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Detection of AFM_1 in Milk and Some Dairy Products in Iraq using different techniques

Muna T. AL-Mossawei[1], Labeeb A. AL-Zubaidi[2], Isam S. Hamza[3] , Saja Y. Abduljaleel[4],
[1]Department of Biology, College of Science for Women, University of Baghdad. Iraq.
[2]Directorate of Environmental and Water, Ministry of Science and Technology. Iraq.
[3]Directorate of Environmental and Water, Ministry of Science and Technology. Iraq.
[4]Department of Biology, College of Education for Women, University of Anbar, Iraq.

Abstract:

The 130 samples of milk and some dairy products were randomly collected from Baghdad markets from September 2014 to June 2015 and distributes into imported and local samples include: liquid and powder milk, white and soft cheese in addition to yoghurt. The samples were analyzed to qualitative and quantitative detection of aflatoxin M_1 (AFM_1) using different techniques {Thin layer chromatography- TLC (qualitative), High performance liquid chromatography- HPLC and Enzyme Linked Immune Sorbent Assay- ELISA (quantitative}. The positive results (contaminated with AFM_1), showed as 50 (38.5%), 65 (50 %) and 70 (53.8%) respectively, furthermore, yogurt and cheese showed more contamination with AFM_1 than other products and the highest concentration of AFM_1 in the local cheese reached 300.7ng/L and 939.67ng/L when detected with HPLC and ELISA techniques respectively. We concluded that ELISA technique was found to be most advisable for detection of low-level AFM_1 contamination in milk and dairy products . On other side the local products were contaminated with AFM_1 than imported products , in addition to yogurt and cheese were more contaminated with AFM_1 than other samples.

Key words: Detection, AFM1, TLC, HPLC , ELISA, Milk, Dairy Products, Iraq

Introduction:

Aflatoxins (AFs) are mycotoxins (secondary metabolites) produced by the aflatoxigenic fungi mainly *Aspergillus flavus* and *A. parasiticus*, while rarely *A. nomius* and *A. pseudotamarii* (Pane *et al.*, 2012). According to the Food and Agriculture Organization (FAO) up to 25% of the world's agricultural commodities involved crops are significantly contaminated with mycotoxin (Yibarek and Tamir, 2014). In addition to contaminate processed food (Songsermsakul, 2015). AFM_1 have been classified as Group 1 carcinogens (IARC.2002). Carcinogenicity of AFM_1 is nearly (2 to 10)% higher than the original form AFB_1 (Iqbal *et al.*, 2013). AFM_1 is associated with milk when lactating animals are feeding on feed contaminated with AFB_1 , it metabolites in the liver as AM_1 and then excreted with milk or urination (Henry *et al.*, 2001). AFM_1 is bound with milk protein particular casein that leads to its presence in dairy products (Prandini *et al.*, 2009). AFM_1 is a hepatocarcinogen 4-hydroxy derivative from metabolized of AFB_1 (*in vivo*), which is formed in liver and excreted into the milk in the mammary glands of both human and lactating animals when ingested contaminated diet with AFB_1.(Gurbay *et al.*, 2010) The residues of AFM_1 are stable enough to survive in raw and processed milk, hence they are known as milk toxins (Mohammadi, 2011). The level of converted AFB_1 into AFM_1 in milk is influenced by many factors including breed, health, physical condition, type of diet, milk production and rate of digestion (Duarte *et al.*, 2013). AFM_1 has hepatotoxic, immunosuppressive, mutagenic, teratogenic and carcinogenic effects. The presence of AFM_1 in milk and its products have a major risk for humans especially infants, the AFM_1 transmitted from the mother to the infant through the milk when mother consumption food-laden with AFB_1 (Dutton *et al.*,2012). Many techniques are being used to detect the presence of AFs, some of these include TLC, ELISA, flourometry, quantitative and qualitative lateral flow assays, HPLC, coupled with UV and mass spectrometry- LC-MS (Shephard *et al.*, 2012). In Iraq there are lack in the data of the natural occurrence on AFM_1 in milk and dairy products, therefore, the current research focused on detection of AFM_1 level in the dairy food samples at the local markets using TLC (qualitative) , HPLC and ELISA (quantitative) techniques.

