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Fate of aflatoxin M_1 in cheese whey processing

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Abstract: Aflatoxin M_1 (AFM_1) is an important mycotoxin frequently found in milk and in dairy products. It is a minor metabolic product of *Aspergillus flavus* and *A parasiticus*. However, it occurs in dairy products as a metabolite formed in cows from aflatoxin B_1 contained in animal feeds. In cheese production, AFM_1 distributes between curd and whey, being present in products derived from cheese whey processing. In this study, cheese whey from dairy processing was artificially contaminated with the mycotoxin at about $0.1\,\mu g\,l^{-1}$. Ultra-filtration experiments of whey were carried out in order to determinate AFM_1 distribution between retentate (protein-rich fraction) and permeate (lactose-rich fraction). Recoveries of AFM_1 in retentate were 72.6–86.4% while, in permeate, recoveries were in the range 2.4–14.7%. Partition coefficients of AFM_1, lactose and protein were calculated to determine whether there was an interaction between AFM_1 and protein. In all experiments, AFM_1 partition coefficient was lower than 1, whilst for lactose coefficients close to 1 were determined, showing an affinity of aflatoxin M_1 to the protein-rich fraction (retentate).

Keywords: aflatoxin M_1; food safety; mycotoxin; ultra-filtration; whey

INTRODUCTION

Aflatoxins are a group of naturally occurring toxins which are secondary metabolites of some *Aspergillus* spp. Aflatoxin M_1 (AFM_1) is a very important toxin in milk and dairy products and is a minor metabolic product of *Aspergillus flavus* and *A parasiticus*.[1] However, it apparently occurs mainly in dairy products as a transformation product of cows of the aflatoxin B_1 contained in animal feeds. Thus, AFM_1 represents a potential hazard to humans via consumption of milk and milk products.[2] AFM_1 is less mutagenic[3] and carcinogenic[4] than AFB_1 but it exhibits high genotoxic activity.[5,6] The maximum admissible level of this mycotoxin in raw milk, heat-treated milk and milk for manufacture of milk-based products[7] range from $0.05\,\mu g\,kg^{-1}$, in the EU, to $0.5\,\mu g\,kg^{-1}$, in the USA.

In cheese processing, two products are derived from milk: cheese and cheese whey: cheese (curd) represents about 10% of the initial mass of milk, the remaining being cheese whey. According to the review of Galvano *et al*,[2] several studies on the partitioning of AFM_1 during cheese manufacture report a wide range of distribution of AFM_1 between whey and curd. Some authors observed that 50–100% of the AFM_1 was in whey. In contrast, others report that most of AFM_1 was in curd (66–100%). These authors refer as possible explanations (1) differences in the methodology for AFM_1 determination, (2) type and degree of milk contamination, (3) differences in the expression of results, or (4) differences in the cheese manufacture process. In addition, Applebaum *et al* (cited by Bakirci[8]) report that, since AFM_1 is a semi-polar component, it has less affinity to serum protein, this being the reason for the lower levels of AFM_1 found in cheese whey than in the original milk used for cheese manufacturing.

Whey is an important by-product from the cheese manufacturing industry. Disposal of liquid whey is costly due to its high biochemical oxygen demand (BOD) content. Utilization of whey protein products is dependent upon increasing the solids content and reducing the ratio of lactose to protein. Ultra-filtration is used to simultaneously fractionate, purify, and concentrate liquid whey for whey protein powder (WPC), which may be accomplished by single or multiple stage continuous processes.[9] Ultra-filtration membranes used for whey protein concentration normally have a molecular weight cut-off in the range 10–50 kDa and readily pass lactose and minerals, but reject proteins and residual milkfat.[9] Since whey proteins have a number of useful nutritional and functional properties, WPC can be used in a wide range of commercial products, such as food additives, or may be fractionated into individual whey proteins.[9] All these products may contribute for AFM_1 intake.

The aim of this work was to study the distribution of aflatoxin M_1 through the retentate and permeate when whey ultra-filtration is carried out. The ultra-filtration permeate contains mainly low molecular weight constituents, such as lactose, acids and ash, and the retentate contains mainly proteins.

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EXPERIMENTAL

Cheese whey powder was obtained from a dairy processing plant. This whey product was produced from defatted sweet cheese whey, by spray drying.

