

## Research Article

# Comparison of Immunoassay and High Performance Liquid Chromatography Methods for Determining Aflatoxin M1 in Raw Milk

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**Abstract:** The objective of this study was to compare methods of immunoassay with High Efficiency Liquid Chromatography methods (HPLC) of processing plants of dairy products. Aflatoxins are a group of organic chemical non-protein compounds of low molecular weight, mainly produced by fungi *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius*. The Aflatoxin M1 (AFM1) is an oxidative metabolite of Aflatoxin B1 (AFB1), produced by animals after the ingestion of this, which appears in milk and urine. The negative impact of aflatoxins in human health is particularly due to their carcinogenicity, by what it is important the detection and quantification of AFM1 in raw milk used as raw material. In the dairy industry it is common to use rapid tests for the determination of the AFM1, but how reliable are these tests? The study discussed 28 raw milk samples taken in times of drought and rain in the South of Cesar, to determine the AFM1 using an immunoassay method with sensitivity of 0.35 µg/kg and for Liquid Chromatography (HPLC) method. The first method data were analyzed with basic descriptive statistics and with the results obtained by chromatography it was estimated the average level of aflatoxin by intervals, AFM1 average estimate was slightly higher in the dry season. It was also noted that results in dry season coincide with the results of the two methods by 42.9% and in the rainy season by 64.3%. The use of immunoassay method has as a disadvantage: the appearance of cross reactions with mycotoxins from the same group.

**Keywords:** Aflatoxigenic, *Aspergillus*, carcinogenicity, metabolite, mycotoxin, sensitivity

## INTRODUCTION

Mycotoxins are defined by FAO (1991) as secondary metabolites of fungi, produced in the final stage of the exponential growth of a fungal colony, causing pathological changes both in human beings like animals and the mycotoxicosis are syndromes resulting from absorption of mycotoxin toxicity. The mycotoxicoses refers to poisoning caused by inhalation, contact or ingestion of food that has been contaminated with mycotoxins (Serrano and Cardona, 2015).

They have very different chemical structures, although they are all are organic compounds of low molecular mass (EFSA, 2004). The main mycotoxins affecting food are: Aflatoxins (produced by fungi of the genus *Aspergillus*), (produced by fungi of the genus *Aspergillus* and *Penicillium*) Ochratoxins, trichothecenes, fumonisins and zearalenone (produced by fungi of the genus *Fusarium*) and Patulin (produced by fungi of the genus *Penicillium*) (Henry *et al.*, 2001).

Aflatoxins are a group of organic non-protein chemical compounds of low molecular weight, whose basic skeleton is a furan ring joined to the core of coumarin, produced mainly by fungi *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius* (Rimblas, 2004). Aflatoxins are carcinogenic activity, teratogenic and mutagenic. Worldwide studies have shown that for years the aflatoxins molecules interact with DNA, as they report it Wang *et al.* (2016). The main syndrome produced is hepatotoxic and it can also cause kidney problems. The main organs affected are: liver, kidney and brain. For this reason, the International Agency for Research on Cancer (IARC), included it as primary carcinogenic compounds (IARC, 2012). AFM1 and M2 are oxidative metabolites of AFB1 and B2 produced by animals after the ingestion of these, they appear in breast (both animal and human) milk, urine and feces (Peraica *et al.*, 1999).

The AFB1 is absorbed in the thin intestine into the portal blood system and is transported to the liver where it is metabolized. A part of aflatoxin is activated

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and attached in liver tissues. Certain water-soluble conjugates the AFB1 metabolite are eliminated within the bile and urine (Gimeno, 2004). Other non-conjugated metabolites are excreted into the blood circulatory system and are dispersed in an organized manner. Potentially, these wastes reach the milk, eggs, muscle and edible tissues (Dennis and Hsieh, 1981).

The milk is the exclusive food of animal origin susceptible to aflatoxin contamination. If dairy cows eat rations contaminated with 300 µg/kg AFB1, 1 µg/L of AFM1 in milk is detected. Toxin disappears in the milk a few hours stop administration of the contaminated animal feed. Although the evacuation of mycotoxins in the milk is low, changes in the blood-milk barrier produced by local and systemic infections, for example, mastitis can allow the passage of mycotoxins (Fink-Gremmels, 2008).

Also when the biota of the rumen is affected in the course of metabolic diseases, such as ruminal acidosis, decreasing degradation ability which favors the unexpected step of toxins not metabolised (Fink-Gremmels, 2008; Pattono *et al.*, 2011). The AFM1 is heat-resistant and is not completely inactivated by pasteurization, sterilization, or other processes of milk processing (Assem *et al.*, 2011). As it can be seen in the study carried out by Iqbal *et al.* (2013), Who analyzed a total 221 samples of milk and dairy products for drew up the presence of AFM1 by HPLC were collected in Punjab, Pakistan. The results revealed presence of AFM1 in 40% of the raw milk, 51% of butter milk UHT, 37% of yogurt, 60% and 43% of the samples of ice cream and 27, 24, 25, 34 and 17% of the samples were above the limit recommended for AFM1, respectively. AFM1 occurrence in milk and dairy products required to implement strict regulations and also the need for continuous monitoring of milk and dairy products to minimize the health risks. Since the thermal treatments did not affect the presence of the AFM1.

