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Rapid and automated analysis of aflatoxin M1 in milk and dairy products by online solid phase extraction coupled to ultra-high-pressure-liquid-chromatography tandem mass spectrometry

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Abstract

This study reports a fast and automated analytical procedure for the analysis of aflatoxin M1 (AFM1) in milk and dairy products. The method is based on the simultaneous protein precipitation and AFM1 extraction, by salt-induced liquid–liquid extraction (SI-LLE), followed by an online solid-phase extraction (online SPE) coupled to ultra-high-pressure-liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) analysis to the automatic pre-concentration, clean up and sensitive and selective determination of AFM1. The main parameters affecting the extraction efficiency and accuracy of the analytical method were studied in detail. In the optimal conditions, acetonitrile and NaCl were used as extraction/denaturant solvent and salting-out agent in SI-LLE, respectively. After centrifugation, the organic phase (acetonitrile) was diluted with water (1:9 v/v) and purified (1 mL) by online C18 cartridge coupled with an UHPLC column. Finally, selected reaction monitoring (SRM) acquisition mode was applied to the detection of AFM1.

Validation studies were carried out on different dairy products (whole and skimmed cow milk, yogurt, goat milk, and powder infant formula), providing method quantification limits about 25 times lower than AFM1 maximum levels permitted by EU regulation 1881/2006 in milk and dairy products for direct human consumption. Recoveries (86–102%) and repeatability (RSD < 3, $n = 6$) meet the performance criteria required by EU regulation N. 401/2006 for the determination of the levels of mycotoxins in foodstuffs. Moreover, no matrix effects were observed in the different milk and dairy products studied.

The proposed method improves the performance of AFM1 analysis in milk samples as AFM1 determination is performed with a degree of accuracy higher than the conventional methods. Other advantages are the reduction of sample preparation procedure, time and cost of the analysis, enabling high sample throughput that meet the current concerns of food safety and the public health protection.

Keywords: Aflatoxin M1, Salt-induced LLE, Online SPE, UHPLC–MS/MS, Milk and dairy products Food safety

1. Introduction

Aflatoxins (AFs) are toxic substances produced by secondary metabolism of several fungal species (*Aspergillus*, *Penicillium* and *Fusarium*), which grow on a large number of commodities, in the field and during storage, under a wide range of climatic conditions [1], [2], [3]. Among all, aflatoxin B1 (AFB1) is the most significant AFs, considering incidence and toxicity. After ingestion, AFB1 is metabolized by the liver of mammals into reactive epoxide or in hydroxylated metabolites. Aflatoxin M1 (AFM1) is the major oxidized metabolite of AFB1, and milk and dairy products may be contaminated by feed carry-over contamination [4]. AFs are mainly produced in cereals and nuts and their by-products, including animal feeds (meal of groundnut, cottonseed and maize) [5]. Climatic and environmental variations as well as poor economic and agriculture practices could easily influence the increase of AFM1 in milk and dairy products. In addition, several studies have

demonstrated the resistance of AFM1 to heat processing (pasteurization, boiling, and ultra-high temperature processing), powder milk and cheese storage [5].
Surveys showed high levels of AFM1 contamination (>0.05 μg L^{-1}) of milk samples from developing countries as compared to developed countries such as France, Italy, Portugal and Spain. These data revealed that developing countries need to develop and implement regulations and control systems that would regulate AFM1 in milk and its products thus ensuring food quality and safety [6].
Less carcinogenic than AFB1 (2–10% of potency), AFM1 was initially classified by the International Agency for Research on Cancer (IARC) as a group 2B agent carcinogenic to humans [7] due to lack of data. However, following further investigations that demonstrated its genotoxic and carcinogenic effects, AFM1 has since been classified as a group 1 human carcinogen [8].
Considering the potential risk of human exposure to AFM1 due to the importance of milk and dairy products as foodstuffs for humans, especially children, the European Union (EU) has set maximum limits (MLs) of 50 and 25 ng kg^{-1} [9] for consumable milk and infant formulae, respectively, aiming to reduce human exposure. As result of very low MLs (parts per trillion range) for milk products and the wide consumption of milk products, powerful analytical tools that combine high accuracy, sensitivity and throughput sample are required for the effective screening and monitoring of AFM1 in foodstuffs.
Conventional methods used to analyze AFM1 in milk samples are based on immunoaffinity chromatography (IAC) followed by high pressure liquid chromatography with fluorescence detection (HPLC–FLD) [10]. However, according to the EC Decision 657/2002 [11] a confirmatory method based on the mass spectrometry (MS) is required due to its ability to provide full or complementary information for the unambiguous identification of analytes. Although IAC allows a highly selective isolation of AFM1 from milk, it is expensive, in terms of time and material consumption. Thus, it is necessary to develop automated procedures able to minimize the number of steps and reduce time analysis and errors, getting more reproducible and accurate results [12].
Online Solid Phase Extraction (online SPE) coupled to liquid chromatography is particularly suitable when large numbers of samples have to be analyzed routinely [13], [14]. As compared to offline SPE, it offers a series of advantages (high throughput, no loss of analytes, small consumption of organic solvent, reusable cartridges, minimal sample handling) which affect the precision and sensitivity of the analytical methods [13], [14].
Continuing our studies on fast and automated procedures for analysis a large number of samples in food mycotoxins survey and monitoring programs [15], [16], [17], [18], [19], [20] a novel fast and robust method to high throughput analysis of AFM1 in milk and dairy products was developed. Salt-induced liquid–liquid extraction (SI-LLE) was selected to remove the milk proteins and water-soluble compounds and simultaneously to extract AFM1 from milk samples. Successively, online SPE coupled UHPLC–MS/MS was used to perform the purification of the SI-LLE extract and to achieve a selective and sensitive detection of the analyte.
The experimental parameters affecting the extraction efficiency and the selectivity of the method (dilution factor of LLE extracts, SPE sorbent, HPLC conditions, type and flow of wash solvent, injection volume) were carefully studied and optimized. Special emphasis was placed on the elimination of matrix effects, which could have negative effect on the accuracy and precision of the method.
The proposed method was validated according to EU regulation 401/2006 on different milks types (cow and goat milk) and dairy products (yogurt, powder milk) and finally applied to different brand names of samples.

