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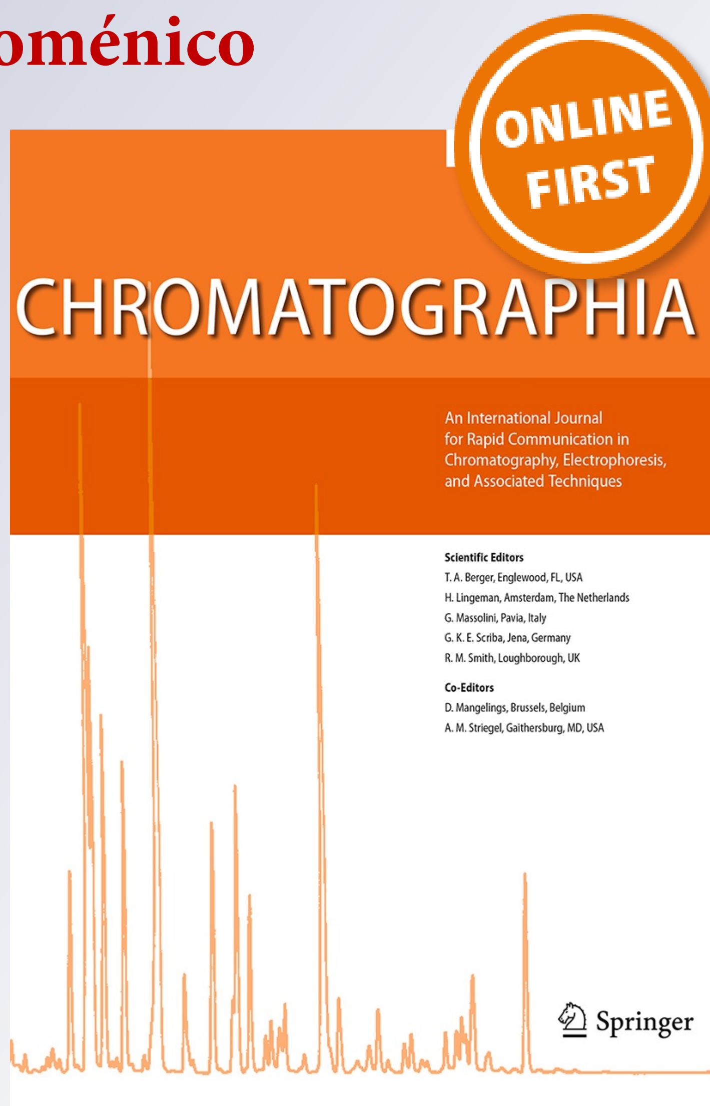

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Multiclass Compatible Sample Preparation for UHPLC–MS/MS Determination of Aflatoxin M1 in Raw Milk

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Abstract A sample preparation method for aflatoxin M1 (AFM1) determination in raw milk was optimized following the quick, easy, cheap, effective, rugged and safe (QuEChERS) strategy, as an alternative to the classic immunoaffinity column clean-up (IAC). The method was adapted to address the complexity of the milk matrix, and to be suitable for final determination by ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC–MS/MS). This approach proved also to be compatible with the simultaneous extraction of pesticide residues and other contaminants (mycotoxins). Regarding AFM1, satisfactory linearity was achieved and appropriate sensitivity was maintained, using matrix-matched calibration to compensate for the heavy ion suppression. The accuracy and precision, which were determined through recovery studies, were 70–95 %, with the relative standard deviation below 15 % in all of the cases. The limit of detection (LOD, 0.002 $\mu g\ L^{-1}$) and limit of quantification (0.007 $\mu g\ L^{-1}$) are compatible with current worldwide regulations (maximum levels of 0.5 and 0.05 $\mu g\ L^{-1}$). The procedure was applied to samples that were naturally contaminated with a range of AFM1 at LOQ–0.187 $\mu g\ L^{-1}$, with comparable results to IAC clean-up, which was employed as a reference method. Therefore, AFM1 determination in raw milk by UHPLC–MS/MS detection through the present QuEChERS extraction constitutes a reliable alternative to IAC clean-up and exhibits advantages related to cost, accessibility of materials and simplicity of operation.

Keywords LC–MS/MS · QuEChERS · Mycotoxins · Pesticides · Milk

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Introduction

Aflatoxins are natural toxic secondary metabolites mainly produced by *Aspergillus* genus moulds (i.e., *A. flavus* and *A. parasiticus*). They are generically classified as International Agency for Research on Cancer (IARC) group I carcinogens [1]. Aflatoxin B1 (AFB1) is of particular interest because it is one of the most potent natural toxins that are bio-transformed in mammalian liver to aflatoxin M1 (AFM1). When mammals consume aflatoxin-contaminated feedstuffs, contaminants can accumulate in muscles and internal organs or be excreted with body fluids (milk and also urine), enabling their intake by consumers. Approximately 0.3–6.2 % of AFB1 initially present in the food consumed by animals may be found as AFM1 in milk [2, 3]. These compounds, chemically classified as difurocoumarocyclopentenone aflatoxins, are slightly soluble in water (10–30 $\mu g\ mL^{-1}$), insoluble in non-polar solvents and freely soluble in moderately polar organic solvents (e.g.,

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chloroform, methanol, acetonitrile, acetone and dimethylsulfoxide) [1].

