Enhanced specificity for targeted analysis of aflatoxin G2 in plant-based meat using MRM^3

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This technical note demonstrates the application of MRM^3 to improve the specificity of detecting aflatoxin G2 (AFG2) in complex matrices such as plant-based meats. Using the QTRAP functionality on the SCIEX 7500 system, the MS/MS/MS (MS^3) fragmentation of an AFG2 precursor ion provided dual mass filtering through monitoring the transition comprised of first- and second-generation product ions. AFG2 was not detected during MRM monitoring of the commonly used *m/z* 331 > 313 transition due to coeluting interferences in the plant-based chicken extract. However, during MRM^3 acquisition, these interferences were removed, resulting in cleaner baselines and improved signal-to-noise (S/N) (**Figure 1**).

Key benefits of the MRM^3 workflow for complex food analysis

- **Reduced background interferences:** The dual fragmentation in an MS^3 scan provided a more compound-specific transition for monitoring, resulting in cleaner MRM^3 chromatograms without any co-eluting interferences.
- **Increased S/N from improved specificity.** S/N enhancements in MRM^3 enabled more reliable peak integration and potentially lower limits of quantitation (LOQs), especially for transitions prone to matrix interferences in MRM.
- **Software tools for easy MRM^3 optimization:** The SCIEX OS software provided automated and guided optimization for parameter tuning during MRM^3 method development.

