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# Development and validation of a simple solid-liquid extraction protocol coupled with LC-ESI-MS/MS for the determination of aflatoxin M1 in products of colostrum-based supplements and whey protein-based sports food

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ABSTRACT

Special categories, such as infants and athletes, rely heavily on milk-based products. However, aflatoxin M1 (AFM1) contamination is a prevalent risk. This study aims to develop and validate a simple solid-liquid extraction protocol coupled with LC-ESI-MS/MS for AFM1 determination in colostrum-based supplements and whey protein-based sports foods. Extracts of acetonitrile-water (3:2, *v/v*) were directly injected into LC-ESI-MS/MS in a positive-ion mode. Separation was performed on the Agilent Poroshell 120 EC-C18 column with core-shell properties in a total analysis run time of 7.5 min. Among the tested extraction solvents, acetonitrile-water (3:2, *v/v*) provided an efficient direct extraction without any further treatments. AFM1 was eluted at a $t_R$ of 4.20 ± 0.05 min using the Agilent Poroshell column, and a tolerable ME% was successfully achieved. In both commodities studied, the recovery percentage, repeatability, intermediate precision, LOD, LOQ, linearity range, and linear regression coefficient ($R^2$) results were 94.3–104.1% with RSDs of 4.70–8.40%, 4.49–9.7%, 7.89–10.6%, 0.0015 μg/kg, 0.005 μg/kg, 0.005–1.000 ng/mL, and 0.9999, respectively. Applicability was demonstrated on two proficiency testing (PT) samples and 60 domestic samples. Results have confirmed the practicality of the proposed assay protocol, with concentrations ranging from 0.010 to 4.423 μg/kg for positive samples. Out of the tested samples, 4.8% of the whey protein-based sports foods violated the EU's established limits. This validated assay protocol would help increase sample processing capacity while also ensuring effective and regular oversight by national regulatory authorities.

## 1. Introduction

Aflatoxins are secondary metabolites produced by a variety of fungal strains, including *Aspergillus Flavus* and *Aspergillus Parasiticus*, and have been found as contaminants in a variety of foods and feeds (Marchese et al., 2018). Aflatoxin B1, B2, G1, and G2 are the most common aflatoxins, with aflatoxin B1 being the most potent naturally occurring carcinogen (Du et al., 2019). Aflatoxins, particularly M1, are most likely present in dairy products as a B1 metabolite as a result of animals being fed grain-based feed contaminated with *Aspergillus* fungi (Islam et al., 2021). This usually results in approximately 0.30 to 6.2% of B1 being converted to the corresponding M1 in the liver (i.e., a hydroxylated metabolite) (Fink-Gremmels, 2008; Veldman et al., 1992). Aflatoxin M1 (AFM1) is a heat-stable compound with a high detection rate in pasteurized milk as well as other dairy products derived from contaminated raw milk. This includes many products like whey protein, infant

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formulas, and colostrum-based products (Iha et al., 2013; Wood et al., 2020).

According to the International Agency for Research on Cancer (IARC), AFM1 is a possible human carcinogen (Group 2B) (International Agency for Research on Cancer, 2002). Far from this, a variety of serious health threats and complications, such as hepatotoxicity, teratogenicity, and immune-toxicity, were also reported (Izzo et al., 2022; Nguyen et al., 2020).

Owing to global safety concerns, AFM1 is typically monitored and analyzed in milk and milk-based dairy products; however, foodstuffs such as colostrum-based supplements and whey protein-based sports food, which are heavily consumed by special categories such as infants and athletes, have not yet been investigated for AFM1 (Jiang et al., 2018). According to the EU Commission Regulation No. 165/2010/EC (European Union, 2010) and the recently promulgated binding technical rules by the national regulatory agency (NFSA, 2022), limits of 0.05 μg/kg and 0.025 μg/kg were applied for milk-based products and dietary foods intended specifically for infants (e.g., food for special medical purposes) such as whey protein-based sports food and colostrum-based supplements, respectively.

In a continuous context, the prevalence of AFM1 along with conservative regulatory limits constituted an immense barrier to efficient analysis and accurate determinations in the aforementioned complicated commodities. Previous literature have described thin layer chromatography (TLC) (Filazi et al., 2010) and high performance liquid chromatography coupled to fluorescence detector (HPLC-FLD) techniques (Lee and Lee, 2015; Shuib et al., 2017) for the quantitative determination of AFM1 in various commodities, including milk and milk-based dairy products. However, a set of limitations, such as poor separation and unsatisfactory quantification accuracy, were reported for TLC methods. Likewise, additional cleanup procedures are required for mycotoxin determinations at μg/kg levels in various commodities using HPLC methods (Shuib et al., 2017). On the other hand, although mycotoxins have been extensively analyzed using the enzyme-linked immunosorbent assay (ELISA) technique in several previously published reports (Shaneshin et al., 2018; Tadesse et al., 2020), relying on obtained results may afford false-positive particularly when the obtained concentrations are less than 50 ng/kg. Consequently, any positive tested sample should be further confirmed by the HPLC-FLD reference method (Esam et al., 2022; Maggira et al., 2021). Since the last decade, LC-MS/MS has been widely applied for the reliable and accurate determination of AFM1 (Chen et al., 2022; Flores-Flores and González-Peñas, 2017; Iqbal et al., 2023). This is because of its high separation efficiency, improved selectivity, and enhanced sensitivities along with accurate quantitation results obtained over other reported analytical techniques (Magdalena Pisoschi et al., 2023).

Sample preparation, on the other hand, is of critical importance for providing efficient extraction with the minimum introduction of interfering substances while preserving the analyte of interest. From this perspective, several pretreatments for milk and milk-based dairy products have been reported to overcome the complexity of such commodities owing to their rich composition of fats, proteins, amino acids, and other ingredients (Shuib et al., 2017). Among these applied protocols are solid-phase extraction (SPE) (Jiang et al., 2018) and immune-affinity columns (IAC) (Shuib et al., 2017). Despite their ability to achieve selective analyte determination in complex matrices, IAC and SPE exhibit several drawbacks, including lengthy procedures, high costs, the use of hazardous solvents, and a requirement for well-trained personnel (Zhao et al., 2020). Dispersive solid-phase extraction protocols (d-SPE), particularly the quick, easy, cheap, effective, rugged, and safe (QuEChERS) method (Michlig et al., 2016; Rodríguez-Carrasco et al., 2018), and extraction methods based on QuEChERS principles were also employed for AFM1 extraction and sample enrichment (Chen et al., 2022). While QuEChERS protocols offer a cost-effective and high-recovery alternative to IAC, they necessitate an additional step of sample enrichment through solvent exchange (Chen et al., 2005; Michlig et al., 2016). Besides, for QuEChERS to perform well with some commodities of variable and complicated composition, several modifications were demonstrated prior to proceeding with the method (Vaz et al., 2020). Therefore, and in light of the presented drawbacks owing to sample processing for AFM1 extraction, developing a streamlined sample preparation protocol coupled with sensitive and accurate determination techniques such as LC-MS/MS for obtaining reliable test results from such complicated commodities is essential.