2 Materials and Methods

2.1 Samples Collection.

The 130 samples of milk and some dairy products were randomly collected from Baghdad markets from September 2014 to June 2015 and designated into imported and local samples include liquid and powder milk 50 samples; yogurt 40 sample ,white and soft cheese 40 sample. All of the samples was immediately transported to the laboratory into ice-packs and stored at -20 ^{0}C until analysis.

2.2: AFM_1 Extraction from Milk and Dairy Products Samples

A- Milk

The AFM_1 Extraction from milk samples were carried out according to Charoenpornsook *et al.* (2006). The sample must be homogenization in the storage tank, was determined from approximately 100 ml, the milk fat was separated from sample by centrifuging at 3500 rpm for 15 min in maximum 10 ^{0}C. The skim milk was filtrated by filter paper and then passed through an immunoaffinity column (C_{18} column). The column was washed in water 40 ml to remove non specific material, the AFM_1 was released by the elution with acetonitrile-methanol 2.5 ml 3/2 v/v and methanol 2.5 ml, then the elute was evaporated instrument under vacuum (Shundo *et al.*, 2004).

B- Cheese

The cheese sample 10 g was cut to small pieces and blended for 2 min at high speed and then mixed with 80 ml dichloromethane. The mixture was filtered by millipore filtrate, the filtrate was evaporated to dryness using the evaporated instrument under vacuum then the residue was dissolved in methanol-water-hexane (1, 30, 50 v/v) and transferred to separate funnel to manual shaking for 2 min. The water phase (lower layer) was collected and the hexane phase (upper layer) was then washed twice with 10 ml water and the water phase was also collected subsequently. Both water phases were homogenized and then applied into immune-affinity column (C_{18} column). The toxin was then eluted from the column using 10 ml methanol (Elgerbi *et al*., 2004).

C- Yogurt

AFM_1 execrated was performed according to Stublefied (1990) with some modification. Samples execrated were carried out by taking about 50 g from yogurt sample and mixed with 10 ml NaCl solution saturated at 35 ^{0}C and warmed at 120 ml chloroform at 38^{0}C and mixed the solution with sample and salt solution in separated funnel for 2 min. Therefore, the mixture was centrifuging at 4000 rpm for 10 minutes, the chloroform phase was separated well. The chloroform layer was filtered through filter paper Whitman No. (1) into graduated cylinder. The filtrate was treated with hexane (v/v) in separated funnel and then the chloroform phase (lower layer) was collected. The filtrate applied to an immune-affinity column (C_{18} column), the AFM_1 was eluted with 2.5 ml acetonitrile-methanol (3/2 v/v) and methanol 2.5 ml, then, the elution was evaporated an instrument under vacuum (Grosso *et al.,* 2004 ; Shundo *et al.,* 2004).

2.3: Preparation of sample for ELISA technique

A- Liquid Milk

The milk liquid can be used directly for the assay after centrifuging the milk for 10-20 min. at 4000 rpm. The lower layer was used for analysis.

B- Powder Milk

The milk sample 1 g was placed in a suitable container and adding water 10 ml then dissolving by shaking and then using centrifuge for separating the fat layer at 10 min , the lower layer was used for analysis.

C- Cheese

To 1 g of finely grated cheese, adding 4 ml of 100% methanol. Vortex vigorously for 5 min manually or using a multi vortex. Centrifuge the samples for 10 min at 4000 rpm. Transfer 1ml of the supernatant to a new tube and dry to completion using a rotary evaporated at 70 ^{0}C or by blowing nitrogen gas the sample. To each dried sample, add 800 µl of (1x PBS), vortex for 1 min. Use 200 µl of the sample per well for the assay.

D - Yogurt

Take out 0.5 ml of the sample into a vial, add 0.5 of 1x Milk Extraction Buffer. Vortex for 3 min. at maximum speed. Centrifuged for 5 min. at 4000 rpm. Use 200 µl of the lower aqueous layer for the assay (avoid contact with the top fat layer). To determination of AFB_1 can be calculated using special program with Excel functionality for Bio-scientific Company.

2-4: Detection of AFM_1

A- **preparation of Aflatoxin M_1 Standard Curve.**

The standard AFM_1 solution was prepared according to AOAC (2000) with some modification in acetonitrile at a concentration of 0.25 µg/ml to prepare stock solution and kept at -20 ^{0}C. The standard curve drawn with concentrations (1, 2, 3 and 4) ng/ ml of AFM_1 using HPLC technique Figure (1).