2. Experimental

2.1. Standards and materials

Reference standard solutions in acetonitrile of AFM1 0.5 μg mL^{-1} were obtained from LGC Promochem GmbH (Wesel, Germany). Stock solutions of AFM1 (5 and 50 ng mL^{-1}), used for

spiking procedure and for preparation of working calibration solutions, were prepared in acetonitrile and stored in amber glass vials at −20 °C. Analytical-grade acetonitrile (MeCN) and sodium chloride (NaCl) were obtained from Carlo Erba (Milan, Italy). Ultrapure water (18 MΩ) was obtained by a Milli-*Q* purification system (Millipore, Bedford, USA). MS-grade H_2O and methanol (MeOH) were supplied by Romil (Cambridge, UK) and MS-grade ammonium formate (NH_4CO_2H) and formic acid (HCOOH) were provided by Sigma-Aldrich (Milan, Italy). The following online cartridges ZIC-cHILIC 20 × 2.1 mm, 5 µm particle size (Merk, SP, Brazil); Atlantis HILIC Si, 50 × 2.1 mm, 3 µm particle size (Waters Corporation, Milford, MA, USA); Oasis HLB, 20 mm × 2 mm, I.D., 25 µm particle size (Waters Corporation, Milford, MA, USA), BioBasic C18 and C8, 20 mm × 2.0 mm I.D., 5 µm particle size (Thermo Fisher Scientific, Milan, Italy), were evaluated during the online SPE optimization.

2.2. Safety considerations

The AFM1 is a carcinogenic compound; consequently, solutions and extracts should be handled with extreme care. Gloves and other safety precaution clothing were worn as safety precaution during the handling of the compounds, to guarantee the protection of research workers. To avoid any risks, all stock solutions were prepared under a laminar airflow fume hood. Glassware and any material used for standard or sample preparation were treated with 5% aqueous sodium hypochlorite to destroy AFM1 residue before cleaning and reuse. Aflatoxin is subject to light degradation thus all analytical work was protected from daylight keeping in amber vials.

2.3. Samples

Different milk and dairy product types (whole, skimmed cow milk, powder milk, goat milk and yogurt) from different brands were purchased in supermarkets of the Campania region (Italy). Milk samples were processed directly without any pretreatment. Powder milk samples (20 g) were dissolved with warm ultrapure water (120 mL, 60 °C) and stirred until a homogeneous solution was obtained. Yogurt samples were diluted in ratio of 1:1 with water immediately before the processing. Samples used in optimization and validation studies, were earlier analyzed by IAC followed by HPLC-FLD [10] to verify the absence of AFM1 contamination. Spiked samples were prepared by adding specific volumes of AFM1 stock solutions (5 and 50 ng mL^{-1}) to 50 g of analyte free samples to achieve the required contamination levels used in the optimization and validation studies (200, 100, 50, 25 and 10 ng kg^{-1}). After spiking, fortified samples were left to equilibrate by stirring at room temperature for 2 h and stored in the dark at 4 °C for maximum three days.

2.4. Salt-induced liquid–liquid extraction (SI-LLE)

In order to remove the protein and water-soluble substances from milk, our previous method was applied [19]. Briefly, NaCl (1 g) was dissolved into 5 g of milk, then MeCN (3.8 mL) and HCOOH (50 µL) were added to the samples and manually shaking for 10 s. Subsequently the mixture was centrifuged for 5 min at 13000 rpm (SL 16 centrifuge, Thermo Fisher Scientific, Milan, Italy). Thereafter, 1 mL of upper phase (2.9 ± 0.2 mL, MeCN), containing AFM1, was diluted at 10 mL with deionized water and transferred into amber vials for the online SPE UHPLC–MS/MS analysis.