These sample processing protocols most commonly include simple solvent extraction approaches in which a solid-liquid extraction step is performed directly to transfer the analyte to the liquid phase (Castilla-Fernández et al., 2022). The major advantages of the solid-liquid extraction protocols are simplicity, minimal analyte losses, high sample throughput, and the number of analyte classes included, along with their wide applicability to a variety of matrixes, including food, biological, and environmental (Greer et al., 2021). While various protocols have outlined simple solid-liquid extraction methods for extracting several mycotoxins from various commodities (Dada et al., 2020; Malachová et al., 2018; Sulyok et al., 2020), no research, to our knowledge, has specifically examined AFM1 in milk or dairy products.

Hence, this study aimed to develop a simple solid-liquid extraction protocol for efficient extraction of AFM1 from colostrum-based supplements and whey protein-based sports food, followed by rapid and accurate determination with an LC-ESI-MS/MS system. In addition, performing method validation as per EU guidelines (European Commission, 2021; Magnusson and Örnemark, 2014) so as to comply with the EU regulatory limits (European Union, 2010). Validation parameters include selectivity, recovery percentages, repeatability, intermediate precision, LOD, LOQ, linearity range, and linear regression coefficient ($R^2$). Practicality testing of the proposed assay via its application to real commercial samples and proficiency testing samples (PT).

## 2. Materials and method

### 2.1. Chemicals and standard solutions

Methanol (MeOH) of an LC-MS grade with a purity ≥ 99.99% was purchased from Supelco® (Darmstadt, Germany). Acetonitrile (MeCN) of HPLC grade (99.9%) was obtained from Carlo Erba (Val-de-Reuil, France). Formic acid (FA) of HPLC grade and purity ≥ 99.0% was purchased from Carlo Erba (Val-de-Reuil, France,). De-ionized water was generated by the Milli-Q UF-Plus purification system, with a resistivity > 18.0 MΩ×cm and a total organic carbon (TOC) < 5ppb (Millipore, Darmstadt, Germany). A reference standard stock solution of AFM1 obtained from DR. EHRENSTORFER was dissolved in MeCN at a concentration of 500 ng/mL (LGC, Wesel, Germany). Working standard solutions were prepared by diluting the appropriate volume of the standard stock solution with MeCN to achieve concentrations of 1 and 50 ng/mL. The 50 ng/mL working standard solution is employed for spiking samples in quality control testing and for preparing a set of matrix-matched calibration levels (MMCs) at concentrations of 1.0 and 0.5 ng/mL. These MMCs are fortified extracts of blank samples prepared just before instrumental analysis. The 1 ng/mL working standard solution is used to prepare the remaining calibration levels, spanning the range of 0.005 to 0.1 ng/mL. All stock and working standard solutions were stored in the dark at − 20 ± 2 °C. Before routine work analysis commenced, working mixtures were kept in the dark at ambient temperature (23 ± 2 °C).

### 2.2. Instrumentation and analysis conditions

An Exion LC™ system coupled with Sciex QTrap 6500 + tandem mass spectrometer instrument was obtained from (Applied Biosystems/Sciex, Toronto, Canada). An Agilent Poroshell 120 EC-C18 column (50 × 4.6 mm, 2.7 μm) was purchased from (Agilent, Santa Clara, USA) and maintained in the column compartment at a temperature of 40 ± 2 °C.

The unit mass resolution was set for Q1 and Q3. MS/MS parameters were set as follows: Turbo Spray Ion Drive was used as the ion source, with an Ion Spray voltage of 4500 V and a temperature of 550 °C. Nitrogen (purity 99.999%) was used as the medium collision gas. The curtain gas pressure was 25 psi. Both ion source gases 1 and 2 had pressures of 50 and 60 psi, respectively. The flow rate was 0.4 mL/min, the injection volume was 5 μL. The sample tray was kept at a cool temperature of 5 °C. The mobile phase is composed of 10-mM ammonium formate in water with MeOH (9:1, *v/v*) (pH 5.5 ± 0.05) in a reservoir (A) and an LC-MS-grade MeOH in a reservoir (B). The gradient was accomplished as follows: start at 20% B for 1.5 min, linearly increase to 40% B till 1.6 min, remain in this condition till 3.5 min, linearly increase to 85% B till 3.6 min, then remain at 85% B till 4.5 min, and finally return to the initial conditions in 0.05 min, holding another 2.95 min for equilibration, with an overall run time of 7.5 min. Samples were analyzed in MRM with positive ion modes, in which the first transition was used for quantitation and the other one was used for confirmation as per EU guidelines for confirmatory methods (European Commission, 2021). These guidelines mandate that the signal-to-noise ratio (S/N) of both ionic transitions shall be > 3 and that the analyte's ion ratio aligns with that of the MMC at comparable concentrations, measured under identical conditions, within a ± 40% relative deviation. The main MS parameters for AFM1 determination were a declustering potential of 91 V and collision energies of 35 and 51 V for quantifier and qualifier ions, respectively. The most intense signal was used as the quantifier ion (329.1 → 273.2 *m/z*), while the less intense signal was used as a qualifier ion (329.1 → 259.1 *m/z*), with a qualifier-to-quantifier ion ratio of 50.4%. Analyst software 1.7.2 (Applied Biosystems/Sciex, Toronto, Canada) was used for data processing and calculations.

The auxiliary apparatuses included Spex™ sample prep 2010 Geno/Grinder™, a versatile high-throughput automated plant and animal tissue homogenizer/cell lyser with speeds ranging from 500 to 1750 strokes/min, purchased from Thomas Scientific (Metuchen, USA). For sample centrifugation, a centrifuge Z 446 K with a relative centrifugal force (rcf) of 16020g for 10 × 50 mL has been obtained from HERMLE Labortechnik GmbH (Wehingen, Germany). Extraction-assisting apparatuses included a shaking thermostatic bath (BSH model) with adjustable temperature from room temperature to 100 °C, adjustable shaking speed of 10–150 rpm, and programmed incubation duration (0–99.5 h) purchased from Raypa (Barcelona, Spain). In addition, an ultrasonic cleaning unit equipped with high-performance 37 kHz sandwich transducer systems, a rotating knob for establishing continuous and short-period operation from 1 to 30 min, and temperature-controlled ultrasonic operation from 30 to 80 °C (applicable only to units with heating) has been obtained from Elmasonic S 60 (H) (Singen, Germany).

