

## Preparation and characterization of Aflatoxin M1- Fluoresceinamine conjugate for AFM1 immunoassay in milk

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**Abstract:** *AFM1-oxime-Fluoresceinamine conjugate was prepared and characterized. For that purpose first was obtained and purified AFM1-oxime. UV-Vis scan spectral analysis of the obtained AFM1-oxime-Fluoresceinamine conjugate was made. The activity of AFM1-oxime-Fluoresceinamine conjugate was measured. It was proved that the obtained conjugate had high activity and potential possibility for detection of AFM1 in milk by immunofluorescent assay.*

**Key words:** *AFM1-oxime-Fluoresceinamine conjugate, AFM1-oxime, AFM1, milk, immunofluorescent assay*

### Introduction

Aflatoxins are a group of toxic and carcinogenic secondary metabolites produced by different *Aspergillus* species such as *Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus nomius*. [2]. They have been found to contaminate different agricultural commodities such as nuts, grain, several food and feedstuffs. Aflatoxin M1 (AFM1) is derived from Aflatoxin B1 following ingestion of feed contaminated with Aflatoxin B1 (AFB1). AFM1 is transferred to milk and consequently milk products destined for human consumption [4]. AFM1 has been found to have high genotoxic activity. And it is also known to be hepatotoxic and carcinogenic [5]. Therefore, the presence of AFM1 in milk and dairy products may pose a threat, especially to infants and children as they are the major consumers of milk and dairy products in some countries. According to the European Union regulations, the maximum level of AFM1 in raw milk, heat-treated milk and milk-based products should not exceed 50 ng/kg. For infant milk and follow-on milk no more than 25 ng/kg are allowed (Commission Regulation, 2006). The production processes do not essentially affect the concentration of AFM1, due to its heat and light stability. Therefore, the main strategy to diminish exposure risk, both for animals and human beings, is an appropriate preventive monitoring program.

### MATERIALS AND METHODS

#### Reagents and chemicals

All the used reagents were purchased from the following companies: Aflatoxin M1 from LKT laboratories, INC; O-(Carboxymethyl)hydroxylamine hemihydrochloride (CMO), Pyridine, Dicyclohexylcarbodiimide (DCC); Fluoresceinamine isomer I, Acetonitrile, Albumin from chicken egg white (OVA) from Sigma-Aldrich; Aflatoxin M1-antibody from MyBiosource; Sigma-Aldrich, Tween 80 from Scharlau Chemie S.A., Tetrahydrofuran (THF) and Dimethylformamide (DMF) from Merck.

#### Modification of AFM1

Since AFM1 has no reactive groups for coupling reactions it was converted to AFM1-O-Carboxymethyl oxime (AFM1-oxime) by the method described by Dean et al in their steroid work [1]. 200 µg Aflatoxin M1 and 1,6 mg CMO were dissolved in 2 ml pyridine :water :methanol (1:1:4 v/v). The mixture refluxed for 2,5 h at 110 °C and was incubated overnight at room temperature in complete darkness. After that it was concentrated on a rotary evaporator and the yellow colored residues were dissolved in 1 ml chloroform:methanol (9:1 v/v).

#### Purification of AFM1-oxime

The reaction mixture dissolved in chloroform:methanol (9:1, v/v) was loaded onto a silica gel (mesh size 60–120) column (20 cm x1 cm) pre-equilibrated with chloroform. The column was initially eluted with chloroform followed by a mixture of chloroform–methanol (9:1, v/v). Fractions (2 ml) were collected and the absorbance of each fraction was read at

260 and 365 nm in a double-beam spectrophotometer (6900 UV/Vis spectrophotometer Jenway) The column was finally washed with methanol. The fractions were pooled according to the absorbance peak and the resultant AFM1-oxime was concentrated and used for further work.

#### Preparation and purification of AFM1-oxime-Fluoresceinamine conjugate

The obtained AFM1-oxime was used for the preparation of AFM1-oxime-Fluoresceinamine conjugate. AFM1-oxime was dissolved in 200  $\mu$ l THF and mixed with 100  $\mu$ l DCC dissolved in DMF (10 mg/ml) and 200  $\mu$ l Fluoresceinamine in DMF (5mg/ml). The mixture was incubated overnight at 4°C in complete darkness. The reaction mixture was loaded onto a silica gel (mesh size 60–120) column (20 cm x 1 cm) preequilibrated with chloroform:methanol (9:1, v/v). The column was initially eluted with a mixture of chloroform–methanol (9:1, v/v), followed by a mixture of chloroform: methanol: acetic acid (40: 10: 3, v/v). Fractions (2 ml) were collected and the absorbance of each fraction was read at 260 and 494 nm in a double-beam spectrophotometer 6900 UV/Vis spectrophotometer Jenway.