2.5. Online SPE–UHPLC–MS/MS analysis

Online SPE and chromatographic analysis were performed with an Ultimate 3000 UHPLC system (Thermo Fisher Scientific, Milan, Italy) constituted of a degasser, a dual pump, a column compartment, an autosampler fitted with a 5 mL injection loop, and a programmable Rheodyne® ten-port switching valve between the load and inject position. The chromatographic column was interfaced to a TSQ Quantum Ultra (Thermo Fisher Scientific, Milan, Italy) triple quadrupole mass spectrometer. The mobile phase of both pumps (left and right) consisted of H_2O (A) and MeOH (B) all buffered with 2 mM NH_4CO_2H and 0.1% HCOOH. In the optimal conditions, a BioBasic C18 online cartridge was connected to a ten-port switching valve. The chromatographic analyses were

performed using a Kinetex PFP column (100 × 2.1 mm I.D., 2.6 μm, Phenomenex, Bologna, Italy) thermostated at 30 °C. Details of online SPE–UHPLC conditions, time programing of the valve and chromatographic gradient were reported in Table 1. The online SPE system setup (Fig. 1S of Supplementary data) consisted of three steps (I-III). I step (load, 0–1 min), 1 mL of diluted SI-LLE extracts were loaded onto the cartridge fitted into the loading position of Rheodyne® 10 port switching valve. The left pump was used to load the extracts at high flow (1 mL min^{-1}, 5% B) on the trapping cartridge. II step (wash, 1–6 min), SPE cartridge was washed using 5 mL of 10% B (1 mL min^{-1}) and the sample matrix interferences were flushed to waste, while the analyte was retained on the SPE cartridge. Simultaneously, the UHPLC column was equilibrated with the right pump. III step (injection, 6 min), after washing step the valve switched to injection position and the analyte was transferred in the back-flush mode to the UHPLC column by the gradient elution (right pump). After analytical separation, the switching valve returned to the loading position to washing (15.5–20 min, 95% B) and re-equilibrating (20–22.5 min, 5% B) of online SPE cartridge.

The mass spectrometer was equipped with a heated electrospray ionization (H-ESI) source and operated in positive ionization mode. The MS and MS/MS parameters were selected by flow-injection analysis (FIA) of AFM1 standard solution at 1 μg mL^{-1} (MeOH/H_2O 1:1, 0.1% HCOOH) at flow rate of 5 μL min^{-1}. The mobile phase and flow rate-dependent source parameters were optimized at the chromatographic conditions by injecting standard solution 1 ng mL^{-1}. The optimal conditions were: spray voltage, 3500 V; capillary temperature, 280 °C; vaporizer temperature, 250 °C; tube lens, 100; sheath and auxiliary gas pressure, 15 and 5 units, respectively; collision gas pressure, 0.8 bar. Nitrogen (99.9% purity) was used as auxiliary and sheath gas in the H-ESI source and argon (99.9999% purity) as collision gas in the collision cell. For identification, confirmation and quantification of the analyte, selected reaction monitoring (SRM) mode was used, choosing the precursor/product ion transitions *m*/*z* 329.1 → 258.9 (I_1); 329.1 → 273.0 (I_2), collision energy 25. SRM parameters for all transitions were scan width (*m*/*z*), 0.200; scan time (ms), 0.01; Q1 and Q3 resolution (FWHM) 0.6. AFM1 quantification was carried out using both SRM transitions and the identification was accomplished by comparing the retention time and SRM transition ratio of the analyte obtained in the matrix with those of standard solutions. Positive identification was achieved when retention time agreement was within 1% and when the relative abundance of the two selected ion transitions was within a margin of ±10% of the expected ratio (I_2/I_1 3.1 ± 0.2). Excalibur software version 2.2 was employed to collect and process the data.

2.6. Method performance and matrix effect evaluation

Calibration solutions were prepared by diluting appropriate volumes of AFM1 stock solution with 10% MeCN (solvent curve) or the diluted SI-LLE extracts of analyte-free samples (matrix-matched curves). Linearity of the solvent and matrix-matched curves was evaluated in the working range of 0.86–34.5 pg mL^{-1} (corresponding to the range 5–200 ng kg^{-1}), with six calibration levels, each injected in triplicate. The regression curves were tested with the analysis of variance (ANOVA) and linear model was found appropriate over the tested concentration range. For the evaluation of matrix effects (ME), signal suppression or enhance, matrix-matched curves (AFM1 peak area versus ng kg^{-1}) of analyte-free milk matrices (whole and skimmed cow milk, goat milk, yogurt and powder milk) were compared with the solvent curve in the same concentration range. For matrix effect experiments, three independent SI-LLE extracts of each matrix were spiked with AFM1 levels corresponding to the contamination range 10–100 ng kg^{-1} and analyzed by online SPE–UHPLC–MS/MS. The slope of calibration curves was considered to evaluate the matrix effects [21], [22]. ME values around 100% point out to little differences between the matrix-matched and solvent curves. Values below 80% or above 120% displayed a significant ME (signal suppression or enhance, respectively).Method detection limits (MDLs) and method quantification limits (MQLs) were calculated using analyte-free milk samples fortified at low levels (1, 2, 5 and 10 ng kg^{-1}). Each level was processed in triplicate by the optimized analytical procedure, and the MDLs and MQLs were calculated by extrapolation of the

concentrations giving a signal-to-noise ratio (*S*/*N*) of 3 and 10, respectively, from a linear regression (*S*/*N* versus concentration).
Recovery experiments were performed on whole and skimmed cow milk, goat milk, yogurt and powder milk samples spiked at four AFM1 levels: 10, 25, 50 and 100 ng kg^{-1}, each processed in triplicate. The AFM1 concentration was determined using solvent curve. The intra-day precision (expressed as relative standard deviation, RSD) was determined with the same experiments.