Analytical methods applied to the study of mycotoxins are focused on three groups: chromatographic methods, immunoassays and sensors-biosensors. Chromatographic methods are based on the analysis of the retention time of the analyte to a standard, in the particular case of mycotoxins methods with gaseous mobile phase (GC) and Liquid: High Performance (HPLC) or Thin Layer Chromatographic (TLC) (Skoog *et al.*, 2001). Immunoassay methods are based on the principle of ELISA (trial immuno absorbent enzyme-linked). These methods are often used for routine screening and field analysis. There are different versions that vary in the rapidity of the procedure and the quantification of the results. Detection of the effects of matrix is relevant to validation, there is a risk that arise false positives, by what the results require ratification by chromatographic methods. Some authors have studied the correlation of

ELISA methods with the chromatographic methods. Published limits of detection by ELISA procedure were always higher than obtained by chromatographic methods (Beaver *et al.*, 1991; Dreher and Usleber, 1996).

In this context it is necessary to emphasize the importance of a systematic monitoring of the contamination of milk and its products to ensure that AFM1 levels do not exceed the MRLs established by current regulations. Dairy companies opt for the quick methods, but how reliable are these methods, to determine whether raw (raw milk), is accepted or rejected on ground?

The objective of this study was to compare immunoassay with liquid chromatography HPLC methods as a methods of processing plants of milk products, to determine if the raw material complies with applicable legal requirements as to the concentration of the AFM1 and in this way to guarantee the safety of the products.

## MATERIALS AND METHODS

Taking into account that the Niño phenomenon started in the quarter of February, March and April 2015, when temperatures were at +0.5°C. In March-April-May rose to +0.7°C; in April-May-June, +0.9°C and came to +1.0°C in the quarter May-June-July; it would intensify in the last months of the year to the first quarter of 2016 (Henriquez, 2015), the suppliers of raw milk for livestock feed used different feed, therefore dairy determined 13 herds to take samples of raw milk in dry season and rainy season as the target population of study, located in the southern region of Cesar, Colombia.

For this study we used two methods for determination and quantification of AFM1: the method of immunoassay to determine the presence of AFM1, is a rapid test Reveal AFLATOXIN M1, which is based on the specific reaction was used as Antigen-antibody and immunochromatographic for the detection of AFM1 in milk fresh, valid for the milk of cow, sheep and goat, raw and mixed. The results are displayed in a Table 1 as the presence or absence of AFM1.

On the other hand, to quantify the AFM1 it was used liquid chromatographic methods HPLC of reference NTC 5219:2003. The AFM1 is extracted by passing the portion to analyze through an immunoaffinity column. The column contains specific antibodies linked to a solid support. As the sample passes through the column, the antibodies selectively bind with AFM1 (Antigen) forming an antigen-antibody complex. Other components of the sample matrix are washed with water column. The AFM1 is then eluted from the column, collecting the eluate. The amount of this in this eluate AFM1 is determined by High Performance Liquid Chromatography (HPLC) with fluorometric detection.

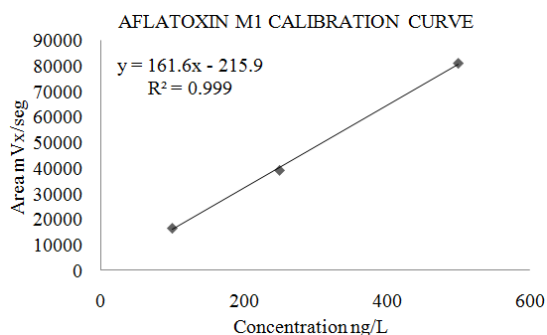


Fig. 1: Aflatoxin M1 calibration curve, Authors

It is necessary to stress that for the quantification of AFM1 in raw milk, the respective curve linear regression was built with its mathematical model, Fig. 1. We must also mention that the results of the concentration of AFM1, applied the statistical t-student for estimating intervals AFM1 averages according to dry and rainy seasons.

### RESULTS AND DISCUSSION

Detection of the AFM1 for raw milk in dry season was performed using two different methods (Table 1) where it can be seen that the results of both methods in some samples, showing positive immunoassay with sensitivity of  $\geq 0.35 \mu\text{g/kg}$ , but when applying the HPLC method the result are below this value. On the other hand, it shows 025 casts a severely positive immunoassay method, whereas in chromatography HPLC it was not detected presence of the AFM1. Sample 041 was reported as doubtful in the immunoassay method since it was not possible to distinguish whether there was presence or not, by not showing any of the two options as indicated the test instructions. Table 1 presents the comparative results of AFM1 of raw milk sampled in the dry season by the methods used.