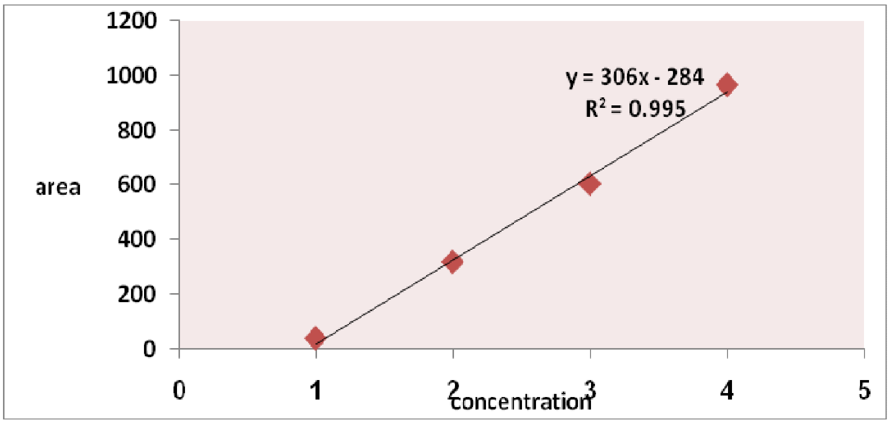

Fig.1: Standard curve of AFM_1 concentrations using HPLC technique

B- **Qualitative detection of AFM_1**

The AFM_1 extracts were re-dissolved in 150 µl of chloroform. The sample and standard solution were spotted on florescent silica gel plate at (20 X 20) cm as 10 µl of drop with many drops and then the plate was developed in chloroform-acetone-isopropanol (87: 10: 3 v/v). After drying the plate, it was examined under UV light. 366 nm wavelength (Shundo and Sabino, 2006).

C- **Quantitative detection of AFM_1.**

The quantitative analysis of AFM_1 were detect using fluorescent HPLC according AOAC (1990). The concentration of aflatoxin for each sample could be measured by application area of any peak from HPLC analysis in the standard curve equalities to gain the AFM_1 concentration of the samples. For determination AFM_1 by ELISA. The reagent and samples must be prepared according to the recommended Bio-scientific Kit instruction.

2.6 Statistical Analysis

The statistical analysis was conducted to extract the Mean ± Standard Error. The averages were tested using polynomial Duncan test (Duncan, 1955). Test the differences between the averages in the experiences of the effectiveness of different Numbers separately compared to the control using T-test .(Steel and Torrie, 1980)

3- Results and Discussion

3.1 Qualitative detection AFM_1 by TLC Technique.

Table (1) shown that out of 130 study samples were AFM_1 detected at 50(38.5%) positive samples, while 80(62.1%) negative samples using TLC technique Figure (2).

Table (1): Results of AFM_1 detection in different samples of milk and their products using TLC technique

Sample Category	Source of Sample	No. Sample	Positive Sample		Negative sample	
			No.	(%)	No.	(%)
Milk	Imported	25	5	20	20	80
	Local	25	10	40	15	60
Cheese	Imported	20	10	50	10	50
	Local	20	5	25	15	75
Yogurt	Imported	20	10	50	10	50
	Local	20	10	50	10	50
Total	130		50 (38.5%)		80 (61.5%)	

Also, it appears in the same table the yogurt is imported and local samples were more contaminated than other dairy products.

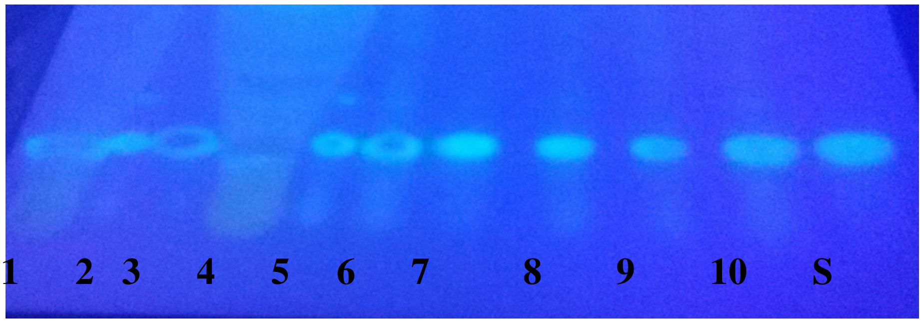

Fig. (2): The results showed the all samples were contaminated with AFM_1 when compared with standard, the spots 1-4 for imported and local milk samples, 5-8 for imported and local cheese samples and 9-10 for (imported, local) yogurt samples.

