was found with the presence of histamine, tryptamine and putrescine throughout the storage. Though, BAs are present within safe limit in all the milk and selected milk products but their presence indicates a serious quality concern.

**Key words:** Biogenic amines, Fermented milk products, HPTLC, Milk.

## INTRODUCTION

Safety of food products are one of the emerging challenge. Establishing hazard identification checks based on experience and qualitative reasons is important tool for identifying, evaluating, determining and justifying the selection or rejection of control measures to reduce risk (Mortimore, 2000). One major known considered biochemical hazard indicator is BAs in food products (Lee and Hathaway, 1998; Mortimore, 2000).

BAs are primarily produced due to decarboxylation of amino acids by microbial enzymes. Numerous microorganisms reported to produce the BAs in food products (Onal, 2007). BAs occur in various dairy products as a result of the metabolism of several amino acids. The precursor amino acids are found naturally in milk or produced by the hydrolytic activity of proteases, peptidases and amino peptidases during fermentation, ripening and storage (Anli and Bayram, 2008). The presence of BAs in milk and milk products have reported as serious concern. Milk provides sufficient medium because of their rich and balanced chemical composition for the growth of almost all microorganisms, including those that produce toxic metabolites. Therefore, dairy products are often associated with food borne illness due to contamination of preforms microbial toxins, including bacterial toxins, mycotoxins and BAs. Increasing concern for presence of BAs is due to the frequent detection of their high levels in the wide variety of dairy products (Novella-Rodriguez *et al.,* 2002).

Various biochemical changes occur during the ripening of cheese are responsible for the typical flavor of cheese (Andic *et al*., 2010). In cheese ripening process, the microbial enzymes such as lipase, protease, peptides, amino acid decarboxylase and deaminases are important. Beside these, proteases and exopeptidases are produced by lactic acid bacteria and other microflora are also responsible for various hydrolytic reactions (Linares *et al.,* 2012; Loizzo *et al.,* 2013). In ripened cheese, the soluble nitrogen steadily increases to 40% and the free amino acids and low molecular weight peptides (<1000 Da) contribute about 12% of the total nitrogen. So, the free amino acids are produced during the proteolysis process and causes amino acid decarboxylation. Several outbreaks of amine intoxications are apparent due to the consumption of cheese. The BAs such as histamine, tyramine and tryptamine are abundantly found in cheese (Halasz *et al.,* 1994; Tarakci *et al.,* 2004). Because of the potential toxicity of BAs and also as a spoilage indicator, their presence determines the quality of food (Onal, 2007). Therefore, it is need of the day to assess the various BAs to address the quality of milk and milk products.

## MATERIALS AND METHODS

### Sample collection

Milk and milk products assessed for the BAs were procured from the local market of Anand, Gujarat (Table 1). All the samples were stored at 4°C.

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### Analysis of BAs

Sample preparation and analysis of BAs were done by using HPTLC method (Shalaby, 2000).

### Extraction of BAs

Samples (50 g) are extracted with 40 ml of 5% trichloroacetic acid (TCA) using a Waring blender. Each blended mixture was centrifuged for 15 min at 8000 rpm. The fat floating on the supernatant was separated after centrifugation. The supernatant was then filtered using Whatman No. 1 filter paper. The volume was adjusted to 250 ml with 5% TCA. The equivalent of 1 g of sample, as the TCA extract (5 ml), was added into a screw-capped tube and washed thrice with an equal amount of diethyl ether to remove the acid. The ether remaining with the aqueous extract was removed by heating in water bath. Two drops of concentrated HCl were added to the washed extract and the solution was evaporated just to dryness using a current of air and hot water bath. Residue was stored at 4°C and maximum for one week.

