Quantitation of aflatoxin M1 using AP-MALDI coupled with with SCIEX QTRAP 5500 triple quadrupole MS

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Overview

Atmospheric pressure matrix-assisted laser desorption/ionization (AP-MALDI) was coupled with SCIEX QTRAP 5500 MS for the quantitative determination of metabolite aflatoxin M1 (AFM1) in milk samples. The developed method was validated with UHPLC-FLD analysis. This method combines the rapid, chromatography-free and high throughput AP-MALDI analysis with the high sensitivity and selectivity of the QTRAP MS. AP-MALDI and ESI sources can be interchanged within minutes. AP-MALDI methods complement, and add value to existing LC-MS workflows for high throughput aflatoxin screening and quantitation.

Introduction

Aflatoxins (AFs) are toxic compounds (mycotoxins) produced by the genera *Aspergillus* sp., *Fusarium* sp. and *Penicillium* sp. (Vaz *et al.* 2020). Aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1) and aflatoxin G2 (AFG2) comprise the four classes of aflatoxins that naturally contaminate food and feed products (Bellio *et al.* 2016). They are considered as secondary metabolites, which differ in chemical structure, biosynthetic origins, and biological effects among themselves. Aflatoxins can act as hepatotoxins, nephrotoxins, neurotoxins and immunotoxins. The exposure to aflatoxins through ingestion, inhalation, or absorption through skin, even in miniscule concentrations can be carcinogenic, hepatotoxic, teratogenic, and mutagenic for humans and animals (Vaz *et al.* 2020, Bellio *et al.* 2016). Among the four classes of aflatoxins, AFB1 is the most potent naturally occurring carcinogen, and categorized as a group I carcinogen (Vaz *et al.* 2020). AFB1 is converted to its hydroxymetabolite, aflatoxin M1 (AFM1) in the liver of lactating

animals that have consumed contaminated feeds, and is expressed in the milk. Approximately, 0.3% to 6.2% of the ingested AFB1 is metabolized to AFM1 (Vaz *et al.* 2020, Bellio *et al.* 2016). It is less lethal than AFB1 but has hepatotoxic and carcinogenic effects and is comparatively stable during the processing of milk. The presence of AFM1 in milk and dairy products possess a major risk to human health (Vaz *et al.* 2020). The maximum limit (ML) of AFM1 in milk and dairy products is regulated. As per the European Union (EU) legislation, the limit for AFM1 in raw milk, heat-treated milk and dairy products should be less than 0.05 µg/kg for adult, and 0.025 µg/kg for infants and young children. The Codex Alimentarius Commission and United States Food and Drug Administration (US FDA) defined the regulatory limit of 0.5 µg/kg for milk (Vaz *et al.* 2020, Bellio *et al.* 2016). Due to the toxicity and carcinogenicity of AFM1 and considering the defined ML values, different analytical methods have been developed for the AFM1 analysis (Vaz *et al.* 2020). The methods for AFM1 detection can be classified into two main categories- immunochemical and chromatographic methods. Immunochemical methods such as enzyme-linked immunosorbent assay (ELISA), immunoaffinity column assays (ICA), sequential injection immunoassay (SIIA) and radioimmunoassay (RIA) were developed for rapid screening of AFM1 in milk. Chromatographic methods such as thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC) have been developed for the quantitative determination of AFM1 after rapid screening tests. Generally, immunochemical methods require specific antibodies for the detection of the analytes that can be prohibitively expensive. Chromatographic methods are time-consuming with lower throughput (Bellio *et al.* 2016). Hence, there is a need to develop a simple and rapid analysis method. The SCIEX QTRAP 5500 is an industry standard mass spectrometer that is routinely used for the detection of toxic compounds in food and feed products. AP-MALDI coupled with SCIEX 5500 QTRAP increases the throughput of the method significantly. Sample preparation for AP-MALDI is relatively simple and rapid without the added step of loading the sample under vacuum conditions (Sundaram *et al.* 2011). In this application note, we demonstrate the rapid analysis of AFM1 in milk samples with AP-MALDI SCIEX QTRAP 5500 that enables scaled up operations for high throughput screening and quantitation in a food testing laboratory.