### 2.3. Sampling

Sixty samples of various brands of colostrum-based supplements and whey protein-based sports food were purchased from the domestic market to test the AFM1 content. All purchased samples were in powder form with various flavors. Out of the studied products, 18 samples were colostrum-based supplements that have been packaged in three-layered sachets made of transparent plastic, aluminum foil, and coated paper and kept inside a cardboard box. The remaining products were 42 samples of whey protein-based sports food. In which 32 products were packaged in a plastic jar with a 1.8–3.2 kg capacity. The other 10 products were packaged in flat-bottom Ziploc plastic bags with a 5.45 kg capacity. All products were kept at storage conditions that had been recommended by the manufacturer until analysis. It should be noted that prior to proceeding to the analysis, samples are allowed to stand at room temperature for 10 min and are thoroughly mixed till complete homogeneity is attained.

### 2.4. Sample preparation

An aliquot of 5 ± 0.02 g from the studied commodities and blank samples were weighed in a falcon 50 mL polypropylene plastic tube. For quality control testing, blank samples were also weighed and spiked at 0.15 ng/mL with an appropriate volume of the 50 ng/mL AFM1 working standard solution to test the recovery percentage. Spiked samples were allowed to stand in the dark at room temperature for 5 min to facilitate standard interaction with the matrix components. Afterward, 15 mL of the extraction mixture MeCN/water (3:2, *v/v*) was directly added. For complete homogeneity, samples were vortex-mixed for 1 min, then vigorously shaken vertically using a mechanical shaker at 700 rpm for 20 min. Complete separation was performed via sample centrifugation at a relative centrifugation force of 16020 (rcf) for 5 min under cooling conditions at 5 ± 3 °C. The obtained supernatants were then filtered through a 0.2 μm Polytetrafluoroethylene (PTFE) syringe filter and transferred to an amber glass vial for direct injection into the LC-ESI-MS/MS system.

### 2.5. Calibration and validation

A set of six calibration levels (0.005, 0.01, 0.05, 0.1, 0.5, and 1.000 ng/mL) were prepared through serial dilution of the working standard solution in MeCN. Likewise, matrix-matched calibration was also constructed via spiking the final extracts of blank samples with appropriate volumes from the working standard solutions to reach the same levels that were prepared in a solvent. Both solvent-based calibrations (SBCs) and MMCs were analyzed as described above. The calibration curves were constructed by plotting the detector response against the known concentration (ng/mL), and the linear regression coefficient ($R^2$) was estimated for each one.

The method was validated following Eurachem 2014 guidelines, where percentage recovery, repeatability, intermediate precision, LOD, LOQ, linearity range, and selectivity were tested (B. Magnusson and U. Örnemark, 2014). Furthermore, an additional confirmation of the trueness was carried out via analyzing two proficiency testing (PT) samples for AFM1 in milk powdered samples. Both samples were supplied by the Food Analysis Performance Assessment Scheme (FAPAS). The obtained AFM1 results were compared to the assigned values reported and evaluated in terms of z-scores, where the final decision is considered satisfactory when z values are ≤ |2|. For measurement uncertainty (MU), the directions of Eurachem CITAC Guide CG 4 were implemented (Ellison, S.L.R., Williams, 2012) to account for the result accepted range.

### 2.6. Matrix effect and quantitation

Matrix effect was evaluated by comparing the slopes of both MMC and SBC curve. The obtained slope difference% were used to account for the magnitude of the ME% as shown in Eq. 1 which demonstrates the ME calculation. As previously reported, ME% is considered tolerable when the slope difference % falls within the range of ± 20%. Moderate and strong effects are considered to occur when the slope difference % exceeds ± 20% but remains within ± 50%, and when it exceeds ± 50%, respectively (Marzouk et al., 2023). All analyzed samples were quantitated using MMC and the obtained results were multiplied by a dilution factor of 3 and expressed as μg/kg.

$$ME\% = [(Slope_{MMC} - Slope_{SBC}) / Slope_{SBC}] \times 100 \quad (1)$$

## 3. Results and discussion

### 3.1. LC-MS/MS optimization

A reference standard solution of AFM1 (20 ng/mL) was prepared in MeCN and directly infused into ESI-MS/MS under automatic full scanning mode. The intensities of the obtained ions in both positive and negative ion modes were recorded. It was found that the positive ion mode scanning condition has resulted in the best sensitivity and maximum ion stability possible. Three transitions (273.2, 259.1, and 229.1 *m/z*) emerged from the protonated parent ion $[M+1]^+$ of 329.1 *m/z*; however, the initial two ions (273.2 and 259.1 *m/z*) were selected for quantitation and further confirmation, respectively. This is because the last transition, which is 229.1 *m/z*, was omitted owing to the observed interferences that arose from real and spiked samples during routine-work analysis. In accordance with EU guidelines for confirmatory methods (European Commission, 2021), four identification points were successfully achieved: 1 for the precursor ion and 1.5 for each product ion. Furthermore, for correct identification and accurate quantification of the studied analyte, four acceptance criteria were considered to avoid false-positive results. These criteria include: chromatographic retention time stability; matching of the retention time of the studied analyte in spiked samples and standard solutions; presence of the relevant transitions from the analyte molecular peak; a S/N > 3 of the ionic transitions; and ion ratio stability between the quantifier and qualifier peak.

On the other hand, owing to the challenges that constrain AFM1 analysis, such as low regulatory limits in the studied commodities, efficient chromatographic resolution of the studied compound is of crucial importance. A set of analysis criteria was taken into consideration to provide prompt and efficient AFM1 separation. Consequently, the studied analyte properties and the columns' characteristics in terms of stationary phase composition, particle size, and column dimensions were the common factors used for selecting the chromatographic column to undergo separation testing. In this regard, candidates of three chromatographic columns from different suppliers and with variable particle sizes were selected and tested under multi-step gradient elution programs. Besides, various injection volumes were also tested at 1, 5, and 10 μL for achieving maximum sensitivity and the best possible peak characteristics.

