APPLICATION NOTE

Liquid Chromatography/ Mass Spectrometry

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# Determination of Aflatoxin M1 in Milk and Infant Formula by QSight LC/MS/MS with Three Fit for Purpose Sample Preparation Methods

## Introduction

Mycotoxins are toxic metabolites generated by fungi growing in foods and animal feeds. Aflatoxins (including aflatoxins B1, B2, G1, G2 and M1) are some of the most carcinogenic mycotoxins.[1-2] Aflatoxin B1 is often found in animal feeds such as corn, cottonseed cake, groundnut meal, and other agricultural commodities. When these contaminated feeds are ingested by milk-producing animals, such as cattle, aflatoxin B1 can be converted to aflatoxin M1 (a hydroxy derivate of aflatoxin B1) by oxidation metabolism, and then excreted in milk.[3-4] To protect consumers from the potential toxicity and carcinogenicity of aflatoxin M1, regulatory agencies have established regulatory limits or action levels for aflatoxin M1 in milk products. According to a U.S. FDA Compliance Policy Guidance, the regulatory action limit of aflatoxin M1 in milk is 0.5 µg/kg (ppb).[5] The European Union (EU) maximum levels of aflatoxin M1 are 0.05 µg/kg in milk and 0.025 µg/kg in baby food (infant formula).[6]

Various analytical methods, such as enzyme-linked immunosorbent assay (ELISA), thin layer chromatography (TLC), and high-performance liquid chromatography (HPLC) coupled with different detection methods, have been used for the determination of aflatoxin M1 in milk and milk products.[7-16] ELISA is a fast screening technique for the determination of aflatoxins owing to its simplicity, high-throughput capability, and low limits of quantification, especially when large numbers of samples need be analyzed in a short period of time.[17] However, one of the major drawbacks of immunoassays is the lack of information on the structure of the analyte. Thus, for regulatory applications, samples detected as positive for aflatoxins (containing toxins above the regulatory limit) by enzyme immunoassay, TLC, or LC-fluorescence often need confirmation by LC/MS methods.[5]

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Recently, with the advancement and availability of LC/MS/MS instruments, LC/MS/MS methods have increasingly become the method of choice for quantification and confirmation of mycotoxins in various food sample matrices owing to its superior sensitivity, selectivity and specificity.[11-16] Different extraction and sample clean-up techniques have been utilized when analyzing aflatoxin M1 in milk and milk products, including salt induced liquid-liquid (L-L) extraction using ethyl acetate or acetonitrile as an extraction solvent.[11-13] To further clean sample matrix components, solid phase extraction (SPE) or on-line SPE has been used after L-L extraction.[14-15] A multi-residue sample preparation technique utilizing QuEChERS extraction and subsequent d-SPE clean-up has also been applied for analysis of aflatoxin M1 in raw milk samples.[16] Immunoaffinity chromatography (IAC) purification is still a reliable and extensively used method for analysis of aflatoxin M1 in milk and milk products, although it is time consuming and involves multiple steps to accomplish the work.[10]

In this study, a simple, fast, selective and sensitive LC/MS/MS method for the analysis of aflatoxin M1 is presented, along with the evaluation of three sample preparation methods. By combining this LC/MS/MS method with different sample preparation approaches, we validated several fit-for-purpose methods to effectively serve different customers and meet various regulation requirements.

## Experimental

### Hardware/Software

Chromatographic separation of aflatoxin M1 from potential interfering components was conducted utilizing a PerkinElmer QSight® LX 50 ultra-high-performance liquid chromatography (UHPLC) system, with subsequent detection achieved using a PerkinElmer QSight 420 or 220 triple quadrupole mass spectrometer with a dual ionization source (ESI and APCI). All instrument control, data acquisition and data processing were performed using Simplicity™ 3Q software.

