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## Assessment of Microbiological Quality and Aflatoxin Levels of Paneer Marketed in Chennai, India

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### **Abstract:**

India is an agrarian country with major proportion of population as vegetarian so paneer is of great value in diet with respect to its food and nutritive value. An attempt was made to evaluate the microbial and toxicity levels of paneer marketed in Chennai city. For this purpose, 40 samples were collected from the supermarket located in South and North Chennai. Standard methods were used for both microbiological assessment and aflatoxin identification. All samples were analyzed for presence of *E. coli*, *Salmonella* spp., *Pseudomonas aureginous*, *Staphylococcus aureus*, total bacterial count and total yeast and mould count. Among all the samples, *E. coli* was found in 50% of the market samples. *Salmonella* spp., *Pseudomonas aureginous* and *Staphylococcus aureus* were found to be absent in all samples. All the 40 samples had bacteriological counts ranging from  $10 \times 10^6$  to  $45 \times 10^6$  cfu/g and fungal counts ranging from  $5 \times 10^5$  to  $25 \times 10^5$  cfu/g. Aflatoxin contamination in paneer was also analyzed by high-performance liquid chromatography (HPLC) using fluorescence detector. 43% of samples were found to be contaminated with aflatoxins at concentrations ranging from 0.03-389  $\mu\text{g}/\text{kg}$ . Overall, this study carried out suggests that microbiological quality of paneer available in the city of Chennai is not within the limits set by the regulatory bodies and contains residual level of aflatoxins which pose public health risk. Therefore, there is a need for continuous monitoring of aflatoxins in paneer and also implementation of HACCP in dairy industry.

**Keywords:** Microbiological assessment, aflatoxin, bacteriological counts, fungal counts, high-performance liquid chromatography

### **1. Introduction**

India has emerged as the largest milk producer in the world with the success of the Operational Flood Programme. About 5% of milk produced in India is converted into paneer (Chandan, 2007). The Indian soft cheese (paneer) is obtained by acid and heat coagulation of milk. It represents one of the soft varieties of cheese family and is used in culinary dishes/snacks. According to Food Safety and Standards (Food Products Standards and Food Additives) Regulations (Part-I), 2011, paneer shall not contain more than 70% moisture and the milk fat content shall not be less than 50% of the dry matter. Bureau of Indian Standards (BIS 1983) also specifies a minimum of 50% fat on dry matter basis but a maximum of 60% moisture in paneer.

Microbiological quality of paneer is known to be dependent on the microbiological quality of milk and upon the post manufacture conditions, particularly, handling, packaging and storage of the product (Khan and Pal, 2011). Pathogens of great public health concerns found in paneer include *E. Coli* particularly serotype O157:H7, *Staphylococcus aureus*, *Salmonella*, *Bacillus cereus*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Yersinia*, *Clostridium* and *Campylobacteria*. Vishweshwaraiah and Anantakrishnan (1985) suggested that Standard Plate Count (SPC) lesser than 5,000/g and higher than 2 lakhs/g can be termed as 'Excellent' and 'Poor' quality paneer respectively. Bureau of Indian Standards (BIS 1983) set limits for microbial count in paneer viz., total plate count  $< 5 \times 10^5/\text{g}$ , yeast and mould count  $< 250/\text{g}$ , and coliform count of  $< 90/\text{g}$ . Kalhan and Grover (1984) also reported that freshly prepared paneer showed absence of any pathogenic microorganism under hygienic conditions. Microbial profile of fresh paneer as reported by Singh et al. (1989) is SPC- $2.3 \times 10^3$ , proteolytic bacteria- $7.4 \times 10^2$ , lipolytic bacteria-  $4.9 \times 10^2$  and fungi-10/g. More than 60% of paneer