The tested columns were Kinetex XB-C18 100 Å (150 mm × 4.6 mm, 3.5 μm), Acclaim™ RSLC Polar Advantage II 120 Å (75 mm × 3.0 mm, 3 μm), and Agilent Poroshell 120 EC-C18 column (50 mm × 4.6, 2.7 μm). An elution system composed of 10-mM ammonium formate in water-MeOH, 9:1 *v/v* (pH 5.0 ± 0.05), and MeOH has been applied as per (Ren et al., 2007). Various gradient elution programs and flow rates were also tested to achieve optimal separation efficiencies along with enough data points of at least 10–12 for each peak over a relatively short analysis run time. Hence, spiked samples at a concentration level of 3 ng/g were chromatographed under the aforementioned conditions. Considering this, three distinct gradient elution programs, each with a gradual increase in the starting ratio of the organic phase (MeOH), were developed and evaluated on the examined columns. As shown in Table S1, the Acclaim column demonstrated optimal chromatographic performance with a high starting organic phase (MeOH) ratio (40%) in the mobile phase composition. The Kinetex XB-C18 columns performed optimally at a moderate organic phase starting ratio (30% MeOH), while Poroshell required a low organic phase starting ratio (20% MeOH) for optimal performance. Separation efficiencies achieved for each column were further optimized by adjusting the flow rate from 0.3 to 0.5 mL/min. The detailed results are presented below.

As shown in Fig. 1, the obtained separation results on the Acclaim™ C18 column exhibited poor peak characteristics (intensity of $1.80e^{+004}$), with an analyte peak asymmetry of 0.57 indicating a peak fronting, and were associated with analyte early elution at 2.93 min. The Kinetex XB-C18 column performed rather well in terms of sensitivity obtained

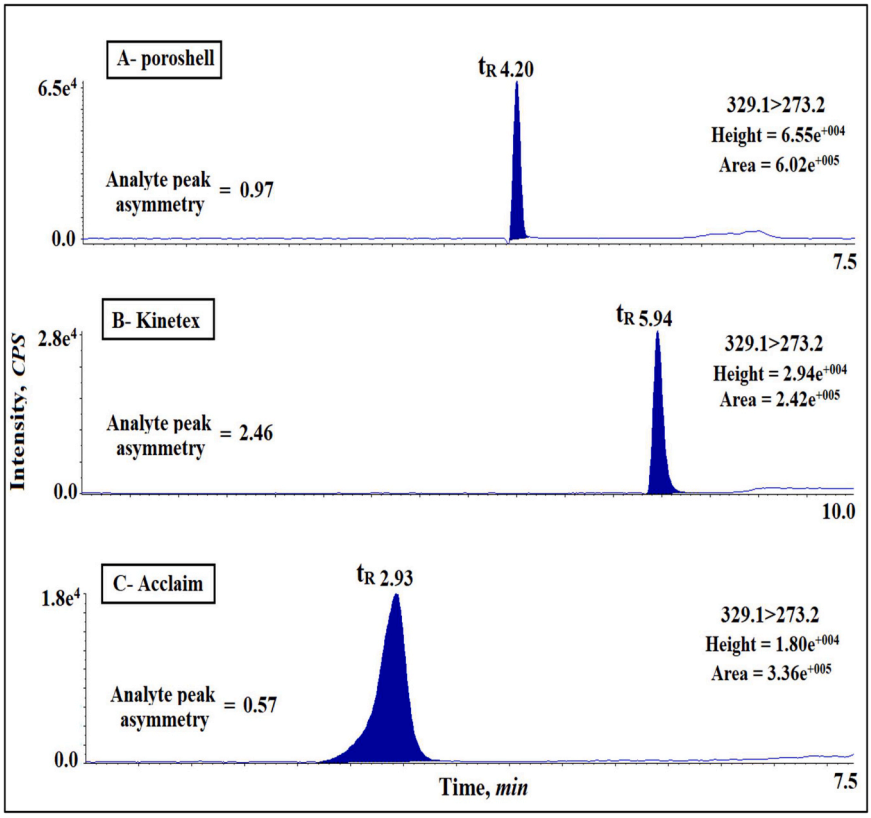


**Fig. 1. :** Comparison of the AFM1 performance on three different columns a) Agilent Poroshell 120 EC-C18 column (50 mm × 4.6, 2.7 μm); b) Kinetex XB-C18 100 Å column (150 mm × 4.6 mm, 3.5 μm); c) Acclaim™ RSLC Polar Advantage II 120 Å column (75 mm × 3.0 mm, 3 μm) using spiked samples at 3 μg/kg.

($2.94e^{+004}$), but late retention elution was obtained at $t_R$ of 5.94 min, leading to analysis run time increments exceeding 10 min for complete separation and column equilibration. Furthermore, the analyte peak asymmetry calculations yielded a value of 2.46, indicating tailing peak features. On the contrary, excellent performance was successfully achieved with the Agilent Poroshell C18 column at a reasonable $t_R$ of 4.2 ± 0.05 min. The overall peak characteristics obtained exhibited an analyte peak asymmetry of 0.97, which indicated a perfect peak shape associated with enhanced accuracy of integration algorithms, resulting in more reliable quantitation of either peak area or height. Besides, a high intensity was also achieved, with a peak height of $6.55e^{+004}$. This exceptional performance could be attributed to the superficially porous core-shell particles of the stationary phase. It should be noted that a flow rate of 0.4 mL/min was proper for providing adequate retention for the studied polar analyte under the early described elution program. Moreover, because of the small particle size of the Agilent Poroshell C18 column used, it helped to reduce column backpressure throughout the analysis run. Fig. 1 demonstrates the chromatographic separation efficiencies and sensitivities of the studied C18 columns for AFM1 analysis in spiked samples at 3 μg/kg.

As shown in Table S2, in agreement with (Jiang et al., 2018), a chromatographic separation run of approximately similar total run time (around 8 min) was successfully achieved upon employing the Agilent Poroshell C18 column for targeted commodities. Likewise, (Chen et al., 2022) have separated the AFM1 from raw milk extracted samples in a total run time of 8.3 min, but with a C18 Waters CORTECS column. On the contrary, a relatively long analysis run time was reported for AFM1 separation from extracts of raw and milk powder samples by (Wang et al., 2011), (Shuib et al., 2017), and (Wang et al., 2012) at 12, 15, and 35 min, respectively. It is worth noting that a total run time of approximately 4 min was achieved by (Michlig et al., 2016) and (Huang et al., 2014) owing to the chromatographic application of the C18 Waters ACQUITY UPLC BEH column.

It deserves mention that a higher injection volume of 10 μL resulted in column overload and peak symmetry deterioration, whereas a peak fronting (i.e., 0.36) was noticed. Dramatic sensitivity loss (≈ 12 times less than the optimal condition described) was observed upon injecting 1 μL of the spiked samples at 0.3 μg/kg, while convenient results were successfully obtained with an injection volume of 5 μL. Fig. S1 depicts the relationship between the injection volume of a spiked sample at a concentration level of 0.3 μg/kg and the observed peak characteristics (symmetry and sensitivity).