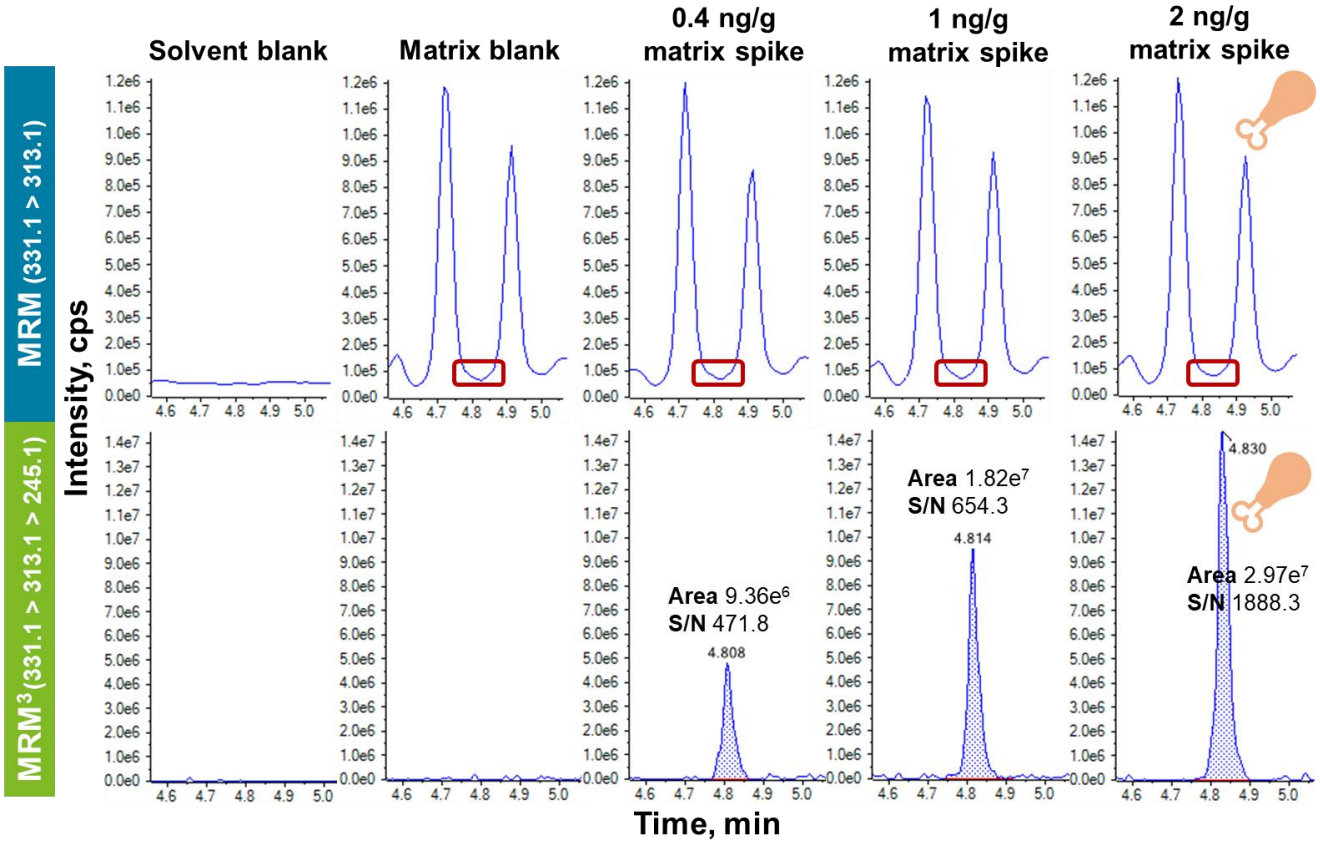

Figure 1. Comparison of the detection of AFG2 in plant-based chicken extracts between MRM and MRM^3 mode. The top panel shows the extracted ion chromatograms (XICs) of the quantifier transition (*m/z* 331.1 > 313.1) of AFG2 in the solvent blank, matrix blank and matrix extracts post-spiked at 0.4, 1 and 2 ng/g acquired in MRM, while the bottom panel shows the XICs for the MS^3 transition (*m/z* 331.1 > 313.1 > 245.1) acquired in MRM^3. MRM^3 significantly improved the specificity of AFG2 in the plant-based chicken extracts due to the removal of co-eluting interferences that were obscuring the detection of the analyte in standard MRM.

Introduction

Aflatoxins represent a family of mycotoxins (AFB1, AFB2, AFG1 and AFG2), primarily produced from molds in the *Aspergillus* species.[1] They are recognized as cytotoxic, genotoxic, hepatotoxic and immunosuppressive agents.[1] Although aflatoxins have been reported in animal- and plant-based foods, higher levels are often found in the latter due to direct exposure from fungal growth on agricultural commodities. This is worth noting, given the recent expansion of plant-based meat alternatives in response to a shift in dietary preferences based on health and sustainability reasons. While the European Commission has established maximum residue levels (MRLs) for mycotoxins in some raw ingredients of plant-based foods,[2] the final plant-based products are not yet regulated. As such, sensitive methods are needed to ensure the safety of plant-based meats.

MRM acquisition is commonly used for targeted quantitation due to the high sensitivity and selectivity from monitoring compound-specific precursor-product ion transitions. However, MRM quantitation can be challenging in complex food matrices like plant-based meat, which contain highly processed ingredients, such as plant protein, fats and polysaccharides, to mimic the organoleptic properties of meat.[3] These components can contribute to high background interferences, compromising LOQs. Sample clean-up and chromatographic optimization can help reduce these interferences but are time-, labour- and consumable-intensive. Here, MRM^3 offered a more selective approach to improving the detection of AFG2 in plant-based meats. **Figure 2** demonstrates how the second in-trap fragmentation produced unique and compound-specific MS^3 transitions comprised of 2 generations of product ions for increased specificity.[4]

Methods

Standard stock preparation: A neat standard of AFG2 was purchased from Evolution Life Sciences. A stock solution of 25 µg/mL was prepared in acetonitrile.

Sample preparation: After adding 10 mL of water to 5 g of homogenized plant-based meat, the sample was vortexed for 3 minutes. 10 mL of acetonitrile was added, vortexed for 10 min and the solution was transferred to a 50 mL tube containing 4 g of $MgSO_4$ and 1 g of NaCl, and vortexed for 10 min. The mixture was centrifuged at 4500 rpm for 10 min, and the supernatant was diluted 1:1 with 50:50 (v/v), acetonitrile/water, followed by spiking with AFG2 at 100 pg/mL, 250 pg/mL and 500 pg/mL for analysis.

