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Short communication

High-performance liquid chromatography with post-column derivatisation and fluorescence detection for sensitive determination of aflatoxin M_1 in milk and cheese

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Abstract

A new HPLC method with fluorescence detection using pyridinium hydrobromide perbromide as a post-column derivatising agent has been developed to determine aflatoxin M_1 in milk and cheese. The detection limits were 1 ng/kg for milk and 5 ng/kg for cheese. The calibration curve was linear from 0.001 to 0.1 ng injected. The method includes a preliminary C_{18}-SPE clean-up and the average recoveries of Aflatoxin M_1 from milk and cheese, spiked at levels of 25–75 ng/kg and 100–300 ng/kg, respectively, were 90 and 76%; the precision (RSD_r) ranged from 1.7 to 2.6% for milk and from 3.5 to 6.5% for cheese. The method is rapid, easily automatable and therefore useful for accurate and precise screening of aflatoxin M_1 in milk and cheese.
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1. Introduction

Aflatoxin M_1 (AFM_1), recently reported as carcinogenic to humans [1], is a hydroxylated metabolite found in milk of lactating animals which have consumed feedstuffs contaminated by aflatoxin B_1 (AFB_1). Because of the binding of AFM_1 to the protein fraction, in particular the association with casein [2], this metabolite can be present also in dairy products with contaminated milk [3]. Many countries have therefore established tolerance limits for AFM_1 in milk for human consumption and in dairy products, such as cheese (Table 1), until 1998 when the European Union has set a maximum residue limit (MRL) of 50 ng/kg for AFM_1 in milk [4–5] and recently also a MRL of 25 ng/kg in baby food [6].

To allow an effective control of the contamination of cow milk and dairy products by this toxin, very sensitive and reliable analytical techniques have been developed [7–13], many of them based on solid phase extraction (SPE) or immunoaffinity chromatography in combination with reversed-phase HPLC and fluorescence detection with or without derivatisation.

Derivatisation with a suitable fluorophore enhances the natural fluorescence of aflatoxins and improves detectability. This approach can be used as a confirmation tool for aflatoxins B_1 and G_1. The pre-column approach uses the formation of the corresponding hemiacetals using trifluoroacetic acid (TFA) [14–15], while the post-column one uses either bromination by an electrochemical cell (Kobra Cell) or addition of bromide or pyridinium hydrobromide perbromide (PBPB) to the mobile phase [16–18] and the formation of an iodine derivative [19].

Regarding AFM_1 only examples of pre-column derivatisation are reported [8,12,20]. Starting from our previous work [12], the present paper reports a more convenient and sensitive method for the determination of AFM_1 by HPLC/Fluorescence, using for the first time PBPB as post-column derivatising agent of this toxin.

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Table 1
Maximum acceptable levels (ng/kg) of AFM_1 in milk and milk products in various European countries and USA

Country	Milk	Cheese	Butter
Switzerland	50	250	20
Austria	50	250	20
Belgium	100	–	–
Germany	50	–	–
The Netherlands	50	200	20
Sweden	50	–	–
France	30 (children's milk) 50 (adult's milk)	–	–
Czech Republic	100 (children's milk) 500 (adult's milk)	–	–
Bulgaria	500	–	–
USA	500	–	–

2. Experimental

2.1. Chemicals

AFM_1 standard was purchased from Supelco (Bellefonte, PA, USA); PBPB from Sigma (St. Louis, MO, USA). SPE-C_{18} cartridges and dichloromethane were supplied by J.T. Baker (Mallinckrodt Baker, Phillipsburg, NJ, USA). Other chemicals and solvents were of analytical or HPLC grade and provided by Carlo Erba (Milan, Italy). Deionized water was purified on a MilliQ system (Waters, Milford, MA, USA).

2.2. Instrumentation

AFM_1 was analysed using an HPLC system consisting of an "Alliance" (Waters), equipped with a Waters 2695 separation module connected to a Waters 2475 Multi-wavelength fluorescence detector through a Waters post column reaction module; excitation and emission wavelengths were set at 353 and 423 nm, respectively. The eluate passes through a Supelcosil LC-18 column (5 μm particle size, 250 mm × 4.6 mm I.D.) maintained at 40 ± 0.1 °C. The system was governed by Waters Empower personal computer software.