### Materials and Methods

#### *Chemicals and Materials*

Aflatoxin M1 stock solution (0.5 µg/mL in acetonitrile) and its stable isotope labeled internal standard (IS) U-[$^{13}C_{17}$]-aflatoxin M1 (0.5 µg/mL in acetonitrile) were obtained from Romer Laboratories, Inc. Immunoaffinity columns (IAC-Aflatoxin M1), QuEChERS extraction kits (AOAC 2007.01), disposable polypropylene syringes (10 mL), syringe filters (0.22 µm), polypropylene centrifuge tubes (15 mL and 50 mL), and amber autosampler vials were obtained from PerkinElmer, Inc. HPLC grade solvents (methanol, acetonitrile and water) and other chemicals such as formic acid, ammonium formate and phosphate buffered saline (PBS, pH 7.4) were obtained from Sigma-Aldrich. Fresh milk and milk-based infant formula samples were purchased from a local store (Toronto, ON, Canada).

#### *Standard Preparation*

To prepare calibration standards and quality control samples, several working standard solutions (100, 10, 5, 1, 0.1 ng/mL) and working internal standard solutions (50 and 5 ng/mL) were prepared from the corresponding stock solutions (0.5 µg/mL) by appropriate dilutions with a diluent of acetonitrile/water (50:50, v/v). Nine levels of calibration standard solutions (0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1, 5, and 10 ng/mL) were prepared from working standard solutions by appropriate dilutions with the same acetonitrile/water diluent and each standard solution contains internal standard 0.25 ng/mL. Two zero standard solutions were also prepared: standard 01 was prepared by adding the diluent directly into an autosampler vial to check the background and potential contamination to the vials; standard 02, containing only the 0.25 ng/mL of IS, was prepared to check the isotope purity of the IS.

#### *Sample Preparation Method One*

To meet the U.S. FDA aflatoxin M1 regulatory limit in milk and milk products (0.5 µg/kg), a simple, fast and less expensive sample preparation method was developed for this application note based on the work published by Kai Zhang and his colleagues from the Center for Food Safety and Applied Nutrition, Office of Regulatory Science, U.S. FDA.[13] The aforementioned method included simple sample dilution, centrifugation, and filtration. Briefly, 0.5 g of the sample (milk, milk powder, or milk-based infant formula) was weighed into a 15 mL disposable centrifuge tube, fortified with 25 µL of the IS working solution (50 ng/mL), and vortexed for one minute. The sample was then extracted with 5 mL of 50% acetonitrile solution (in water, 1:1 in v/v), and agitated for 10 minutes on a shaker. The sample solution was centrifuged at 4 °C for 30 minutes at 4000 rpm, and 2 mL of the supernatant was filtered through a 0.22 µm syringe filter into an autosampler vial for LC/MS/MS analysis.

For method validation, a laboratory reagent blank (LRB) was prepared and tested first to ensure that there was no interference or contamination from reagents or materials used, or from the sample preparation processes. Then, milk and milk-based infant formula blank samples were examined for analyte peak and any interfering components. Finally, to evaluate sample matrix effects and analyte recovery from the sample matrix, laboratory fortified matrix samples (LFM) were prepared. Following the same milk sample preparation procedures described above, using milk and milk-based infant formula as the sample matrix, each matrix was spiked with target analytes at three concentration levels - 0.5, 2.5 and 5 µg/kg. At each spiking level, LFM samples were prepared in triplicates, and no aflatoxin M1 was detected in blank samples used for recovery studies.

#### *Sample Preparation Method Two*

This sample preparation method is based on a QuEChERS (AOAC 2007.01 method) extraction method without dispersive SPE clean-up. Briefly, samples (6 g of liquid milk, or 4 g of milk powder or infant formula) were weighed into a 50 mL centrifuge tube, fortified with 100 µL of IS solution (and suitable amounts of working standard solutions when performing recovery study) and vortexed for one minute. Water (10 mL for infant formula and 4 mL for liquid milk sample) and acetonitrile (10 mL) were then added to the tube and vortexed for two minutes. Next, QuEChERS salts were added to the tube and hand-shaken for two minutes. After centrifugation for 20 minutes at 4 °C and 4000 rpm, 7 mL of the supernatant and 3 mL of hexanes were transferred to a 15 mL centrifuge

tube and then vortex-mixed for 2 min for defatting. After removing the top hexane layer, 5 mL of the sample solution was transferred to a clean graduated test tube, and then the solution was evaporated to 0.5 mL with $N_2$ gas flow at 30 °C. Finally, 0.5 mL of LC grade water was added to the sample tube, and the solution was vortex-mixed and filtered through a 0.22 µm syringe filter before LC/MS/MS analysis.