Several studies showed that the level of AFM_1 in raw milk and their results in the world have presented exceeded regarding the Codex Alimenterious regulatory limit and European Community limit (Dashti *et al.*, 2009; Amer and Ibrahim, 2010; Kamrar *et al.*, 2011; panahi *et al.*, 2011).

In the study of Filazi *et al.*,(2010) recorded that the presence AFM_1 in cheese samples were analyzed by TLC in Turkey, the concentration ranged from (20-2000) ng/kg in 14(28%) out of 50 samples, but only 5(10%) of cheese samples were found to have exceed the legal limit of established by the Turkish food codex (250 ng/kg).

3.2 Qualitative and Quantitative Determination of AFM_1 by HPLC Technique

The results of AFM_1 contamination of milk and dairy products were determination by HPLC technique table (2) out of 130 study samples were detected at 65(50%) positive samples and the different samples were ranged from (0.6 to 300.7) ng/L , the samples considered positive samples when the contamination of AFM_1 in milk above 50 ng/L according to EU regulation (Comission regulation, 2006; Hamid, 2011). In local samples of yoghurt, milk and cheese were contaminated with AFM_1 (75, 60 and 50)% respectively, more than imported samples (50, 40 and 25)% respectively.

The concentration of AFM_1 in local cheese, milk and yoghurt were ranged from (75.35- 300.7, 1.6 - 251.57 and 22.2- 172.9) ng/L respectively, while the mean values were (200.2, 150 and 103.9) ng/L respectively. In the imported cheese, yoghurt and milk ranging from (0.6- 250.3, 30.5-107.4 and 0.0- 96.81) ng/L respectively ,while the mean value were (93.8, 58.37 and 42.35) ng /L respectively.

Table (2): Results of AFM_1 determination in different samples of Milk and their products (ng / L) using HPLC Technique

Sample Category	Source of Sample	No. Sample	Positive		Range	Negative sample
			No	(%)	Max. -Min.	Mean ± SE
Milk	Imported	25	10	40	0.0- 9 6.81	42.35 ± 13.57
	Local	25	15	60	251.57 -1.6	150 ± 44.29
Cheese	Imported	20	5	25	250.3 -0.6	93.8 ± 68.18
	Local	20	10	50	300.7 -75.35	200.2 ± 70.26
Yoghurt	Imported	20	10	50	107.4 -30.5	58.37 ± 21.3
	Local	20	15	75	172.9 -22.2	103.9 ± 38.1
Total	130		65 (50%)		-	

Henry *et al.*,(2001) and Yavoglu *et al.*, (2005) suggested that the level of AFM_1 is relatively stable during raw, processed dried milk, stored, freeze, heat treated and milk products. Also the JiEan *et al.,* (2009) suggested that the milk may be contamination with AFM_1 after manufacture process of milk products or may be results from bad storage that lead to production food unfit human consumption. On the other hand the study of Marina *et al.*, (2007) was tested of AFM_1 in 128 samples of hard cheese by HPLC technique, Eight samples (6.25%) were found to be contaminated level at the maximum permissible level (0.05µg/kg), while 120(93.95%) were not contaminated in Portugal. Sarica *et al.*,(2015) can be detected AFM_1 in milk and dairy products (cheese and yoghurt) in Ankara- Turkey by HPLC-FLD and the percentage were 83% out of 24 milk samples, 92.6% out of 27 cheese samples and 89.5% out of 19 yoghurt samples, the level of AFM_1 ranged from 7.3 to 107.2 ng/kg and only 5 yoghurt samples exceeded the safety limit established by the Turkish food codex milk and yoghurt 50 ng/kg and cheese 250 ng/kg.

On the other hand Trombete *et al*., (2014) reported 30 cheese samples were analyzed by using HPLC Fluorescence detection it was found 18 in 60% contaminated with AFM_1, 8 samples in 26.7% presented AFM_1 above tolerance limit regulated by EC 0.25 µg/kg and all the cheese samples were least the maximum limit regulated by Brazilian legislation for cheese 25 µg/kg and concluded the presences AFM_1 in greater cheese consumed in Brazil were high relatively and that could be provide potential hazard for public health.