samples were found to be contaminated with coliforms from organized dairies and markets in India (Kumar and Sinha, 1989). Aflatoxin M1 contamination in market panner in different localities of Chhattisgarh was observed by Choudhary et al. (2007). Mycotoxins are secondary metabolites produced by microfungi that have potential to cause hazardous disease in humans and animals, eventually causing their death. Aflatoxins (AFs) are one of the most important groups of mycotoxins and are one of the most potent toxic substances that occur naturally. There are at least 16 naturally occurring aflatoxins, among this B1, B2, G1, G2 are major toxins. High temperature and humidity of hot and humid regions are optimal for moulds growth and toxin production (Ventura *et al.*, 2004; Zollner & Mayer-Helm, 2006). Aflatoxins M1 and M2 are the hydroxylated metabolites of aflatoxins B1 and B2 and can be found in milk or milk products obtained from livestock that have ingested Aflatoxin contaminated feed. AFB1 and AFM1 have been classified as carcinogenic agents to humans by WHO International Agency for Research on Cancer (IARC, 2002). AFM1 links with the nucleic acid in toxic ways and manifests its adverse effects in the form of hepatotoxicity and carcinogenicity (Wong *et al.*, 2000). EU countries have 0.05 µg/l (Commission Regulation (EC) N. 466/2001) of AFM1 in milk as lowest allowable concentrations. Other countries have legislation for mycotoxins ten times higher with allowable concentrations of 0.5 µg/l. With this available information, an attempt was made to evaluate the microbial and aflatoxin levels of paneer marketed in Chennai city.

## 2. Materials and Methods

### 2.1. Sampling

40 samples were bought from different dairy collection centres and supermarkets of different areas in Chennai, India. Samples were collected and analyzed during September to October 2014. All samples were analyzed before their expiry date. The samples were collected in presterilized containers and transported to the lab in ice bucket. During the period of analysis the samples were kept in refrigerated condition.

### 2.2. Microbiological Enumeration

For the detection and enumeration of microorganisms, standard media were prepared. All the samples were processed under sterile conditions. All samples were analyzed for the presence of *E. coli*, *Salmonella* spp., *Pseudomonas aureginous*, *Staphylococcus aureus*, total bacterial count and total yeast and mould count (FDA Bacteriological Analytical Manual, 2005, 18th Ed., AOAC, Washington, DC). Serial decimal dilutions were made and the microbial analysis was carried out on agar plates in triplicates.

MacConkey Agar was used for the enumeration of *Escherichia coli* (*E. coli*). *E. coli* was detected by gas and indol production on MacConkey broth. The plates were incubated at 36° C to 38° C for 48 hours. *Salmonella* spp. were estimated by adding of enrichment culture on Selenite F Broth and incubated at 36° C to 38° C for 48 hours. It was followed by inoculation on Bismuth Sulphite Agar and incubation was done at 36° C to 38° C for 18 to 24 hours. *Pseudomonas aeruginosa* were enumerated by plating on Cetrimide Agar Medium and incubated for 35° C to 37° C for 18 to 24 hours. *Staphylococcus aureus* were estimated by plating on Baird Parker Agar and incubating at 35° C to 37° C for 45 to 48 hours. Total bacterial count was estimated by applying Nutrient Agar and incubating at 37° C for 24 hours. Total yeast and mould count were enumerated using Potato Dextrose Agar with antibiotics by incubating the plates at 20° C to 25° C for 5 days. Plates were observed for typical colonies of each microorganism and colonies were counted for total bacterial count and total yeast and mould count with the help of colony counter. The results were recorded as cfu/g and were compared to that of the BIS specification. Table 1 depicts the Indian standards specifications for paneer (IS: 10484-1983). In India, the code of conduct laid down by BIS should be strictly followed by the paneer manufacturers.

Characteristics	Requirement	Method
Bacterial count per gram max.	5x10 <sup>5</sup>	IS : 5402-1969
Coliform count per gram max.	90	IS : 5401-1969
Fungal count per gram max	250	IS : 5403-1969

Table 1: The Indian Standards Specifications for Paneer (IS: 10484-1983)

### 2.3. Aflatoxin Analysis

Estimation of Aflatoxin in paneer was determined by Romer's All Purpose Method (1975). All the chemicals used were of HPLC grade. All the paneer samples were analyzed by HPLC technique for the presence of Aflatoxin. Extraction, filtration, cleanup, partitioning and evaporation were done for each sample as required by Romar's All Purpose Method. After it was evaporated to required level, it was reconstituted with 200 µL of methanol, vortexed and filtered through 0.45 µm nylon membrane filter prior to HPLC analysis. HPLC is one of the most common methods to detect and quantify aflatoxins in food. It has been used jointly with techniques such as UV absorption, fluorescence, mass spectrometry and amperometric detectors (Espinosa-Calderón *et al.* 2011)

- High-Performance Liquid Chromatography- Purified extracts were analyzed by reversed-phase isocratic high performance liquid chromatography (HPLC) from Shimadzu LC 10A using a Platinum C18 column (250 × 4.6 mm id, 5 µm) maintained at 40°C. A fluorescence detector was set at 360 nm (excitation) and 440 nm (emission). The mobile phase applied was deionized water/acetonitrile/methanol in the ratio 6:2:2 with flowrate of 1.0 mL/min and injection volume of 20 µL.
- Validation of analytical method- The analytical method was assessed for linearity, recovery, precision and limit of detection before sample analysis.