AFM1 exposure has been strictly regulated worldwide, with the maximum levels (MLs) in milk set to 0.5 μg L^{-1} by Argentinean regulations [4] and 0.05 μg kg^{-1} (0.025 μg kg^{-1} for baby foods) by the European Union [5]. In Argentina, recent reports indicate a steady high AFM1 incidence of 63.8 % [6] and 76.5 % [7] with concentrations complying with the MERCOSUR (Southern Common Market) legislation but with controversial values at the EU levels [8].

Measurements at these regulated low levels require very sensitive and selective analytical techniques, which involve exhaustive clean-up stages to address interferences from the complex milk matrix. The primary analytical technique for the determination of AFM1 in milk and milk products that has been reported in the literature is the enzyme-linked immunosorbent assay (ELISA) [9], which has the potential to yield false positive results [10]. Therefore, instrumental analytical techniques are required to achieve precise non-ambiguous identification for confirmation of positive results. Chromatographic techniques have the most potential among the suitable instrumental methods. High performance liquid chromatography (HPLC) with fluorescence detection is the second most commonly reported technique [9], and this method is the official AOAC method for the determination of AFM1 in milk [11]. Ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC–MS/MS) is becoming important for the analysis of AFM1 and other mycotoxins due to its high sensitivity and selectivity, which provide greater levels of certainty for the identification as well as lower limits of detection and quantification [12–17]. UHPLC–MS/MS applications for the analysis of mycotoxins in milk are still under development [12, 18].

To take advantage of the analytical benefits of these techniques, especially for very low level detection of AFM1, purification or clean-up steps have been extensively proposed to minimize the effect of interfering matrix compounds. The most common purification and pre-concentration method for the analysis of AFM1 in milk and milk products employs immunoaffinity columns (IAC) with monoclonal antibodies that are specific for the retention of AFM1 and the removal of matrix interferences prior to LC–MS/MS determination [6, 13, 15]. Solid phase extraction (SPE) has also been used as a clean-up method for single or multi-toxin milk analysis [12, 16, 17, 19–21]. Other novel preparative approaches have been proposed including liquid–liquid extraction (LLE) [22], dispersive liquid–liquid microextraction (DLLME) [23], matrix solid phase dispersion (MSPD) [24], stable isotope dilution [25] and dilute-and-shoot or no clean-up techniques [16, 26].

The QuEChERS method is a dispersive-SPE clean-up approach with primary secondary amine (PSA) after acetonitrile extraction, and this method was first developed for the extraction and purification of pesticide residues from fruits and vegetables [27, 28] and further adapted for use with fatty food matrices [29]. Despite its suitability for mycotoxin isolation and clean-up, this approach has been employed on a limited basis to prepare samples for the determination of AFM1 in cow milk by UHPLC–MS/MS. In addition, a QuEChERS-based methodology for the simultaneous multiclass determination of pesticides and mycotoxins in milk has been investigated, and the employed analytical conditions were not suitable for the extraction of AFM1 at the European Union tolerance level [16]. Recently, Rubert et al. [30] applied this procedure to extract AFM1 and other mycotoxins from breast milk samples for UHPLC–MS/MS determination.

The search for simplified sample preparation methods for the determination of AFM1 in milk that are compatible with advanced instrumentation remains a challenge. Adequate analytical approaches must be sensitive, simple and low cost to provide controlled results with sufficient confidence required for extensive monitoring activities. IAC purification is the most reliable and extensively used method. However, this approach requires expensive and proprietary columns that hinder analysis in infrastructurally or economically challenged regions [18]. The aim of this work was to optimize an alternative and reliable UHPLC–MS/MS extraction procedure based on the QuEChERS methodology for the analysis of AFM1 in milk that would comply with ML levels required by European and regional legislations.

Materials and Methods

Reagents

An AFM1 stock standard solution (10 mg L^{-1}) in acetonitrile with a purity of 98.5 % was supplied by Supelco (Sigma-Aldrich, Bellefonte, PA, USA). Optima®-grade water, methanol (MeOH) and acetonitrile (MeCN) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Ammonium acetate (NH_4Ac) (>98 %) and sodium acetate (NaAc) (99.7 %) were obtained from Anedra (Argentina), ammonium formate (NH_4F) (>97 %) was obtained from Alfa Aesar (Ward hill, MA, USA), and formic acid (96 %) was obtained from Tedia (Fairfield, OH, USA). Reagent-grade anhydrous $MgSO_4$ was obtained from Cicarelli (Argentina), and Selectrasorb™ octadecyl endcapped C18 and Enviro-Clean® primary secondary amine (PSA) were purchased from UCT (Brockville, Canada). The IACs

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(AFLAPREP® M) were supplied by R-Biopharm Rhône (Glasgow, Scotland).

A 1 mg L^{-1} AFM1 intermediate standard solution was prepared by dilution of the stock standard solution with MeCN and stored at −18 °C. In the same way, 100 and 50 μg L^{-1} working standard solutions were prepared from the intermediate solution, and these solutions were used to spike samples and in the calibration assays.

Samples

Raw bovine milk samples were removed from the cooling tanks of dairy farms located in the central area of the Santa Fe Province (Argentina) and immediately frozen prior to analysis. Milk from one specific farm was employed as a blank sample for method development experiments after confirming the absence of AFM1 using IAC columns as a clean-up procedure.

Sample Preparation

The frozen samples were placed in a fridge overnight to thaw, and prior to centrifugation, these samples were warmed for a few minutes in a water bath at 37 °C and homogenized by occasionally gently inverting the containers by hand. Homogenized milk was transferred to suitable tubes and centrifuged for 15 min (4 °C, 1300*g*). Skimmed milk was carefully taken from the middle portion of the tubes with a syringe and needle and then filtered through cellulose filter paper (Boeco, Hamburg, Germany) under vacuum.