### 3.2. Sample preparation optimization

#### 3.2.1. Sample size and dilution magnitude

In conjunction with introducing the least amount of interferences into the final extract, achieving acceptable recovery percentages, and obtaining precise results of low CV%, maintaining the lowest possible levels for LODs and LOQs is of critical importance. As a result, appropriate selection of the proper sample size and dilution factor is essential for developing an efficient sample preparation protocol capable of providing reliable test results from representative analytical samples. Therefore, sample sizes of 2.5 and 5.0 g from the studied commodities were spiked at a concentration level of 0.3 μg/kg in triplicates and subsequently extracted with various volumes of MeCN-water 3:2 *v/v* by the aid of a mechanical shaker, as will be discussed later, representing a dilution factor of 1X to 5X, respectively.

Interestingly, at 3X, 4X, and 5X in both commodities tested, higher recovery percentages were achieved in the range of 93.3% to 95.8%, with CV% < 11%, in addition to getting a tolerable ME for each tested sample size. On the other hand, low recovery percentages of < 65% and intermediate signal suppressions of less than − 36% were obtained at 2X. At 1X, for each tested portion in both commodities tested, inconvenient test results were obtained owing to the complexity of sample processing at such a low dilution level.

Despite the fact that, at 4X and 5X dilution levels, acceptable high recoveries and tolerable ME were obtained, they were not selected owing to the inability to achieve lower LOQ levels, as will be described later. In addition, it has been decided not to perform testing for small sample sizes such as 0.5 g and 1.0 g test portions so as to avoid any possible fluctuations that may arise at ultra-low level determinations as the targeted analyte has strict regulatory limits in both national and EU regulations (European Union, 2010).

In fact, sample sizes of 2.5 g and 5.0 g were found appropriate for conducting AFM1 testing in whey protein-based sports food and colostrum-based supplements at a 3X dilution level while maintaining tolerable MEs and attaining lower LOD and LOQ values. However, the latter sample size was considered in the current study to provide reliable test results for various commodities with different compositions.

#### 3.2.2. Extraction optimization and matrix effect study

Initially, a frequently used extraction solvents such as MeOH and MeCN were tested at different mixing ratio with water (1:1, 3:2, 7:3, and 4:1, *v/v*), each for direct extraction of AFM1 from whey protein-based sports food and colostrum-based supplements. Spiked samples at a concentration level of 0.30 μg/kg were analyzed in triplicates using the proposed extraction mixtures. Results were assessed in terms of achieving tolerable matrix effects (ME) and acceptable recovery percentages, complying with the EURACHEM 2014 guidelines (B. Magnusson and U. Örnemark, 2014). Except for colostrum–based supplements, a solvent mixture of MeOH–water (1:1, *v/v*) was found inappropriate for extracting AFM1 because of the limited two-phase separation, even if high centrifugation forces were applied.

Furthermore, all MeOH–water extraction mixtures provided poor recovery percentages in the range of 18 to 33%, and 20.1% to 47.8% for whey protein–based sports foods and colostrum–based supplements, respectively. Also, all recorded ME% for extracting solvents composed of MeOH–water mixtures had different ratios ranging from − 66.0% to − 78.0%, and − 63% to − 72.8% (Fig. 2). Thus, correcting the obtained results to the ME% was found mandatory to get acceptable results. However, strong signal suppression is not desirable as it affects the end results accuracy. Besides, it limits the capability of the determination method to reach very low concentration levels, resulting in high LOQs surpassing the EU-MLs of AFM1. It should be noted that all resultant chromatograms obtained for AFM1 extracts with MeOH–water mixtures suffered from high background noise and a profound reduction in the compound's obtained sensitivities.

On the other hand, acceptable recovery percentages, as well as tolerable ME% of ≥ −19.1 and ≤ −10%, were obtained for all tested commodities regardless of the MeCN–water mixing ratio (Fig. 2). Because of limited sample agglomeration during sample processing procedures, a mixing ratio of MeCN–water (3:2, *v/v*) was found to be optimal for efficient AFM1 extraction in all studied matrixes. This could be attributed to the compromise reached between the amounts of MeCN and water used for the extraction, as increasing the MeCN limits the water penetration capacity to sample components. Although a direct extraction for AFM1 with MeCN was successfully achieved from powdered milk samples in previous reports (Huang et al., 2014), additional sample treatments, including SPE and a pre-concentration step via vacuum evaporation, were found to be essential to mitigate the ME% to intermediate signal suppressions of − 35% to − 32% (Table S2). This has confirmed that the addition of a well-studied amount of water to the MeCN would inevitably result in a ME% reduction to tolerable levels. Thus, and as shown in Fig. S2, high water content in the extraction solvent mixture (i.e., MeCN-water, 1:1 v/v) yielded chromatograms with additional interfering peaks and a well-noticed reduction in the compound's obtained sensitivity.

In agreement with (Wang et al., 2011), AFM1 extraction from powdered milk products has resulted in tolerable ME% of 21.2% to − 16.8% and acceptable recovery percentages of 88.8% to 100.6% (Table S2). Nevertheless, quite long sample processing procedures