During method validation, an LRB sample was prepared and tested, together with milk samples, to ensure that no interference or contamination from the sample preparation procedures were present. Recoveries from sample matrices (liquid milk and milk-based infant formula, in this study) were evaluated by fortification of aflatoxin M1 at concentrations of 0.025 and 0.05 µg/kg, respectively, in the sample matrices. At each fortification level, samples were prepared in triplicates, and no aflatoxin M1 was detected in blank samples used for recovery studies.

### *Sample Preparation Method Three*

This sample preparation method used an immunoaffinity column (IAC, PerkinElmer P/N FOOD-1506-01) for sample clean up based on antibody-antigen specific interactions. Sample preparation procedures for liquid milk, milk powder, infant formula, and other dairy foods were described in detail in the PerkinElmer IAC user's manual for Aflatoxin M1 analysis.[18] Briefly, samples (4 g of liquid milk, or 1 g of milk powder or infant formula) were weighed into a 50 mL centrifuge tube, fortified with 100 µL of IS solution (and suitable amounts of working standard solutions when performing recovery study), and vortexed for one minute (milk powder or infant formula need dissolved in 4 mL of hot water). 10 mL methanol was then added to the tube, and vortexed for three minutes. After centrifugation for 20 minutes at 4 °C and 4000 rpm, 7 mL of the supernatant was diluted, and mixed with 40 mL of PBS solution and then loaded on an IAC for sample clean up and analyte purification. After washing the sample tube and IAC with 10 mL of water, the analyte was eluted from the column with acetonitrile twice (2 mL/time) and collected in a clean graduated test tube. The eluate was evaporated to 0.5 mL with $N_2$ gas flow at 30 °C. Finally, 0.5 mL of LC grade water was added to the sample tube, the solution was vortex-mixed and filtered through a 0.22 µm syringe filter before LC/MS/MS analysis.

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### *LC Method and MS Source Conditions*

The LC method and MS source parameters are shown in Table 1. Two PerkinElmer LC columns were evaluated for analyte separation during the method robustness study. The multiple reaction monitoring mode (MRM) transitions of aflatoxin M1 (AflaM1) and its internal standard (AflaM1-IS), and their optimized parameters, are shown in Table 2. Multiple MRM transitions were monitored to evaluate potential interfering components for certain transitions in real samples, which will help confidently identify analytes in complex sample matrices, reduce the potential for false positives and false negatives in the results, and increase the accuracy of analyte quantification. Optimization of MS/MS parameters, such as collision energies (CE), entrance voltages (EV), and lens voltages (CCL2), was done by infusion of standards and use of the autotune feature in the Simplicity 3Q software. MS source parameters, including gas flows, temperature and probe position settings, were optimized for maximum sensitivity by flow injection analysis (FIA). Dwell time for each MRM transition was set at 100 ms.

*Table 1.* LC Method and MS Source Conditions.

| LC Conditions | |
|---|---|
| LC Column | Quasar SPP C18 (50 x 3.0 mm, 2.6 µm, P/N9308915) or Brownlee, SPP C18 (50 x 3.0 mm, 2.7 µm, P/N9308408) |
| Mobile Phase A | 0.1% formic acid and 5 mM ammonium formate in water |
| Mobile Phase B | 0.1% formic acid and 5 mM ammonium formate in methanol |
| Mobile Phase Gradient (Flow Rate: 0.3 mL/min) | Start at 40% mobile phase B and increase B to 100% at 3 min and then keep at 100% B for 1 min to clean the column, finally return to initial condition at 4.1 min and keep running at initial conditions to 6 min. |
| Column Oven Temperature | 35 °C |
| Auto Sampler Temperature | 8 °C |
| Injection Volume | 10.0 µL |
| **MS Source Conditions** | |
| ESI Voltage (Positive) | 4500 |
| Drying Gas | 120 |
| Nebulizer Gas | 200 |
| Source Temperature | 400 °C |
| HSID Temperature | 280 °C |
| Detection Mode | MRM |