For the recovery studies, defatted and whole milk were spiked with the AFM1 working standard solution to yield analyte concentrations of 0.03, 0.05 and 0.1 μg L^{-1}. The spiked milk was gently stirred for 4 h at ambient temperature to allow appropriate contact between the added aflatoxin and the matrix.

QuEChERS Procedure

A volume of 10 mL of milk was placed into 50 mL polypropylene tubes, and 1 mL of a 1 M Na_2EDTA solution plus 10 mL of MeCN (1 % acetic acid v/v) were added followed by vortex shaking for 30 s. Then, 4 g of anhydrous magnesium sulfate and 1 g of sodium acetate were added, and manual vigorous shaking was applied for 3 min for partitioning. Then, the tubes were centrifuged for 6 min (20 °C, 1000*g*). An 8 mL aliquot of the MeCN layer was transferred to a 15 mL centrifuge tube containing 200 mg of $MgSO_4$, 67 mg of PSA and 180 mg of C18 followed by manual shaking for 2 min prior to centrifugation (20 °C, 1000*g*). Finally, 5 mL of the cleaned extract was dried under a gentle stream of N_2 at 50–55 °C, then reconstituted in 0.5 mL of the mobile phase (A:B, 80:20) and filtrated through a 0.2 μm nylon filter before injection into the UHPLC system. If a multiresidue determination of pesticides was also intended, the latter concentration step was avoided, and one aliquot of the cleaned extract was filtered and injected into the chromatographic system.

IAC Clean-Up Procedure

According to the manufacturer's instructions, 50 mL of defatted milk was loaded into 60 mL syringe barrels placed on top of the IAC columns. Milk was passed through the columns under vacuum at a flow rate of 1–2 drops per second. Then, the columns were washed with two 10 mL portions of water to flush the matrix components followed by drying under air for 30 s to avoid dilution of the bound analyte. Then, AFM1 was eluted into amber glass vials with 2 × 1.25 mL aliquots of MeCN:MeOH (60:40). The eluate was filtered through 0.2 μm nylon filters and injected into the UHPLC system, and a further concentration step was not required.

Experimental Designs for Optimization

Mobile Phase Optimization

A 3^2 factorial design (two factors studied at three levels involving nine experiments) was carried out to optimize a mobile phase composition that would increase AFM1 sensitivity in terms of its chromatographic area (the studied response). The selected variables were NH_4F concentration (levels 0.5, 5.25 and 10 mM) and formic acid concentration in the mobile phase (levels 0, 0.25 and 0.5 %), both in the aqueous (A) and organic (B) eluents.

AFM1 Recovery Optimization

Based on the QuEChERS methodology proposed by Mertzig et al. [31] for the analysis of pesticides in milk, alternative modifications to enable the application of this method to the determination of AFM1 in the same matrix were investigated. An experimental design was employed to study how the main variables (factors) of the method influenced the AFM1 recovery rates and to determine the optimal conditions that yielded appropriate recoveries. The shaking time in the extraction step (factor A) and the amount of C18 in the clean-up step (factor B) were considered the two most relevant variables, and a factorial design of these two factors studied at three levels was chosen (3^2 design implying 9 experiments). These results were analyzed using response surface methodology (RSM). Factor A was studied at 3, 6.5 and 10 min, and factor B was studied at 50, 125 and 200 mg. In all of the experiments,

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the milk sample was spiked with 0.1 μg L^{-1} AFM1 and the recovery percentage was selected as the studied response. Statgraphics® XV Centurion (StatPoint Technologies Inc., Warrenton, VA, USA) was used for statistical analysis with both experiments.

In addition to the experimental design, the initial sample/MeCN mixing step prior to the addition of the salts was also optimized to ensure a more efficient extraction by considering the potential interaction between AFM1 and casein micelles [36]. Three different extraction modes were investigated as follows: (a) 30 s of vortex shaking, (b) 3 min of manual shaking, and (c) 3 min of ultrasonication. To determine the effectiveness of the extraction, a naturally AFM1-containing milk sample was analyzed by the three alternatives and by IAC extraction as a reference in the same batch. Recovery trials at 0.05 μg L^{-1} for each method were also performed.

Validation Assays

The optimized methodology was validated according to the European guidelines 2002/657/EC [32] and Document SANCO/12571/2013 [33] to determine the following performance parameters: trueness, precision, linearity, specificity, matrix effects, and limits of detection (LOD) and quantification (LOQ).

Trueness and precision were estimated via recovery studies by spiking blank samples (whole or defatted milk) with the AFM1 standard at three concentration levels (0.03, 0.05 and 0.1 μg L^{-1}) in triplicate. The linearity was determined using matrix-matched calibration at concentration levels of 0, 0.25, 0.5, 1, 5 and 10 μg L^{-1} (representing values ten times lower in the milk samples). Matrix effects were calculated with the ratio of the solvent and matrix calibration slopes (ME = matrix-matched slope/solvent slope × 100). The specificity of the method was analyzed by comparing the chromatograms of blank samples with those in the spiked experiments. Finally, the LOD and LOQ were estimated using a signal-to-noise ratio of 3 and 10, respectively, according to SANCO/12571/2013 guidance.

The performance of the optimized method on the extraction of pesticide residues and other contaminants from milk was evaluated to verify its suitability for use in multi-class and multi-residue approaches. A group of 63 pesticides from different families and 2 mycotoxins (aflatoxins B1 and B2) was selected and their recoveries analyzed (10 μg L^{-1} spiking level) using the optimized QuEChERS strategy (Table 5). Although AFB1 and AFB2 are not expected to be found in milk, they were added to the mix of compounds representing the mycotoxin family of contaminants.