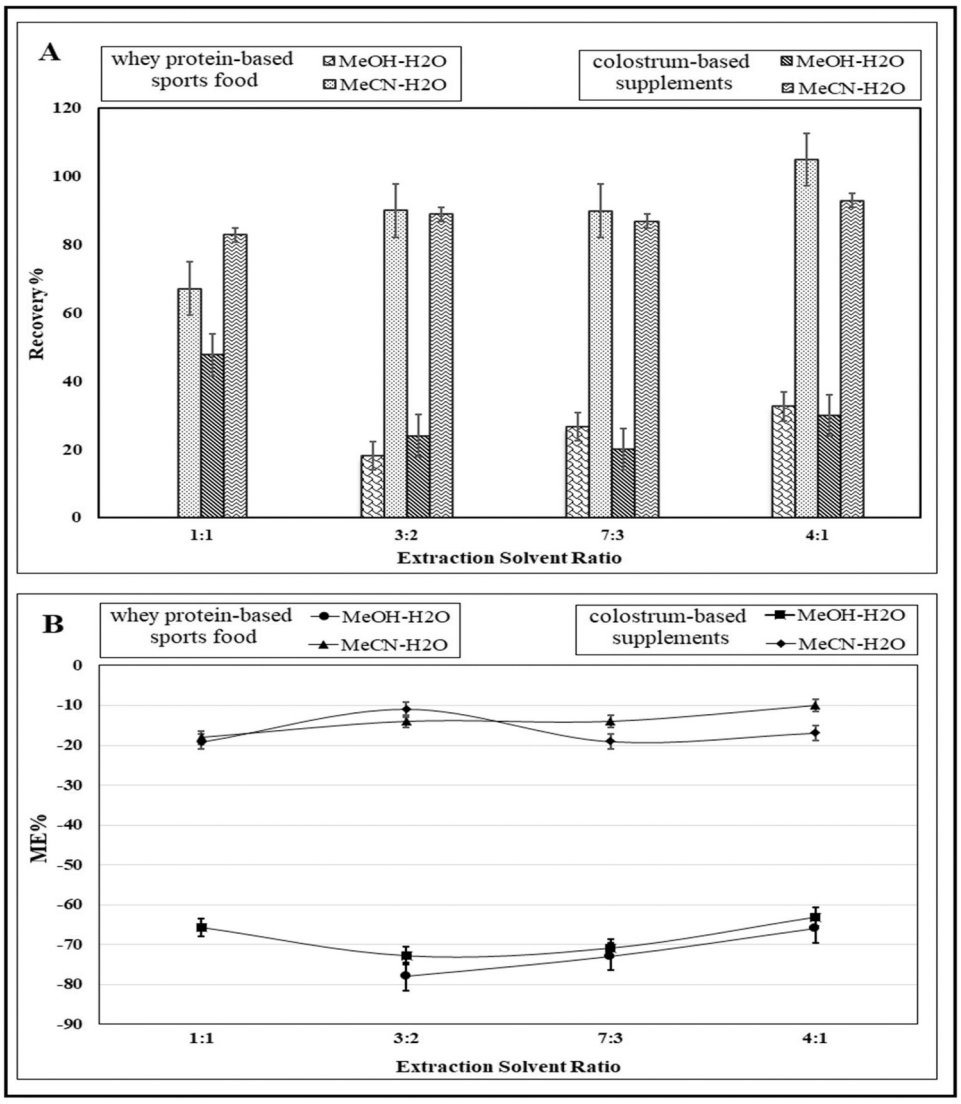


**Fig. 2. :** Relationship between different extraction solvent composition ratios (MeOH-water and MeCN-water) and the obtained recovery% and ME% in whey protein-based sports food and colostrum-based supplements at a concentration level of 0.3 μg/kg (n = 3).

involving additional evaporation steps using vacuum evaporation for a large volume of an extraction mixture composed of a high water ratio were reported (Table S2). Unlike other reported methods (Jiang et al., 2018; Michlig et al., 2016; Shuib et al., 2017; Wang et al., 2011, 2012), our proposed assay protocol has enabled a rapid and efficient sample processing in only 25 min (Table S2).

### 3.2.3. *Extraction-assisting techniques and sample purification*

In light of the foregoing, the efficiency of selective AFM1 transfer from the sample matrix to the employed extraction solvent of MeCN-water 3:2 *v/v* was also evaluated. Therefore, the effect of several influential parameters (e.g., temperature and contact time) on recovery percentages and MEs was assessed owing to the applied extraction and separation assisting techniques. These techniques included a heated water bath with a mechanical shaker, an ultrasonic (i.e., heat-inducing apparatus), and a mechanical shaker at the ambient temperature, along with an applied centrifugation force and time.

For instance, in heat-assisted extraction applications, samples are extracted using mild shaking conditions (110 rpm) at different time intervals of 1, 5, 10, 15, 20, 25, and 30 min in a heated water bath at 40, 50, and 60 °C. In addition, a similar extraction procedure was also carried out using a heat-inducing apparatus like an ultrasonic for efficient AFM1 extraction. Surprisingly, poor recovery percentages as well as intermediate MEs of signal suppressions were obtained regardless of the temperature applying techniques for both tested commodities. This might be due to the possible interaction between the tested analyte and the matrix components mediated by temperature. Therefore, mandatory procedures such as salting out steps with a combination of 4.0 g MgSO4 and 1.0 g NaCl or sodium acetate (Huang et al., 2014; Rubert et al., 2014), or employing IAC with monoclonal antibodies specific for AFM1 retention (Shuib et al., 2017) are required to achieve higher recovery percentages (Table S2). Furthermore, the two latter-reported protocols exhibited a set of mutual limitations, such as the consumption of additional chemicals and reagents, high cost, time-wasting, and a need for well-trained personnel able to handle the IAC technique. From this perspective, it is essential to avoid heat treatments when processing powdered products for AFM1 efficient extraction unless additional procedures are applied.

On the other hand, at similar time intervals, extraction-assisted vertical mechanical shaking at mild, moderate, and strong strokes of 500, 700, and 1000 rpm was tested at the ambient temperature. In both commodities studied, optimal extraction efficiencies were successfully achieved in 20 min with moderate strokes of 700 rpm, demonstrating acceptable recovery percentages and tolerable ME results. It should be

noted that an additional matrix component transfer to the extraction solvent, expressed as ME of intermediate signal suppression, was observed at time intervals exceeding 20 min. This might be attributed to the potential formation of emulsions during the prolonged shaking time. At time intervals below 10 min, a significant decrease in the resultant recoveries associated with MEs of intermediate signal suppressions was also observed. Starting from a shaking duration of 10 min, results of acceptable recovery percentages and intermediate signal enhancements were obtained, but with strong vertical mechanical shaking. Similar results were also obtained at mild shaking, but at a shaking duration starting at 20 min

Owing to the complexity of the studied commodities, sample purification through the physical removal of matrix components from the obtained extracts has been studied using a cooling centrifuge at different times and speeds following a mechanical shaking step at 700 rpm for 20 min. Therefore, speeds of 6000, 9000, 12000, and 16020 rcf were tested at 1, 5, 10, 15, and 20 min, respectively. In only 5 min, optimal performances were obtained at a centrifugation speed of 16020 rcf for both tested commodities. Although all tested times/speeds have resulted in tolerable MEs, the optimized condition of centrifugation was selected as a practically convenient step, resulting in complete phase-out separation (i.e., a sharp interface between the two phases).

In all, the emerging results demonstrate the feasibility of mechanical shaking at ambient temperature for efficient extraction of AFM1 from powdered products, while 15 mL of the optimized extraction solvent ratio of MeCN-water (3:2, *v/v*) was applied to a 5 g sample size (i.e., the dilution factor is 3X), followed by two-phase separation with 16020 rcf in 5 min. This optimized simple extraction protocol has enabled compliance with EU regulatory limits (European Union, 2010).

### 3.3. Method validation

#### 3.3.1. Selectivity

The proposed method of analysis was applied to samples of whey protein-based sports food, and colostrum-based supplements. The samples that exhibited the absence of AFM1 or minor detection at levels well below the LOD were deemed blank samples, as we will discuss later. Samples spiked at a concentration level of 0.015 μg/kg were compared to blank samples to investigate the test method selectivity. The obtained results revealed that no interfering peaks were detected in the retention time region of the studied compound. Fig. 3 presents a comparison of the AFM1′s spiked sample at a concentration level of 0.015 μg/kg versus the blank sample using the proposed method of analysis.