Bovine serum albumin (BSA; minimum 98%) and AFM_1 stock solution were obtained from Sigma (Lisbon). HPLC grade solvents were delivered from Merck (Lisbon).

Cheese whey reconstitution

Cheese whey was reconstituted to the desired concentration (7, 8 or 10% w/v) by dissolving the appropriate amount of powder in double-distilled water. AFM_1 contamination was accomplished by the addition of an accurate volume of a $500\,\mu g\,l^{-1}$ AFM_1 standard solution in methanol. The final spiked AFM_1 concentration was $0.1\,\mu g\,l^{-1}$.

Ultra-filtration experiments

Ultra-filtration experiments were performed using a Pellicon® XL filter with membrane Biomax™ 10 (Millipore Ibérica SA, Madrid), with a cut-off of 10 kDa. Reconstituted cheese whey (feed) (500 ml) was pumped with a peristaltic pump at a flow rate of $48\,ml\,min^{-1}$ to the ultra-filtration unit, the permeate and the retentate being collected in different graduate containers for volume measurement. With these volumes, and corresponding AFM_1 concentrations, a material balance was performed to calculate the recovery of the mycotoxin in each fraction. Control of the flow rate of permeate was made by adjusting the pressure at the retentate outlet.

After a first stage in the operation of the ultra-filtration membrane as described above, diafiltration stages were conducted by adding double-distilled water to the retentate in equal volume of the permeate that was collected from the previous filtration. Permeate and retentate were collected as mentioned before.

Solids determination

In order to determinate the dry extract of solids in feed, retentate and permeate, a refractometer (Leica DC 60; El Paso, USA) was used. A calibration curve was made using solutions in the range $0-100\,g\,l^{-1}$ of dried whey powder in water, and a linear regression ($r^2 = 0.99$) was obtained between dry extract and refractive index.

Protein determination

Protein was determined by the method of Bradford.[10] Working solutions containing $0.02-1\,mg\,l^{-1}$ of BSA were prepared in order to construct a calibration curve. Aliquots of protein solution (10 µl) were added to 290 µl of Coomassie reagent in a microplate. After 20 min the absorbance at 595 nm was read in a Spectra & Rainbow Tecan instrument (Salzburg, Austria). Protein was determined in the inlet (feed) and in the two outlets (retentate and permeate) of the ultra-filtration unit. The protein partition coefficient (K_p) was determined as the ratio between the protein concentration in permeate and in retentate.

Lactose determination

Lactose content was determined using the enzymatic kit lactose/D-galactose UV method of Boehringer Mannheim. Lactose was determined in the feed, in retentate and in permeate. Its partition coefficient (K_L) was determined again as the ratio between its concentration in permeate and in retentate.

Aflatoxin M_1 determination

Immunoaffinity clean-up

Aflatoxin M_1 was determined in the feed, in retentate and in permeate as reported by Tuinstra *et al*.[11] Samples of these three solutions were centrifuged at $2500 \times g$ for 15 min and then filtered through a glass microfibre filter (1.6-µm pore size) in order to collect a 25-ml volume sample that was passed through an immunoaffinity column (Aflaprep M, R-Biopharm Rhone Ltd, Glasgow, Scotland, UK). The unbound components were washed twice with 10 ml of distilled water. Elution was done using 1.25 ml of methanol:acetonitrile (20:30) at a flow rate of one drop every 2–3 s. A further 1.25 ml of distilled water was passed through the column resulting in a final eluate volume of 2.5 ml. This eluate was analyzed directly by HPLC.

To perform recovery assays, reconstituted whey was artificially contaminated with AFM_1 at level of $0.1\,\mu g\,l^{-1}$. Analytical recovery average for this method was 98% (CV = 3.3%, $n = 3$).

HPLC quantification

In order to construct a calibration curve, standard working solutions containing $0.1-2\,\mu g\,l^{-1}$ AFM_1 in mobile phase were prepared from a ready-to-use aflatoxin M_1 standard solution ($500\,\mu g\,l^{-1}$; Sigma-Aldrich, Lisbon, Portugal) in methanol. A calibration curve was obtained by linear regression of the least-squares method using peak height of the standard as response. The correlation coefficient was higher than 0.999. The limit of detection was $0.04\,\mu g\,l^{-1}$, as calculated from the sum of intercept and three times the standard deviation of the *y*-residuals of the calibration graph. Therefore, the limit of detection in whey samples was $0.004\,\mu g\,l^{-1}$.