3. Results and discussion

The detection of AFM1 in milk and dairy products remains a challenge because of the very low level contamination and the complexity of matrix. Highly complex composition of milk prevents the direct analysis of the samples by online SPE, therefore a preliminary pretreatment to remove the major milk components (proteins, fats, lactose and minerals) is required. Previously, SI-LLE was used to remove most of milk components and simultaneously to perform the extraction of AFM1 [19]. This pretreatment process provides an exhaustive recovery of the analyte from matrix and offers numerous advantages such as the ease of operation, very short analysis time and low costs. Therefore, in this study SI-LLE was applied to precipitate the proteins, to separate the fats, to remove water-soluble compounds (lactose and minerals) combined with the analyte extraction. Subsequently, SI-LLE upper phase, containing AFM1, was processed by online SPE coupled to UHPLC–MS/MS in the optimization and validation studies.

3.1. UHPLC–MS/MS method

Initially, the elimination of the LC column was considered to simplify and shorten the analytical process. However, subsequent trials showed a significant loss of signal intensity, most probably due to ion suppression induced by co-eluting matrix constituents. The selection of the chromatographic column was performed considering its ability to focus the analyte after the elution from SPE cartridge and to reduce matrix effect phenomena. For this purpose several columns with different stationary phase (kinetex C18 and PFP, Hibar purospher RP-18, and Chromolith RP-18e) and a number of gradient programs were tested, analyzing solvent and matrix-matched AFM1 solution. Kinetex PFP was selected for further method development because the pentafluorophenyl stationary phase with high steric interaction values allowed better retention of analyte resulting in narrow peak. Also the high purity silica of stationary phase in Hibar purospher RP-18 provided a strong retention of AFM1 with good peak symmetry. However, this column showed higher ion suppression when compared with kinetex PFP (Fig. 2S of Supplementary data), where the solid core plus the porous shell gives a larger particle and higher surface area gave a high resolution of analyte from matrix interferents [23]. LC–MS methods require an efficient separation and a sensitive detection of the analytes to minimize the matrix effects and improve the sensitivity of developed method. In order to obtain good peak shapes and get high ionization of analyte, several LC eluents (MeOH/H_2O, MeCN/H_2O and MeOH/MeCN/H_2O) and mobile phase additives (0, 1, and 2 mM of acetic acid, formic acid and ammonium acetate) were investigated. The maximum AFM1 response was obtained using MeOH as eluent solvent and the additives NH_4CO_2H (2 mM) and formic acid (0.1%). For MS detection, positive ionization mode was selected and selected reaction monitoring (SRM) was used to monitor the analyte. According to the 2002/657/EC regulation [11] two characteristic MS/MS transitions were selected to confirm its identity and the ratio between the signal intensities of two SRM transitions was used as additional identity confirmation.

3.2. Optimization of on-line SPE

The performance of method is strongly influenced on the efficiency of SPE process. In order to remove the greatest amount of matrix interferents and to achieve the maximum sensitivity for the determination of AFM1 in milk samples, specific parameters such as SPE sorbent, composition, volume and flow rate of washing solvent, dilution ratio of extract and injection volume were carefully optimized. Experiments were carried out in triplicate using the SI-LLE extracts of blank whole cow

milk spiked at the concentration of 86 pg mL^{-1}, corresponding to the EU MLs (50 ng kg^{-1}) fixed for milk [9].

The first parameter to be investigated in the optimization of an online SPE procedure is the selection of SPE sorbent that depends on the nature of both analyte and matrix. Initially, a hydrophilic interaction liquid chromatography (HILIC) material as SPE sorbent was evaluated due to the nature of SI-LLE extract (acetonitrile). An HILIC SPE column can be employed to trap relatively hydrophilic compounds when they are loaded with a substantial percentage of an organic solvent (typically acetonitrile) [14]. The use of a HILIC sorbent in online SPE would provide high method sensitivity due to the direct injection of extract without dilution. Nevertheless, preliminary experiments showed that the online HILIC SPE setup was not suitable to AFM1 trapping, likely due to poor hydrophilicity of AFM1. Consequently, reversed phase (RP) SPE- and LC setup was investigated. When using an RP SPE, the samples should usually be aqueous or loaded on to the SPE with a non-eluting RP solvent (e.g. 98–90% aqueous solvent) to avoid breakthrough effects [14]. Therefore, spiked SI-LLE extracts were ten times diluted with water to perform the experiments to select RP SPE sorbent. Three disposable RP cartridges, Oasis HLB, BioBasic C8 and C18, were evaluated in terms of peak shape and extraction efficiency of analyte. In these experiments, the injection volume was preliminary set at 500 µL and, after loading and washing (5% MeOH, 5 mL) steps, the analyte was eluted in back-flush mode with UHPLC gradient. BioBasic C18 SPE cartridge was selected as SPE stationary because showed the higher AFM1 recovery (>98%) and stronger refocusing of the analyte band. Oasis HLB and C8 SPE cartridges exhibited the same behavior previously observed for aflatoxins [20].

After the SPE cartridge selection, the washing conditions to remove/reduce the interfering compounds without any loss of the AFM1 were optimized to improve the sensitivity, accuracy and robustness of LC–MS method. In any online SPE–LC–MS method, the removal of co-eluted matrix components is very important, especially in the analysis of food samples. Though proteins and lactose were efficiently removed by SI-LLE, there are still compounds with medium polarity in milk that also could interfere with lifetime of system and create problem in identification and quantification of analyte. In this optimization step, the composition, volume and flow rate of wash solvent were investigated considering the analyte response and matrix effects. Regarding wash solvent composition, methanol aqueous mixtures (5–25% MeOH, 5 mL at flow rate of 0.5 mL min^{-1}) were evaluated comparing AFM1 response in diluted SI-LLE milk extracts and 10% MeCN. As shown in Fig. 1, in solvent AFM1 was efficiently retained on the SPE column up to 10% MeOH, while increasing eluotropic strength the analyte response was beginning to decline until to become drastic after 10%. On the contrary, in spiked matrix sample AFM1 response improved increasing the percentage of MeOH up to 10–15% before decreasing due to analyte breakthrough. This different behavior than to solvent can be explained by the ability of washing solvent to reduce the matrix effect (ion suppression) and thus improve the response of mass spectrometry. On the basis of these results, 10% MeOH was resulted the best balance between removal of interfering compounds and analyte trapping.