### Derivatization of sample

Dansylated derivatives of the amines were prepared by dissolving the residue with 1 ml of saturated sodium bicarbonate solution. The $CO_2$ bubbles forms and it neutralizes the solution. Then, 1 ml of dansyl chloride reagent was added which forms dansylated amines. The sealed tube was immediately mixed for 30 s using a vortex mixer and the reaction mixer was then incubated at 40 for 1 h. During the incubation, the tubes were shaken vigorously every 10 min in order to ensure a complete mixing of dansyl chloride with the extracted amines. The dansylamines were extracted by addition of 10 ml water which leads to change in colour to golden yellow. Further extracting the mixture with several portions of diethyl ether. The combined ether extracts were evaporated to dryness and the residue was dissolved in 1 ml acetonitrile and sonicated for 1 min or vortexed for 30 s. The extract was stored at -20°C until HPTLC analysis.

### Preparation of standards

Five milligram of each reference standard was taken in 10 ml volumetric flask and dissolved in 0.1 N HCl solution prepared in deionized water. A standard solution for analysis was prepared from the stock solution, each stock solution 100 µl was taken for further analysis (0.5 mg $ml^{-1}$). The prepared solution was evaporated to dryness using hot water bath. Dansyl derivative were prepared as described above. The dried residue was dissolved in 1 ml acetonitrile.

### Separation of dansylamines

The HPTLC technique was used to separate the selected BAs extracted from the milk and milk products. The reference standard and dansyl food extract were applied 8 mm from the base of the (20 × 10) TLC plate and at 1 cm interval using 100 µl micro-syringe (CAMAG). Aliquots of mixed dansylated standard applied on TLC plate were 2, 4, 6, 8, 10, 12 µl. While, each dansylated food extract (10 µl) was applied. TLC plate was developed using two solvent systems by CAMAG automatic developing chamber ADC2 and CAMAG mobile phase chamber. The plate was developed in chloroform : benzene : triethylamine (6 : 4 : 1, v/v/v) for 8 cm (10 min). Then, it was removed from the jar and allowed to dry. Further it was developed in the same direction in benzene : acetone : triethylamine (10 : 2 : 1, v/v/v) for 8 cm (10 min). The plate was initially dried at room temperature followed by hot air dryer. The chromatogram was interpreted under CAMAG TLC UV visualizer at 254 nm ultraviolet and 360 nm fluorescence wavelength to detect the dansylamines.

### Quantification of dansylamine

The quantification of dansylamine was carried out by the CAMAG TLC scanner 4 and the absorbance value for each separated spot was recorded at 254 nm and 360 nm wavelength. Calibration curve was prepared for the quantification of the BA using WinCat software. For this, aliquots of working standard solution (1 µg/µl) of BA equivalent to 1.872, 3.744, 5.618, 7.488, 9.36 and 11.232 µg per band were applied on one TLC plate. The TLC plate was developed and scanned as described above. Peak area was recorded at each concentration and were treated by linear least-square regression analysis. The linear regression analysis data for the calibration plots was performed and showed a good linear relationship ($r^2$ =0.9960) concerning peak area in the concentration range of 1.872-11.232 ìg/band was used for quantification of BAs.

## RESULTS AND DISCUSSION

### Qualitative assessment of BAs

Milk and selected milk products were assessed for the presence selected BAs such as histamine, spermidine, spermine, putrescine, cadaverine, tyramine and tryptamine as are reported more prevalent in the selected dairy products.

### BAs in raw and pasteurized milk

The qualitative determination was carried out for the assessment of BAs in the raw milk. The sampling time for the qualitative analysis of BAs was 12 h for the fresh raw

**Table 1:** Milk and selected milk products assessed for BAs

| Types of products | Final products |
|---|---|
| Cow milk | Raw milk |
| | Pasteurized milk |
| Buffalo milk | Raw milk |
| | Pasteurized milk |
| Fermented milk products | Curd |
| | Lassi |
| | Mozzerella cheese |
| | Gouda cheese |
| | Cheddar cheese |
| | Feta cheese |

milk and 24 h for the pasteurized milk. The chromatogram of HPTLC for detection of the selected BAs present in raw milk and pasteurized milk from cow and buffalo confirming the presence of BAs (Fig 1 and 2). Raw as well as pasteurized milk from cow and buffalo found to show the presence of histamine, spermidine and tryptamine (Table 2). While putrescine, spermine, cadaverine, tyramine and tryptamine were found to absent in all milk samples. The presence of these BAs confers the possible health hazards or could indicate quality concerns in the raw milk and pasteurized milk. Therefore, it necessitates the milk to be evaluated further for quantitatively for BAs.