*Table 2.* Retention Time (RT), Optimized MRM Transitions and Parameters.

| Analyte | RT (min) | MRM | CC | EV | CCL2 |
|---|---|---|---|---|---|
| AflaM1 | 1.56 | 329.1/273.2 | -29 | 48 | -168 |
| | | 329.1/259.3 | -28 | 49 | -148 |
| | | 329.1/229.0 | -53 | 43 | -140 |
| AflaM1-IS | 1.56 | 346.1/288.1 | -29 | 48 | -168 |
| | | 346.1/242.2 | -53 | 43 | -140 |

## Results and Discussion

### LC/MS/MS Method Optimization

For mass detection of aflatoxin M1, both positive and negative electrospray ionization (ESI) modes were evaluated initially. The results showed that higher signal intensity and better signal-to-noise ratio were observed for aflatoxin M1 under positive mode, and therefore positive ESI detection was used in this study. For aflatoxin M1 and the IS, several product ions were generated at their corresponding collision energies and thus, multiple MS/MS transitions could be used. The optimized MRM parameters are listed in Table 2.

Two PerkinElmer UHPLC columns available in our laboratory were evaluated for separating aflatoxin M1 from potential interfering components in milk sample matrices. The columns used were a Quasar SPP C18 (50 x 3.0 mm, 2.6 µm) column, and a Brownlee SPP C18 (50 x 3.0 mm, 2.7 µm) column. Both columns provided equivalent results in terms of separation efficiency, retention time consistency and peak shape, and therefore, both are used for the method robustness study. The mobile phase compositions were optimized in a previous study and were used in this work without further modification (see Table 1).[16]

### Calibration Curves and Linearity of the Method

Several sets of calibration curves with concentration levels ranging from 0.01 ng/mL to 10 ng/mL were generated on separate days for aflatoxin M1, based on an internal standard calibration method. All calibration curves show good linearity, with correlation coefficients ($R^2$) greater than 0.999, as demonstrated in Figure 1. The accuracies for most of the calibration points evaluated by the RSD% of the residuals are less than 15% (and less than 20% for the lowest standard).

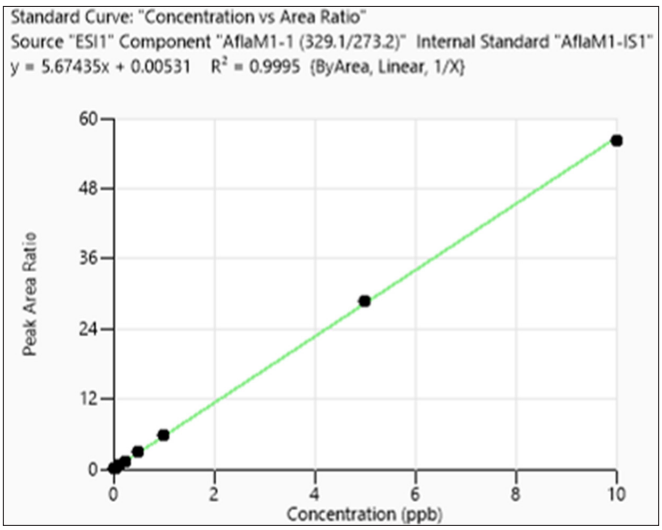


*Figure 1.* Calibration curves for the Aflatoxin M1.

### Method Performance and Comparison

*Sample Matrix Effects, Method's Selectivity and Sensitivity*

Sample matrix effects have received great attention in LC/MS/MS method development and validations as they can significantly affect the data quality and method's selectivity, sensitivity and accuracy, especially for complex sample matrices such as milk and milk-based infant formula samples. To reduce matrix effects, several approaches have been applied to the LC/MS/MS method development, such as simple sample dilutions, isotope dilution with stable isotopically labeled internal standard, matrix-matched calibration method, sample clean-up, use of high efficiency UHPLC columns for better separation, and the use of alternative ionization sources.[19]