#### 3.3.2. Sensitivity

The method's sensitivity was expressed in terms of practical LOD and LOQ obtained. Consequently, the fit-for-purpose approach was followed to determine the lowest calibration level of the proposed assay through injecting further dilutions of the studied compound in both commodities tested. Hence, S/N of 3 is considered the LOD while S/N of 10 is used as LOQ value. For all studied commodities, it was found that the lowest concentrations that would be accurately quantified is 0.005 μg/kg while a concentration level of 0.0015 μg/kg was used to express the LOD value. Upon taking into consideration the method's dilution factor which is 3, then a fortification level of 0.015 μg/kg would be equivalent to the LOQ value that is employed in linearity range of the constructed calibration curve as mentioned below. It should be noted that the obtained results are well below the AFM1 EU regulatory limits for whey protein-based sports food and colostrum-based supplements (European Union, 2010).

Furthermore, in light of the obtained results for both LOD and LOQ, our method provided a more sensitive determination protocol for AFM1

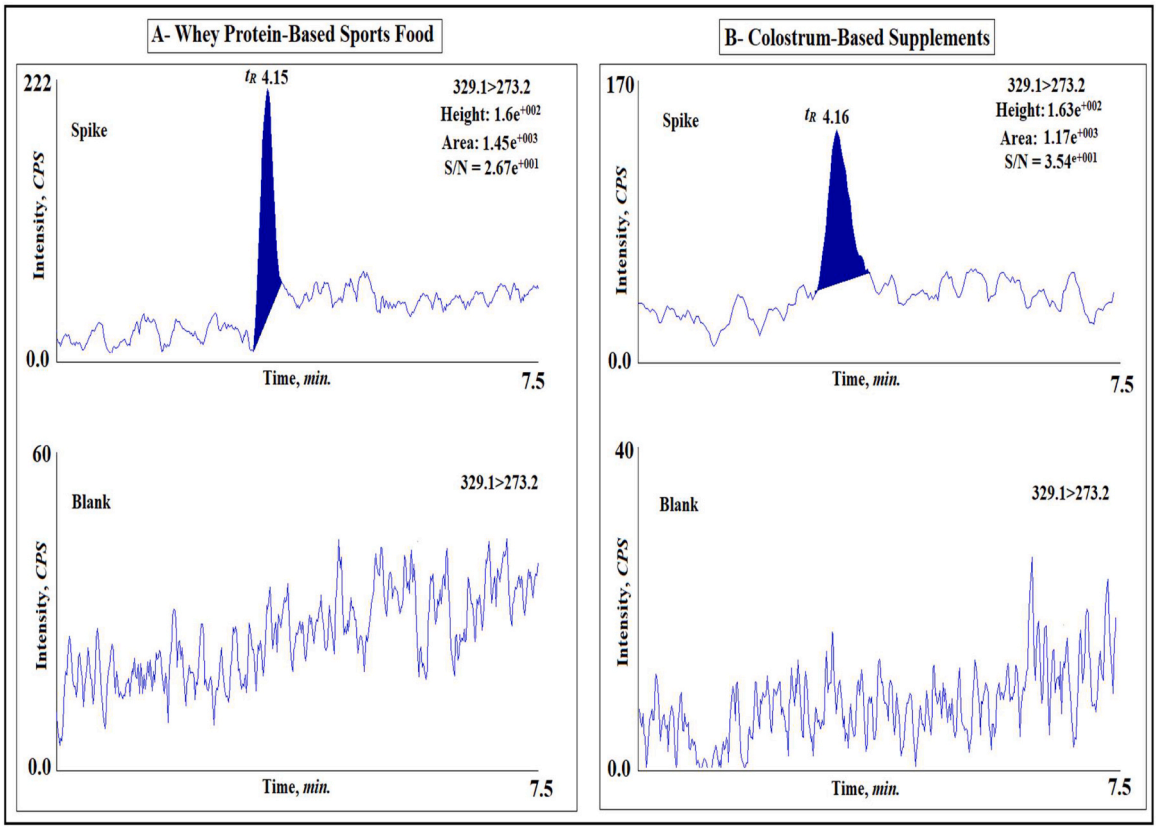


**Fig. 3. :** Blank versus spiked sample with AFM1 at a concentration level of 0.015 μg/kg, exhibiting the proposed assay protocol selectivity in A) whey protein-based sports food and B) colostrum-based supplements.

at approximately 2–5 times less than those previously reported (Jiang et al., 2018; Wang et al., 2011, 2012). Similar to previous protocols (Huang et al., 2014; Michlig et al., 2016; Shuib et al., 2017), comparable LOD and LOQ results were demonstrated. On the contrary, our developed protocol is 2 times less sensitive than (Chen et al., 2022) (Table S2).

### 3.3.3. Linearity range

A multilevel calibration curve of 6 points was successfully constructed over the concentration range of 0.005 to 1.0 ng/mL, with a linear regression coefficient ($R^2$) of 0.9999 and a linear regression equation of $y = 4.41e^{+005}$ x - $1.28e^{+003}$ at the origin being excluded. These results demonstrate the best curve fitting and excellent linearity over the studied range. In addition, this wide-range calibration curve would help provide accurate quantitation of samples with high concentrations without the need for further dilutions.

### 3.3.4. Trueness

For both tested commodities, the test method's trueness was expressed as recovery percentages ± RSD. Spiked samples with AFM1 at 0.015, 0.150, 0.300, and 3.000 μg/kg were analyzed using the proposed assay, 10 replicates each. The average recoveries obtained for whey protein-based sports foods were in the range of 96.5–104.1%, with RSDs ≤ 8.4%. For colostrum-based supplements, recovery percentages of 94.3–102.5 with RSDs ≤ 6.65% were obtained (Table 1). Further confirmation of the trueness results was carried out via conducting the proposed assay on two PT samples of milk powder (round no. 04423, 2021) and (round no. 04444, 2022) supplied by FAPAS. Every sample was analyzed in triplicate, and the obtained average concentration was reported as 0.207 ± 0.0035 and 0.395 ± 0.0031 μg/kg, respectively. As per the FAPAS report, the reported results are compared to the assigned value and considered acceptable when the z-score lies between ± 2. Our reported results exhibited a satisfactory z-score, as shown in Table 2. Furthermore, either individual or average reported values would achieve acceptable z-scores. This is due to the minimal data scatter, resulting in a very low standard deviation, leading to a raised conclusion about the high trueness accomplished.