**BAs in curd and lassi**

Curd and lassi used in the present studies is the standardized product with shelf life of 15 and 90 days respectively. Considering worst case scenario, qualitative analysis of curd and lassi samples were carried out on 15th and 90th days respectively. None of these samples showed presence of selected BAs. These products have low pH and high salt concentrations, as well as the microbes used in the preparation are standardized. These could be a reason for the absence of BAs and product confirms to be of high quality.

**BAs in selected varieties of cheese**

Presence of BAs were assessed in four selected cheese varieties. From the selected cheese varieties, cheddar cheese is unprocessed, while mozzarella, feta and gouda are the processed cheese. The qualitative estimation for BAs were carried out from the samples after 120 days of their manufacture. The results of the presence of BAs are shown in the HPTLC chromatogram (Fig 3). BAs such as histamine, putrescine and tryptamine were detected in the cheese samples (Fig 3, Table 2). Based on results, all cheese samples were further analysed quantitatively for the BAs during storage.

Qualitative analysis of the selected milk and milk products were revealed the presence of BAs in the milk and cheese samples (Table 2). These samples were further studied for the quantitative analysis at various intervals throughout their shelf life.

**Quantitative assessment of BAs**

Selected BAs from raw and pasteurized cow and buffalo milk were identified and quantified throughout shelf life period using HPTLC method.

**Quantitative estimation of BAs in raw milk of cow and buffalo**

It was observed that histamine, spermidine and tryptamine were present in the fresh raw milk of cow as well as buffalo (Table 3). Other BAs such as cadaverine, putrescine, tyramine and spermine were found to be absent in all raw

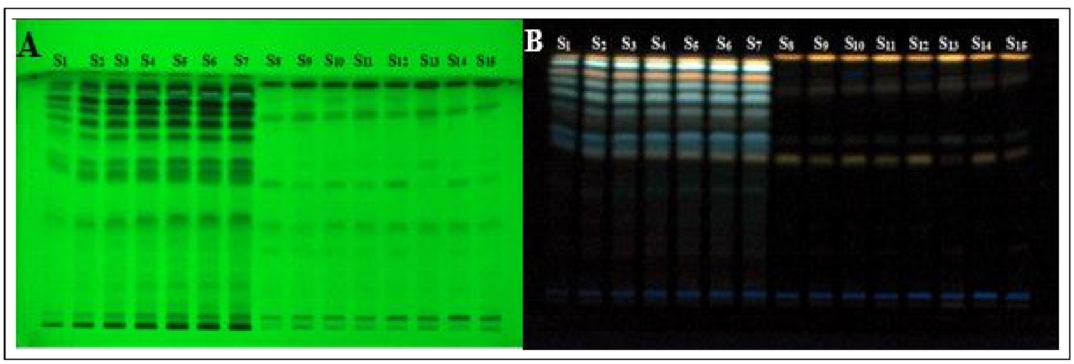


**Fig 1:** HPTLC chromatogram showing presence of BAs in raw milk of cow and buffalo, A; at 254 nm and B; 360 nm wavelength. Note; Lane S1 to S5 are loaded with the mixture of reference standard of BAs under study, while lane S6-S9 loaded with pasteurized cow milk and S11-S14 loaded with pasteurized buffalo milk.