Determination of AFM_1 was carried out by reverse-phase HPLC[11] equipped with a Jasco FP-920 fluorescence detector (360 nm excitation wavelength; 430 nm emission wavelength). Chromatographic separations were performed on a Waters Spherisorb ODS2 (4.6 mm × 250 mm; 5 µm) column, fitted with a pre-column with the same stationary phase operated at 30 °C. The mobile phase used was pumped at $1.0\,ml\,min^{-1}$ and consisted of an isocratic programme as follows: water:acetonitrile:methanol (68:24:8, v/v/v). The injection volume was 100 µl.

Aflatoxin M_1 partition coefficient (K_{AFM}), was determined as the ratio between its concentration in permeate and in retentate.

RESULTS AND DISCUSSION

The AFM_1 concentrations in cheese whey loaded to the ultra-filtration unit (feed) and in both solutions coming out of the same unit (retentate and permeate) were determined (Table 1). The recovery of AFM_1 in permeate and retentate fractions was also calculated (Table 1). Partition of total protein, lactose and AFM_1 between permeate and retentate in the ultra-filtration of cheese whey was determined (Table 2).

AFM_1 was found to favour the retentate fraction, where high molecular weight compounds, such as proteins, are expected to be concentrated. Data in Table 1 show a higher concentration of AFM_1 in retentate than in permeate for all experiments. Recoveries of AFM_1 in retentate were in range 72.6–86.4%, while in permeate the range varied between 2.4 and 14.7% (Table 1). These results suggest that AFM_1 is transferred preferentially to the protein rich fraction, although being a low molecular weight (328.3 $g\,mol^{-1}$) compound. Total mean recovery of 87.4% was obtained. This small loss in AFM1 could be due to fouling in the membrane.

The affinity of AFM_1, lactose and total protein to permeate and retentate was assessed by the determination of their partition coefficients between these fractions.

For the assay using 7% w/v whey powder, lactose partition coefficient (K_L) was determined and found to be near unity (Table 2), meaning that there is similar distribution of lactose between permeate and retentate, which is the common behaviour of lactose in industrial-scale cheese whey ultra-filtration.[9,12]

However, AFM_1 and total protein partition coefficients, K_{AFM} and K_P, respectively, were found to be much lower than one. The very low values reported for K_P were expected, since the molecular weights of these compounds do not allow them to be present in the permeate. Only a small fraction of some small peptides, originated from the hydrolysis of casein proteins during cheese making, were expected to be present in the permeate, and explain the small values of protein concentration that were reported in this fraction.

Partition coefficients of AFM_1 shown in Table 1 cannot be explained based on its molecular weight. This mycotoxin has a molecular weight of the same order as lactose: 328.3 and 442 $g\,mol^{-1}$, respectively. In the absence of any interaction with other compounds present in cheese whey, aflatoxin M_1 should partitioned like lactose between permeate and retentate. The higher affinity of AFM_1 for the retentate fraction can be due to an interaction between this mycotoxin and proteins.

The concentration of AFM_1 in permeate does not differ from its concentration in the original whey. However, since this retentate has a lower lactose content, it is expected that after drying it will originate a higher AFM_1 concentration in WPC than in the original whey protein powder.

Table 1. Aflatoxin M_1 distribution between retentate and permeate

		Feed	Retentate		Permeate	
Dried whey powder (% w/v)	Stage[a]	Aflatoxin M1 ($\mu g\,l^{-1}$)	Aflatoxin M1 ($\mu g\,l^{-1}$)	Recovery (%)	Aflatoxin M1 ($\mu g\,l^{-1}$)	Recovery (%)
10	UF	0.098	0.094	80.2	0.015	2.4
	DF	0.076	0.077	86.4	0.025	5.1
8	UF	0.096	0.089	72.6	0.029	6.5
	DF	0.066	0.069	85.5	0.031	8.6
	DF	0.055	0.058	84.4	0.033	12.0
7	UF	0.097	0.089	73.0	0.026	5.5
	DF	0.068	0.067	76.4	0.033	10.9
	DF	0.050	0.049	74.8	0.031	14.7

[a] Stage: UF = ultra-filtration; DF = diafiltration.