3.3 Qualitative and Quantitative determination of AFM_1 by ELISA Technique.

The high concentration of AFM_1 recorded by ELISA technique in the local and imported samples rang reached (0.3 to 939.67) ng /L respectively, and out of 130 study samples 70(53.8%) were positive results. The imported samples of yoghurt , milk and cheese were (100 , 25 and 25)% respectively, while in the local samples were (75, 60 and 50)% respectively. In the local samples of cheese, yoghurt and milk have high concentration of AFM_1 were range from (0.3 to 939.67, 29.25 to 505 and 32.1 to 380) ng/L respectively, while the mean values were (438.3, 215 and 210.4) ng /L respectively. The range and mean values for imported yoghurt, cheese and milk were reached from (232 to 432.3, 0.6 to 273.8 and 0.0 to 50.2)ng /L respectively, whereas mean values (333.5, 107.5 and 15) ng/L, respectively. (Table 3)

The incidence of AFM_1 in seven type of powder milk in Iraq reported by Al-Sowaf *et al.,* (2012) were found 82.8% contamination with AFM_1 and the range of contamination different from these types. AFM_1 in Multi, Melgro, Nido, Dielac, Lona, Angolas and Al-Mudhish from (200 to 640, 135 to 534, 50 to 280, 30 to 310, 10 to 270, 80 to 160 and 32 to 44) ng/ kg respectively. In the study of Darsanaki *et al*., (2013) they tested raw milk for AFM_1 by ELISA technique the presence of AFM_1 at concentration was between (2.1-131) ng/L in 56 out of 90 raw milk samples and they observed that the level of AFM_1 in 23 samples (31.11%) was higher than the maximum tolerance level (50 ng/L). Another study in Iraq by Najim *et al*., (2013) recorded the presence AFM_1 100% in milk and dairy products, they were taken raw milk, locally produced soft white cheese, locally produced yoghurt and imported pasteurized milk 30 samples for each were contaminated with AFM_1 ranging from (0.15 to 86.96, 31.84, 89.44, 0.16 to 42.74 and 0.18 to 85.66) ng/kg respectively. In addition to study of Ghalampour Azizi *et al*., (2007) showed that AFM_1 was 100% in milk sample, the concentration from 193 to 259 ng/L by ELISA technique. In the study of Barjestch *et al*., (2010) and according to ELISA result, it was found that 100% of pasteurized and local yogurt samples in (Northern Iran) were positive with AFM_1, but 2.5% out of 40 pasteurized yogurt sample and 10% out of 10 local yogurt sample contaminated above the limit of European Community Regulation (50 ng/L) where is 10% and 30% from pasteurized and local yogurt samples other than 25ng/L (the standard limit for milk children).

Table (3): Results of AFM_1 determination in different samples of Milk and their products using ELISA Technique (ng /L).

Sample Category	Source of Sample	No. Sample	Positive Samples		Range	Mean ± SE
			No	(%)	Max. – Min.	
Milk	Imported	25	5	25	50.2 – 0.0	15 ± 1.5
	Local	25	15	60	32.1 – 380	210.4 ± 26.3
Cheese	Imported	20	5	25	273.8 - 0.6	107.5± 13.4
	Local	20	10	50	939.67 – 0.3	438.3 ± 54.7
Yogurt	Imported	20	20	100	432.3 – 232	333.5 ± 41.7
	Local	20	15	75	29.25 – 505	215 ± 26.9
Total	130		70 (53.8%)		-	

There are many reported described the contamination cheese with AFM_1 as: Oliveira *et al*., (2011) were found the value of AFM_1 ranging from 0.04 to 0.31 µg/kg. Ertas *et al*., (2011) in Turkey were recorded presence of AFM_1 in 135 (64%) out of 210 analyzed samples of different dairy products. Amer and Ibrahim, (2010) were examined 150 samples of different type of cheese in Egypt and found maximum value of 0.25 µg/kg of AFM_1. In Italy virdis *et al*., (2008) were examined 41 cheese samples and found about (10%) positive for AFM_1 and highest value found was 0.39 µg/kg. In addition to study of Elzupir and Elhusse in (2010) ; Elkak *et al*., (2012) ; Anfossi *et al*., (2012) and Tavakoli *et al*., (2012) all of these studies described the persecuted of AFM_1 in other dairy products specifically different varieties of cheese.