- Estimation of aflatoxins by HPLC- Aliquots of standard aflatoxins B1, B2, G1, G2 were run first, adopting suitable HPLC conditions. The area under each peak was determined to ascertain concentration versus area. The sample aflatoxin extract was then run under the same HPLC conditions. The concentration of each aflatoxin could be obtained from the area under peaks, relative to those of standards.

#### 2.4. Statistical Analysis

The data were analyzed using ANOVA method to know the effect of presence of microbial levels in paneer. All statistical analyses were performed using IBM SPSS (version-20) software and MS Excel (Microsoft, USA). Results of microbial analysis are presented as means  $\pm$  standard error (SE) and statistical significance was set at  $p \leq 0.05$ .

### 3. Results and Discussion

#### 3.1. Microbiological Enumeration

The results of microbial analysis, including *E. coli*, *Salmonella spp.*, *Pseudomonas aureginous*, *Staphylococcus aureus*, total bacterial count and total yeast and mould count from 40 samples are depicted in Table 2. *E.coli* was present in 50% of the samples tested. The presence of coliforms especially the *E. coli* in the sample is a reflection of poor hygiene and sanitary conditions at different stages of handling. *Salmonella spp.*, *Pseudomonas aureginous* and *Staphylococcus aureus* were found to be absent in all samples. All the 40 samples had bacteriological counts ranging from  $10 \times 10^6$  to  $45 \times 10^6$  cfu/g and fungal counts ranging from  $5 \times 10^5$  to  $25 \times 10^5$  cfu/g. There is a significant ( $p \leq 0.05$ ) affect of microbial count found in paneer. This result indicated that the values are very well above the limit set by both BIS (IS: 10484-1983) and Food Safety and Standard Authority of India (FSSAI). According to Food Safety and Standards (Food Product Standards & Food Additives Regulations, 2011 (Part-II) Total Plate Count should not be more than 30,000/g, coliform should be less than 10/g and *E. coli* should be absent/g. Results similar to those described in this study have also been reported in various other parts of India. Vaishnavi *et al.* (2001) obtained SPC in the range of  $3 \times 10^2$  to  $9.7 \times 10^{10}$  cfu/g in paneer samples sold in Chandigarh, India. Godbole *et al.* (2013) obtained bacteriological counts ranging from  $1 \times 10^6$  to  $8.2 \times 10^6$  cfu/g and fungal counts ranging from  $1 \times 10^5$  to  $6.6 \times 10^5$  cfu/g in paneer samples sold in Nagpur, India. The occurrence of microbial contamination may be due to lack of knowledge of various practices relevant to paneer production and the possible health hazards may occur if food safety is not followed by the workers and vendors. The application of HACCP to identify the critical control points for coliforms and *Staphylococcus spp.* has indicated that the contamination is due to food handlers using bare hands to remove excess water in paneer (Bhat R.V. *et al.*, 2000). Microbial contamination seems to be more effectively controlled by Good Manufacturing Practices (GMP) and hygienic rules as well as HACCP during handling and paneer processing. The present study also supports this observation and projects that microbiological quality of paneer in India needs continuous monitoring.

