Agricultural Research Department
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IDF collaborative study on the determination of Aflatoxin M1 in milkpowder, using immuno-affinity columns

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IDF COLLABORATIVE STUDY ON THE DETERMINATION OF AFLATOXIN M_1 IN MILKPOWDER, USING IMMUNO-AFFINITY COLUMNS

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ABSTRACT

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In the framework of the aim of the International Dairy Federation (IDF) to test a method for low aflatoxin M_1 concentration in milk a collaborative study was performed.
The study involved 16 participants from 11 countries. The method, chosen after a comparison of several methods by a preparatory group, uses an immuno-affinity column binding aflatoxin M_1 from the milk passing through the column. Acetonitrile elutes the aflatoxin M_1 from the column after washing other compounds from that column. Final determination was carried out by RP-HPLC and fluorescence detection.
Over the tested range (80-600 ng aflatoxin M_1/kg milkpowder) a RSD(R) of about 20% was obtained by analyzing 24 samples (blind duplicates) of which two samples were blanks.

Keywords: Aflatoxin M_1, immuno-affinity, milkpowder, HPLC, fluorescence.

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INTRODUCTION

In March 1989 the working group on mycotoxins within the IDF decided to perform a collaborative study of a method based of immuno-affinity chromatography clean-up with HPLC determination. The aim was to test a method applicable for low aflatoxin M_1 concentrations in milk (< 50 ng/kg milk).

COLLABORATIVE STUDY

16 Laboratories each received six randomly coded samples of milkpowder with a low fat content (about 1%) and six randomly coded milkpowder samples with a high fat content (about 28%). These latter six samples were remainders of batches of milk powders, used to prepare certified reference materials (1,2), hence the aflatoxin M_1 contents of these samples were known. The samples with low fat contents originated from the Netherlands Institute for Dairy Research (NIZO, Ede) and were used for their own purposes as internal check samples.

All types of samples were present as blind duplicates, in quantities just enough for one extraction procedure according to the method protocol i.e. 10 g milkpowder. Two of the six low fat samples were blanks.
The contamination level of aflatoxin M_1 in the other 10 samples varied from 0.08 - 0.6 μg/kg powder i.e. 80 - 600 ng/kg reconstituted milk.

Furthermore collaborators received a practice sample (20 g, sufficient for two analyses) with a known aflatoxin M_1 content, an unknown practice sample (10 g), fifteen immuno-affinity columns and a standard solution aflatoxin M_1 in chloroform (1 μg/μl). The result obtained with the unknown practice sample was to be returned by fax to the organizer, before the participant received an o.k. to proceed with the other unknown samples.
Participants received detailed instructions about the procedure to follow, including a test for the linearity of the HPLC system.

1 METHOD

Principle

Aflatoxin M_1 is extracted from the milk sample by passing it through an immuno-affinity column. The column contains specific monoclonal antibodies bound onto a solid support material. As the sample passes through the column, the antibodies selectively bind with any aflatoxin M_1 (antigen) present

and form an antibody-antigen complex. All other components of the sample matrix are washed off the column with water. Then aflatoxin M_1 is eluted from the column with acetonitrile, which is collected. The amount of aflatoxin M_1 present in this eluate is determined by means of high performance liquid chromatography coupled with fluorimetric detection.

2 Reagents

All reagents shall be of recognized analytical quality. The water used shall be distilled water or water of at least equivalent purity.

2.1 Immuno-affinity column (containing monoclonal antibodies against aflatoxin M_1) (Rhône Poulenc Diagnostics ltd., Glasgow, Scotland).

2.2 Acetonitrile

2.2.1 25% (v/v) acetonitrile in water. Dilute 250 ml of acetonitrile (2.2) to 1000 ml with water (degas before use).

2.2.2 10% (v/v) acetonitrile in water. Dilute 100 ml of acetonitrile (2.2) to 1000 ml with water.

