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Testing the suitability of different high-performance liquid chromatographic methods to determine aflatoxin M1 in a soft fresh Italian cheese

T.M.P. Cattaneo*, L. Marinoni, S. Barzaghi, K. Cremonesi, L. Monti

CRA-FLC Centro di ricerca per le produzioni foraggere e lattiero-casearie (Fodder and Dairy Productions Research Centre), Via A. Lombardo 11, 26900 Lodi, Italy

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ABSTRACT

Aflatoxin M1 (AFM1) is a toxic undesirable compound in milk. AFM1 affinity for caseins causes a concentration effect during milk process for dairy transformation. In spite of this, no official method of analysis, nor maximum tolerance level for aflatoxin M1 in cheese have been established. Thus, the aim of this work was to test the suitability of different HPLC methods for the AFM1 quantification in soft cheese samples at three different contamination levels (low, medium and high, at respectively nearly 30, 100 and 250 ng/kg). Nine participants were selected among Italian laboratories accredited by the Italian accreditation body (ACCREDIA) for HPLC toxin analysis. They were asked to analyze samples applying the method routinely used. The different applied methods were compared, and precision and accuracy parameters were evaluated. The main differences among HPLC procedures were registered at the level of extraction step. The use of an enzymatic digestion for the extraction of the toxin from cheese seemed to be particularly advantageous and the use of immunoaffinity columns seemed to be determinant for the improvement of sensitivity at low contamination levels. In general, the applied methods well discriminated the 3 levels of contamination, even though they performed better at the medium and high concentration levels (100 and 250 ng/kg) than at the low one (30 ng/kg). In fact relative standard deviation for reproducibility at low level was higher (60.1%) than the same value at medium and high levels (22.8% and 28.9%, respectively).

1. Introduction

Milk may be contaminated by aflatoxin M1 (AFM1) when dairy cattle have consumed feeds containing aflatoxin B1. Due to the known adverse health effects of AFM1 [1], the European Union established that milk exceeding 50 ng/kg, the maximum level set out in CEC Regulation 1881/2006 [2], shall not be placed on the market for human consumption nor be processed for dairy transformation. It is well known that AFM1 binds to casein [3], has a high stability and concentrates in curd during cheese production, in different proportions according to the applied technology [4–17]. Its great stability has been also demonstrated during cheese ripening and storage [4–17].

Nevertheless, the detection and determination of AFM1 in dairy products, particularly in cheese, has not been fully ruled yet. The European Union has not imposed a maximum tolerance level for aflatoxin M1 in cheese, but it has only established that changes of the concentration of the contaminant, caused by processing, shall be taken into account [2]. As a consequence of the aflatoxin emergency occurred in Italy on maize grain in the summer 2003, the Italian Ministry of Health has posed a provisional limit of 450 ng/kg for AFM1 in hard, long ripening cheese [18]. Other countries tried to regulate the maximum permissible AFM1 concentration in cheese: for example Switzerland and Turkey, that introduced a legal limit of 250 ng/kg, or Moldova and Ukraine that set a higher limit of 500 ng/kg [19]. One of the factors contributing to the promulgation of mycotoxin regulations is the availability of reliable analytical methods which can allow an effective control of the possible contamination of commodities by mycotoxins, providing exposure data [20]. For milk analysis, immunological methods such as enzyme-linked immunosorbent assay (ELISA) are commonly used for screening purpose, but positive results have in any case to be confirmed using more sensitive and reliable methods, like reversed-phase High-Performance Liquid Chromatography (HPLC) analysis with fluorescence detection with or without derivatization, or liquid chromatography–tandem mass spectrometry [21–26].

In the case of complex food matrices, methods must be developed and validated for toxin-matrix combinations, and in the case of cheese, few methods have been investigated [27–35].

The aim of this study was to compare different HPLC procedures, commonly used to determine the amount of AFM1 in soft cheese

* Corresponding author. Tel.: +39 0371 450011; fax: +39 0371 35579.
E-mail address: tiziana.cattaneo@entecra.it (T.M.P. Cattaneo).

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samples, and to evaluate their precision and accuracy parameters. This could be considered a first step in generating an official method of analysis, satisfying a need particularly felt by both the producers and the consumers.

2. Materials and methods

2.1. Preparation of milk batches

AFM1 naturally contaminated milk (nearly 150 ng/kg) was produced at the experimental farm of the University of Piacenza by feeding animals with aflatoxin B1 naturally contaminated feed, within the accomplishment of a project on the carryover of aflatoxin from feed to milk in dairy cows [36]. The research protocol and animal care were in accordance with the EC Council Directive guidelines for animals used for experimental and other scientific purposes [37].

Three batches of milk at different levels of contamination (low, about 10 ng/kg; medium, about 30 ng/kg; high, about 80 ng/kg) were prepared by diluting contaminated milk with uncontaminated raw bulk milk ($AFM_1 < 5$ ng/kg), collected at the experimental farm of the Fodder and Dairy Productions Research Centre (CRA-FLC) in Lodi. Milk at different levels of contamination and uncontaminated milk, previously analyzed for their toxin content by HPLC analysis [38], were used to produce soft fresh cheese.