#### 3.2. Aflatoxin Analysis

Aflatoxins were detected in paneer at concentrations ranging from 0.03-389  $\mu\text{g}/\text{kg}$ . Of the 40 analyzed paneer samples, 17 samples (43%) were contaminated with aflatoxins that might contribute to health hazards for humans. Aflatoxin B1 was found in 6 samples (15%) ranging from 3-389  $\mu\text{g}/\text{kg}$ . Aflatoxin B2 was found in 12 samples (30%) ranging from 0.03-125  $\mu\text{g}/\text{kg}$ . Aflatoxin G1 was found in 8 samples (20%) ranging from 5-135  $\mu\text{g}/\text{kg}$ . Aflatoxin G2 was found in 11 samples (28%) ranging from 1-380  $\mu\text{g}/\text{kg}$ . Linearity graph for aflatoxin G1, G2, B1, B2 are depicted in Figure 1, Figure 2, Figure 3 and Figure 4, respectively. The average retention time for AFG2, AFG1 peak was 7.448 and 8.979, respectively. The average retention time for AFB2, AFB1 peak was 9.658 and 11.847, respectively as projected in chromatogram of standard aflatoxins (Figure 5). In Figure 6, the HPLC chromatogram of a sample no. 1 is projected. Total aflatoxin levels in Paneer samples from Chennai, India are depicted in Figure 7.

From Figure 7, it was clearly understood that the total aflatoxin content in paneer sample collected in Chennai city is ranging from 0.03-389  $\mu\text{g}/\text{kg}$ . This is an alarming situation for consumer as the figure is very high than the limit (0.05  $\mu\text{g}/\text{kg}$ ) set by Codex Alimentarius Commission (Codex Alimentarius Commission action, 2001) and FSSAI for milk and milk products. Presence of aflatoxins B1, B2, G1, G2 in paneer sample implies that the animals are fed with feeds higher in aflatoxin content. Being only the first study of aflatoxin B1, B2, G1, G2 in paneer marketed in India, there are no any previous works to compare the contamination level of this study. This is the first study of its kind and lots more is to be revealed in the future. However, several other studies on detection of aflatoxin M1 on milk and milk products have been carried out by other researches. Unusan (2006) detected occurrence of AFM1 in UHT milk in Turkey at levels of 0.543  $\mu\text{g}/\text{kg}$ . Ghazani (2009) found AFM1 contamination in pasteurized milk in Tabriz as high as 0.259  $\mu\text{g}/\text{kg}$ . Kav *et al.* (2011) detected AFM1 in white-brined Urfa cheese consumed in Turkey at concentration ranging from 0.070 to 0.771  $\mu\text{g}/\text{kg}$ . Kafle *et al.* (2012) observed AFM1 levels in raw and processed milk marketed in Kathmandu valley at concentrations ranging from 0.025 to 0.138  $\mu\text{g}/\text{kg}$ . Hathout *et al.* (2013) study depicted that the maximum AFM1 level in Egyptian White Soft Cheese samples was 0.059  $\mu\text{g}/\text{kg}$ .