Experimental

Standards

Aflatoxin M1 (AFM1) (Certified reference material, > 98% purity) was sourced from Trilogy Analytical Laboratory, USA, India. LC-MS grade solvents methanol and acetonitrile were purchased from Thermo Fisher, USA. α-Cyano-4-hydroxycinnamic acid (CHCA), trifluoroacetic acid (TFA) were procured from Sigma-Aldrich, USA. A water-purification system (Pall India Pvt. Ltd., India) was used to generate HPLC-grade de-ionized water (18.2 MΩ cm^{-1}) used in the preparation of solutions for the experiments. Aflaprep® M 25 immunoaffinity chromatography (IAC) kit was used for sample clean-up.

Preparation of Standard solutions

A primary stock solution of reference standard with concentrations of 1000 µg/kg in acetonitrile was prepared. Serial dilution solutions were prepared to achieve a working concentration range (0.02 to 50 µg/kg) of calibrants. MALDI matrix (10 mg CHCA) was prepared in 70% Acetonitrile: 30% 0.1% TFA in deionized water.

Sample preparation

A generalized workflow for the sample preparation, instrument and software analysis is provided in **Figure 1**. Commercially available buffalo's milk was used for the AFM1 analysis. The milk sample used for AP-MALDI QTRAP 5500 analysis was procured from a local dairy farm. The contamination level of the milk sample was assessed by AOAC official Method of Analysis (2005), Ch. 49.3.07 Method, 2000.08. AFM1 levels were found to be less than 0.05 ng/mL, and hence it was considered as residue-free milk. 50 mL of the milk sample was homogenized at 4000 rpm for 5 min, heated and centrifuged at 7000 rpm for 5 min to separate the milk solids. The upper lipid layer was discarded. 30 mL of the remaining milk sample was aliquoted into three 50 mL centrifuge tubes, with 10 mL in each tube. To these tubes, 10 mL of deionized water was added and vortexed for 1 min. This sample was further processed for the extraction of AFM1 via IAC method. The recovery percentage of AFM1 obtained with QTRAP 5500 was analysed by spiking the residue-free milk samples with AFM1 at 0.5 µg/kg and 1 µg/kg concentration. 10 mL of this diluted milk sample was then loaded onto an IAC column cartridge packed with monoclonal antibodies of AFM1, at a flow rate of about 1-2 drops/s. AFM1 was eluted

with 1 mL methanol and collected in a glass tube, to be used for AP-MALDI MS/MS analysis. An equal volume of sample (AFM1 standard and milk samples) and the CHCA matrix was premixed and 1 µL of the mixture was spotted onto a stainless steel MALDI target plate. The samples are spotted randomly. The target plate was air-dried prior to the analysis. A representative figure for AP-MALDI target plate with sample scheme is provided in **Figure 3**. The samples were also analysed using UHPLC-FLD on Acquity H-Class UHPLC-FLD (Waters Corporation, Manchester, UK) instrument. The FLD comprised a large volume (13 µL) flow cell, and the chromatographic separation was achieved by using an Acquity UPLC® BEH C_{18} column (2.1 × 50 mm, 1.7 µm, Waters Corporation, Bangalore, India). The column temperature was maintained at 40 ºC and the flow rate was optimized at 0.4 mL/min with injection volume of 10 µl. The mobile phase was composed of 0.1 % acetic acid in water (A) methanol (B) and Acetonitrile with an isocratic mode (64:18:18) was used in order to enable the chromatographic baseline separation of aflatoxin M1 within 5 min. The excitation wavelength was 365 nm and the emission wavelength was set at 456 nm.