2.2. Cheese production

All cheese productions were carried out at the experimental pilot plant of the CRA-FLC. Each batch (about 15 kg) of raw whole milk was pasteurized for 2 min at 70 °C and then immediately cooled to 40 °C. Cattle liquid rennet (80% chymosine, 20% pepsin), strength 1:10,000 [Caglificio Clerici S.p.A., Cadorago (CO), Italy], and starter culture (EZAL™, Rhodia Food, Dangé Saint Romain, France) were added. The coagulation and acidification processes were carried out during a 40-min period. Afterwards, curd was cut and after 20 min milk-whey was drained: the curd was placed in perforated moulds to obtain a complete draining, and turned twice every 30 min.

In accordance with AFM1 affinity for casein [3], cheese production provoked an enrichment of the toxin in curd: cheeses at three different contamination levels (low, medium and high), confirmed by HPLC analysis [32], were produced.

Cheese samples were then portioned and stored at 4 °C for 7 days. After this period, samples were divided into aliquots, frozen at −20 °C, coded and randomly assigned to the participating laboratories.

2.3. Homogeneity testing of cheese batches

Homogeneity testing was made on cheese samples before sending them to labs for proficiency testing: 10 samples were randomly taken from each batch and analyzed in duplicate for their AFM1 content at 0 and 15 days of storage at −20 °C by using the HPLC method [38]. The obtained concentrations were subjected to statistical one-way analysis of variance (ANOVA), in order to determine if samples could be considered homogeneous.

2.4. Organization of the collaborative study

Participants were selected among Italian laboratories specialized in HPLC toxin analysis and ranged from official food control Institutions, private Laboratories and research Organizations. The list of the participants in alphabetical order is reported:

- Analysis S.r.l., Todi (Perugia);
- ARAL – Associazione Regionale Allevatori della Lombardia – Laboratorio latte e agroalimentare Crema (Cremona);
- Chemical Control S.r.l., Madonna dell'Olmo (Cuneo);
- Chemservice S.r.l., Novate Milanese (Milano);
- CNR, Istituto di Scienze delle Produzioni Alimentari, Bari;
- Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta – U.O. ricerca residui, Torino;
- Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise, Teramo;
- Neotron S.r.l., S. Maria di Mugnano, Modena;
- Università Cattolica del Sacro Cuore, Istituto di Scienze degli alimenti e della Nutrizione, Facoltà di Agraria, Piacenza.

In this paper, laboratories will remain anonymous and will be numbered from 1 to 9, independently from the above list.

Each participant received by express delivery 10 frozen samples, 6 of which corresponding to blind duplicates of cheese deriving from milk batches at three levels of AFM1 contamination, and 4 uncontaminated samples to perform blank analysis and to evaluate the recovery of its home-developed HPLC method. Participants were asked to use their own aflatoxin M1 solutions to build up calibration curves and to perform recovery tests. They were also asked to send results with one significant figure and uncorrected for recovery.

2.5. AFM1 analysis

Laboratories analyzed the aflatoxin M1 content of the samples by applying the method routinely used. Since there is no-official method of analysis for AFM1 detection in cheese, each laboratory developed its own procedure which should be confidential: for this reason only a summary of the procedure principles is shown in Table 1. Contaminated samples were thawed overnight at 4 ± 2 °C. The morning after, cheeses were analyzed: for each sample, a single extraction was performed, while the HPLC separation was repeated twice. For recovery calculations, blank samples were thawed overnight at 4 ± 2 °C, spiked using AFM1 standard solutions (100, 250 and 500 ppt), homogenized, kept for one night at 4 ± 2 °C, extracted and then analyzed with the same procedure used for the analysis of contaminated samples.

2.6. Data processing

Raw data were corrected for recovery values and submitted to statistical evaluation according to ISO Standard 5725-2 [39] to identify straggling and outlying data and to assess performance of both labs and procedures. The examination of results for consistency and outliers was performed both using a graphical technique and numerical outliers tests. For the former, Mandel's h and k statistics were used to calculate the between-laboratory and the within-laboratory consistency, respectively. Cochran test at 1% and at 5% critical value was performed in order to detect the presence of significantly too high differences between replicates intra-lab, while Grubbs' test and double-Grubbs' test at 1% and at 5% critical value were applied to identify straggling and outlying values inter-labs means. Repeatability (r) and reproducibility (R) values, repeatability and reproducibility standard deviations (s_r; s_R), repeatability and reproducibility relative standard deviations (RSD_r; RSD_R) were calculated. Finally, z-score values (z) were calculated for each sam-

Table 1
Summary of the method principles (step by step) used by different labs for AFM1 determination in cheese.