Experimental conditions: injection volume, 0.5 mL; wash volume, 5 mL; wash flow rate, 0.5 mL min^{-1}; elution, chromatographic conditions of Table 1; (n = 3). Error bars mean ± SD.

Once selected the eluotropic strength, the maximum volume of washing solvent without the analyte breakthrough was assessed (5–15 mL; 10% MeOH, flow rate, 0.5 mL min^{-1}). Losses of AFM1, related to its elution during the washing step, were observed exceeding the 5 mL (data not showed). Finally, the optimization of flow rate of wash solvent was carried out in order to reduce the time of analysis (0.5–2 mL min^{-1}; 10% MeOH, 5 mL). In the range from 0.5 to 1 mL min^{-1} no loss of analyte and no excessive cartridge backpressure were observed, whereas over 1 mL min^{-1}, despite the backpressure of system was still acceptable, a drastically decrease of AFM1 recovery occurred (Fig. 3S of Supplementary data). Based on these data, 5 mL of 10% MeOH at the flow rate of 1 mL min^{-1} was selected as optimal wash conditions. The capability of washing process to remove interfering was also confirmed after 50 injections of spiked real sample, under the optimized condition. No

increase of backpressure and significant variations of AFM1 response (RSD < 5%) were observed, which means that matrix impurities were removed by online SPE.
Subsequently to improve the sensitivity of online SPE method, the dilution factor of SI-LLE extract and the injection volume were investigated. In order to trap AFM1 on the RP SPE cartridge in the loading step, the dilution of the SI-LLE extract (acetonitrile) was necessary. The maximum amount of SI-LLE extract in loading solution was evaluated processing spiked extract diluted in three different ratios with water (9:1, 8:2 and 7:3, v/v). The results showed broad signals with consequent decrease of *S*/*N*, for ratios 8:2 and 7:3, thus the dilution factor was kept at 9:1 (data not shown). Thereafter, to compensate the dilution of SI-LLE extract the injection volume was studied. Generally, the concentration factor and the sensitivity of the online methods are amplified increasing the injection volume. However, the high column load may easily lead to column overloading, generating a suppression/enhanced of MS signal and a loss of the accuracy of method. The impact of the injection volume on the sensitivity and accuracy of method was evaluated in the range 0.5–3 mL. The trend-line slopes generated from the MS signal versus injected volume (Fig. 2) showed a good linearity ($R^2 > 0.999$) for both, solvent and matrix. The analyte response linearly increased with the injection volume and no matrix effects were observed for whole cow milk sample. These data demonstrated that online SPE allows the improvement of the sensitivity and detection limit of AFM1 in milk products, without inducing significant matrix effects and compromising chromatographic resolution. However, an injection volume of 1 mL enabled to detect AFM1 at levels lower than MLs and it is suggested for the routine analysis of food samples to extend column life and protect instrument.

3.3. Matrix effect evaluation

In the development of quantitative LC-MS methods, the accuracy and precision can be seriously affected by suppression or enhancement of the analyte signals (matrix effect, ME) due to co-eluting matrix components [21], [22]. Therefore, ME was carefully assessed to establish the most suitable quantification method.
Absolute ME was determined for different dairy products (whole, semi-skimmed, skimmed cow milk, goat milk, yogurt and powder milk), comparing matrix-matched curves (9:1, v/v, diluted SI-LLE) with solvent curve (10% MeCN). No relevant absolute MEs were estimated for tested milk products (95–108%); in fact all matrix-matched curves were comparable with the solvent curve (Fig. 4S of Supplementary data). Moreover, the evaluation of the relative ME (difference in response between various lots of post-extraction spiked milk) [19] was not revealed statistically significant RSD difference (data not shown).
In our previous research on the analysis of AFM1 in milk by SI-LLE combined with dispersive liquid–liquid microextraction (DLLME) [19], a negligible matrix effect was also observed. These results prove that SI-LLE efficiently remove macromolecules (proteins) and interfering compounds with wide polarity from a complex matrix as milk. Consequently, the accurate AFM1 quantification was not disturbed by matrix effects and can be carried out avoiding the use of internal standards, as labeled surrogate, or matrix matched calibration, decreasing the cost and time of the analysis. On the contrary, other methods present in literature for the analysis of AFM1 in dairy products reported significant matrix effects due to the presence of matrix interferents in final extracts [24], [25]. In these cases the procedures were proposed as screening methods and required the use of labeled internal standards [24] or labor sample preparation procedure (IAC) [25].

3.4. Analytical performance

The proposed analytical procedure was validated in terms of selectivity, linearity, sensitivity, recovery, accuracy and precision, according to the European Commission Decision 657/2002 [11]. To evaluate the applicability of the method to different milk products, the method validation was performed on five different milk matrices (whole and skimmed cow milk, goat milk, yogurt and powder milk).