2.3 Nitrogen

2.4 Chloroform, stabilized with 0.5 to 1.0% of ethanol, by mass

2.5 Aflatoxin M_1 (Sigma, St. Louis (USA) or Makor Chemicals, Jerusalem, Israël)

2.5.1 Stock solution (0.1 μg/ml aflatoxin M_1 in chloroform)

Prepare the aflatoxin M_1 solution in chloroform and dilute to a 0.1 μg/ml aflatoxin M_1 stock solution. The stock solution should be well stoppered and wrapped in aluminum foil to exclude light and stored in a refrigerator at a temperature below 5°C in the dark.

2.5.2 Preparation of working dilutions of aflatoxin M_1 standards

Before preparing working dilutions of the aflatoxin M_1 standard, it is essential that the 0.1 μg/ml solution (2.5.1), having previously been stored in a refrigerator, is allowed to attain ambient temperature before removing aliquots of the solution for subsequent dilution.

By means of a pipette (3.4) transfer 1.0 ml of the 0.1 μg/ml aflatoxin M_1 solution (2.5.1) to a 20 ml conical tube (3.9). Evaporate the solution to dryness using a constant stream of inert gas (2.3) and

dissolve the residue obtained in 20.0 ml of 10% acetonitrile (2.2.2). Shake occasionally during a period of 30 min. This solution contains 0.005 μg/ml of aflatoxin M_1.

Use this diluted solution (0.005 μg/ml) for the preparation of a series of dilutions of aflatoxin M_1 standard solution, which contain 0.1, 0.2, 0.4 and 0.8 ng aflatoxin M_1/ml respectively. The dilutions shall be made using 10% acetonitrile (2.2.2) as the diluent.

3 Equipment

3.1 Disposable syringes - capacity 10 ml and 50 ml

3.2 Vacuum system (i.e. Büchner flask, Vac-Elut system or peristaltic pump)

3.3 Centrifuge (capable of generating 1000 g, for defatting of the milk)

3.4 Pipettes-capacity 1.0, 2.0 and 50.0 ml

3.5 Glass beakers-capacity 250 ml

3.6 Volumetric flask-capacity 100 ml

3.7 Water bath capable of operating at 50°C

3.8 Whatman #4 filterpaper

3.9 Graduated conical glass tubes with ground glass neck and stopper-capacity 10 ml and 20 ml

3.10 HPLC-apparatus, consisting of an injector fitted with a 500 μl loop, solvent delivery system, fluorescence detector capable of operating as follows: λ ex: 360 nm and λ em: 435 nm and a reversed phase analytical column plus guard column.

Note:
A Spherisorb S5 ODS2 (12% C loading), (Phase Separations Inc. Norwalk, CT 06854, USA) 25 x 4.6 mm i.d has been found suitable, as well as a Chrompack HPLC cartridge, (Chrompack, Middelburg, Netherlands) 5 μm Chromspher-C18, length 20+1 cm (including guard column), inner diameter 3 mm. A guard column filled with reversed phase material may be used.

4 Procedure

See remarks 5.1; 5.2 and 5.3

4.1 Immuno affinity column preparation

Attach the barrel of a 50 ml disposable syringe (3.1) to the top of an immunoaffinity column (2.1). Connect the immuno-affinity column to the vacuum system.

4.2 Extraction

4.2.1 Milk

Warm the milk to 35-37°C and either filter enough milk through Whatman #4 filterpaper(s) (3.8) or centrifuge at 1000 g for 15 minutes. Collect at least 50 ml milk (if necessary use several filters). Pipette 50 ml of milk into the syringe barrel and allow it to pass through the immuno-affinity column at a slow steady flow rate of 2 to 3 ml per min., controlling the flow rate by means of the vacuum system. Continue with 4.2.3.