Lab code	Extraction	Clean-up	HPLC analysis
1	10 g of sample; extraction with dichloromethane; solvent evaporated to dryness; residue dissolved in methanol; addition of water and hexane	Water phase purified by immuno-affinity column clean-up; elution with acetonitrile/methanol; filtrate evaporated.	Residue dissolved in water/acetonitrile/methanol; reversed-phase HPLC with fluorescence detection (λ_{exc} 360 nm–λ_{em} 430 nm); eluent: water/acetonitrile/methanol
2, 8	10 g of sample; extraction with acetone; solvent evaporated to dryness; residue dissolved in hexane methanol, water.	Water phase purified by immuno-affinity column clean-up; elution with methanol; filtrate evaporated.	Residue dissolved in water/methanol; reversed-phase HPLC with post-column derivatization with Kobra Cell system and fluorescence detection (λ_{exc} 362 nm–λ_{em} 440 nm); eluent A: KBr, nitric acid, acetonitrile, methanol; eluent B: acetonitrile.
3, 9	5 g of sample; enzymatic digestion using a 0.2% pepsin solution in 0.1 N HCl, for 16 h at 42 °C. Sample centrifuged, filtrated and neutralized with 1 N NaOH.	Filtrate purified by immuno-affinity column clean-up; elution with methanol (Lab.9 used acetonitrile); filtrate evaporated.	Residue dissolved in water/acetonitrile (Lab 9 + methanol); reversed-phase HPLC with fluorescence detection (λ_{exc} 365 nm–λ_{em} 440 nm); eluent: water/acetonitrile (Lab 9 + methanol).
4	20 g of sample; extraction with chloroform; solvent evaporated to dryness; residue dissolved in methanol; addition of water and hexane.	Water phase purified by immuno-affinity column clean-up; elution with methanol; filtrate evaporated.	Residue dissolved in water/acetonitrile; reversed-phase C18 HPLC with fluorescence detection (λ_{exc} 360 nm–λ_{em} 440 nm); eluent: water/acetonitrile/methanol.
5	20 g of sample; extraction with chloroform.	Solid phase extraction of the extract using polar weakly basic silica column; elution with acetone/water; filtrate evaporated.	Residue dissolved in toluene/acetonitrile; TFA pre-column derivatization and reversed-phase C18 HPLC analysis and fluorescence detection (λ_{exc} 366 nm–λ_{em} 430 nm); eluent: water/methanol/acetonitrile.
6	10 g of sample; extraction with dichloromethane; solvent evaporated to dryness; residue dissolved in methanol; addition of water and hexane.	Water phase purified by immuno-affinity column clean-up; elution with methanol; filtrate evaporated.	Residue dissolved in water/acetonitrile; reversed-phase C18 HPLC with fluorescence detection (λ_{exc} 360 nm–λ_{em} 435 nm); eluent: water/acetonitrile + 1% acetic acid.
7	10 g of sample; extraction with dichloromethane/acetone; solvent evaporated to dryness; residue dissolved in methanol; addition of phosphate buffer.	Extract purified by C18-SPE clean-up; elution with dichloromethane/acetone; filtrate evaporated.	Residue dissolved in water/acetonitrile; reversed-phase C18 HPLC with fluorescence detection (λ_{exc} 365 nm–λ_{em} 435 nm); eluent: water/acetonitrile.

ple separately to establish the individual laboratory performance [40]; applying the following formula:

$$z = \frac{x - X}{\sigma}$$

where "x" is the measured AFM1 concentration in a test material; "X" is the median which is considered to be the true reference value; and "σ" is the target value for standard deviation.

3. Results and discussion

3.1. Homogeneity of cheese samples

Cheese production provoked a nearly 3-fold enrichment of the toxin in curd: these values are in good agreement with literature data related to the concentration factor of AFM1 in fresh cheese. Different authors found AFM1 levels about 3-fold higher in curd and cheese samples than those in respective milk samples: for example, Kiermeier and Buchner [11] found a 3.2-fold AFM1 increase for Camembert, Yousef and Marth [15], defined an enrichment factor ranging from 2.5 to 3.5 for soft type of cheeses and Cattaneo et al. [10] found an enrichment factor of 2.6 in a soft fresh Italian cheese. HPLC analysis [32] confirmed contamination of cheese at three different contamination levels (low, medium and high) (Table 2).

One-way analysis of variance (ANOVA) involving an F-test was carried out in order to investigate about the existence of significant differences between replicates taken from each batch, or values decay in two weeks at −20 °C. Statistical analysis revealed that the F-value for different concentration levels was below the critical value of $F_{9,10}(p = 0.05) = 3.02$. It was therefore concluded that no significant differences among samples existed and thus that replicates inside each batch of samples could be regarded as being homogeneous.

Once verified that the test was successful, frozen samples were mailed to the participant labs.

3.2. Results of the collaborative study

3.2.1. Comparison of HPLC procedures

The main differences among HPLC procedures are reported in Table 1. Seven of 9 labs used the same method for cleaning-up step, based on a purification on an immuno-affinity column, only two made this step by using SPE method. Chromatographic separation was carried out in all cases by RP-HPLC coupled with a fluorescence detector; one lab used a post-column derivatization step, based on the Kobra® Cell system, an electrochemical cell using a potassium bromide salt in the mobile phase as derivatization agent precursor, and another lab used a TFA pre-column derivatization procedure. Main differences were reported about the extraction step, in

Table 2
Homogeneity testing for cheese batches at three contamination levels: n = number of samples; mean = values of mean concentration for each contamination level at time (t) 0 and 15 days of storage; std dev = standard deviation; F = calculated F value; F_{crit} = F tabulated critical value at the 95% confidence level.

		n	Mean (ng/kg)	Std dev (ng/kg)	F	F_{crit} (p = 5%)
Level I	t = 0 days	10	36.1	1.7	2.99	3.02
Level I	t = 15 days	10	34.1	1.9	1.71	3.02
Level II	t = 0 days	10	106.9	1.6	2.96	3.02
Level II	t = 15 days	10	103.82	2.0	2.29	3.02
Level III	t = 0 days	10	262.4	2.7	1.64	3.02
Level III	t = 15 days	10	256.9	2.5	1.41	3.02