E. coli	Salmonella spp.	Pseudomonas aureginous	Staphylococcus aureus	TBC*, cfu/g, x10 <sup>5</sup>	TYM*, cfu/g, x10 <sup>6</sup>
-	-	-	-	12 ± 3.0 <sup>a</sup>	5 ± 1.0 <sup>b</sup>
-	-	-	-	23 ± 3.6 <sup>a</sup>	8 ± 2.0 <sup>b</sup>
+	-	-	-	44 ± 3.6 <sup>a</sup>	13 ± 3.6 <sup>b</sup>
+	-	-	-	33 ± 5.3 <sup>a</sup>	19 ± 1.7 <sup>b</sup>
+	-	-	-	34 ± 5.3 <sup>a</sup>	11 ± 2.6 <sup>b</sup>
+	-	-	-	45 ± 2.0 <sup>a</sup>	22 ± 2.6 <sup>b</sup>
-	-	-	-	24 ± 3.5 <sup>a</sup>	9 ± 4.6 <sup>b</sup>
-	-	-	-	25 ± 3.5 <sup>a</sup>	5 ± 2.0 <sup>b</sup>
+	-	-	-	44 ± 3.6 <sup>a</sup>	25 ± 2.6 <sup>b</sup>
-	-	-	-	16 ± 3.6 <sup>a</sup>	6 ± 1.7 <sup>b</sup>
-	-	-	-	10 ± 2.6 <sup>a</sup>	5 ± 1.0 <sup>b</sup>
-	-	-	-	25 ± 5.6 <sup>a</sup>	10 ± 3.6 <sup>b</sup>
+	-	-	-	45 ± 4.4 <sup>a</sup>	15 ± 4.6 <sup>b</sup>
+	-	-	-	35 ± 3.6 <sup>a</sup>	20 ± 4.0 <sup>b</sup>
+	-	-	-	35 ± 6.2 <sup>a</sup>	10 ± 2.6 <sup>b</sup>
+	-	-	-	45 ± 3.5 <sup>a</sup>	20 ± 2.6 <sup>b</sup>
-	-	-	-	25 ± 2.6 <sup>a</sup>	10 ± 1.7 <sup>b</sup>
-	-	-	-	25 ± 2.6 <sup>a</sup>	5 ± 1.7 <sup>b</sup>
+	-	-	-	45 ± 2.6 <sup>a</sup>	25 ± 4.4 <sup>b</sup>
-	-	-	-	15 ± 2.6 <sup>a</sup>	5 ± 2.0 <sup>b</sup>
-	-	-	-	11 ± 4.4 <sup>a</sup>	7 ± 1.7 <sup>b</sup>
-	-	-	-	23 ± 3.6 <sup>a</sup>	12 ± 2.6 <sup>b</sup>
+	-	-	-	44 ± 2.6 <sup>a</sup>	17 ± 1.7 <sup>b</sup>
+	-	-	-	32 ± 1.7 <sup>a</sup>	18 ± 2.6 <sup>b</sup>
+	-	-	-	36 ± 2.6 <sup>a</sup>	9 ± 1.7 <sup>b</sup>
+	-	-	-	43 ± 3.0 <sup>a</sup>	19 ± 3.6 <sup>b</sup>
-	-	-	-	27 ± 1.0 <sup>a</sup>	12 ± 1.7 <sup>b</sup>
-	-	-	-	26 ± 1.7 <sup>a</sup>	5 ± 1.7 <sup>b</sup>
+	-	-	-	43 ± 1.7 <sup>a</sup>	24 ± 3.6 <sup>b</sup>
-	-	-	-	12 ± 2.6 <sup>a</sup>	7 ± 1.7 <sup>b</sup>
-	-	-	-	10 ± 1.7	8 ± 1.0
-	-	-	-	27 ± 2.6 <sup>a</sup>	13 ± 1.7 <sup>b</sup>
+	-	-	-	45 ± 1.7 <sup>a</sup>	12 ± 3.0 <sup>b</sup>
+	-	-	-	33 ± 4.6	22 ± 2.0
+	-	-	-	37 ± 1.7 <sup>a</sup>	13 ± 1.7 <sup>b</sup>
+	-	-	-	42 ± 2.6 <sup>a</sup>	21 ± 1.0 <sup>b</sup>
-	-	-	-	27 ± 2.0 <sup>a</sup>	11 ± 1.7 <sup>b</sup>
-	-	-	-	27 ± 2.6 <sup>a</sup>	7 ± 1.7 <sup>b</sup>
+	-	-	-	42 ± 2.0 <sup>a</sup>	23 ± 2.0 <sup>b</sup>
-	-	-	-	14 ± 1.7 <sup>a</sup>	6 ± 2.6 <sup>b</sup>

Table 2: Microbiological Counts of Paneer Samples from Chennai, India (samples=40)

\* TBC - Total bacterial count, TYM - Total yeast and mould count

Values expressed are means (n=3) ± standard deviation; a-b, means in the same row with different superscripts were significantly (p≤0.05) different