Additional verification assays were performed to analyze samples (bulk raw whole milk from the cooling tanks of selected dairy establishments) naturally contaminated with AFM1. The AFM1 concentrations of the samples determined with the proposed methodology were compared to the concentrations of the same samples determined with the IAC clean-up procedure as the reference method.

Equipment and Analytical Conditions

UHPLC was employed using an ACQUITY UPLC™ System (Waters, Milford, MA, USA) coupled to a triple quadrupole mass spectrometer (Micromass TQ Detector from Waters, Manchester, UK) through an orthogonal-Z-spray ionization source. The separations were performed using an ACQUITY UPLC® BEH C18 RP Shield (1.7 μm 2.1 × 100 mm) column from Waters at a flow rate of 0.3 mL min^{-1} and a temperature of 40 °C. The mobile phase consisted of A (5 mM NH_4F + 0.1 % formic acid in water) and B (5 mM NH_4F + 0.1 % formic acid in MeOH) programmed with a time gradient that started at 20 % B for 1 min, then linearly increased to 100 % B in 1.6 min, remained in pure MeOH for 2.4 min, and finally returned to the initial conditions in 1 min. The column was allowed to re-equilibrate for one additional minute prior to the next injection, which resulted in a total run time of 7 min. The injection volume was 10 μL.

The instrumental parameters for the MS/MS analysis were as follows: nitrogen (N_2) was employed as the desolvation and cone gas at a flow rate of 900 and 48 L h^{-1}, respectively. For ionization, the electrospray ionization (ESI) source was operated in the positive ion mode with a capillary voltage of 3.2 kV at 120 °C for the source and 390 °C for desolvation. The cone voltage was 40 V, and argon gas (Ar) was used in the collision cell at a flow rate of 0.14 mL min^{-1} (1.3 e−5 mbar) for ion fragmentation. MassLynx v4.1 software (Waters, Manchester, UK) was employed for instrumental operation, data acquisition and analysis.

Results and Discussion

Conditioning of the UHPLC–MS/MS Determination

The separation step in the UHPLC–MS/MS system was performed using a reversed phase C18 column, which is an extensively used system for the satisfactory separation of pesticides and mycotoxins in food. With respect to the mobile phase selection, initially various combinations of water–MeCN and water–MeOH were assayed and results indicated that better peak resolution and greater sensitivity for AFM1 were obtained using water and MeOH combinations as eluent solvents. Secondly, the use of different modifiers that promote ionization like ammonium formate (AmF), ammonium acetate (AmAc) and formic acid (FA)

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was evaluated. Mobile phases with modifiers such as AmF or acidified with FA in both eluents (water and MeOH) have been reported in literature for multi-pesticide analysis but are less frequently employed in milk AFM1 analysis. In our study, both ammonium salts were assayed to evaluate the AFM1 chromatographic sensitivity. When 0.5 mM NH_4Ac was changed to 0.5 mM NH_4F, a 75 % increase in the areas of the two ion transitions (*Q* and *q*) was observed at a 50 µg L^{-1}AFM1 concentration, and when the concentration of NH_4F was increased from 0.5 to 5 mM, an additional 50 % increase in the areas was observed. Respective increases in the signal-to-noise ratios were also observed at the same percentages. A sensitivity increase in the AFM1 chromatographic behavior has also been previously reported for multi-toxin-related applications [30, 34].

These results led us to optimize the mobile phase composition through an experimental design using ammonium formate and formic acid as modifiers. The concentration of formic acid was selected as a second variable due to the high variability in the bibliography reported concentrations. ANOVA analysis indicated that both factors exhibited effects that were significantly different from zero ($P = 0.088$ for NH_4F concentration and $P = 0.932$ for % formic acid) with a 95 % confidence level. The AFM1 response was nearly proportional to the ammonium formate concentration (the higher the concentration level, the greater response), and the formic acid concentration correlated with an increase in the AFM1 area at the extreme levels studied (i.e., 0 and 0.5 %). The optimal experimental conditions required to maximize the response included 10 mM NH_4F without the addition of any formic acid (pH = 5). However, because the extraction methodology presented in this study was focused on a multi-class approach (i.e., to be able to efficiently extract AFM1 and pesticides residues from milk), additional mobile phase analyses were performed to evaluate the pesticide behavior. The sensitivity of a group of 63 pesticides from different families was evaluated under two chromatographic conditions (mobile phases). The first set of conditions was optimized for AFM1 (10 mM NH_4F in water and in methanol with no formic acid), and the second set of conditions involved those typically used to analyze pesticides in multi-residue approaches (5 mM NH_4F in water and in methanol with the addition of 0.1 % formic acid). The results indicated an overall better performance with the latter mobile phase for pesticide sensitivity with no considerable loss on the AFM1 signal compared to the optimized conditions. Therefore, the final chromatographic conditions that were adopted were as follows: mobile phase A (5 mM NH_4F + 0.1 % FA in water) and B (5 mM NH_4F + 0.1 % FA in methanol) with the previously described program. The use of mobile phases consisting of combinations of ammonium formate and formic acid has been previously employed for multi-residue and multi-class analyses of pesticides and mycotoxins [34].