Data acquisition was carried out in SRM mode with two characteristic fragments of $[M + H]^+$ ion of AFM1. In this manner, the method satisfied EU guidelines with four identification points for the confirmation of analytes with LC–MS/MS detection [11]. Additionally, the SRM intensities ratio was used as additional identification criterion with a tolerance of less than 10% of the expected ratio. Selectivity was experimentally evaluated analyzing blank and spiked samples. No interfering peaks in the elution region of AFM1 were observed for all tested matrices. Chromatograms of dairy products spiked to MLs or naturally contaminated are shown in Fig. 3 A and B.
The linearity range was estimated by solvent (MeCN 10%, v/v) calibration curves. AFM1 response was found to be linear in the concentration range corresponding to 5–200 ng kg^{-1} in milk products, with a correlation coefficient greater than 0.99 for a linear model ($y = 1.3 \times 10^7 \times +181.7$) of the calibration curve (ANOVA test).
The method sensitivity was experimentally estimated by the analysis of dairy products spiked at low levels and the evaluation of the signal-to-noise ratio (*S*/*N*). The calculated method detection and quantification limits (MDLs and MQLs) are listed in Table 2. Fig. 3C displays the chromatogram of a real milk sample contaminated to level close to MQL (4.0 ng kg^{-1}). For all tested milk matrices, MQLs were lower than the MLs imposed by current EU regulation for milk intended for direct human consumption and infant formulae milk (50 ng $kg^{-1}$and 25 ng kg^{-1}, respectively) [9] and comparable to our limits previously reported [19]. The sensitivity could be improved increasing the injection volume up to 2 mL without considerable matrix effects.

Accuracy and precision of the whole analytical procedure were established processing the analyte-free milk products, each spiked at four concentrations levels (10, 25, 50 and 100 ng kg^{-1}). The results of the recovery and intra-day precision (expressed as RSD) experiments ($n = 3$ independent analysis) (Table 2) are in agreement with EU regulation [26] regarding the performance criteria of the analysis methods for the official control of the levels of mycotoxins in foodstuffs. The proposed method provides an exhaustive analyte recovery, improving the accuracy of the analysis, unlike our previous study [19] where the low affinity of AFM1 with DLLME extraction solvent provided recovery in the range of 61–75%.
Finally, the proposed procedure was applied to analysis of AFM1 in dairy products (fifty-five) obtained on the local market in Campania region (Table 1S of Supplementary data). All analyzed samples contained AFM1 concentrations that were well below the EU MLs. AFM1 was detected in 76% of the milk and dairy samples at concentration ranges between <MQL −6.5 ng kg^{-1}. The contamination levels found are in agreement with official data of Italian products and they result from the continuous monitoring for public health and to reduce consumer exposure.

4. Conclusions

A novel automated online SPE–UHPLC–MS/MS method for the sensitive and accurate determination of AFM1 in dairy products has been developed. After a simple protein precipitation simultaneous to analyte extraction by SI-LLE, the online analysis allows the accurate AFM1 determination in less than 20 min. In the optimized condition, the proposed analytical procedure was validated on different dairy products and its performance fulfils the criteria required for methods of analysis of mycotoxins in foodstuffs (EC Decision 657/2002 and Regulation 401/2006).
Compared to other offline SPE procedures, comparable and/or lower MQLs were achieved and no significant matrix effects were observed by virtue of the sample preparation efficiency. Additionally, the method minimized the sample preparation procedure, producing a rapid reliable and robust method that can be used for routine screening and quality control programs in food chain and occurrence studies.

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Table 1. Timetable of online SPE and UHPLC system and switching valve position.

	SPE (Pump left)		UHPLC (Pump right)		
Time (min)	**Flow rate (µL/min)**	**Solv B (%)**	**Flow rate (µL/min)**	**Solv B (%)**	**Valve position**
0.0	1000	5	10	10	Load
1	1000	5	400	10	
6.0	1000	10	400	10	
9.0	10	95	400	40	Inject
12.0	10	95	400	60	
14.0	10	95	400	60	
15.0	10	95	400	95	
15.5	1000	95	600	95	Load
20.0	1000	95	600	95	
20.1	1000	5	10	10	
22.5	1000	5	10	10	

Table 2. Analytical performance of proposed method in different milk products.

	MDLs	**MQLs**	**Recovery ± SD[a] (intra-day precision, RSD[b]) (*n* = 3)**			
Milk product			**Level (ng kg^{-1})**			
	ng kg^{-1}		**10**	**25**	**50**	**100**
Whole cow milk	0.6	2.0	96 ± 8 (4)	98 ± 8 (7)	95 ± 5(3)	95 ± 6 (2)
Skimmed cow milk	0.6	1.8	98 ± 7 (4)	96 ± 7 (8)	92 ± 7 (6)	89 ± 9 (6)
Goat milk	0.6	2.0	89 ± 9 (7)	101 ± 4 (4)	88 ± 8 (6)	88 ± 6 (4)
Powder milk	0.7	2.4	101 ± 8 (4)	102 ± 2 (3)	93 ± 3 (3)	91 ± 12 (5)
Yogurt	0.5	1.5	92 ± 7 (4)	86 ± 3 (4)	95 ± 6 (4)	92 ± 5 (4)

a SD: standard deviation.

b RSD: relative standard deviation.