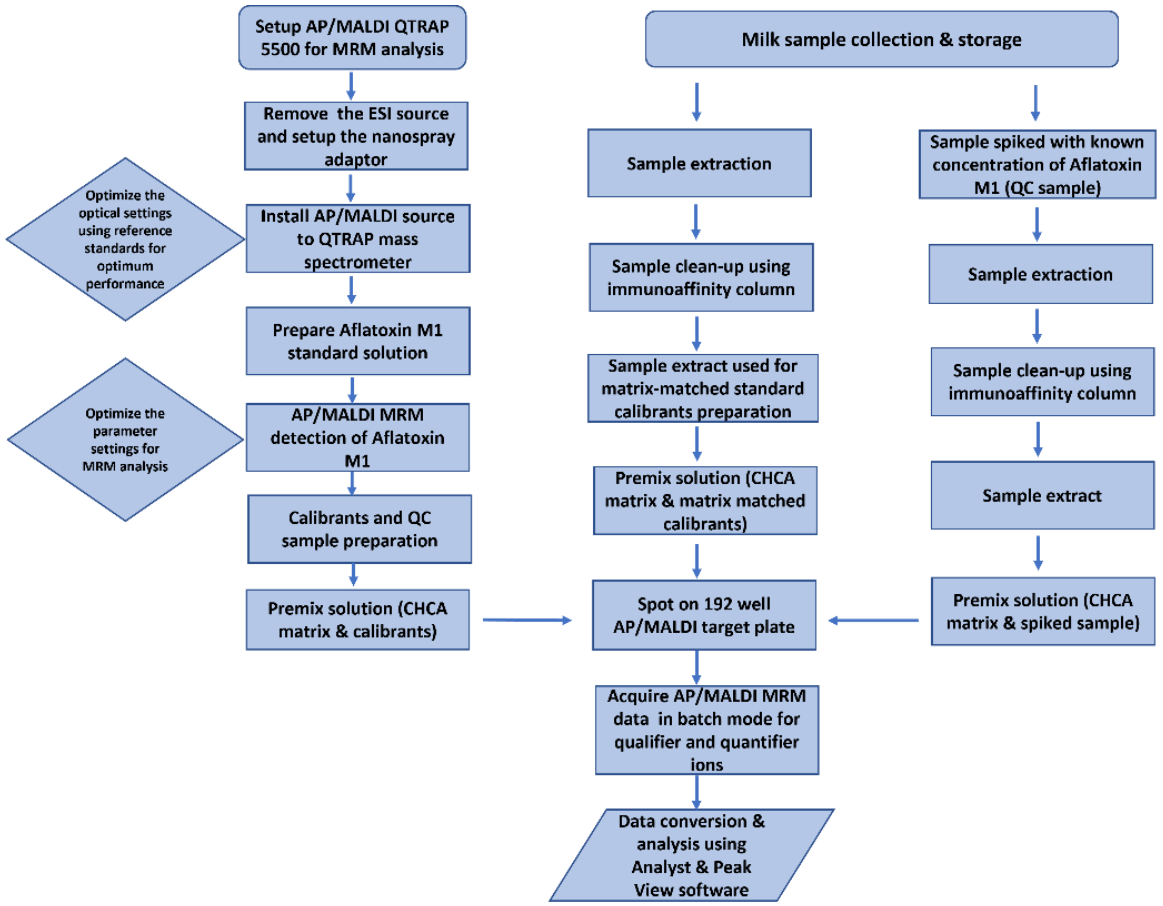

Figure 1. A generalized workflow demonstrating sample preparation, instrumental and software analysis of aflatoxin AFM1 with AP-MALDI MRM.

Figure 2. Separate images of extenders at top and bottom, developed by MassTech for interfacing with SCIEX NanoDCI Interface and Optiflow source inlets. Image on the right shows AP-MALDI hyphenated with SCIEX QTRAP 5600+ system and the image on left shows SCIEX QTRAP 5600+.as a standalone instrument.