The instrumental conditions for optimal ionization of AFM1 were adopted from the literature data and adjusted by injection of the analyte solution into the system. Ionization using the ESI(+) mode was more suitable in terms of providing the highest sensitivity for the AFM1 $[M + H]^+$ precursor ion. ESI(−) has been employed for AFM1 detection by other authors [15] but resulting in a less sensitive ionization than in the positive mode. This was confirmed in the present work by testing both ionization modes. Using selected reaction monitoring (SRM), the more sensitive transition SRM1 (*m/z* 329.0 > 273.0) (*Q*) was employed for quantification, and the less sensitive transition SRM2 (*m/z* 329.0 > 259.0) (*q*) was employed for the qualitative analysis. These transitions have been previously used for milk analysis [12, 13]. Identification and quantification measurements were performed using the following criteria: (a) Analyte retention times in the samples should correspond to that of the calibration standard with a tolerance of ±0.2 min; (b) peaks with S/N of ≥3 and S/N ≥10 were employed for the SRM2 (*q*) identification transition and SRM1 (*Q*) quantification transition, respectively; (c) *q*/*Q* ratio with a maximum tolerance ≤30 %; and (d) observation of peak shapes [33].

Optimization of the Sample Preparation Procedure (QuEChERS)

The extraction of compounds with high or medium polarity from milk is always a challenge, especially when generic multi-class extraction of compounds is required. Despite the current method being focused on the determination of AFM1 as a unique analyte, its suitability for extension to other classes of contaminants (e.g., mainly pesticides and other mycotoxins) was also considered in the design.

The QuEChERS extraction was based on the method proposed by Mertzig el al. [31] for the analysis of pesticides in milk, and modifications were introduced to allow for AFM1 determination. Although methanol has been the primary solvent of choice for aflatoxin extraction, MeCN-was selected to take advantage of its ability to selectively isolate compounds over a wide polarity range, which makes it compatible with multi-residue methods. Therefore, the acetonitrile buffered extraction strategy was adopted with the addition of 10 mL of MeCN (1 % acetic acid) to the milk sample and simultaneous liquid–liquid partitioning with salts ($MgSO_4$ and NaAc). As a complement to this mixture of reagents, the addition of 1 mL of 1 M Na_2EDTA was adopted to improve the extraction of protein-bounded mycotoxin, which is favored by the dissolution of casein micelles via the sequestration effects on colloidal calcium phosphate [35]. This addition has been shown to improve

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Table 1 Extraction performance of AFM1 by three different methods prior to partitioning using the QuEChERS methodology

Extraction method	AFM1-incurred sample (μg L^{-1})*,**	Recovery (0.05 μg L^{-1})* (%)
(a) Vortex 30 s	0.184	84
(b) Manual agit. 3 min	0.180	106
(c) Ultrasonication 3 min	0.178	75
IAC	0.187	111

* Mean values ($n = 3$)

** ANOVA $P = 0.58$ (95 % confidence)

Table 2 Comparison of recoveries obtained for AFM1 using the QuEChERS method for spiked defatted and whole milk

	Spiking level (μg L^{-1})					
	0.03		0.05		0.1	
	% Rec	RSD[a] (*n*)	% Rec	RSD[b] (*n*)	% Rec	RSD[a] (*n*)
Defatted milk	66	4 (3)	96	11 (9)	98	13 (3)
Whole milk	83	10 (3)	81	15 (6)	70	13 (3)

n number of independent replicates, *% Rec* recovery percentage, *RSD* relative standard deviation

[a] RSD intra-day repeatability: runs consisted of three replicates at each level

[b] RSD inter-day repeatability: runs consisted of three replicates on 3 and 2 different days for defatted and whole milk, respectively

the extraction of pesticides in milk matrices [31]. The importance of shaking in the extraction procedure and liquid–liquid partitioning with MeCN is well known. Manual or mechanical shaking has been extensively used, and in the current study, manual shaking of the tubes was adopted. The shaking time was expected to be an important variable and it was included as a factor in the optimization design of the experiment.

A solvent/matrix ratio of 1:1 (10 mL/10 mL) was used for the defatted and whole milk assays because trials with a smaller amount of sample (5 mL) did not result in good partitioning. Milk is typically classified as low fatty matrix (2–20 %) by most residue analysis protocols. However, the fat content may present serious problems because it could be transferred to the organic phase and affect the analytical response during LC–MS/MS or the maintenance and behavior of the chromatographic systems. Therefore, a special adaptation of the dispersive-SPE clean-up of the QuEChERS method has been proposed based on the addition of C18 to the sorbent mixture. The originally proposed amount was 50 mg of C18 to a solvent/matrix ratio of 1:1 [29]. In the current study, the amount of C18 was optimized by including this variable in the statistical design of the experiment.

The shaking time in the extraction step and the amount of C18 in the clean-up step were optimized through experimental design at the previously described conditions, evaluating their effect on the AFM1 recovery rate. The recovery results from all the combinations of factors varied from 59 to 109 %. The analysis of variance (ANOVA) indicated that the amount of C18 was the unique significant source of variation at a 95.0 % confidence level ($P = 0.032$). Therefore, the remaining factors and interactions were not significant at the same confidence level ($P > 0.05$). The optimized values that were adopted included 3 min of shaking and 180 mg of C18. Under these optimized experimental conditions, a further evaluation of the clean-up agitation times at 2, 5 and 10 min was performed, and 98, 83 and 98 % recoveries, respectively, were obtained. Finally, a 2-min time was adopted to minimize the operation time.