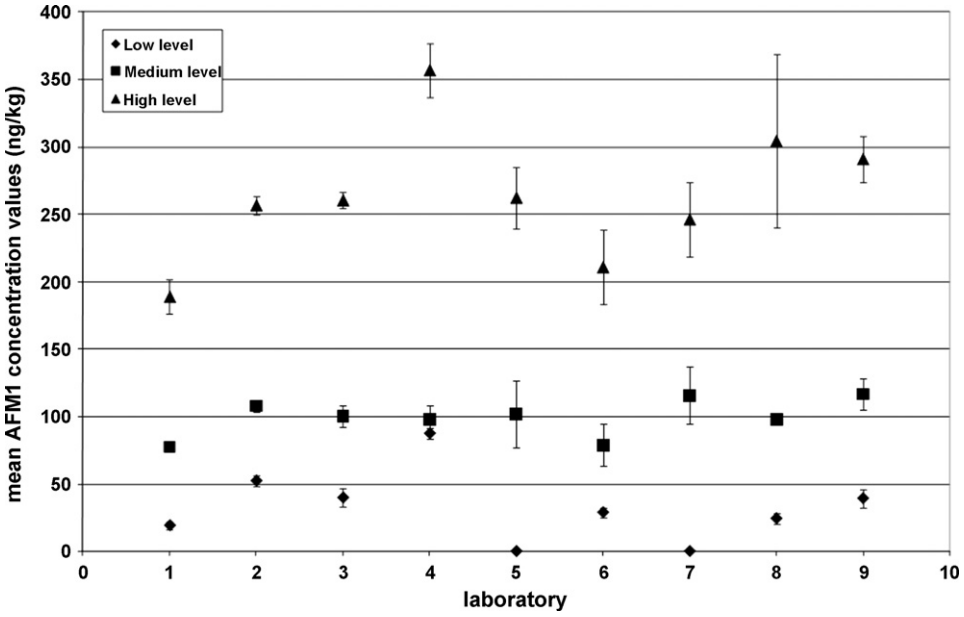

Fig. 1. Plot of mean values and standard deviations for each level and each lab.

particular two labs used dichloromethane, two chloroform, two acetone, two an enzymatic digestion, and the last one a mixture dichloromethane/acetone. Sample extraction is a critical and time-consuming step in quantitative analysis and there is an increasing demand for extraction techniques reducing organic solvents consumption: the use of an extraction step based on an enzymatic digestion in an aqueous phase could certainly be a first step in this sense. Sample preparation method can also have some influence on recovery values and on final quantitative results: post-column derivatization of aflatoxins can increase detectability and/or selectivity of responses for the HPLC detector, but for example the relative instability of the TFA-derivatives has to be considered [41]. Moreover, the choice of the appropriate organic solvent for aflatoxin extraction from cheese should be considered, too, since the rate of extraction can vary greatly according to the solvents used, just like residues which could interfere with the analysis [42].

3.2.2. Recovery

The accuracy of the applied procedures was evaluated by recovery experiments, spiking the matrix of the test material with known amounts of AFM1 (Table 3).

Mean recovery values for each lab on three levels of contamination showed large variation, ranging from 50.5% to 95%. All values were over 50%, and none was discarded.

The highest recovery values (95%) were obtained by Lab No. 9, using the enzymatic digestion procedure. It had already been hypothesized that AFM1 is not covalently bound to casein, and that hydrophobic interactions could occur in aflatoxin binding to casein. Proteolysis could cleave proteins in a way that less hydrophobic areas of the casein micelle could exist, thus facilitating toxin release and extraction. This mechanism had already been considered responsible also for apparent increase in toxin recovery during cheese shelf-life [4]. Anyway, it has to be noticed that another Lab (No. 3), using the same method, obtained lower recovery values (57–81%), probably underlining the importance of the operator, apart from the adopted procedure. The same happened for Labs No. 2 and No. 8, that used the same method but obtained mean recovery values of 69% and 52%, respectively.

Mean recovery values for each level of contamination were calculated, too. Raw data from each lab were corrected for its mean recovery value (Table 4), in order to compensate for losses which can occur, to correctly compare results obtained in different laboratories, and to also consider the wide range of the recorded recovery values.

Data referring to blank samples were very surprising: laboratories No. 4, 5, 6 and 7 found values under the detection limit of the method, while the others found values ranging from 5 to 23 ng/kg.

3.2.3. Quantitative determination of AFM1

Not all methods applied were able to quantitatively determine AFM1 concentration in cheeses at low contamination levels. Laboratories No. 5 and No. 7 indicated that low level samples (409 and 664) were under the detection limit of the method (<5.0 ng/kg). These two laboratories used solid-phase extraction (SPE) columns for the purification of cheese extracts, in contrast with the other laboratories which used immunoaffinity cartridges for cleaning-up purposes: the use of immunoaffinity columns could be determinant for the improvement of sensitivity at low contamination levels. In fact, due to their high specificity, immune-affinity columns produce cleaner extracts with a minimum level of interfering matrix components and excellent signal-to-noise ratios compared to less selective SPE sorbent materials. The same samples, as analyzed by other laboratories, showed an AFM1 concentration from 19 to 88 ng/kg. The amount of aflatoxin detected, ranged from 78 to 117 ng/kg and from 189 to 357 for medium (793–321), and high level (863–594) samples, respectively.