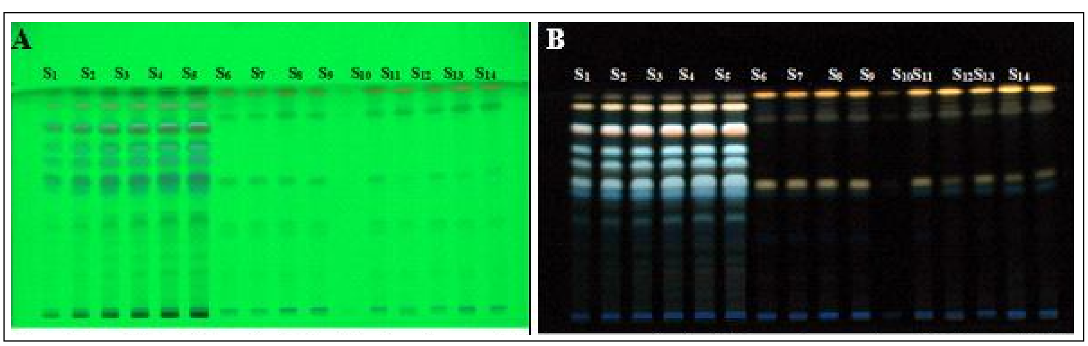


**Fig 2:** HPTLC chromatogram showing presence of BAs in pasteurized milk of cow and buffalo, A; at 254 nm and B; 360 nm wavelength. Note; Lane S1 to S7 are loaded with the mixture of reference standard of BAs under study, while lane S8-S11 loaded with raw cow milk and S12-S15 loaded with raw buffalo milk.

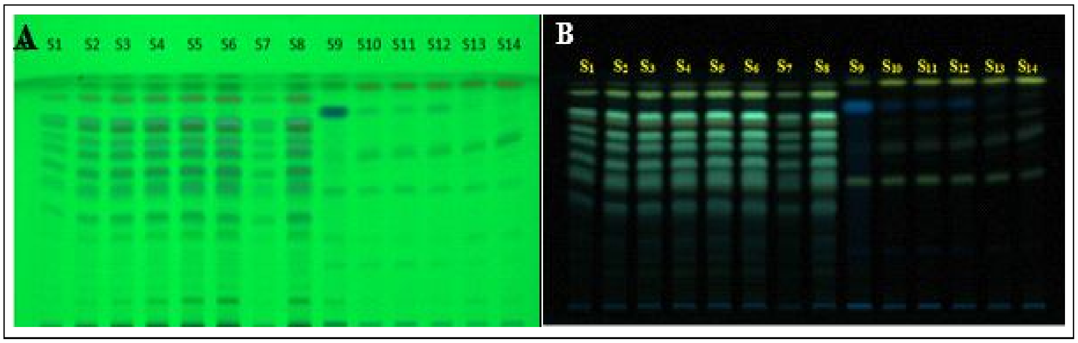

**Fig 3:** HPTLC chromatogram showing presence of BAs in selected cheese varieties, A; at 254 nm and B; 360 nm wavelength. Note; Lane S1 to S6 are loaded with the mixture of reference standard of BAs under study, while lane S7-S8 loaded with cheddar cheese; S9-S10 loaded with mozzarella cheese; S11-12 loaded with feta cheese and s13-14 loaded with gouda cheese.

**Table 2:** Assessment of BAs in milk and selected milk product.

| Products | Sampling time | Biogenic amines | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | HIS | SPD | CAD | PUT | TRY | TYR | SPE |
| Raw milk of cow (h) | 12 | + | + | - | - | + | - | - |
| Raw milk of buffalo (h) | 12 | + | + | - | - | + | - | - |
| Pasteurized cow milk (h) | 24 | + | + | - | - | + | - | - |
| Pasteurized buffalo milk (h) | 24 | + | + | - | - | + | - | - |
| Curd (days) | 15 | - | - | - | - | - | - | - |
| Lassi (days) | 90 | - | - | - | - | - | - | - |
| Cheddar cheese (days) | 120 | + | - | - | + | + | - | - |
| Mozzarella cheese (days) | 120 | + | - | - | + | + | - | - |
| Feta cheese (days) | 120 | + | - | - | + | + | - | - |
| Gouda cheese (days) | 120 | + | - | - | + | + | - | - |

+ presence of BA, - absence of BA.