Additionally, the effectiveness of AFM1 extraction by acetonitrile was assayed evaluating recovery rates and the AFM1 concentration of one naturally contaminated milk sample by three different extraction modes in the initial sample/MeCN mixing step (vortex extraction, manual shaking and ultrasonication) using IAC in parallel as reference. The ANOVA indicated no significant difference in the concentration means from the four experiments, and the recovery values were within the acceptable range in all of the cases. However, ultrasonication was the least effective mode (Table 1). These results led to the selection of a 30-s vortex extraction mode based on practical reasons (i.e., shorter analysis time and sample manipulation).

Because the fat in the matrix influences IAC extraction, its effects on the QuEChERS performance using defatted and whole milk were investigated. The recovery results from both matrices were within the usual range for mycotoxin analysis [33] (Table 2). Despite the presence of fat in whole milk, no significant differences were observed in the chromatogram profiles or background. Therefore, the use

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Table 3 AFM1 regression parameters resulting from the calibration study in solvent and matrix extracts (whole raw milk) using the optimized QuEChERS method

	Rt	Slope (S_s)	Intercept (S_i)	r^2	LR (μg L^{-1})	ME[a] %	LOD (μg L^{-1})	LOQ (μg L^{-1})
Matrix-calibration	2.86	218.282 (*1.211*)	−5.629 (*3.516*)	0.9998	0.25–10	75	0.002	0.007
Solvent-calibration	2.85	288.833 (*1.731*)	5.751 (*9.689*)	0.9996	0.25–10			

Rt retention time, S_s slope standard error, S_i intercept standard error, *LR* linear range, *ME* matrix effect, *LOD* limit of detection, *LOQ* limit of quantification

[a] Matrix effect % calculated as (matrix-slope/solvent-slope) × 100

Table 4 Trueness and precision results for aflatoxin M1-spiked whole milk using the adopted QuEChERS methodology

	Spiking level (μg L^{-1})			
	0.03	0.05	0.1	0.5
Mean recovery	70 %	95 %	83 %	78 %
Intra-day RSD	13 %	15 %	10 %	14 %
Inter-day RSD	NE	16 %	NE	NE
n	3	10	3	3

Intra-day repeatability consisted of three replicates per level, and the value for each replicate was an average from four repeated injections

Inter-day repeatability consisted of replicates at the 0.05 μg L^{-1} level on 10 different days during 4 months

RSD relative standard deviation, *NE* not evaluated, *n* number of independent replicates

of whole milk was adopted to avoid an additional defatting step and to ensure the procedure's compatibility with pesticide multi-residue approaches.

Validation of the Method

Matrix-matched calibration curves at the same levels as the solvent calibration curves (0.25, 0.5, 1, 5 and 10 μg L^{-1}) indicated a significant ion suppression effect with a decrease in the signal on the order of 25 % in the QuEChERS clean-up of whole milk. The matrix effect was investigated by comparing the calibration slopes resulting a value of 75 % (Table 3). Signal suppression was higher for the QuEChERS clean-up compared to that for the IAC clean-up, which exhibited a ME value of 85 % (i.e., 15 % signal decrease). In the QuEChERS procedure, the presence of fat did not contribute in a differentiated mode to the interference compared to the defatted matrix. In addition, defatting did not prevent the substantial influence on the signals obtained with both techniques (i.e., IAC and QuEChERS). This result reflects the complexity of a heterogeneous matrix, such as milk, which severely influences the ESI mechanism. The effect of the matrix on the ESI response of diverse compounds occurs primarily due the co-elution of the analyte with different matrix components. To compensate matrix interference, matrix-matched calibration curves were adopted for validation and quantification of the method employed in our studies.

The linear regression parameters for the multilevel matrix-matched calibration functions are shown in Table 3. The linearity acceptability criterion was based on visual inspection of the plotted lines with residual values lying ≤20 % throughout the range and coefficients of regression (r and r^2) being close to one. The conditions were satisfactorily achieved with ordinary linear regression models, and the weighted linear regression lines (1/x) according to SANCO/12571/2013. LOD were calculated as the lowest analyte concentration that produced a peak with a S/N ratio of 3 for the SRM1 (Q) transition. The LOQ was calculated as the lowest analyte concentration that produced a S/N ratio of 10 for the same transition, whereas the S/N of the SRM2 (q) transition was higher than 3 in both cases. The calculations also met the conditions for quantification and confirmation previously mentioned regarding Rt and the q/Q ratio. The LOD and LOQ calculated values for AFM1 in whole raw milk using the optimized QuEChERS method were 0.002 and 0.007 μg L^{-1}, respectively. These values indicate the ability of the procedure to comply with the ML specifications of most of the important regulations worldwide (e.g., 0.5 μg L^{-1} regulated for milk in MERCOSUR, United States, China and other countries, and 0.05 μg kg^{-1} in the European Union).

Mean recoveries from multilevel spiked raw milk samples were used as an estimation of trueness. The design included independent triplicates of four spiked levels (0.03, 0.05, 0.1 and 0.5 μg L^{-1}) with a focus on the European Union ML for AFM1 at 0.05 μg kg^{-1}. Satisfactory values ranging from 70 to 95 % for whole milk were observed (Table 4). Repeatability precision was evaluated by calculating the relative standard deviation (RSD) from intra-day replicates. The results for all four levels in the intra-day runs were less than 15 %. The inter-day variability, which was calculated from repeated measurements on different days at a level of 0.05 μg L^{-1}, was 16 % (Table 4). The evaluation of the responses in the reagent blank and blank control samples (samples without AFM1) confirmed the