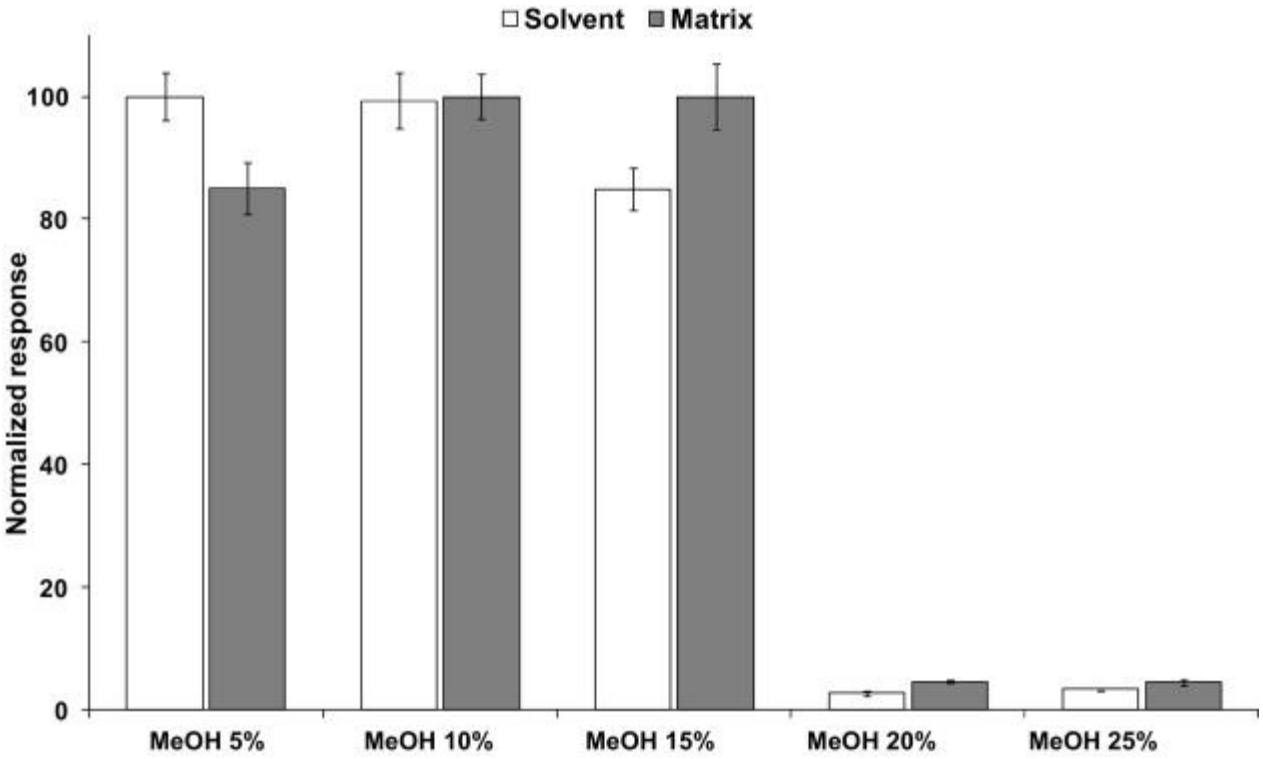

Fig. 1. Normalized AFM1 response under different wash solvent compositions.