4.2.2 Milkpowder

Weigh to the nearest 0.1 g, 10 g of milkpowder into a 250 ml beaker. Take 50 ml of water warmed to 50°C and add this in small amounts to the milk powder. Mix using a stirring rod, until a homogeneous mixture is obtained (remark 5.4). Allow the solution of milkpowder so obtained to cool to 20°C and then quantitatively transfer it to a 100 ml volumetric flask using small amounts of water. Make the volume of the milkpowder solution up to the mark. Filter enough reconstituted milk through Whatman #4 filterpaper(s) (3.8) or centrifuge at 1000 g for 15 minutes and transfer 50 ml of the solution into the syringe barrel and allow it to pass through the immuno-affinity column at a slow steady flow rate of 2 to 3 ml per min., controlling the flow rate by means of the vacuum system. Continue with 4.2.3.

4.2.3 Remove the 50 ml syringe barrel and replace by a clean 10 ml syringe barrel. Wash the column with 10 ml of water. The water should be passed through the column at a steady rate and after the washing, blow the column completely dry. Disconnect the column from the vacuum system. Elute any aflatoxin M_1 slowly from the column by passing 4 ml of acetonitrile (2.2) through the column using a 10 ml syringe. The acetonitrile should take about 60 seconds to pass through the column and the flow rate is controlled by means of the syringe plunger. Collect the eluate in a 10 ml conical tube (3.9). Reduce the volume of the eluate to 300-500 µl at 30°C, using a stream of nitrogen (2.2) (warning: losses may occur if evaporated to complete dryness). Make up to 5.0 ml with water (remark 5.5).

4.3 High Performance Liquid Chromatography

4.3.1 Pump the eluent (2.2.1) at a constant flow rate through the HPLC column. Inject in sequence 500 µl of the aflatoxin M_1 standard solutions which contain 0.05, 0.1, 0.2 and 0.4 ng aflatoxin M_1. Prepare a calibration graph by plotting the peak area or peak height for each standard against the quantity of aflatoxin M_1 injected.

Note:
If necessary, (depending on the type of column used), the acetonitrile - water - ratio of the HPLC eluent (2.2.1) may be adjusted to ensure an optimal separation of the aflatoxin M_1 from any other extract components. The flow rate of the eluent (2.2.1) also depends on the column (3.10). As a guideline for conventional columns (length approximately 25 cm, internal diameter approximately 4.6 mm) a flow rate of approximately 1 ml/min gives optimal results; for HPLC columns with internal diameter of 3 mm a flowrate of 0.5 ml/min is optimal. It is advisable to ascertain optimal conditions using a sample extract (preferably free from aflatoxin M_1) which is injected in combination with an aflatoxin M_1 standard and as such.

4.3.2 Inject 500 µl of the sample extract into the HPLC apparatus via the injection loop and separate any aflatoxin M_1 present, using the same conditions as for the standard solutions. Determine the area of the aflatoxin M_1 peak of the sample extract.
From the calibration graph determine the amount of aflatoxin M_1 in the sample extract in ng. If the peak area or height of aflatoxin M_1 in the sample extract is greater than that of the highest standard solution, dilute the extract quantitatively with water and re-inject into the HPLC apparatus.

Note:
Where a series of sample extracts is to be injected one after the other, it is recommended that an aflatoxin M_1 standard is injected after every five injections of sample extracts.

4.4 Calculation

4.4.1 Milk
The aflatoxin M_1 content of the sample may be calculated using the following formula:

$$\frac{a}{Vm} \times \frac{Vext}{M} = \mu g/l \text{ aflatoxin } M_1$$

4.4.2 Milkpowder

The aflatoxin M_1 content of the sample in μg/kg may be calculated using the following formula:

$$\frac{a}{Vm} \times \frac{Vext}{M} = \mu g/kg \text{ aflatoxin } M_1$$

where in each case

a = amount in ng of aflatoxin M_1 corresponding to the area of the aflatoxin M_1 peak of the sample extract

Vm = volume of sample extract injected (500 μl)

Vext = volume in which the sample extract is dissolved (5000 μl)

M = volume of milk (50 ml) or mass of milkpowder (5g), passing through the column

This formula is applicable only when no dilution has been carried out. Otherwise this should be taken into account.