2.3. Sample preparation

Milk: A sample of milk was homogenized and centrifuged at $3000 \times g$ for 10 min. Then, 10 ml of the aqueous phase, diluted with the same volume of deionized water, were purified on SPE [12]. Briefly, a SPE-C_{18} cartridge was conditioned with acetonitrile (5 ml) and deionized water (10 ml). After applying the diluted samples and washing with water (10 ml), acetonitrile/water (20:80, v/v) (20 ml) and then *n*-hexane (10 ml), AFM_1 was eluted with dichloromethane/acetone (95:5, v/v) (6 ml), the eluate was evaporated under a gentle stream of nitrogen and the residue dissolved in acetonitrile (200 μl); an aliquot (10 μl) of the AFM_1 extract was analysed by HPLC.

Cheese: A sample of 10 g, cut into small pieces, was extracted with dichloromethane/acetone (1:1, v/v) (50 ml) by UltraTurrax, with addition of sodium chloride (10 g). After centrifugation, 10 ml of the organic extract were evaporated to dryness under a gentle stream of nitrogen, the residue was dissolved in methanol (0.5 ml) and 0.01 mol/l sodium phosphate-buffered saline (PBS), pH 7.2–7.4 (20 ml) and *n*-hexane (10 ml) were added. After shaking, the lower layer was quantitatively collected and cleaned up on C_{18}-SPE as for milk.

2.4. HPLC separation and post-column derivatisation

Standard curve solutions of AFM_1 were prepared by diluting stock solution with acetonitrile to obtain final concentrations in the range 0.1–10 ng/ml. The mobile phase was acetic acid/acetonitrile/2-propanol/water (2:10:10:78, v/v/v/v). Isocratic HPLC was performed at 1.2 ml/min. As a post-column derivatising agent an aqueous solution of 50 mg/l PBPB [17–18] was used: flow rate, temperature and volume of reaction coil were properly optimized.

Identification of AFM_1 was based on its retention time. Further identity confirmation was carried out by re-injection of standard and samples without derivatisation: under these conditions, the AFM_1 peak decreases, unlike other co-eluted peaks. The calibration curve, i.e. the peak area versus concentration, was linear and data were fitted by the least-squares method. The line of regression calculated has been used to compute the amount of the analyte in sample extracts by interpolation, using external standard method.

2.5. Validation

Analytical performance was studied according to regulatory in force [21–22]. The selectivity of the method was evaluated by analysing 20 blank samples and checking for any interferences in the region where AFM_1 is eluted. Recovery and repeatability were determined spiking a AFM_1-free milk at 25–75 ng/kg (0.5–1.5 MRL) and, likewise, a AFM_1-free cheese at 100–300 ng/kg (0.5–1.5-fold the MRL for AFM_1 in cheese set in various European countries), for a total of 18 analyses. To estimate within-laboratory reproducibility, these steps were repeated on two other occasions by different operators. To evaluate the correlation between the post-column derivatisation and the pre-column technique, naturally contaminated samples of milk were analysed using both the proposed method (bromination of AFM_1) and pre-column derivatisation with TFA, according to [12]. The Mann–Whitney *U*-test (non-parametric independent two-group comparison) was used to compare the data.

3. Results and discussion

The chromatograms shown in Fig. 1 illustrate the efficiency of the proposed method: there are no interferences