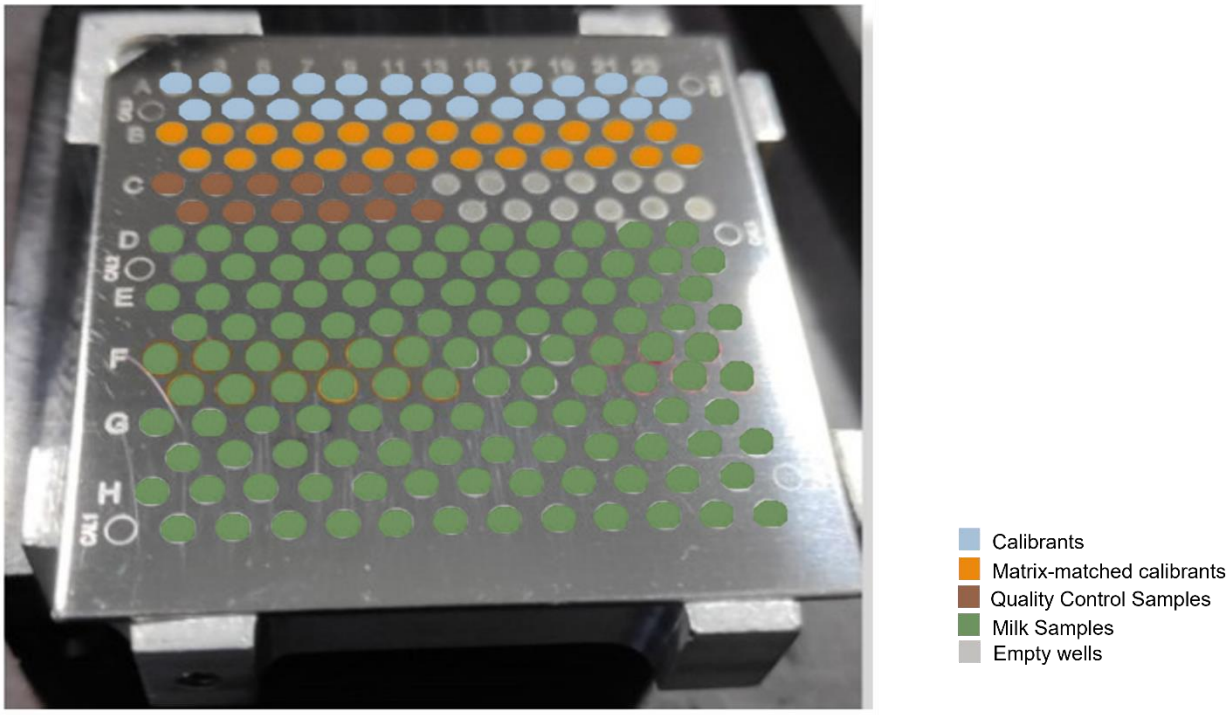

Figure 3. Schematic of the target plate containing all the calibrants in solvent systems, matrix-matched calibrants and quality control (QC) samples spiked and extracted from milk spotted together and analyzed in batch mode.

AP-MALDI MS/MS Detection

All experiments were performed on a SCIEX QTRAP 5500 mass spectrometer (SCIEX) coupled to an AP-MALDI (ng) UHR ion source (MassTech Inc., Columbia, MD USA) with a 355 nm Nd: YAG laser source. A representative figure of AP-MALDI hyphenated with SCIEX QTRAP 5500 is shown in **Figure 2**. For MRM analysis of AFM1, ion spray voltage was optimized to 4000 V, interface heater temperature was set at 220 °C and declustering potential (DP) was set to 91 V for best signals. The collision energy (CE) was optimized at 33 eV for 273.1 and 45 eV for 229.2 to achieve fragmentation. The laser energy of AP-MALDI ion source was optimized at 80% for the analysis.

Software and Data processing

AP-MALDI SCIEX QTRAP data was acquired in MRM mode using the Analyst 1.7.1. The qualitative data analysis was performed using Analyst software.

Results and Discussion

Optimization of AP-MALDI SCIEX QTRAP 5500

Parameters such as laser energy, declustering potential (DP) and collision energy (CE) were optimized for the analysis. The MRM transitions of AFM1 (Q1/Q3; 329.1/273.1) and (Q1/Q3; 329.1/229.2) were observed and optimized with reference standard solutions.