5 REMARKS

5.1 The method described in this protocol requires the use of solutions of aflatoxin M_1. This compound has been shown to be a carcinogen for some rodent strains. This compound should be handled as if it represents a carcinogenic risk to man.

5.2 Protect the laboratory, where the analyses are performed, adequately from daylight and keep aflatoxin standard solutions protected from light by using aluminum foil.

5.3 The use of non-acid washed glassware (e.g. tubes, vials, flasks, beakers, syringes) for aqueous aflatoxin solutions may cause loss of aflatoxin. Particular care should be taken with new glassware and disposable glassware such as autosampler vials and pasteur pipettes. Therefore laboratory glassware coming into contact with aqueous solutions of aflatoxin should be soaked in dilute acid (e.g. sulphuric acid, 2 mol/l) for several hours, then rinsed well with distilled water to remove all traces of acid (check with pH-paper).

5.4 In case the milkpowder is not completely dissolved place the beaker in a waterbath of 50°C for at least 30 minutes. Mix regularly.

5.5 When the acetonitrile content of the injected extract containing aflatoxin M_1 exceeds the 10% (v/v) limit, peak broadening will occur. However, a water content of over 90% (v/v) has no influence on the peakshape.

6 RESULTS AND DISCUSSION

Results, not corrected for recovery as reported by each laboratory, are presented in table 1 for the practice samples and in table 2 for the samples in the final study.

Observations:

Lab 1 reported problems with the sensitivity of the detection system and therefore carried out a pre-column derivatization with TFA (trifluoroacetic acid). Results of the known practice sample were much too low (approx. factor 10). The recovery experiments with lab's own milkpowder were low. At the end it appeared that the elution of the immuno-affinity column for the unknown samples was carried out with acetone instead of acetonitrile because of the need for precolumn derivatization. Therefore it was decided not to include the reported data (table 2) in the statistical calculations.

Lab 2 reported slow filtration of the reconstituted milk made from high fat milkpowder. Lab 4 noticed the same even after centrifuging.

Lab 5 centrifuged all samples and then used filtration to remove any large particulates from the drained milk. Furthermore the energy of the fluorescence detector lamp significantly decreased during the analysis.

Lab 6 reported no or low recovery for aflatoxin M_1 in the practice samples. Experiments with aflatoxin M_1 in water gave also no recovery. Therefore new practice samples were sent as well as, straight from the producer, a new batch of affinity columns. Results were the same; no recovery of aflatoxin M_1 occurred and no data were reported.

Lab 7 reported an increase of retention time (10-20%) during the HPLC measuring period on one day. The temperature in the lab increased to 33°C.

Lab 8 applied for the elution of the column gravity only instead of vacuum.

Lab 9 used a syringe pump for eluting the column. As the reconstituted milk passed easily through the column, no filtration nor centrifugation was used.

Lab 14 only had a calibration curve for 3 data points instead of at least 4. The lowest quantity as proposed in the procedure to be injected (0.05 ng was not injected. This quantity, according to the procedure, is equivalent to 100 ng/kg milk and appeared later to be the limit of detection for this lab.

Lab 15 concentrated the acetonitrile fraction from the column to 300 μl and filled up to 2000 μl i.e. the ratio CH_3CN : H_2O is about 1 : 5.7. According to the method protocol the CH_3CN content should be below 10%, to avoid peak broadening. This indeed showed up in the chromatograms of lab 15.

Participants were asked to provide a chromatogram of a relevant sample and a chromatogram of a M_1 standard solution. Most participants sent the chromatogram of sample E or F. From these the limit of detection (LOD) (signal: noise ratio is 3:1) was derived by the authors. From 5 out of the

remaining 14 participants chromatograms of samples B or D were requested at a later stage to better compare the LOD obtained from the extrapolation with the LOD obtained from sample B or D.