In one case, some results were not available because of technical problems: laboratory No. 6 had troubles with auto-sampler coupled to HPLC system, so samples, prepared in duplicate, were then analyzed just one time, except for a sample at medium level (321).

Fig. 1 shows the data plot referring to mean values and standard deviations for each level and each laboratory. Higher the contamination level, higher the standard deviation values. Compared to other laboratories, the No. 4 found higher values for low and high contamination levels (highlighted by circles in Fig. 1).

3.2.4. Outliers

Data were critically examined in order to identify and treat outliers. Missing data because of loss of sample or mistake in performing the measurement were ignored.

Table 3
Recovery values obtained by each lab through spiking of blank cheese samples (B1, B2, B3) with AFM1 standard solutions at three contamination levels.

Laboratory code	B1			B2			B3			Mean recovery for each laboratory (%)
	Added AFM1 (ng/kg)	Obtained concentration (ng/kg)[a]	Recovery (%)	Added AFM1 (ng/kg)	Obtained concentration (ng/kg)[a]	Recovery (%)	Added AFM1 (ng/kg)	Obtained concentration (ng/kg)[a]	Recovery (%)	
1	100	87.1	87.1	250	225.5	90.2	500	434.0	86.8	88.0
2	101.4	75.5	74.5	253.4	157.5	62.2	506.8	354.5	70.0	68.9
3	100	81.0	81.0	250	173.0	69.2	500	287.0	57.4	69.2
4	100	79.8	79.8	250	218.9	87.6	500	443.2	88.6	85.3
5	–	68.0	80.0	–	143.0	62.0	–	307.0	65.0	69.0
6	100	52.9	52.9	250	119.2	47.7	500	255.0	51.0	50.5
7	100	83.7	83.7	250	178.6	71.4	500	366.1	73.2	76.1
8	100	60.5	60.5	250	111.5	44.6	500	250.5	50.1	51.7
9	100	96.1	96.1	250	237.0	94.8	500	474.5	94.9	95.3
Mean recovery value for each added concentration			77.3			70.0			70.8	

[a] Mean value of two analysis; blank value was subtracted.

Table 4
AFM1 concentration results (ng/kg) obtained by each lab for cheese samples at three different levels of AFM1 contamination. Raw data were corrected for mean recovery value for each lab.

Laboratory code	Blank		Low level					Medium level					High level				
			$n1 = 409$		$n2 = 664$		m'	$n1 = 321$		$n2 = 793$		m'	$n1 = 863$		$n2 = 594$		m'
1	16.3[a]	17.7[a]	17.6	15.8	22.2	21.2	19.2	76.5	78.5	81.0	74.3	77.6	202.2	197.8	176.5	179.9	189.1
2	22.0	22.0	54.4	46.4	54.4	54.4	52.4	112.5	102.3	109.6	107.4	107.9	265.6	248.9	256.9	255.4	256.7
3	15.0	15.0	44.9	46.4	31.9	36.2	39.9	105.8	108.7	91.3	95.7	100.4	263.8	266.7	258.0	253.6	260.5
4	<10	<10	91.0[b]	87.5[b]	90.0[b,a]	82.2[b,a]	87.7	108.8	100.4	83.9	98.0	97.8	340.9[b]	368.6[b]	339.0[a]	378.9[a]	356.9
5	neg	neg	ND	ND	ND	ND	ND	76.8	85.5	115.9	129.0	101.8	244.9	288.4	242.0	273.9	262.3
6	<5	na	26.2	na	31.6	na	28.9	86.2	89.7	61.1	na	79.0	230.4	na	191.5	na	210.9
7	<5	<5	ND	ND	ND	ND	ND	93.8	101.2	133.9	134.3	115.8	286.6	241.9	230.7	226.4	246.4
8	6.0	5.0	28.6	25.9	19.8	23.1	24.4	102.0	100.0	97.3	93.3	98.1	246.1	251.4	356.1	363.9	304.4
9	22.4	23.0	42.2	47.5	34.9	32.6	39.3	122.7	128.1	114.5	101.6	116.7	310.6	299.8	281.4	272.2	291.0
m							32.4					99.5					264.2
SD							27.2					13.9					49.7
M							28.9					100.4					260.5

$n1$, $n2$ = codes of blind duplicates for the same level; ND = not detectable; na = not available; neg = negative value; SD = standard deviation; m' = mean value between duplicates; m = mean value among labs; M = median.
[a] Mandel's k statistic straggler.
[b] Mandel's h statistic straggler.