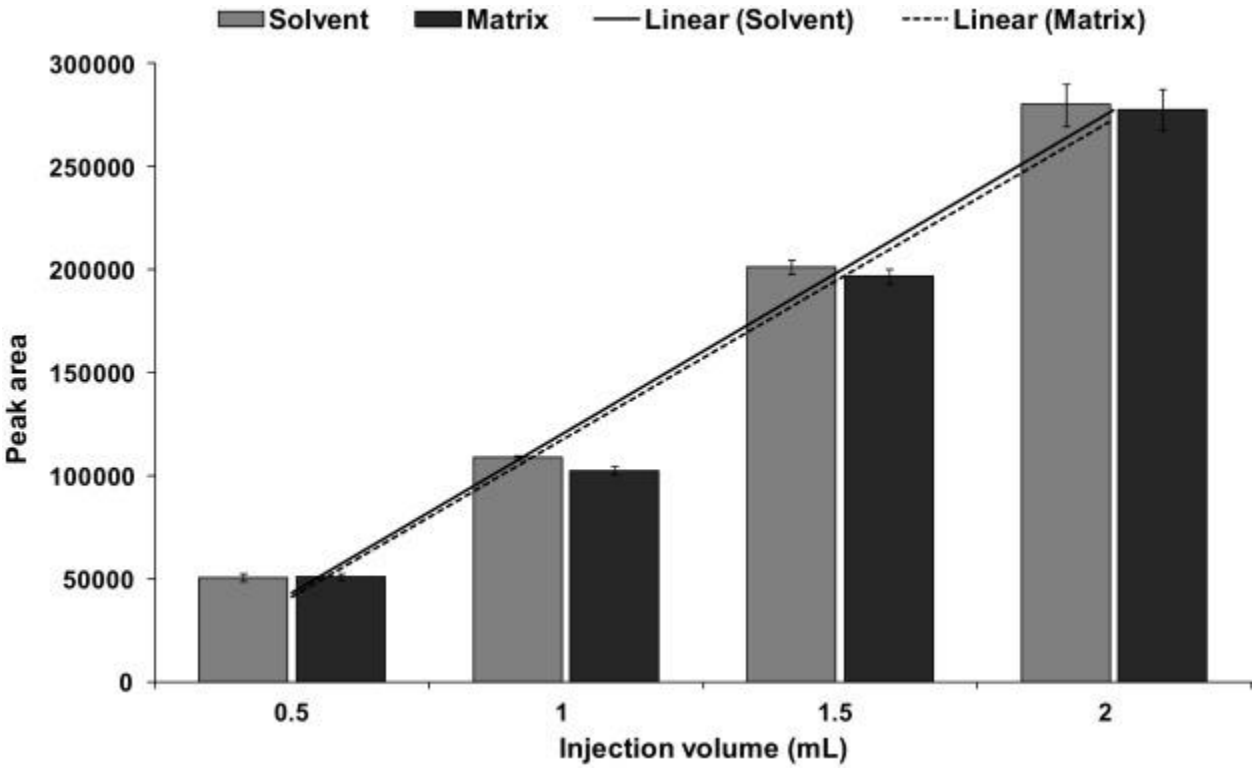

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Fig. 2. Effect of the injection volume on AFM1 response in solvent (MeCN 10%) and blank whole cow milk spiked at EU MLs (50 ng kg^{-1}). Experimental conditions: wash solvent, MeOH 10%; wash volume 5 mL; flow rate 1 mL min^{-1}; elution, chromatographic conditions of Table 1; (n = 3). Error bars mean ± SD.

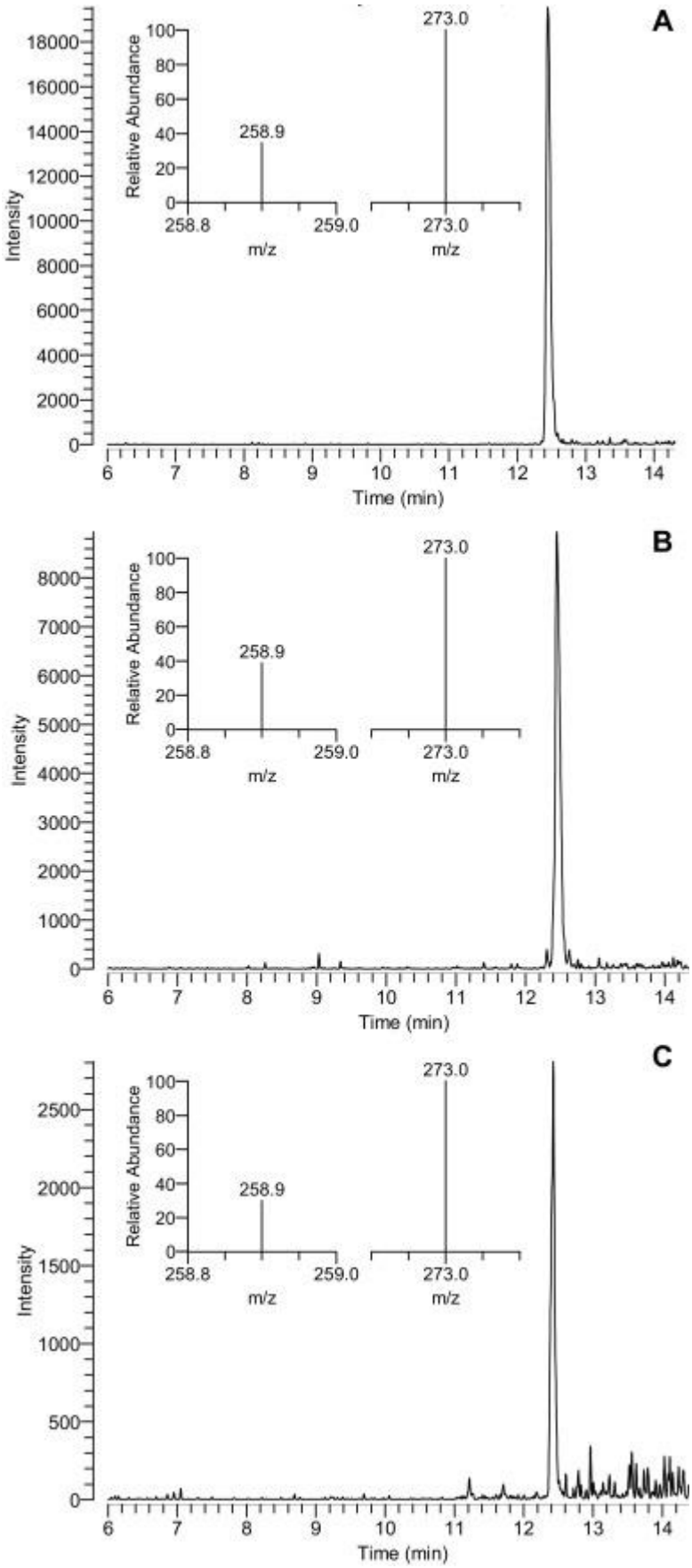

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Fig. 3. UHPLC–MS/MS chromatograms of (A) yogurt spiked at the EU ML for milk products (50 ng kg^{-1}), (B) powder milk spiked at the EU ML for infant formula (25 ng kg^{-1}), and (C) real milk sample contaminated at AFM1 level close to MQL (4.0 ng kg^{-1}).