The resulting LOD's are reported in the last column of table 2. It is surprising that differences in LOD range over at least one order of magnitude.

The LOD is influenced by the sensitivity of the detector as such for the given compound but also influenced by the noise (chemical and electronical). If the chemical noise is negligable the LOD can be improved by injecting more sample. In this exercise it appeared that a few labs injected a smaller quantity of sample than described in the protocol (an amount equivalent to 0.5 g milkpowder was requested). Nevertheless the LOD was clearly below the reported results of these few laboratories. The labs 5,12,14,15,17 reporting results around or below LOD level, injected the requested quantity equivalent to 0.5 g milkpowder. With one exception (lab 12) all these laboratories used filter detectors. From the data of the aflatoxin M_1 standard as such it appeared that these laboratories indeed had a poorer LOD than the other laboratories using detectors with monochromators. To what extent participants used electronic filters is not known.

Sample A was a blank sample, nevertheless lab 5 and especially 15 reported in one of the blind duplicates the presence of aflatoxin M_1. In view of the level reported by lab 15 an interchange of samples seems likely, but could not be explained.

Statistical analysis was carried out according to ISO 5725 to determine outliers and to compute repeatability and reproducibility figures from the raw data as given in table 1 and 2. However data from lab 1 are completely eliminated from the statistical analysis. In table 3 results are given for the practice- and unknown milkpowder samples after eliminating Cochran and the (double) Grubbstest outliers.

With exception of the RSD(R) (relative standard deviation for the reproducibility) for sample E it seems that over the tested range for the low fat and high fat milkpowders the RSD(R) is in the order of 20%. Sample E gives most outliers, three labs by the Cochran test and one by the Grubbstest.

The RSD(r) (relative standard deviation for the repeatability) is in the order of 10-15% with the exception of the RSD(r) of samples D and E, for which lower values were found. In view of the low aflatoxin M_1 levels these results should be considered as very good.

Not known of course to the participants, was the fact that the known practice sample was identical to sample F, and the unknown practice sample was identical to sample E. Participants found indeed essentially the same values. The samples B and C were used, as said, as internal check samples. The aflatoxin M_1 content of these samples, corrected for recovery, is according to NIZO 80 and 150 ng/kg respectively. As the recovery is always between 90 and 100%, the participants in this study, whose data are not corrected for recovery, obtained a good result .

The aflatoxin M_1 contents in samples D, E and F, made from remainders of batches of milkpowder, used to prepare certified reference materials, and corrected for recovery, are respectively:
90 +40/-20, 310 $\pm$ 60 and 760 $\pm$ 50 ng/kg. (1,2)
The indicated 95% confidence interval covers the true value. Compared to the results of the participants (not corrected for recovery) in this collaborative study, there seem to be great differences in results, especially for samples E and F. The recovery correction used in the BCR study (1,2) however can account for up to 20% for material F.
Therefore reckoning with a general RSD(R) of about 20% in this collaborative study (table 3), there is a good overlap between the mean contents in sample D, E and F found in this collaborative study and the BCR exercise. Nevertheless the values for sample E and F obtained in the BCR exercise tend to be a little bit higher. Separately performed recovery experiments carried out at RIKILT-DLO at the aflatoxin M_1 levels of samples E and F did not give any indication, that the capacity of the immuno assay column was the reason.

7 CONCLUSIONS

Sixteen participants from eleven countries participated in a collaborative study for the determination of aflatoxin M_1 in milkpowder. Five samples, containing aflatoxin M_1 levels in the range of 80-600 ng/kg milkpowder, were analysed as blind duplicates. The overall RSD(R) is in the order of 20%, being a very acceptable result.
Hence, immuno-affinity columns are to be considered as an appropriate tool for aflatoxin M_1 determination at low levels of contamination.