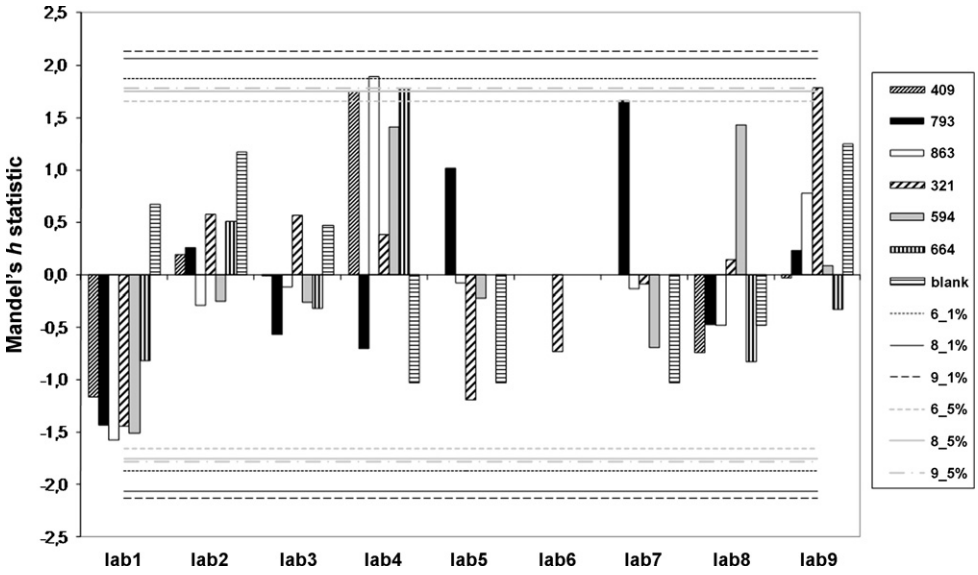

Fig. 2. Plot of the between-laboratory consistency Mandel's *h* statistic, grouped by labs.

Graphical consistency technique was applied: Mandel's *h* and *k* statistics were used to describe the method variability and to make laboratory evaluation.

The between-laboratory consistency statistic *h*, grouped by laboratories, is plotted in Fig. 2: all laboratories have both positive and negative *h* values at different levels of contamination, suggesting that no source of systematic laboratory bias exists.

Lines are drawn on the *h* plot corresponding to indicators at 1 (black) and 5% (grey) significance level. No outliers were found, but stragglers were highlighted in laboratory No. 4: low level samples (409 and 664) had an *h* value greater than its 5% critical value and less than its 1% critical value, calculated for 6 laboratories at that level, while high level sample 863 had a value greater than its 5% critical value and less than its 1% critical value, calculated for 8 laboratories at that level.

Fig. 3 represents the within-laboratory consistency Mandel's *k* statistic, grouped by laboratories: also in this case lines are drawn on the plot corresponding to indicators at 1% (black) and 5% (grey) significance levels. Value of blank sample of laboratory No. 1, calculated on 8 laboratories, and values of low level (664) and high level (594) samples of laboratory No. 4, calculated on 8 and 6 laboratories, respectively, were stragglers.

Cochran's test indicated no stragglers or outliers. Grubbs' test and double-Grubbs' test at 1% and 5% critical value were applied to cell means. No single or double stragglers or outliers were found. On the basis of results obtained by applying statistical tests, stragglers were retained anyway, and no laboratory was eliminated.

3.2.5. *Precision figures*

Retained data were submitted to computation of means and preliminary values of precision for each level separately. Table 5 summarizes the calculated parameters: repeatability, reproducibility, standard deviations and relative standard deviations are indicated.

In general, the applied methods well discriminated the 3 levels of contamination, even though they performed better at the medium and high concentration levels than at the low one. In fact

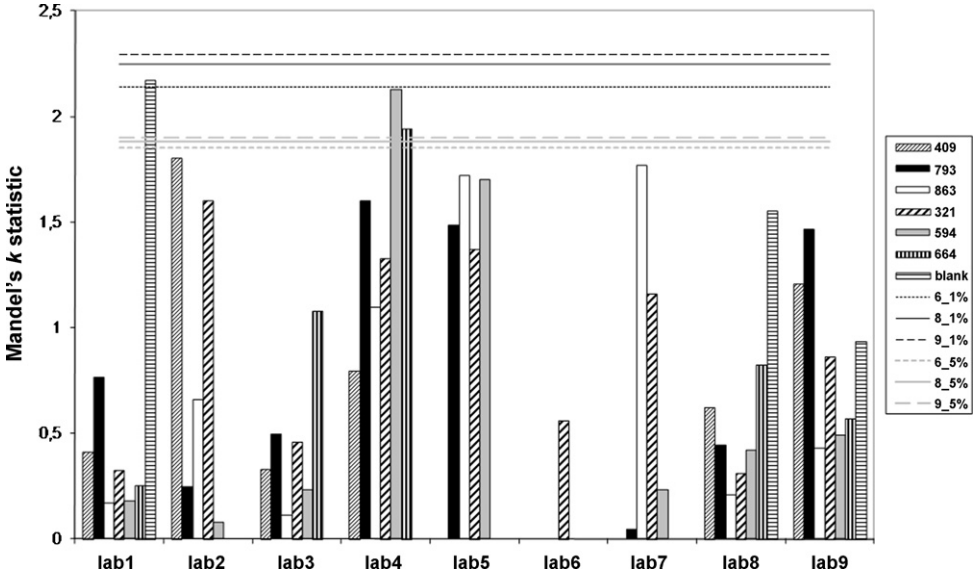

Fig. 3. Plot of the within-laboratory consistency Mandel's *k* statistic, grouped by labs.

Table 5
Statistical summary for precision parameters.

	Average (ng/kg)	r (ng/kg)	R (ng/kg)	s_r (ng/kg)	s_R (ng/kg)	RSD_r (%)	RSD_R (%)
Low level	32.4	13.8	71.7	4.9	25.6	11.6	60.1
Medium level	99.5	37.7	63.9	13.5	22.8	13.5	22.8
High level	264.2	78.9	216.3	28.2	77.2	10.5	28.9

r = repeatability; R = reproducibility; s_r = repeatability standard deviation; s_R = reproducibility standard deviation; RSD_r = repeatability relative standard deviation; RSD_R = reproducibility relative standard deviation.