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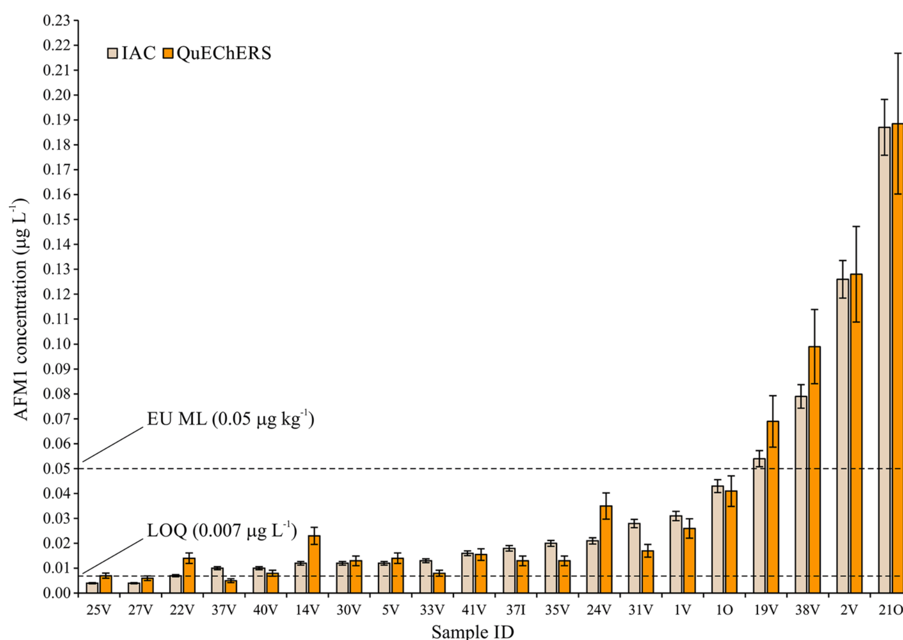

Fig. 1 Comparison of the AFM1 concentration found in 20 naturally occurring raw milk samples analyzed using the optimized QuEChERS procedure and IAC. The *error bars* estimate the uncertainty of the results given by the relative standard deviation of the methods

specificity of the method and the absence of interference peaks at the retention time in the chromatogram.

Analysis of Real Samples

The current method development was carried out as part of a seasonal monitoring project to study the relationships between the occurrence of aflatoxins in animal foodstuffs and the presence of their transformation products (AFM1) in the produced milk. For this study, the previously described IAC–LC–MS/MS methodology was applied. The samples were collected from dairy farms located in the Santa Fe Province in the central region of Argentina, which has a substantial interest in milk production and the dairy industry. Twenty positive AFM1 samples from this parallel project were selected and analyzed using the proposed QuEChERS method, and these values were compared to those obtained with the IAC reference procedure. The levels of naturally contaminated milk were in the range of <LOQ to 0.187 µg L^{-1}. Four samples had AFM1 concentrations greater than the EU ML (0.05 µg kg^{-1}), and thirteen samples had concentrations between the QuEChERS' LOQ and the EU ML. In addition, the other three samples had concentrations located between the LOD and LOQ of the proposed method. Acceptable agreement between the results from the two procedures was obtained, especially when the AFM1 concentration was greater than 0.025 µg L^{-1} (Fig. 1). These results are in compliance with the 2002/657/EC guidelines for the trueness specifications for the quantitative methods. At very low concentrations (below 0.025 µg L^{-1}), more variability was observed, which was most likely due to a less efficient ionization process of AFM1 in the presence of a large variety of interfering compounds that were present in the complex matrix (i.e., whole raw milk). Figure 2 shows the representative AFM1 chromatograms obtained by applying the adopted QuEChERS method to spiked and natural milk samples.

Pesticide Behavior with the Optimized QuEChERS Procedure

As previously mentioned, the current methodology was intended not only to efficiently extract AFM1 from milk but also to allow extraction of multiple pesticides and other mycotoxins in a multi-residue approach. The recovery of 63 pesticides from different families and two other aflatoxins (AFB1 and AFB2) was evaluated along with recovery of AFM1 in whole raw milk using the optimized QuEChERS procedure at a 10 µg L^{-1} (0.05 µg L^{-1} for AFM1) level. For most of the compounds, the obtained recoveries were within the acceptable range (i.e., 70–120 %) [33], indicating good performance for the analyzed pesticides and applicability for this method's use as a multi-residue and multiclass methodology (Table 5).

Conclusions

An alternative methodology for the determination of AFM1 in raw milk based on the QuEChERS strategy has been proposed. The principal aim of this study was to optimize an extraction method for UHPLC–MS/MS determination of AFM1 in raw milk that can replace or complement IAC clean-up, which is very reliable and extensively used but