Table 1: MRM transitions of AFM1.

Compound	Precursor Ion	Product Ion (Quantifier)	Product Ion (Qualifier)	Collision Energy (CE)
AFM1	329.1	229.2	273.1	45 / 33

Detection of AFM1 from milk samples

AFM1 was detected using MRM mode. The MRM transitions of AFM1 (Q1/Q3; 329.1/273.1) and (Q1/Q3; 329.1/229.2) were observed from the spiked milk samples (Rodríguez-Carrasco, Y. *et al.* 2018). To determine the performance of the method, matrix-matched calibrants were analyzed across a concentration range of 0.01 µg/kg to

25 µg/kg. The solvent standard calibration and matrix-matched calibration curve of AFM1 was plotted between the absolute intensity of MRM and the concentrations of AFM1. The solvent standard **(Figure 4a-b)** and matrix-matched calibration curve **(Figure 4c-d)**, both shows good linearity across the concentration range of 0.02 to 50 µg/kg and 0.01 to 25 µg/kg. The regression coefficient (R^2) was observed to be 0.99, with low to acceptable RSD values. A limit of detection (LOD) of 0.01 µg/kg was achieved from matrix-matched calibrants with triplicates. The slope and intercept of the matrix-matched calibration model was used for the estimation of concentration of different spiking levels. Table 2 shows MRM transitions, RSD values, estimated concentration and % recovery. The study reveals that there is no interference observed by the food matrix. The recovery data indicates the reproducibility and accuracy of the method. Based on the recoveries, Q1/Q3 329.1/273.1 and Q1/Q3 329.1/229.2 was considered as qualifier and quantifier ions, respectively. These results clearly indicate the compatibility of AP-MALDI with SCIEX QTRAP 5500 for the detection of AFM1 in milk and milk-based products. The triple quadrupole method was validated with ion ratios from matrix-matched calibrants, spiked quality control samples and milk control were estimated with the following formula:

$$Ion\ ratio = Intensity\left(\frac{Qualifier\ Ion}{Quantifier\ Ion}\right) * 100$$

The results are summarized in Table 3. %RSD for all the samples varied between 1.1-7.6% (*n*=3) demonstrating excellent rigor of the method. The ion ratio from food matrices must be within 30% RSD and with AP-MALDI, ion ratio is within 1.1-7.6%, thereby validating the triple quadrupole analysis.