milk samples. The histamine and spermidine concentrations were found to be below 1 mg/kg and remains unaffected during the storage as shown in Table 3. While, tryptamine was found to increase during the storage of the raw milk of caow and buffalo. Cadaverine, putrescine, tyramine and spermine were absent in all raw milk sample. Spermidine is one of the BAs naturally present in milk (Novella-Rodriguez *et al*. (2000). While the presence of mesophilic bacteria such as LAB, *Pseudomonas* and *Acinetobacter* are responsible for the accumulation BAs and increment in their concentration during storage (Serio *et al*. (2007). It was observed that the concentration of tryptamine increases with increase in the storage time of the milk samples shown Table 3.

Microbial activity, storage conditions and biochemical parameters such as protein, pH, fat and salt are some of the major factors that are responsible for the production of BAs in milk. The concentration of tryptamine slightly increases in first 4 h in both cow and buffalo raw milk sample. But after 4 h, more amount of tryptamine is found in buffalo raw milk as compared to cow raw milk. This is might be due to the presence of higher amount of protein and fat present in buffalo milk than the cow milk. The increase in tryptamine production not only signifies the improper hygienic storage condition of milk but also affect its shelf life. The tryptamine concentration above 10 mg/kg limit is signifies health hazard (Novella-Rodriguez et al., 2000). The maximum level of BAs (2.97 mg/kg) present in raw buffalo milk is even suitable for consumption as per the safe limit. However, the tryptamine content in cow and buffalo milk raise an indication of some issues pertaining to the quality and safety.

### Quantitative estimation of BAs in pasteurized milk of cow and buffalo

Pasteurized cow and buffalo milk were analyzed quantitatively for selected BAs. It was observed that histamine, spermidine and tryptamine were present (Table 4). While, cadaverine, putrescine, tyramine and spermine were absent in the pasteurized milk. Similar results were observed after 4, 6 and 8 h of storage. The concentrations of the BAs detected were >1.00 mg/kg and therefore is safe for consumption. Pasteurization is responsible for reducing the microbial load in milk and therefore microbial transformation for production of BAs is prevented (Novella-Rodriguez *et al*., 2002). BAs have been detected but are in safe limit in pasteurized milk as compared to raw milk. Considering this, it is apparent that pasteurized milk is less susceptible to hazards of BAs, if initial load of BAs in raw milk is satisfactory. Study reveals that the pasteurization and good hygiene condition during processing are very important to prevent the formation of BAs.

**Table 3:** Evaluation of selected BAs in the raw milk during storage.

| Products | Sampling time (h) | Biogenic amines (mg/kg of sample) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | HIS | SPD | CAD | PUT | TRY | TYR | SPE |
| Raw milk of cow | 0 | >1.00 | >1.00 | - | - | 1.73±0.01 | - | - |
| | 4 | >1.00 | >1.00 | - | - | 1.87 ±0.02 | - | - |
| | 8 | >1.00 | >1.00 | - | - | 1.92±0.02 | - | - |
| | 12 | >1.00 | >1.00 | - | - | 1.97±0.03 | - | - |
| Raw milk buffalo | 0 | >1.00 | >1.00 | - | - | 1.82±0.00 | - | - |
| | 4 | >1.00 | >1.00 | - | - | 1.93±0.01 | - | - |
| | 8 | >1.00 | >1.00 | - | - | 2.07±0.02 | - | - |
| | 12 | >1.00 | >1.00 | - | - | 2.97±0.03 | - | - |

- absence of BA.

**Table 4:** Evaluation of selected BAs in the pasteurized milk during storage.

| Products | Sampling time (h) | Biogenic amines (mg/kg of sample) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | HIS | SPD | CAD | PUT | TRY | TYR | SPE |
| Pasteurized cow milk | 0 | >1.00 | >1.00 | - | - | >1.00 | - | - |
| | 4 | >1.00 | >1.00 | - | - | >1.00 | - | - |
| | 6 | >1.00 | >1.00 | - | - | >1.00 | - | - |
| | 8 | >1.00 | >1.00 | - | - | >1.00 | - | - |
| Pasteurized buffalo milk | 0 | >1.00 | >1.00 | - | - | >1.00 | - | - |
| | 4 | >1.00 | >1.00 | - | - | >1.00 | - | - |
| | 6 | >1.00 | >1.00 | - | - | >1.00 | - | - |
| | 8 | >1.00 | >1.00 | - | - | >1.00 | - | - |

- absence of BA.