In this study, isotope dilution with stable isotope labeled internal standard was applied to all three sample preparation methods to compensate for sample matrix effects and any variations in analytical procedures (including variations in sample preparation and instrument performance). Sample matrix effects could be reduced by a simple sample dilution method, as in ***sample preparation method one*** (to which, a dilution factor of 10 was applied). As illustrated in Figure 2, no significant matrix components were observed from both milk and infant formula samples fortified with 0.5 µg/kg of Aflatoxin M1 (the U.S. FDA regulatory limit). However, this sample dilution leads to limited sensitivity of the method. To improve the method's sensitivity, sample concentration steps are needed, as described in ***sample preparation method two*** and ***sample preparation method three.*** As shown in Figure 3 and Figure 4, the target analyte peak as low as 0.025 µg/kg could be determined from both milk and infant formula sample matrices, owing to analyte concentration steps. However, the significant difference between ***method two*** and ***method three*** is obvious when comparing Figures 3 and 4. Several matrix component peaks were observed from Figure 3 (***method two***), which could interfere with the analyte peak, and affect the method's selectivity and accuracy if they were not separated from the analyte peak by the UHPLC method, although this method has improved sensitivity. In ***method three,*** sample matrices were cleaned-up by using an IAC cartridge based on antibody-antigen specific interactions, and thus no matrix peaks were observed from both the milk and infant formula samples, as shown in Figure 4, demonstrating superior selectivity and sensitivity of the method.

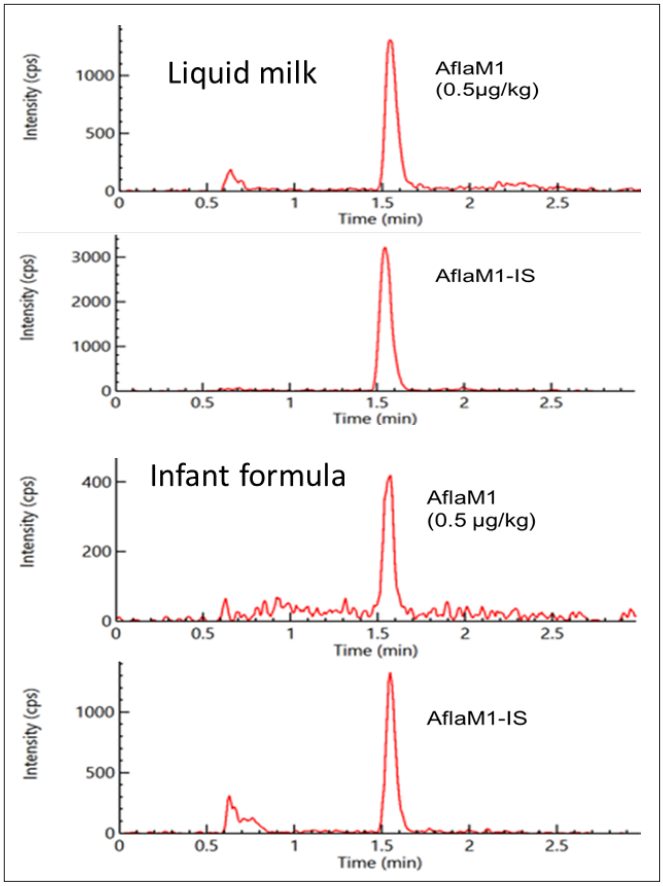


*Figure 2.* MRM chromatograms of aflatoxin M1 (fortified at 0.5 µg/kg) and its IS in milk (upper) and in infant formula (lower), obtained by sample preparation method one.