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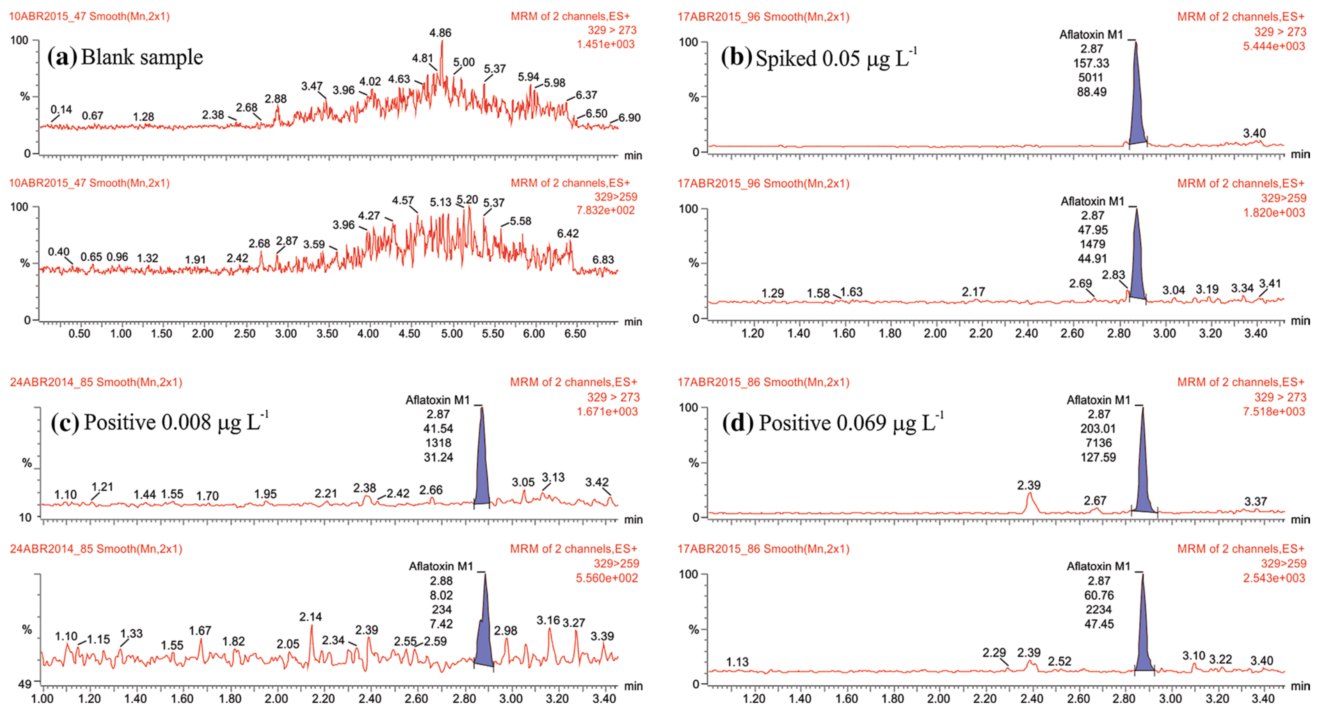

Fig. 2 Representative chromatograms from the analysis of raw milk samples that contained naturally occurring AFM1 using the optimized QuEChERS procedure. **a** Blank sample, **b** spiked milk at the EU ML (0.05 µg kg^{-1}), **c** AFM1-positive milk at a concentration near the LOQ (0.007 µg L^{-1}) and **d** AFM1-positive milk at a concentration near the EU ML (numbers under de AFM1 peak flag are: retention time, area, height and calculated signal-to-noise)

Table 5 List of pesticides and mycotoxins analyzed using the optimized QuEChERS as a multi-residue multi-class methodology

Recovery range (%)	Compound name			
44–70	2,4-D	Abamectin	Amitraz	Chinomethionate
	2,4-DB	Acephate	Anilazine	Cyromazine
70–90	Azinphos-Me	Fenthion	Phosmet	Tebuconazole
	Carboxin	Fipronil	Pyraclostrobin	Terbufos
	Chlorpyriphos-Me	Flusilazole	Pirimiphos-Me	Triadimenol
	Clofentezine	Flutolanil	Prochloraz	Triazophos
	Diazinon	Linuron	Profenofos	
	Diflubenzuron	Methoxyfenozide	Propiconazole	
90–105	Atrazine	Chlorpyriphos	Metalaxyl	Pirimicarb
	Azoxystrobin	Clethodim	Methamidophos	Propargite
	Bentazone	Dichlorvos	Methidathion	Thiabendazole
	Carbaryl	Dinotefuran	Methomyl	Thiamethoxam
	Carbendazim	Fenpropathrin	Methoprene	Triadimefon
	Chlorantraniliprole	Fludioxonil	Metsulfuron-Me	
	Chlorimuron-Et	Mecarbam	Nitenpyram	
105–120	Acetamiprid	Imidacloprid	Aflatoxin B1	
	Bendiocarb	Metribuzin	Aflatoxin B2	
	Clothianidin	Thiacloprid		
	Flonicamid			

Compounds categorized according to recovery ranges

Assayed concentration level: 10 µg L^{-1}

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has severe limitations due to provision times, duration of kit use and elevated costs. These aspects restrict the extension of monitoring systems, especially in infrastructurally or economically challenged countries.

Suitable linearity conditions were achieved through matrix-matched calibration to compensate for ion suppression while maintaining adequate sensitivity. The recovery studies exhibited satisfactory performance for the accuracy, repeatability and intermediate precision with values ranging from 70 to 95 % and with RSD below 15 % in all cases. The LOD and LOQ values were calculated to be 0.002 and 0.007 μg L^{-1}, respectively, with the standard criteria for confirmation and quantification using UHPLC–MS/MS techniques. A complementary verification of accuracy and overall performance was performed by applying the methodology to naturally contaminated samples, and satisfactory results were obtained compared to those of the reference IAC clean-up method. The method exhibited suitable performance in compliance with applicable EU validation guidelines and current MRLs of most milk food regulations.

Based on the simplicity of the QuEChERS strategy, this method could replace or complement existing IAC approaches for enhancing throughput and decreasing costs to improve monitoring of AFM1 in milk. The proposed methodology is also suitable for use as a multi-residue and multiclass method to expand its scope to compounds from diverse chemical families, such as pesticides and other mycotoxins.

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