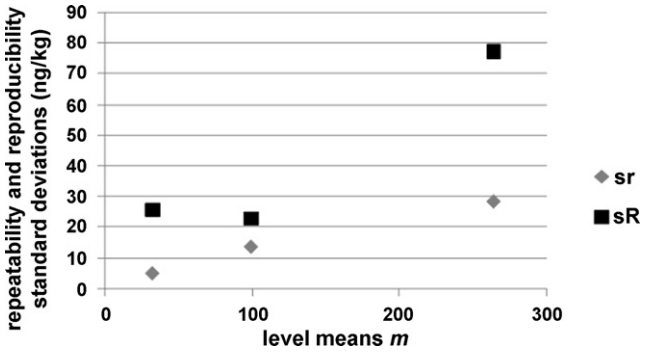

Fig. 4. Relationship between m and precision values.

relative standard deviation for reproducibility at low level was higher (60.1%) than the same value at medium and high levels (22.8% and 28.9%, respectively).

Finally, data were investigated to see whether a relationship between final values of precision and the level means m may exist. s_r and s_R data were plotted against m data, as shown in Fig. 4.

For repeatability, a straight line not passing through the origin seemed to be adequate, confirming that the precision values were dependent from the contamination level of the material.

The following linear relationship was found:

$$s_r = 0.116m + 0.245$$

For reproducibility, conversely, no linear relationship between the level means and the standard deviation was found, suggesting also a certain influence of the adopted procedure on final results.

Finally, the ability of each laboratory was evaluated by means of z-scores. The true values "X" for each batch were calculated as the median of the mean results from all laboratories: the median was chosen in accordance to [43], since it was considered a simple type of robust mean, which is not affected by extreme values. As for "σ", it was calculated as the standard deviation from the mean value for each level of contamination.

In Fig. 5, z-scores are plotted for the three levels of contamination. All laboratories for the three levels of contamination considered showed z-scores lower or equal to 2 ($|z| \leq 2$) and thus satisfactory results.

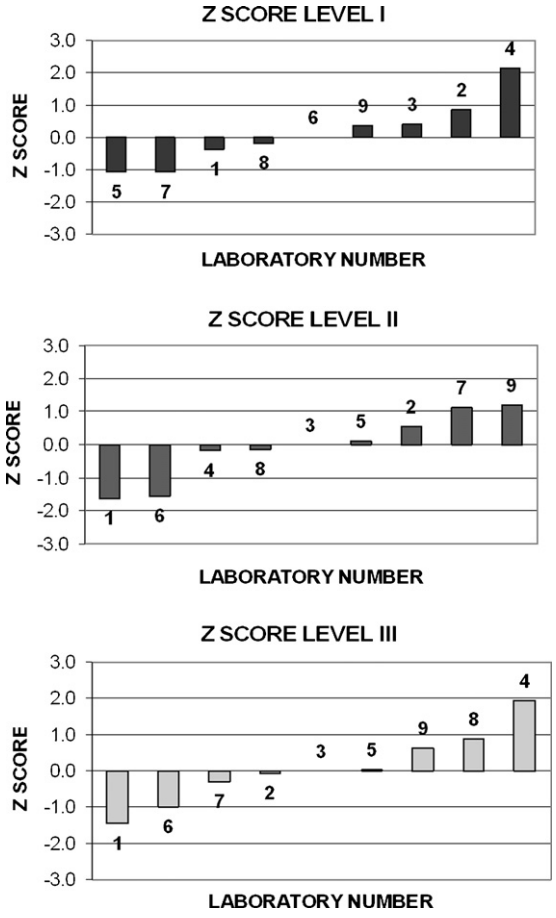

Fig. 5. z-Score for each laboratory for each level of AFM1 contamination.

4. Conclusions

On the basis of the obtained results, this work could give some hints to draft an HPLC procedure for the determination of AFM1 in cheese, In particular, the extraction step made by applying an enzymatic digestion gave the highest recovery values and proved to be the most environment-friendly technique, thus meeting the actual tendency for the reduction in the use of solvents. Moreover, the use of immunoaffinity columns in the cleaning-up step seemed to be determinant for the improvement of sensitivity at low contamination levels. These simple changes could be easily applied to HPLC procedures commonly used in different laboratories with the attempt to normalize procedures normally performed. Anyway, laboratories involved in this study showed to have sufficient background in order to satisfy acceptable precision indices and good laboratory practice with satisfactory repeatability values.

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References

[1] IARC Monograph, IARC Publication No. 82, WHO/IARC Press, Lyon, France, 2002.
[2] Regulation 1881/2006/EC, Off. J. Eur. Commun., L364, 2006.
[3] R.E. Brackett, E.H.Z. Marth, Lebensm. Unters. Forsch. 174 (1982) 439.
[4] R.E. Brackett, E.H.Z. Marth, J. Food Prot. 45 (1982) 549.