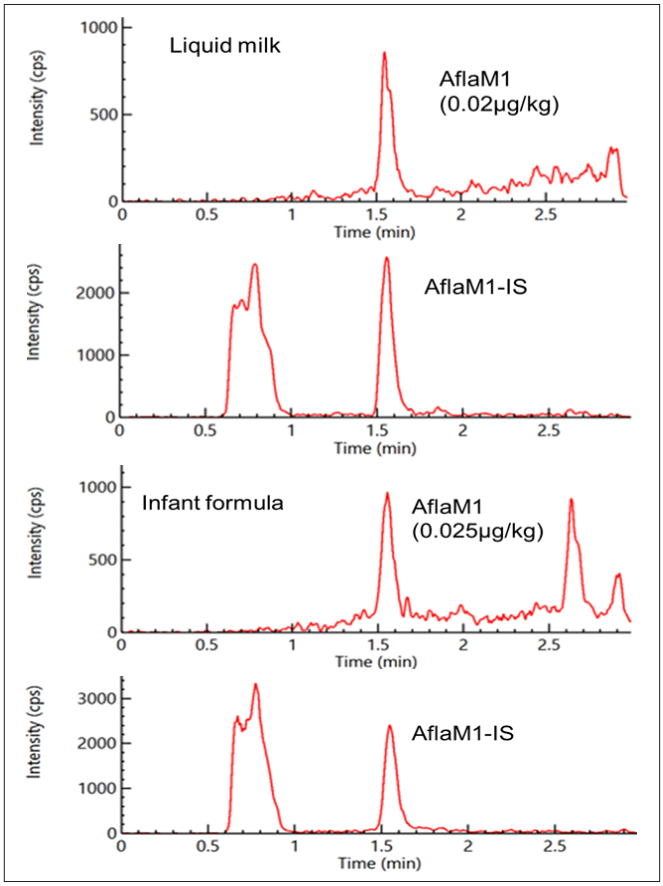


*Figure 3.* MRM chromatograms of aflatoxin M1 (fortified at 0.025 µg/kg) and its IS in milk (upper) and in infant formula (lower), obtained by sample preparation method two.

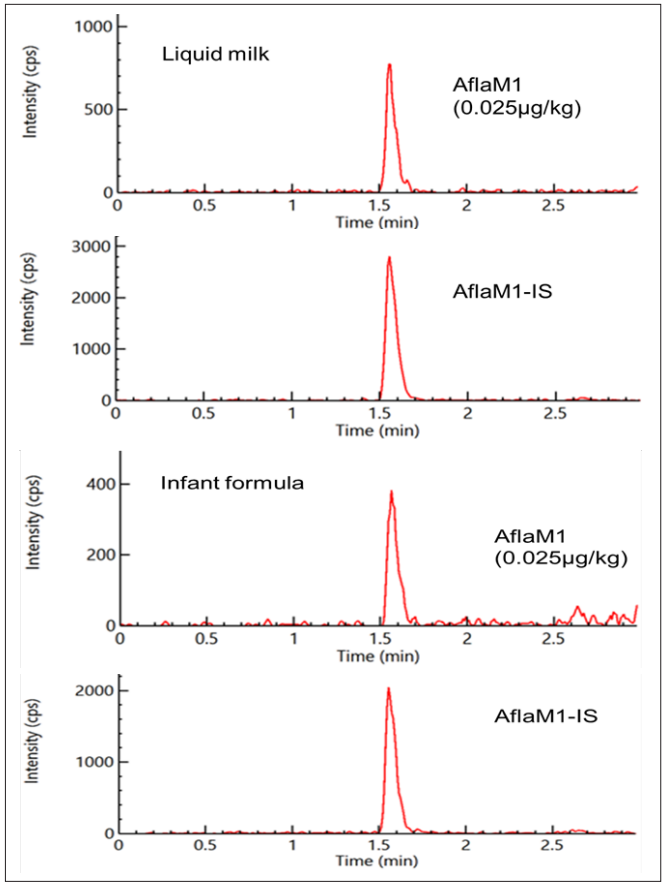


*Figure 4.* MRM chromatograms of aflatoxin M1 (fortified at 0.025 µg/kg) and its IS in milk (upper) and in infant formula (lower), obtained by sample preparation method three.

The method's selectivity and analyte confirmation from milk-based samples can be evaluated by comparing the analyte retention time and mass spectrum information (such as the peak area ratios of qualifier to quantifier ions of the analyte) between the reference standard and samples. According to regulatory guidance on analytical method validation, at least two MS/MS transition ion pairs should be used in an LC/MS/MS method.[20-22] In this study, three MS/MS ion pairs were employed for aflatoxin M1 in the method. As shown in Figure 5, which details a quality control milk sample spiked at 0.025 µg/kg, the ion ratios of 259.3 to 273.2 and 229.0 to 273.2 are quite consistent with those of reference standard, demonstrating good selectivity of the method for aflatoxin M1 analysis.