Similarly for the rainy season as shown in Table 2, where it can be see that do not match the results of the two methods in some samples, where it yielded positive immunoassay with  $\geq$  sensitivity test  $0.35 \mu\text{g/kg}$ , but when applying the HPLC method the values are below this value. Table 2 presents the comparative results of AFM1 of raw milk sampled in the rainy season by the used methods.

Table 3 shows the estimation of levels AFM1 averages by the HPLC method, by comparing the results obtained with respect to the concentration of the AFM1 in raw milk, in contrast to the study conducted for raw milk in Croatia, by Bilandzic *et al.* (2010), where they found that the concentration was statistically higher between January and April ( $0.036$  to  $0,059 \mu\text{g/L}$ ), corresponding to the winter and spring, between June and September ( $0.012$  to  $0.015 \mu\text{g/L}$ ), belonging to the summer and the autumn. This could occur because the feed for animal consumption is more common in winter time and by high atmospheric

Table 1: Comparison of results in dry season

Sample cod	Immunoassay in drought	HPLC dry season
1	Negative	0.1087
2	Positive	0.0120
3	Positive	0.0033
7	Positive	0.0135
8	Negative	0.0198
9	Negative	0.0184
16	Positive	0.0069
17	Positive	0.0091
25	Severely positive	N.D.
27	Positive	0.0596
28	Positive	0.0150
30	Positive	0.0117
40	Negative	0.0874

Authors; Positive:  $\text{AFM1} \geq 0.35 \mu\text{g/kg}$ ; Negative:  $\text{AFM1} < 0.35 \mu\text{g/kg}$ ; N.D.: Not detectable

Table 2: Comparison of outcomes in rainy season

Sample cod	Immunoassay in drought	HPLC rainy season
1	Positive	N.D.
2	Negative	N.D.
3	Positive	0.0172
7	Negative	0.0065
8	Positive	0.0086
9	Negative	0.0079
16	Positive	N.D.
17	Negative	N.D.
25	Negative	0.1000
27	Positive	N.D.
28	Negative	N.D.
30	Positive	0.0110
40	Positive	N.D.

Authors; Positive:  $\text{AFM1} \geq 0.35 \mu\text{g/kg}$ ; Negative:  $\text{AFM1} < 0.35 \mu\text{g/kg}$ ; N.D.: Not detectable

Table 3: Estimation of levels of AFM1 by HPLC method

Sample	Interval AFM1 ( $\mu\text{g/kg}$ )
Dry season raw milk	$0.01430 < \mu < 0.04350$
Rainy season raw milk	$0.00045 < \mu < 0.01995$

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humidity, factors that encourage the growth of fungi. In the region of the South of Cesar, the results of samples in time dry season for the concentration of the AFM1 are superior ( $0.0143$  to  $0.0435 \mu\text{g/kg}$ ) and in the rainy season ( $0.00045$  to  $0.01995 \mu\text{g/kg}$ ) are much lower. It is likely due to the high consumption of silage in the dry season due to the lack of vegetation in the area.

In other studies such as those conducted by Capelli (2014), who used an immunoassay technique, they were obtained that levels between  $0.005$  to  $0.08 \mu\text{g/L}$  of Aflatoxin M1, they were found in 18 samples of milk, bringing the connotation that it is important to corroborate the results with more reliable methods such as chromatographic. Therefore, staying only with the result of the immunoassay test is not sufficient to decide the criterion of acceptance or rejection of raw material in plant.

### CONCLUSION

In the dry season it was presented for raw milk a range of  $0.0143$  to  $0.0435 \mu\text{g/kg}$  for the AFM1, but

23% does not comply with values permitted for export to the European Economic Community (0.05 µg/L). It should be noted that the values are higher when compared with the results of the rainy season. This is due to the increase in the food supply for livestock as silage of corn and palm kernel oil contaminated with *A. flavus* and *A. fumigatus* that became apparent in the present study, it could get to produce aflatoxin B1 in feeding stuffs to be later transformed into AFM1.

Differences in the results arose from applying both methods of detection of AFM1, the rapid test for the detection of AFM1 and the method by immunoaffinity column High Efficiency Liquid Chromatographic (HPLC) coupled with a fluorescence detector; in dry season the results agree with the results of the two methods by 42.9% and in the rainy season by 64.3%.

It should be noted that there is a risk from receiving at the plant, raw material with which is not safe to used immunoassay method. It has as a disadvantage: the appearance of cross reactions with mycotoxins from the same group. It is suitable in the dairies to replace the technique of rapid test kit to determine the presence of AFM1, by laboratory analysis accredited with the method of immunoaffinity column High Efficiency Liquid Chromatography (HPLC) coupled with fluorescence detector; to ensure reliability in the results.

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