From Fig (2 and 3) illustrate the ELISA technique has high positive percentage (53.85%) while HPLC technique was50% and in TLC technique was reached 38.5% alone. The ELISA technique has the highest performance then HPLC because by this technique can be detected high of AFM_1 concentration were reached 939.67 ng/L in local cheese. The less concentration of AFM1 can be detected in ELISA and HPLC were (0.3 and 0.6)ng/L in imported and local cheese respectively. The incidence of AFM1 in yoghurt more than dairy products and then milk, but more concentration can be recorded in local cheese (939.67 and 300.7) ng/L in ELISA and HPLC technique respectively.

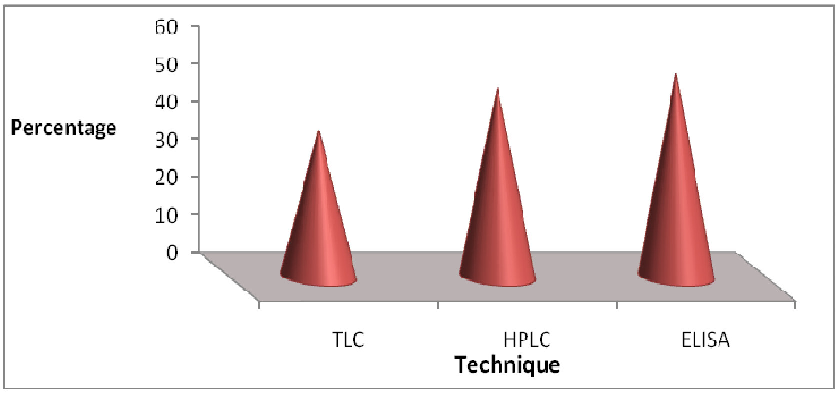

Fig. (3): Comparison results of TLC, HPLC and ELISA Technique for determinate the AFM_1

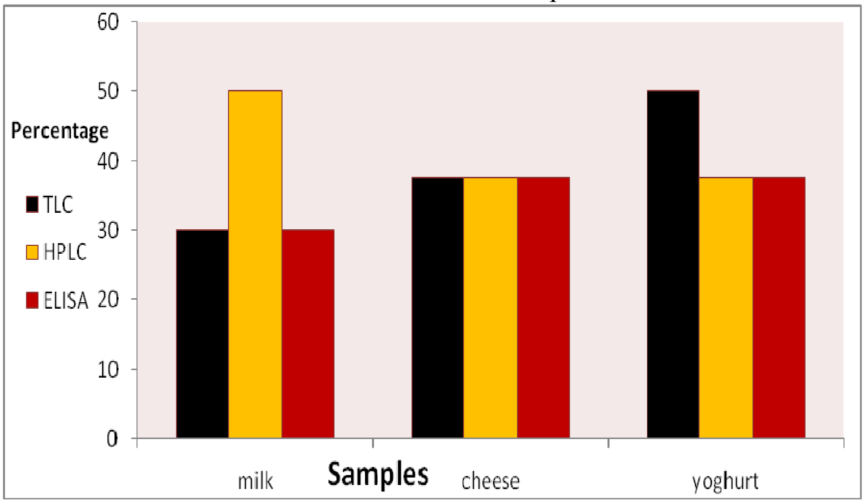

Fig.(4):The compression percentage results of contamination samples with AFM1 using TLC, HPLC and ELISA

Barjesteh *et al*., (2010) concluded that the limit detected by HPLC and ELISA technique was (10 and 2)ng/ml, respectively, HPLC and ELISA techniques were nearly similar in limit detection. Gurby *et al*., (2006) found that AFM_1 in 22 yogurt samples out of 40 samples ranging from (61.61 to 365)ng/kg were tested by ELISA technique. Incidence of AFM_1 in cheese may be cause to three reason: contaminated raw milk with AFM_1 results from contaminated cow feed with AFB_1, produce aflatoxin (B_1, B_2, G_1 and G_2) on cheese by growing *A. flavus,* and *A. parasiticus* (Zertiridis, 1985) and presence of these toxin in dried milk used to enriched the milk which used to synthesis of cheese (Blanco *et al*., 1988) . However, increased AFM_1 in cheese has been explained by the affinity of AFM_1 for casein (milk protein) (Brackett and Marth, 1982). We concluded that ELISA technique was found to be most advisable for detection of low-level AFM_1 contamination in milk and dairy products .
. On other side the local products were contaminated with AFM_1 than imported products , in addition to yogurt and cheese were more contaminated with AFM_1 than other samples.

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