The method's sensitivity depends on the instrument sensitivity (0.01 ng/mL), sample matrix effects (signal suppression or enhancement), and sample preparation methods (sample dilution factors). In this study, signal suppression or enhancement effects were evaluated by comparing the responses of the same amount of IS spiked in solvent (such as in calibration standards) and in milk sample matrices. The results showed that there were no significant differences in IS responses between calibration standards and milk samples prepared by the three different methods, and thus, the sensitivity of the method would not be affected by the sample matrices. Therefore, the limit of quantification (LOQ) of the method can be determined by the instrument sensitivity (0.01ng/mL) and sample preparation methods (sample dilution factors), as listed in Table 3.

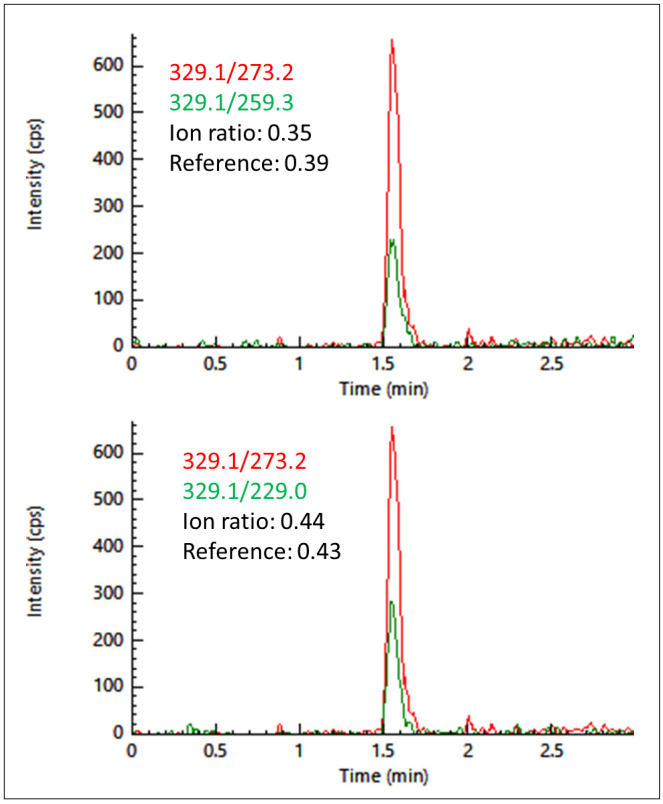


*Figure 5.* Overlapped MS/MS chromatograms and their ion ratios of qualifier/quantifier ions for aflatoxin M1 (fortified at 0.025 µg/kg) in a milk sample, obtained by sample preparation method three.

*Table 3.* The Estimated LOQ and Linear Range for Aflatoxin M1 in Milk-based Samples.

| | LOQ (Liquid Milk) | LOQ (Milk Powder/ Infant Formula) | Linear Range |
|---|---|---|---|
| **Instrument** | 0.01 ng/mL | 0.01 ng/mL | 0.01 - 10 (ng/mL) |
| **Sample Prep Method 1** | 0.1 µg/kg | 0.1 µg/kg | 0.1 - 100 (µg/kg) |
| **Sample Prep Method 2** | 0.004 µg/kg | 0.005 µg/kg | 0.004 - 5 (µg/kg) |
| **Sample Prep Method 3** | 0.005 µg/kg | 0.01 µg/kg | 0.005 - 5 (µg/kg) |

## *Precision, Recovery and Accuracy*

Method precision was assessed based on replicate analyses of a middle level standard and spiked milk samples (3 replicates). The precision was then calculated based on the coefficient of variation (RSD %) of the collected data. The RSDs were 8.3% for the standard, and < 12.6% for the spiked milk samples, respectively.