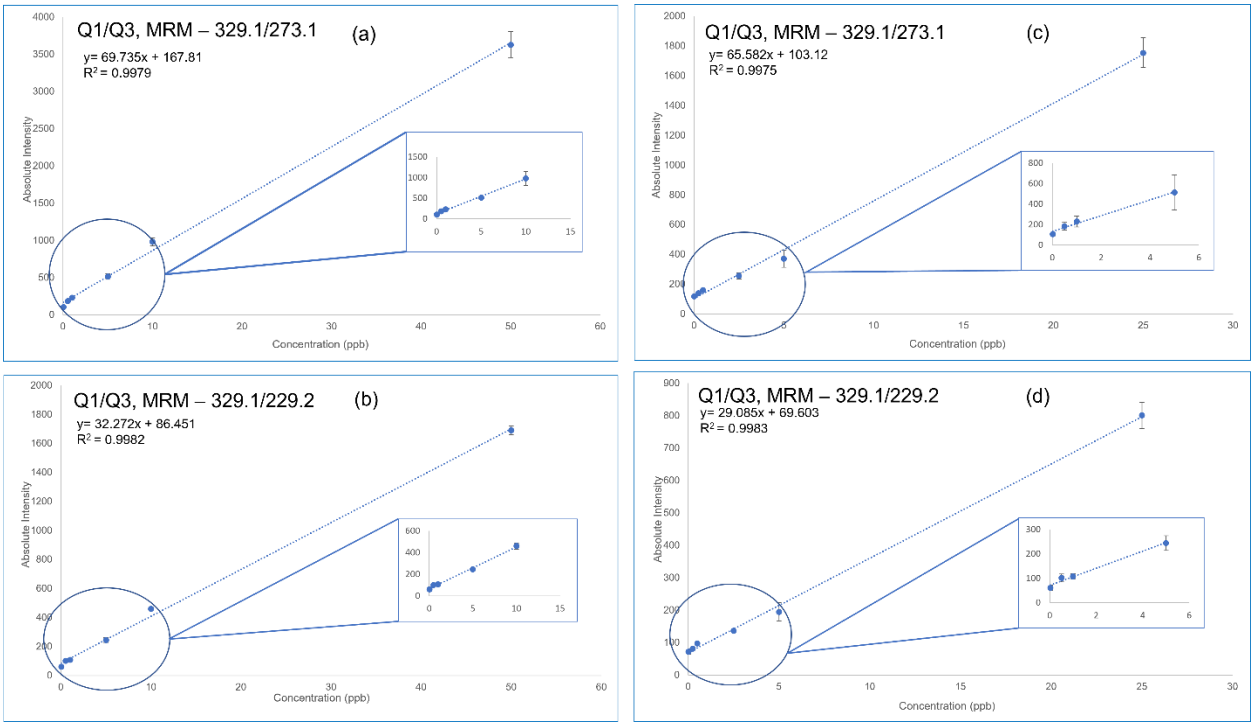

Figure 4. Solvent standard calibration model for aflatoxin M1 (a) Q1/Q3, MRM- 329.1/273.1 & (b) Q1/Q3, MRM- 329.1/229.2 across a concentration range of 0.02-50 ppb and their insets showing 0.02-10 ppb. Matrix-matched calibration model for aflatoxin M1 (c) Q1/Q3, MRM- 329.1/273.1 & (d) Q1/Q3, MRM- 329.1/229.2 across a concentration range of 0.01-25 ppb.

Table 2: Estimated concentration and % recovery of AFM1 from 2-fold diluted milk samples.

Conc. (ppb)	MRM (Q1/Q3)	RSD	% Recovery
Control	329.1/273.1	5.3	
	329.1/229.2	4.1	
0.25 ppb	329.1/273.1	25.4	170.5
	329.1/229.2	26.9	106.0
0.5 ppb	329.1/273.1	4.2	100.2
	329.1/229.2	1.9	97.4

Table 3: Ion ratios (IR) of qualifier (273.1u) to quantifier (229.2u) for AFM1 from matrix-matched calibrants, quality controls and milk control samples

Conc. (ppb)	Ion ratio (quantifier:qualifier) (*n*=3)			Avg.	SD	RSD (%)
25	221.2	219.7	216.4	219.1	2.4	1.1
5	188.8	189.0	192.4	190.1`	2.0	1.1
2.5	184.3	200.9	174.5	186.6	13.3	7.1
0.5	175.3	161.1	157.1	164.5	9.6	5.8
0.25	169.1	179.9	161.2	170.1	9.4	5.5
0.01	160.8	161.9	169.4	164.0	4.7	2.9
Two fold diluted QC samples and milk control						
Control	164.4	157.2	164.8	162.1	4.3	2.6
0.25*	157.5	171.0	183.3	170.6	12.9	7.6
0.5*	165.3	156.6	165.0	162.3	4.9	3.1

*Note: The amounts spiked in milk were 0.2 and 0.4 ppb. After diluting the milk matrix, concentrating during clean-up procedure and premixing with MALDI matrix, the amounts correlate to 0.25 and 0.5 ppb.

Summary

With the above results we demonstrated the compatibility of AP-MALDI with SCIEX QTRAP 5500 and establish MRM assay for the analysis of AFM1 in milk samples. The developed method provides confirmation of AFM1's presence in extracted milk samples. The AP-MALDI MRM analysis provides good sensitivity and accuracy for the analysis, with minimal sample preparation and no requirement of internal standard. Additionally, the method was validated with the qualifier to quantifier ion ratios from calibrants spiked in milk and control milk sample. The AP-MALDI QTRAP high throughput analysis allows analysis of AFM1 in a variety of milk samples in a short period of time.

References

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