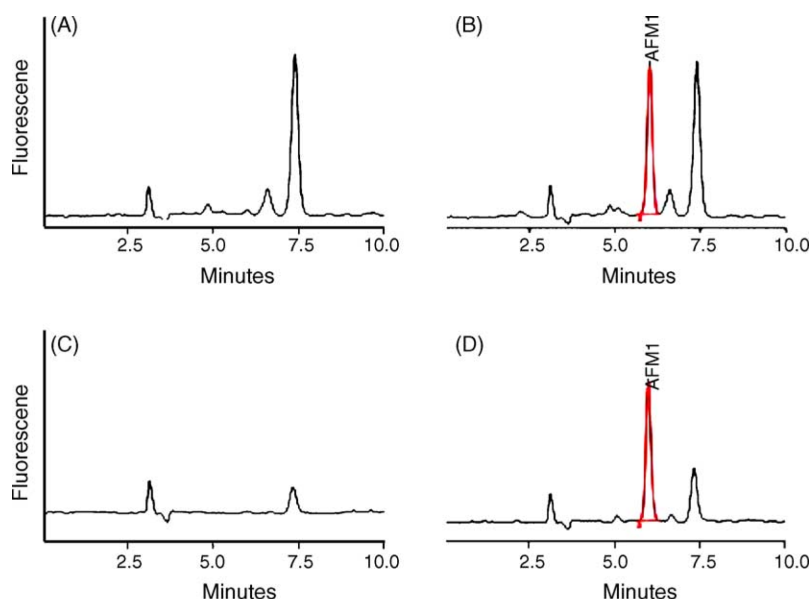

Fig. 1. Chromatograms of AFM_1-free milk (A) and cheese (C), milk spiked with AFM_1 at 50 ng/kg (B) and cheese spiked with AFM_1 at 200 ng/kg (D). Mobile phase, acetic acid/acetonitrile/2-propanol/deionized water (2:10:10:78). Flow rate: 1.2 ml/min.

in the region where AFM_1 is eluted. The retention time of about 6 min is short, but still ensures there will be no interference from unretained compounds, permitting a high sample throughput.

The AFM_1 peak intensity is affected by the mobile phase composition and maximum value was observed with acetic acid/acetonitrile/2-propanol/water (2:10:10:78, v/v/v/v), at a flow rate of 1.2 ml/min.

Concerning post-column derivatisation, the maximum detectability was achieved by using PBPB 50 mg/l at a flow rate of 0.3 ml/min; upon increasing the flow to 0.4–0.5 ml/min, the signal was reduced by 10%. The temperature of the reaction coil affects the derivatisation: best performance was achieved at 30 °C under controlled temperature (±0.5 °C) conditions. The coil volume of 1000 µl causes effective mixing with minimal bandspreading, maintaining analytical resolution from high performance column. Post-column derivatisation by PBPB was found to be rapid, simple and easy to automate. The derivatising agent used has such physical-chemical properties that the natural fluorescence of AFM_1 is enhanced, which results in a higher detectability: the signal of AFM_1 increases three-fold after derivatisation.

The detection limits were 1 ng/kg for milk and 5 ng/kg for cheese (signal-to-noise ratio, 3); the detection limit for milk is lower than that determined earlier [12].

The HPLC standard calibration curve was linear over the range of concentrations of AFM_1 injected. The equations for the calibration curves of AFM_1 in milk and in cheese, with 95% confidence interval of slope and intercept estimates, were: $y = 671867(\pm 17611)x - 425(\pm 951)$ $(r = 0.9988)$ $(n = 18)$ and $y = 105217(\pm 6454)x + 754(\pm 1394)$ $(r = 0.9934)$ $(n = 18)$, respectively.

Table 2
Analytical parameters for the determination of AFM_1 in spiked milk and cheese: repeatability and recovery percentages

Milk		Cheese	
AFM_1 added (ng/kg)	Recovery (%)	AFM_1 added (ng/kg)	Recovery (%)
25	90 ± 1.5	100	79 ± 3.0
50	90 ± 2.5	200	75 ± 5.0
75	89 ± 1.5	300	75 ± 2.5

Based on results for spiked samples, the mean recovery ± SD was found to be 90 ± 2% $(n = 18)$ for milk and 76 ± 4% $(n = 18)$ for cheese. Therefore, the method is accurate enough for use in the survey. Table 2 shows the homogeneity of the percent recovery and relative standard deviation for repeatability (RSD_r) data at the three levels of spiking for both matrices. The relative standard deviation for within-laboratory reproducibility (RSD_R) ranged from 2.5 to 4% and from 3 to 8.5%, respectively, suggesting a very acceptable precision at the present AFM_1 target levels. As regards the correlation between the two techniques, the results obtained by the proposed method and in accordance with [12] did not differ $(p > 0.05)$ according to the Mann–Whitney *U*-test.

4. Conclusions

The HPLC method with post-column derivatisation proposed in this paper improves AFM_1 analysis compared with earlier methods. It is simple and easy to automate, the reproducibility and ruggedness are improved and the analysis time is shorter. The detection limits of 1 ng/kg for milk and 5 ng/kg for cheese are 50-fold lower than the MRL for AFM_1 in milk

and 40-fold than the maximum acceptable level for AFM_1 in cheese set by various European countries.

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