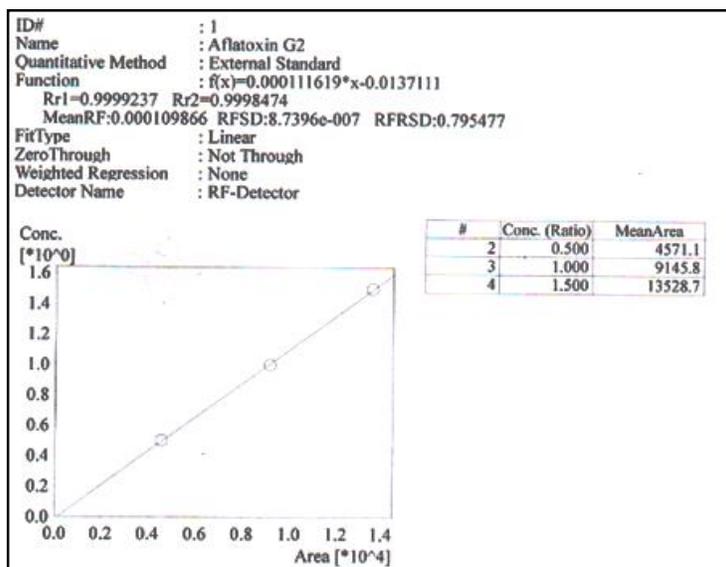


Figure 1: Calibration curve for aflatoxin G2 in HPLC method

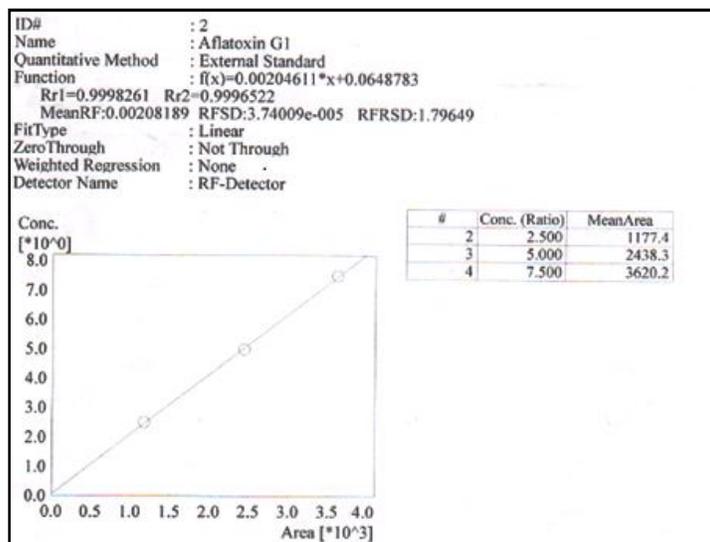


Figure 2: Calibration curve for aflatoxin G1 in HPLC method

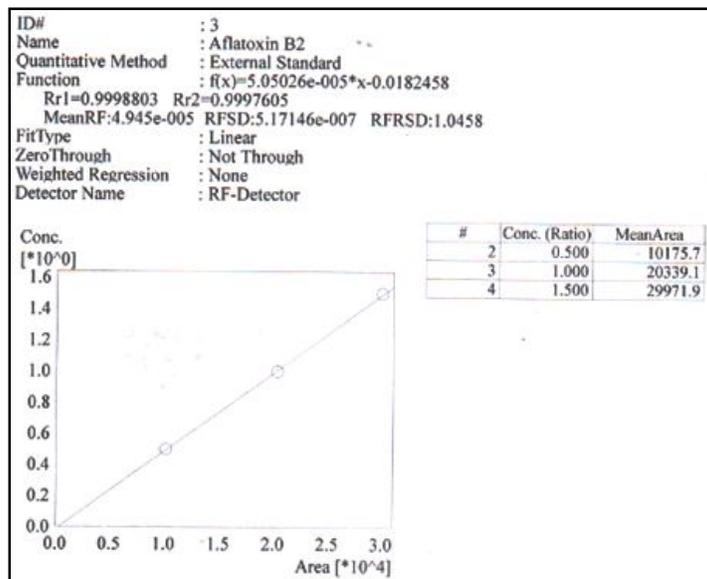


Figure 3: Calibration curve for aflatoxin B2 in HPLC method

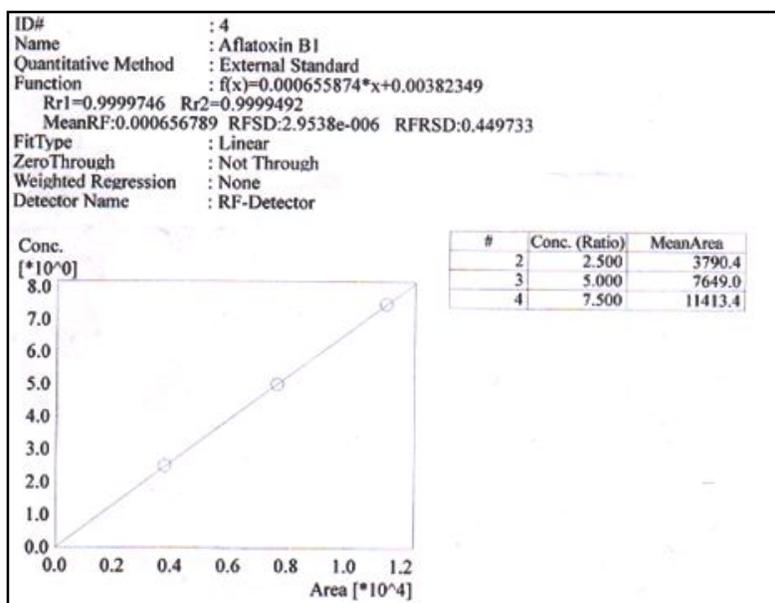


Figure 4: Calibration curve for aflatoxin B1 in HPLC method

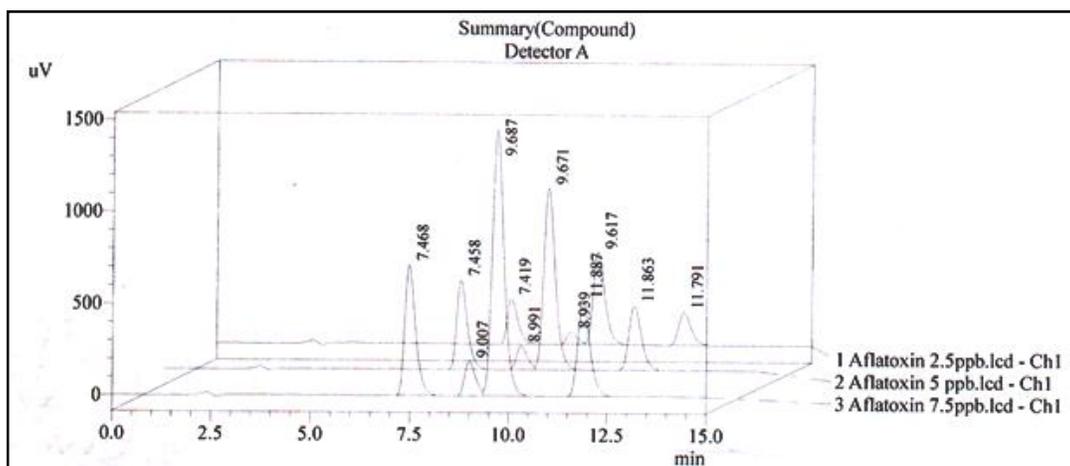


Figure 5: HPLC chromatogram of standard aflatoxins G2, G1, B2, B1

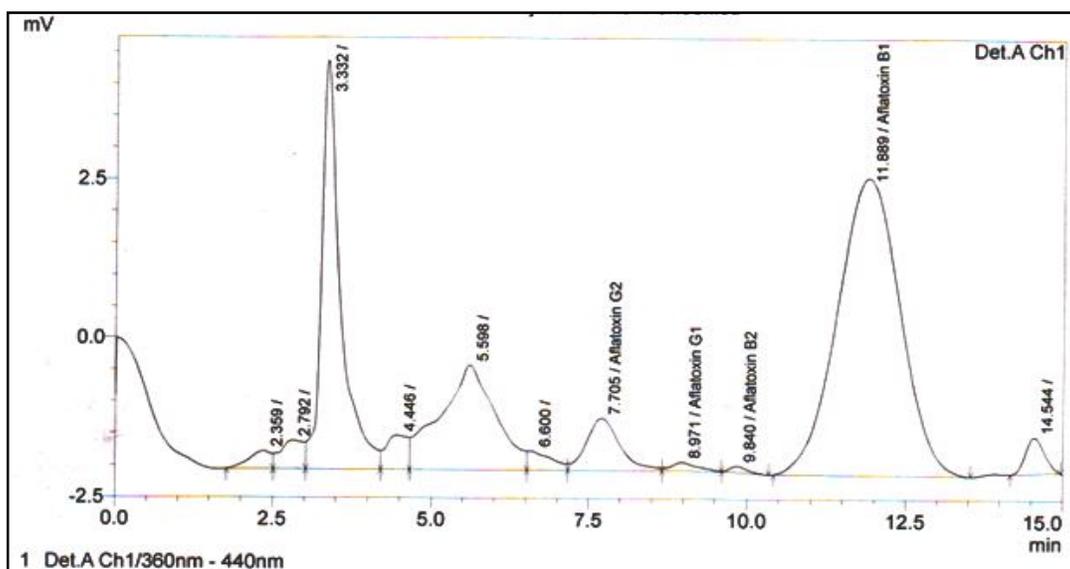


Figure 6: HPLC chromatogram of sample no. 1

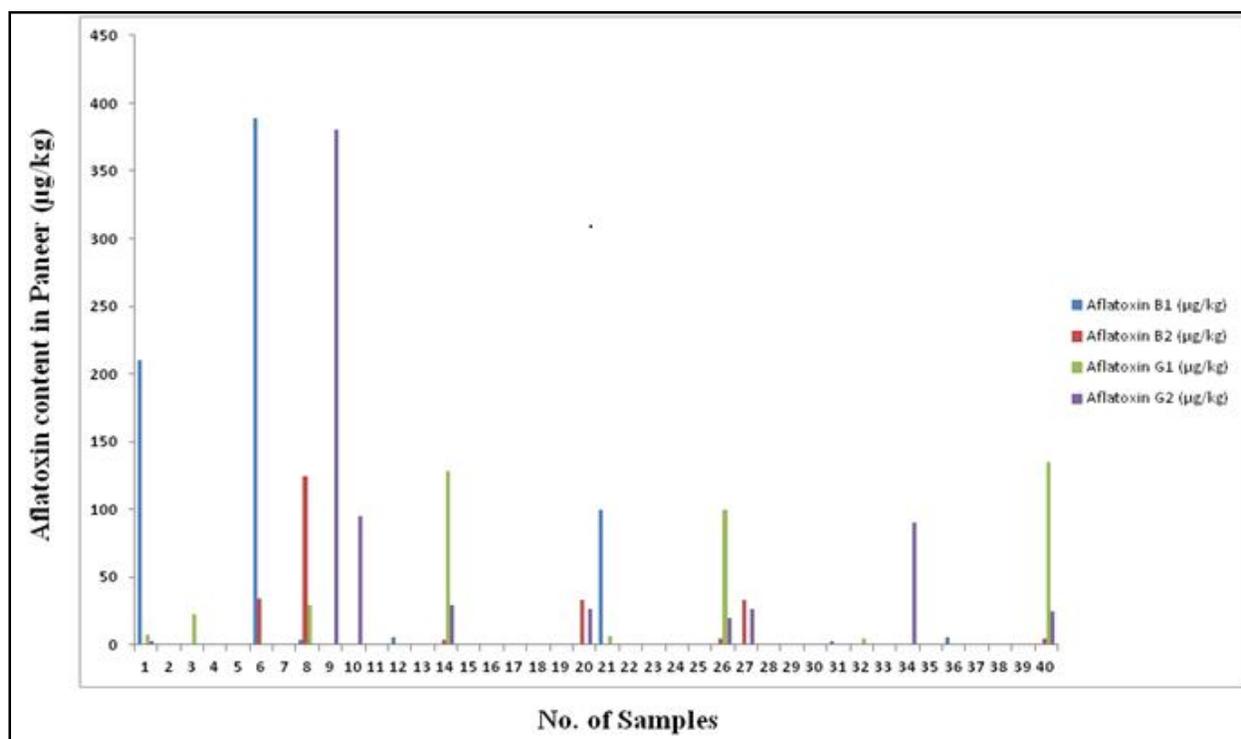


Figure 7: Total aflatoxin levels in Paneer samples from Chennai, India (samples=40)

## 5. Conclusion

Overall, this study carried out suggests that microbiological quality of paneer available in the city of Chennai is not within the limits set by the Food Safety & Standard Authority of India (FSSAI), Bureau of Indian standards (BIS) and the International Committee on Microbiological Specification for Foods (ICMSF) standards. Thus it is necessary to incorporate the HACCP and Microbial Risk Assessment (MRA) plans for prevention of contamination of raw milk and paneer. Due to the increase of aflatoxins in many food consumptions, studies on the presence of aflatoxins in dairy products have been increasing globally as well as in India. Mycotoxins have serious effects on humans and animals. This finding also reflects that paneer marketed in Chennai, India contains residual level of aflatoxins and pose public health risk. Therefore, there is a need for continuous monitoring of aflatoxins in paneer. The high occurrence of aflatoxins emphasizes the need for regular monitoring and a more stringent food safety system in order to control the aflatoxins at the lowest possible level. Precautions must be taken in the storage of feed commodities. Low moisture content, low temperature and low humidity conditions should be maintained during storage because these depress the fungus growth and thus eliminate aflatoxin contamination. Analysis of aflatoxin at µg/L or kg level needs high technology laboratories equipped with highly precised instrumentation.

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