#### Measuring AFM1-oxime-Fluoresceinamine conjugate activity by Fluorescent Immunoassay

A fluorescent immunoassay was performed to measure the activity of the AFM1-oxime-Fluoresceinamine conjugate. AFM1-antibody was immobilized on polystyrene microtiter plate. The following dilutions from antibody stock solution (15  $\mu$ g/ml) were made: 10; 50; 250; 500; 1000 and 1250 times. 100  $\mu$ l of the antibody were set in each well and was incubated overnight at 4 °C. On the next day the plate was washed three times with phosphate buffer saline containing 0,1 % Tween 80 (PBST) and the wells were blocked with 200  $\mu$ l 1% OVA in PBST for 1 hour. Then another washing step was performed. After that 100  $\mu$ l of the conjugate (10  $\mu$ g/ml) was set in each well and incubated for 40 minutes at 37 °C. The solution was pipetted out and fluorescence was measured on Cary Eclipse Fluorescence Spectrophotometer (USA).

## RESULTS AND DISCUSSION

### Modification of AFM1

AFM1 was converted to AFM1 - O-Carboxymethyl oxime to introduce free carboxyl groups suitable for binding of Fluoresceinamine .Figure 1 shows the reaction of AFM1 with CMO.

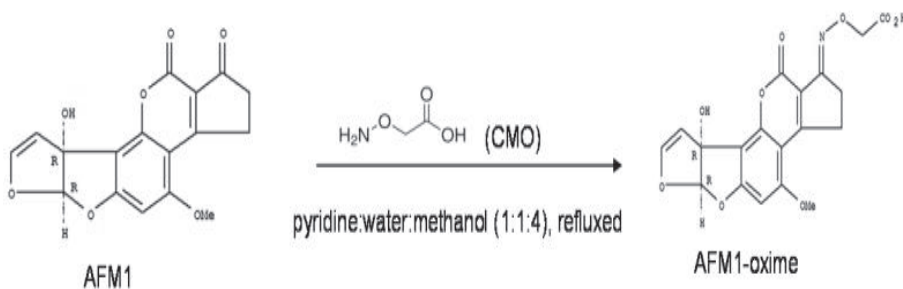


Fig. 1 Modification of AFM1 with CMO to AFM1-oxime

The obtained AFM1-oxime was purified by silica gel column chromatography. The sample was loaded into the column and was eluted with a mixture of chloroform–methanol. The absorbance of the fractions was measured at 260 and 365 nm and the results are shown on Figure 2.

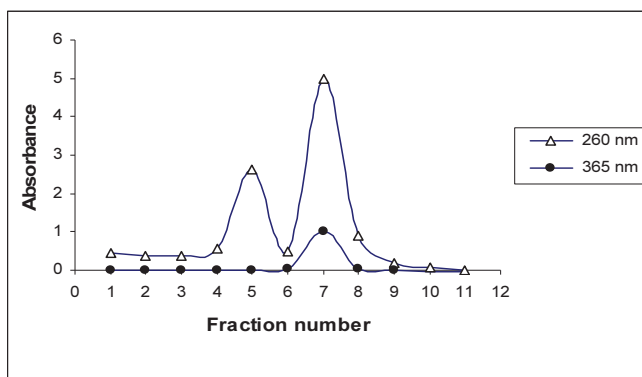


Fig. 2 Absorbance of fractions collected following silica gel column chromatography at 260 and 365 nm.

Fraction numbers 6, 7, and 8 that gave maximum absorbance at both the wavelengths were collected. These fractions were AFM1-oxime. Fraction numbers 4 and 5 that gave maximum absorbance only at 365 nm were the unreacted AFM1. UV-visible scan spectrum analysis was performed for AFM1-oxime. For comparison such analysis was performed for commercial AFM1. The results of UV-visible scan spectrum analysis are presented on Figure 3. The spectrum scan of purified AFM1-oxime is quite similar to that for standard AFM1, proving that the heterocyclic chromophore of AFM1 is not altered during the reaction [3]. The spectra of AFM1-oxime showed two peaks at  $\lambda_{\max}$  365 and 260 nm. Standard AFM1 showed peak only at 260 nm.

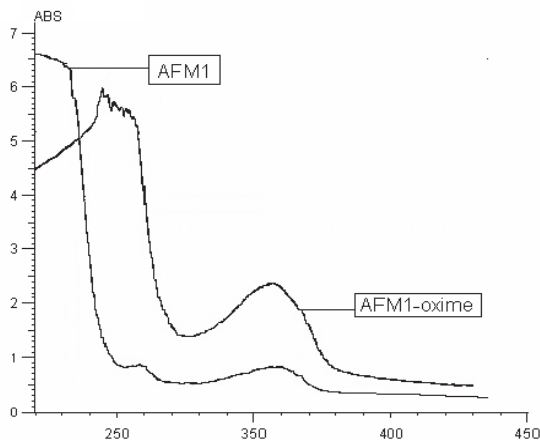


Fig. 3 UV-Vis spectra of the obtained AFM1-oxime and standard AFM1

#### Preparation and purification of AFM1-oxime-Fluoresceinamine conjugate

The obtained AFM1-oxime was used for the preparation of AFM1-oxime-Fluoresceinamine conjugate. The purification step was the same as the purification of the AFM1-oxime. Only the elution mixture was different, it contained acetic acid due to the need of more polar solvent. Fractions following silica gel column chromatography were measured at 260 and 494 nm. These wavelengths were chosen because AFM1-oxime gave maximum absorbance at 260 nm and fluoresceinamine isomer I gives maximum