### 3.3.5. Precision

At four spiking levels of 0.015, 0.150, 0.300, and 3.000 μg/kg, the method's repeatability was measured for both commodities studied. While the lowest concentration level of 0.015 μg/kg was selected to conduct the intermediate precision testing owing to the maximum variability expected, 10 replicates were analyzed per each level on the same day for repeatability testing and on three successive days for intermediate precision experiments. In both experiments, RSDs were calculated and recorded. The RSDs achieved for repeatability testing in whey protein-based sports food and colostrum-based supplements were 5.1–9.7% and 4.49–6.45%, respectively. Intermediate precision, on the other hand, had RSDs of 10.6% and 7.89% in whey protein-based sports food and colostrum-based supplements, respectively. (Table 1).

### 3.3.6. Uncertainty measurement

According to Eurachem guidelines, CITAC Guide CG 4 (Ellison, S.L. R., Williams, 2012), estimation of the uncertainty measurement can be determined correctly by systematically taking into consideration all relevant influencing factors possibly affecting the measurement results. In both tested commodities, the expanded uncertainty calculation has revealed a value of approximately ± 30% dispersion of the values that could be reasonably attributed to the measurand (Table 1).

## 3.4. Application to real samples

The obtained results of AFM1 analysis in the 60 tested real samples revealed that 54.7% and 50% of AFM1-tested positive samples were whey protein-based sports food and colostrum-based supplements, respectively. In both commodities, all samples detected positives (data not shown for tested brands) exhibited concentration ranges of 0.010–4.423 μg/kg and 0.022–1.076 μg/kg, respectively (Fig. S3). In light of the EU regulatory limits set at 0.05 and 0.025 μg/kg for whey protein-based sports food and colostrum-based supplements, respectively, the violation percentages were calculated taking into account the manufacturer specifications concerning the serving size and the labeled instructions of preparation. In this regard, all the colostrum-based supplements were accepted, while the tested products of whey protein-based sports food exhibited violating percentage results of only 4.8% (2 samples), with obtained concentrations of 2.04 and 4.42 μg/kg. This presents the practicality and applicability of the validated test method for AFM1 determination in various commercial real samples. Table 3 demonstrates the Prevalence of AFM1 in various commercial real samples purchased from the domestic market in Egypt.

## 4. Conclusions

An optimized ratio of MeCN-water (3:2, *v/v*) facilitated a streamlined extraction of AFM1 from whey protein-based sports food and colostrum-based supplements. This validated extraction protocol, together with LC-ESI-MS/MS, ensured accurate determinations at ultra-low concentrations with tolerable MEs. The proposed assay has been confirmed to be more sensitive than previously reported protocols for powdered milk product analysis, yielding LOD and LOQ values of approximately 2 to 5 times less. The method has so far proved applicable via PT and real

**Table 1**
Results of validation requirements as per Eurachem 2014 guideline for AFM1 in products of whey protein-based sports food and colostrum-based supplements using the proposed assay protocol.

| Compound | Spiking Level, μg/kg | Mean Recovery% ± RSD (n = 10 replicates, each) | Repeatability (n = 10 replicates, each) | | Intermediate precision at 0.015 μg/kg (n = 30 replicates) | | linear Dynamic Range, ng/mL | $R^2$ | LOD, ng/g | LOQ, ng/g | MU % |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Mean Conc., μg/kg | RSD | Mean Conc., μg/kg | RSD | | | | | |
| AFM1 | Whey Protein-Based Sports Food | | | | | | | | | | |
| | 0.015 | 102.1 ± 8.40 | 0.0154 | 9.70 | 0.0153 | 10.60 | 0.005-1.000 | 0.9999 | 0.0015 | 0.005 | 30 |
| | 0.15 | 104.1 ± 6.50 | 0.156 | 7.10 | | | | | | | |
| | 0.3 | 102.5 ± 7.60 | 0.297 | 9.20 | | | | | | | |
| | 3 | 96.5 ± 6.34 | 2.820 | 5.10 | | | | | | | |
| | Colostrum-Based Supplements | | | | | | | | | | |
| | 0.015 | 102.5 ± 6.65 | 0.0157 | 6.44 | 0.0124 | 7.89 | 0.005-1.000 | 0.9999 | 0.0015 | 0.005 | 29.5 |
| | 0.15 | 94.3 ± 6.39 | 0.1405 | 6.45 | | | | | | | |
| | 0.3 | 101.6 ± 4.70 | 0.311 | 4.49 | | | | | | | |
| | 3 | 94.3 ± 5.70 | 2.770 | 6.20 | | | | | | | |

**Table 2**
The obtained results of two PT milk powder samples purchased from FAPAS.

| Round No./Date | Commodity | Assigned Value, μg/kg | $\sigma_p$ | Average Conc., of the Reported Value (3 replicates),μg/kg ± SD | z-score | Comment |
|---|---|---|---|---|---|---|
| 04423, September 2021 | Milk powder | 0.238 | 0.0524 | 0.207 ± 0.0035 | -0.6 | ***Satisfactory*** |
| 04444, June 2022 | Milk powder | 0.340 | 0.0749 | 0.395 ± 0.0031 | 0.7 | ***Satisfactory*** |

**Table 3**
Prevalence of AFM1 in various commercial real samples purchased from the domestic market in Egypt.

| Product Tested | AFM1 Contamination Level | | | | | |
|---|---|---|---|---|---|---|
| | No. of Tested Samples | % of Samples Detected Positive | Concentration Range, μg/kg | Violating Concentration Range, μg/kg | % of Violating Samples[a] | EU Regulatory Limits (165/2010/EC) |
| Whey Protein-Based Sports Food | 42 | 23 (54.7%) | 0.010–4.423 | 2.042 and 4.423 | 4.8% (2 samples) | 0.05 μg/kg |
| Colostrum-Based Supplements | 18 | 9 (50%) | 0.022–1.076 | 0 | 0% < EU-limit | 0.025 μg/kg |
| **Total** | 60 | 32 (53%) | | | 2 | |

a, violation % was calculated taking into account the manufacturer specifications concerning the serving size and the labeled instructions of preparation and daily dose regime.

domestic samples analysis. This efficient analysis protocol would help increase sample processing capacities, particularly in routine work-based laboratories. Besides, it would allow effective and regular oversight of AFM1 in the targeted products by the national regulatory agencies, resulting in the maximum safety possible for Egyptian consumers.

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## CRediT authorship contribution statement

Muhammad El-Sayed Ismail Abdul-Hay: Methodology, Validation, Writing - Original Draft, Amr H. Shendy*: Conceptualization, Writing - review & editing, Formal analysis, Methodology, Data curation, AlaaEldean Fathy Ahmed Aboelhassan: Methodology, Ahmed M. Gomaa: Conceptualization, Supervision, Review, Eman H. Ismail: Conceptualization, Supervision, Review.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data Availability

Data will be made available on request.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jfca.2023.105933.

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