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2. Addendum to report EUR 10412

Table 1. **REPORTED AFLATOXIN M_1 CONTENT IN THE PRACTICE SAMPLES (ng/kg MILKPOWDER)**

Laboratory	Known sample (580 ± 170 ng aflatoxin M_1/kg)	Unknown sample
	HF*	HF*
1	1)	1)
2	646	184
3	593	200
4	570	190
5	505	315
6	1)	1)
7	645	211
8	686	246
9	530	207
10	716	182
11	588	185
12	586	204
13	608	190
14	500	223
15	485	190
16 2)		
17	593	244

1) Results not included, see text
2) participation cancelled
* HF = milkpowder with high fat content

Table 2. **REPORTED AFLATOXIN M_1 CONTENTS IN THE SAMPLES OF THE FINAL STUDY (ng/kg MILKPOWDER) AND LIMIT OF DETECTION (LOD) (SEE TEXT)**

Laboratory	Sample A	B	C	D	E	F	LOD (ng/kg milkpowder)
	LF[1)]	LF	LF	HF[2)]	HF	HF	
1*	n.d.	144	156	34	84	352	
	n.d.	100	n.d.	97	176	520	
2	n.d.	65.9	132	88.4	220	602	25
	n.d.	80.8	134	88.8	205	608	
3	n.d.	77.2	153	84.3	190	571	10
	n.d	78.1	153	82.1	189	592	
4	n.d.	67.0	155	85.3	191	572	40
	16.9	72.5	148	72.6	204	607	
5	46	112	69	54	167	427	50 - 60
	n.d.	61	145	48	253	339	
6**							
7	n.d.	89	155	93	200	630	10
	n.d.	85	156	91	214	586	
8	n.d.	91	168	106	220	597	10
	n.d.	85	168	94	210	698	
9	n.d.	69	141	75	172	569	30
	n.d.	77	128	71	183	649	
10	n.d.	66.6	126	66.9	166	536	10
	n.d.	73.0	136	74.0	190	558	
11	n.d.	70.7	141	70.7	186	539	30
	n.d.	79.6	150	79.6	190	575	
12	n.d.	102	172	77.6	250	809	80
	n.d.	124	179	89.4	253	740	
13	n.d.	71.7	163	77.2	204	650	40
	n.d.	85.2	151	82.6	202	654	
14	n.d.	125	215	100	210	470	100
	n.d.	n.d.	180	294	186	690	
15	279	62	n.d.	64	322	400	50
	n.d.	45.6	105	58.8	128	390	
16***							
17	n.d.	104	192	104	800	454	150
	n.d.	n.d.	260	96	276	708	

n.d. not detectable (below limit of detection), according to participant
* all data eliminated for statistical analysis
** no results received, see text
*** participation cancelled
1) LF milkpowder with low fat content
2) HF milkpowder with high fat content

Table 3 **SUMMARY OF STATISTICAL ANALYSIS OF COLLABORATIVE DATA FOR THE HPLC DETERMINATION OF AFLATOXIN M_1 IN MILKPOWDER, USING IMMUNO-AFFINITY COLUMN CLEAN-UP**

	dim	known	unknown	B	C	D	E	F
n		14	13[1)]	13[2)]	14	13[3)]	11[4)]	14
m	ng/kg	589.36	204.31	81	150	80	202	580
r	ng/kg			23	60.1	15	27	200
R	ng/kg			52	98	41	61	310
RSD(r)	%			9.9	14.0	6.8	4.7	12.5
RSD(R)	%	11.7	10.6	23.0	22.7	18.3	10.8	19.1
SD(r)	ng/kg			8.05	21.68	5.39	9.52	72.59
SD(R)	ng/kg	69.04	21.66	18.63	35.08	14.62	21.84	110.53

1) lab 5 Grubbstest outlier
2) lab 5 Cochran outlier
3) lab 14 Cochran and Grubbstest outlier
4) lab 5, 15 and 17 Cochran outlier, 17 also Grubbstest outlier