absorbance at 494 nm. Absorbance of all the fractions is shown on Figure 4. Fractions from the first to the fifth showed absorbance at 260 nm but not at 494 nm. This proves that in these fractions there is no fluorescent dye. It was suggested that these fractions contained unreacted AFM1-oxime and DCC. From eleventh to the fifteenth fraction there were absorbance peaks at 260 and 494 nm. The highest values of absorbance were measured in fractions 13, 14 and 15 and these fractions were collected. These fractions were AFM1-oxime-Fluoresceinamine conjugate. UV-Vis scan spectral analysis of the obtained AFM1-oxime-Fluoresceinamine conjugate was made, Figure 5. The spectrum showed peaks at the expected wavelengths (260 and 494 nm). The other wavelength that AFM1-oxime gave absorbance was displaced from 365 to 330 nm due to the binding to Fluoresceinamine.

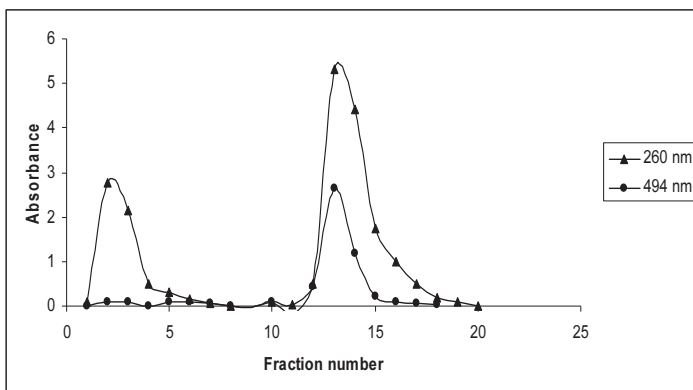


Fig. 4 Absorbance of fractions following silica gel column chromatography, measured at 260 and 494 nm

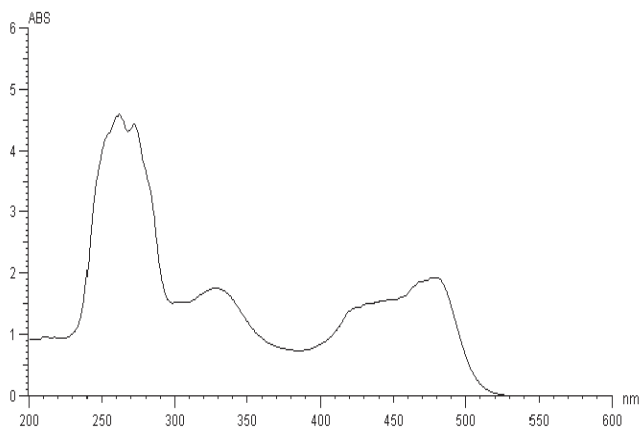


Fig. 5 Absorbance spectrum of AFM1-oxime Fluoresceinamine conjugate

### Measuring the activity of AFM1-oxime-Fluoresceinamine conjugate by Fluorescent Immunoassay

The obtained AFM1-oxime-Fluoresceinamine conjugate will be used for detection of AFM1 in milk by immunofluorescent assay. For performance of this analysis the activity of

the AFM1-oxime-Fluorescenamine conjugate is very important. For that purpose the activity of AFM1-oxime-Fluorescenamine conjugate was measured. The analysis was performed using six different antibody dilution and conjugate at concentration of 10 µg/ml for all the samples. The residual fluorescence in the solution was measured. The results are shown on Figure 6. The measured residual fluorescence at each different antibody dilution was subtracted from the blank sample (without antibody). Obviously the conjugate reacts with the antibody at all the concentrations, and even at antibody dilution of 1:1250 the antigen-antibody reaction proceeded.

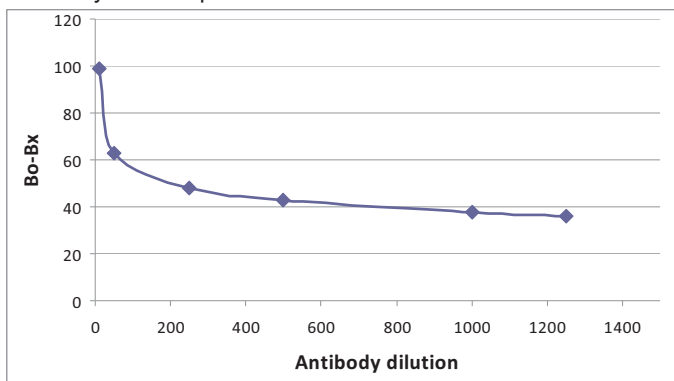


Fig. 6 Relation of antibody dilution and fluorescence

### CONCLUSION

- AFM1-oxime-Fluorescenamine conjugate was obtained and characterized.
- It was proved that the obtained conjugate had high activity and potential possibility for detection of AFM1 in milk by immunofluorescent assay.

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**This paper has been reviewed**