**Table 5:** Evaluation of selected BAs in the different varieties of cheese during storage.

| Products | Sampling time (days) | Biogenic amines (mg/kg of sample) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | HIS | SPER | CAD | PUT | TRY | TYR | SPE |
| Cheddar cheese | 0 | >1.00 | - | - | - | >1.00 | - | - |
| | 30 | >1.00 | - | - | - | >1.00 | - | - |
| | 60 | >1.00 | - | - | >1.00 | >1.00 | - | - |
| | 90 | >1.00 | - | - | >1.00 | >1.00 | - | - |
| | 120 | >1.00 | - | - | >1.00 | >1.00 | - | - |
| Mozzarella cheese | 0 | >1.00 | - | - | - | >1.00 | - | - |
| | 30 | >1.00 | - | - | - | >1.00 | - | - |
| | 60 | >1.00 | - | - | >1.00 | >1.00 | - | - |
| | 90 | >1.00 | - | - | >1.00 | >1.00 | - | - |
| | 120 | >1.00 | - | - | >1.00 | >1.00 | - | - |
| Feta cheese | 0 | >1.00 | - | - | - | >1.00 | - | - |
| | 30 | >1.00 | - | - | - | >1.00 | - | - |
| | 60 | >1.00 | - | - | >1.00 | >1.00 | - | - |
| | 90 | >1.00 | - | - | >1.00 | >1.00 | - | - |
| | 120 | >1.00 | - | - | >1.00 | >1.00 | - | - |
| Gouda cheese | 0 | >1.00 | - | - | - | >1.00 | - | - |
| | 30 | >1.00 | - | - | - | >1.00 | - | - |
| | 60 | >1.00 | - | - | >1.00 | >1.00 | - | - |
| | 90 | >1.00 | - | - | >1.00 | >1.00 | - | - |
| | 120 | >1.00 | - | - | >1.00 | >1.00 | - | - |

- absence of BA.

**Quantitative of estimation of BAs in selected varieties of cheese**

Selected BAs from cheddar, mozzarella, feta and gouda cheese verities were identified and quantified by using HPTLC method during their storage up to 120 days. It was observed that histamine and tryptamine were present in the fresh samples of all the cheese verities under study. Other BAs such as spermidine, cadaverine and spermine were found to be absent in the fresh samples. However, after 60 days, putrescine was detected in all the samples along with histamine and tryptamine. The results were found similar to the study conducted by Bunkova *et al*. (2010).

The presence of various BAs in different types of cheese is shown in Table 5. The concentration of histamine, putrescine and tryptamine found in all cheese samples, were below the >1.00 mg/kg, which is safe and it was not increased significantly up to the storage period of 120 days confers them safe from any hazards due to BAs.

## Conclusion

Presence of the BAs not only indicates the improper hygienic storage condition and processing of milk and selected milk product and affect the shelf life. The presence of tryptamine in raw cow and buffalo milk is a quality concern. While presence of low concentrations of BAs in pasteurized milk refers to the good quality and thus better for consumption as compared to raw milk. Study reveals that good hygiene condition during milk processing and pasteurization are the key factors for the BAs formation. The quantitative estimation of BAs in cheese variants revealed the presence of histamine, tryptamine and putrescine but at safe limit for consumption. However, this alarms the quality and safety aspects. Present study was conducted on the milk and selected milk products from the well-organized reputed make and therefore, there is abnormalities in terms of concentration BAs is not significant so far as the quality norms are concerned. However, considering market presence of the traditional and small unorganized players there is stringent need for monitoring BAs in the milk and milk products to ensure safety.

**Conflict of interest:** None.

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