As shown in Table 4, no interference or contamination from reagents, glassware, sample tubes, or IAC cartridges were observed in this study, as demonstrated by the LRB sample results. Method accuracy assesses how close the experimental value is to the expected value. The method's accuracy was evaluated by the recovery of a known amount of analyte spiked into a sample (LFM samples). To meet the U.S. FDA action limit of 0.5 µg/kg, the accuracy of the simple ***sample preparation method one*** was evaluated by analyzing both liquid milk and infant formula samples fortified with analyte at 0.5, 2.5 and 5.0 µg/kg, respectively. To meet the more stringent EU regulatory limits of 0.05 µg/kg for milk-based products and 0.025 µg/kg for baby food, ***sample preparation method two*** and ***method three*** were examined by spiking sample matrices with the analyte at lower concentration levels. As shown in Table 4, the recoveries of aflatoxin M1 from the spiked LFM samples were between 84% and 114%, demonstrating good accuracy of the methods.

*Table 4.* Aflatoxin M1 Recovery from Spiked Milk and Infant Formula Samples.

| Sample ID | Spiked (µg/kg) | Recovered (µg/kg) | Recovery (%) |
|---|---|---|---|
| ***(Sample Preparation Method One)*** | | | |
| **LRB** | 0 | 0 | 0 |
| **LFM1_Liquid Milk** | 0.5 | 0.497 | 99.4 |
| **LFM2_Liquid Milk** | 2.5 | 2.58 | 103 |
| **LFM3_Liquid Milk** | 5.0 | 5.30 | 106 |
| **LFM1_Infant Formula** | 0.5 | 0.470 | 94.0 |
| **LFM2_Infant Formula** | 2.5 | 2.82 | 113 |
| **LFM3_Infant Formula** | 5.0 | 5.52 | 110 |
| ***(Sample Preparation Method Two)*** | | | |
| **LRB** | 0 | 0 | 0 |
| **LFM1_Liquid Milk** | 0.025 | 0.023 | 92.0 |
| **LFM2_Liquid Milk** | 0.05 | 0.049 | 98.0 |
| **LFM1_Infant Formula** | 0.025 | 0.029 | 114 |
| **LFM2_Infant Formula** | 0.05 | 0.045 | 89.0 |
| ***(Sample Preparation Method Three)*** | | | |
| **LRB** | 0 | 0 | 0 |
| **LFM1_Liquid Milk** | 0.025 | 0.026 | 104 |
| **LFM2_Liquid Milk** | 0.05 | 0.053 | 106 |
| **LFM3_Liquid Milk** | 0.5 | 0.549 | 110 |
| **LFM1_Infant Formula** | 0.025 | 0.021 | 84.0 |
| **LFM2_Infant Formula** | 0.05 | 0.046 | 92.0 |
| **LFM3_Infant Formula** | 0.5 | 0.478 | 95.6 |

## *Method Robustness*

Method robustness is the capacity of a method to remain unaffected by small, deliberate variations in method parameters. In this study, these parameters include different sample preparation methods, mobile phase compositions, and different HPLC columns. All these parameters and their variations on the performances of method were evaluated. The results show that the performances of the method were not affected by the changes of these parameters, and thus confirmed the robustness of the method. In addition, the method was validated on both QSight 220 and 420 LC/MS/MS systems, with the equivalent results obtained.

## *Stability of Standards and Samples*

According to the recommendation from the supplier, the aflatoxin M1 standard and IS stock solutions may be stored in a freezer for up to one year. Working standard and IS solutions are stable for a month, if kept in a dark place and in a freezer after preparation. Calibration standards and sample extracts are stable for at least a week, if kept in a dark place and in a refrigerator after preparation.

## Determination of Aflatoxin M1 in Milk and Infant Formula Samples

The method was applied to the determination of aflatoxin M1 in two milk samples and one infant formula sample, no analyte was found in the tested samples.

## Conclusions

In this study, a simple, fast, sensitive and selective LC/MS/MS method for the determination of aflatoxin M1 was developed by coupling UHPLC with tandem mass spectrometry. Three sample preparation methods were evaluated during the method's validation. By combining this LC/MS/MS method with different sample preparation approaches, we can effectively serve different customers and meet various regulation requirements. The simple and fast sample preparation method (***method on***e) is suitable for customers to meet the U.S. regulatory limit, while the sample preparation ***method two*** and ***method three*** should be used to meet more stringent regulatory requirements. The IAC sample clean up method (***method three***) can provide high quality results, especially for analyte at low concentration levels in complex sample matrices.

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