[5] R.E. Brackett, R.S. Applebaum, D.W. Wiseman, E.H.Z. Marth, J. Food Prot. 45 (1982) 553.
[6] R.E. Brackett, E.H.Z. Marth, J. Food Prot. 45 (1982) 597.
[7] H.H. Oruc, R. Cibik, E. Yilmaz, O. Kalkanli, Food Addit. Contam. 23 (2006) 190.
[8] H.H. Oruc, R. Cibik, E. Yilmaz, E. Gunes, J. Food Safety 27 (2007) 82.
[9] A. Pietri, T. Bertuzzi, M. Moschini, G. Piva, Ital. J. Food Sci. 15 (2003) 301.
[10] T.M.P. Cattaneo, L. Monti, E.V. Panarelli, S. Francolino, T. Bertuzzi, A. Pietri, Ital. J. Food Sci. 20 (2008) 463.
[11] F. Kiermeier, M. Buchner, Z. Lebensm. Unters. Forsch. 164 (1977) 87.
[12] O. Deveci, Food Control 18 (2007) 1103.
[13] R.S. Applebaum, E.H. Marth, J. Food Prot. 45 (1982) 903.
[14] J.L. Blanco, L. Domingues, E. Gomez-Lucia, J.F.F. Garayzabal, J. Goyache, G. Suarez, J. Food Sci. 53 (1988) 1373.
[15] A.E. Yousef, E.H. Marth, in: H.P. van Egmond (Ed.), Mycotoxins in Dairy Products, Elsevier, London, 1989, pp. 127–161.
[16] A. Pecorari, G. Gambini, M. Nocetti, T. Bertuzzi, A. Mulazzi, A. Pietri, Sci. Tecn. Latt. – Cas. 60 (2009) 125.
[17] A.C. Manetta, M. Giammarco, L. Di Giuseppe, I. Fusaro, A. Gramenzi, A. Formigoni, G. Vignola, L. Lambertini, Food Chem. 113 (2009) 595.
[18] Italian Health Department, D.G.V.A/IX/25664/f.5.b.b.2/P, Rome, 2004.
[19] H.P. Van Egmond, M.A. Jonker, Food and Nutrition, Food and Agriculture Organization of the United Nations, Rome, 2004, Paper 81.
[20] H.P. Van Egmond, R.C. Schothorst, M.A. Jonker, Anal. Bioanal. Chem. 389 (2007) 147.
[21] A.E. Yousef, E.H. Marth, J. Assoc. Off. Anal. Chem. 68 (1985) 462.
[22] C. Cavaliere, P. Foglia, E. Pastorini, R. Samperi, A. Lagana, J. Chromatogr. A 1101 (2006) 69.
[23] A. Carisano, G. Della Torre, J. Chromatogr. 355 (1986) 340.
[24] R.M. Beebe, D.M. Takahashi, J. Agric. Food Chem. 28 (1980) 481.
[25] J.-M. Fremy, B. Boursier, J. Chromatogr. 219 (1981) 156.
[26] G. Cirilli, Microbiol. Aliments Nutr. 1 (1983) 199.
[27] P. Chambon, S.D. Dano, R. Chambon, A. Geahchan, J. Chromatogr. 259 (1983) 372.
[28] A.C. Manetta, L. Di Giuseppe, M. Giammarco, I. Fusaro, A. Simonella, A. Gramenzi, A. Formigoni, J. Chromatogr. A 1083 (2005) 219.
[29] H.M. Martins, S.A. Magalhães, I. Almeida, M. Marques, M.M. Guerra, F. Bernardo, Rev. Portuguesa Ciênc. Vet. 102 (2007) 321.
[30] M. Kokkonen, M. Jestoi, A. Rizzo, Food Addit. Contam. 22 (2005) 449.
[31] S. Dragacci, J.M. Fremy, J. Food Prot. 59 (1996) 1011.
[32] A. Pietri, T. Bertuzzi, P. Fortunati, G. Piva, in: M. Miraglia, C. Brera (Eds.), Proceedings of the 1st National Conference: Mycotoxins in agri-food chain, Rapporti ISTISAN 05/42, Rome, 2005, p. 318.
[33] C. Cavaliere, P. Foglia, C. Guarino, F. Marzioni, M. Nazzari, R. Samperi, A. Laganà, J. Chromatogr. A 1135 (2006) 135.
[34] M. Sharman, A.L. Patey, J. Gilbert, J. Chromatogr. 474 (1989) 457.
[35] S. Dragacci, E. Gleizes, J.M. Fremy, A.A.G. Candlish, Food Addit. Contam. 12 (1995) 59.
[36] F. Masoero, A. Gallo, M. Moschini, G. Piva, D. Diaz, Animal 1 (2007) 1344.
[37] European Community 1986, 1986/609/EC, Official Journal L 358, 1–28.
[38] D.N. Mortimer, J. Gilbert, M.J. Shepherd, J. Chromatogr. 407 (1987) 393.
[39] ISO 5725-2: Accuracy (Trueness and Precision) of Measurement Methods and Results, Part 2: Basic Method for the Determination of Repeatability and Reproducibility of a Standard Measurement Method, International Organization of Standardization, Geneva, 1994.
[40] M. Thompson, R. Wood, J. AOAC Int. 76 (1993) 926.
[41] W.Th. Kok, J. Chromatogr. B 659 (1994) 127.
[42] G.S. Shephard, Anal. Bioanal. Chem. 395 (2009) 1215.
[43] M. Thompson, S.L.R. Ellison, R. Wood, Pure Appl. Chem. 78 (2006) 145.