Table 2. Ratio between aflatoxin M_1 concentration (K_{AFM}), lactose concentration (K_L) and protein concentration (K_P) in permeate and in retentate

Dried whey powder (% w/v)	Stage[a]	Aflatoxin M_1 (K_{AFM})	Lactose (K_L)	Protein (K_P)
10	UF	0.16	nd	0.03
	DF	0.33	nd	0.04
8	UF	0.33	nd	0.05
	DF	0.44	nd	0.03
	DF	0.56	nd	0.03
7	UF	0.29	0.94	0.09
	DF	0.49	1.14	0.05
	DF	0.64	0.99	0.05

nd = not determined

[a] Stage: UF = ultrafiltration; DF = Diafiltration

CONCLUSIONS

The fate of AFM_1 during cheese whey fractionation by ultra-filtration was assessed. Based on its molecular weight, and in the absence of interactions with other whey components, it was expected that AFM_1 would permeate through the membrane together with other low molecular weight components of cheese whey, such as lactose. Under the experimental conditions used in this work, lactose distributes evenly between permeate and retentate, whilst AFM_1 exhibits a preference for the retentate. This behavior, which is not explained by its molecular weight, could be due to an interaction with the protein content in this fraction. This interaction apparently contradicts the low affinity of AFM_1 for serum proteins cited by Bakirci.[8] However, Dosako *et al*[13] report that this affinity for serum proteins is lower when compared to its affinity for caseins. Since the latter are almost absent from cheese whey, an interaction between AFM_1 and serum proteins can occur.

These results suggest that the production of whey protein powder by ultra-filtration could lead to a higher concentration of AFM_1 than in the original whey.

REFERENCES

1 Van Egmond HP, *Mycotoxins in Dairy Products*. Elsevier, London, pp 24–25 (1989).

2 Galvano F, Galofaro V and Galvano G, Occurrence and stability of aflatoxin M1 in milk and milk products. A worldwide review. *J Food Prot* **59**:1079–1090 (1996).

3 Barnes JM, Aflatoxin as health hazard. *J Appl Bacteriol* **33**:285–298 (1970).

4 Lafont P, Siriwardana M and Lafont J, Genotoxicity of hydroxy-aflatoxins M_1 and M_4. *Microbiol Aliment Nutr* **7**:1–8 (1989).

5 Sinnhuber RO, Lee DJ, Wales JH, Landers MK and Keyl AC, Hepatic carcinogenisis of aflatoxin M_1 in rainbow trout. *J Natl Cancer Inst* **53**:1285–1288 (1974).

6 Canton JH, Kroes R, Van Logten MJ, Van Schothorst M, Stavenuiter JFC and Verhulsdonk CAH, The carcinogenicity of aflatoxin M_1 in rainbow trout. *Food Cosmetics Toxicol* **13**:441–443 (1975).

7 CAST, *Mycotoxins: Risks in Plant, Animal, and Human Systems*. Council for Agricultural Science and Technology, Ames, Iowa (2003).

8 Bakirci I, A study on the occurrence of aflatoxin M_1 in milk and milk products produced in Van province of Turkey. *Food Control* **12**:47–51 (2001).

9 Morr CV, Whey proteins: manufacture, in *Developments in Dairy Chemistry*, ed by Fox PF. Elsevier, London, pp 245–258 (1989).

10 Bradford MM, A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**:248–254 (1976).

11 Tuinstra LGM, Roos AH and Van Trijp JMP, Liquid chromatographic determination of aflatoxin M_1 in milk powder using immunoaffinity columns for cleanup: interlaboratorial study. *J AOAC Int* **76**:1248–1254 (1993).

12 Bylund G, *Dairy Processing Handbook*, TetraPak Processing Systems AB, Lund, Sweeden, 436 pp (1995).

13 Dosako S, Kaminogawa S, Taneya S and Yamauchi K, Hydrophobic surface areas and net charges of s_1-k casein, casein complex. *J Dairy Res* **7**:123–129 (1980).