Chromatography: A Shimadzu Nexera Prominence LC system was used with a Phenomenex Kinetex C18 column (100 x 2.1 mm, 2.6 µm, 100 Å, P/N: 00D-4462-AN). The gradient conditions used are shown in **Table 1.** The injection volume was 5 µL and the column oven temperature was 40°C.

Table 1: Chromatographic gradient for the analysis of AFG2 in plant-based meat

Time (min)	Flow rate (mL/min)	A (%)	B (%)
0.0	0.5	95	5
1.0	0.5	95	5
5.5	0.5	60	40
7.5	0.5	5	95
8.5	0.5	5	95
8.6	0.5	95	5
10	0.5	95	5

*Mobile phase A: 0.1% **(v/v)** formic acid in water*
Mobile phase B: Acetonitrile

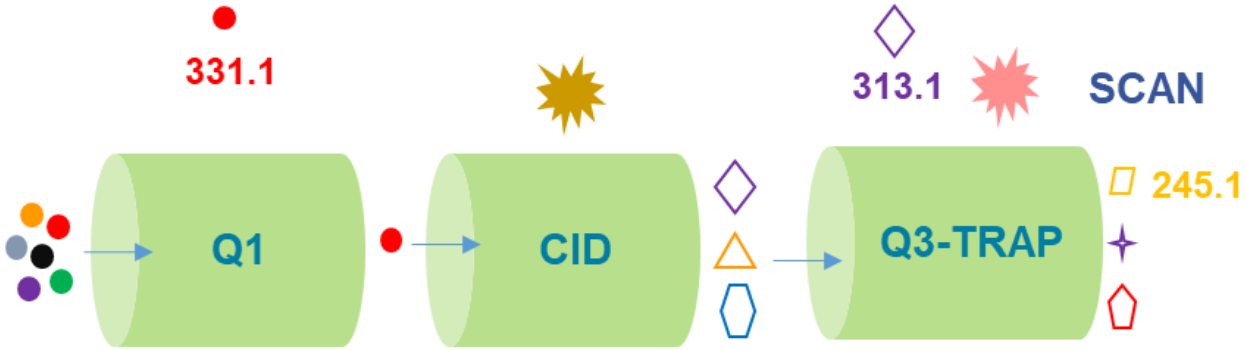

Figure 2. Schematic demonstrating the MRM^3 workflow based on the dual fragmentation using a MS^3 scan. In MS^3, the initial selection of the precursor ion (*m/z* 331.1) in the first quadrupole (Q1) and its subsequent fragmentation by collision-induced dissociation (CID) in the Q2 collision cell is identical to the MRM pathway. The difference occurs in the linear ion trap (LIT) where first-generation product ions are trapped before a single ion, such as *m/z* 313.1, is isolated for secondary fragmentation. The final second-generation product ions, such as *m/z* 245.1, are subsequently ejected to the detector.

Mass spectrometry: Analysis was performed in both MRM and MRM^3 mode with positive electrospray ionization on the SCIEX 7500 system. **Table 2** shows the source and gas parameters for both MRM and MRM^3 modes. **Table 3** shows the compound-dependent parameters for MRM acquisition. Optimization of MRM^3 parameters was performed using the infusion-based guided optimization feature in the SCIEX OS software. MRM^3 data were acquired using 2 looped MS^3 experiments using a scan speed of 10,000 Da/s, a fixed fill time of 40 ms and an excitation time of 25 ms with Q0 trapping enabled **(Table 4)**. For larger MRM panels, optimization for shorter fill times or dynamic fill time is recommended to maintain the cycle time for acquiring enough data points across each LC peak.

Table 2. Source and gas parameters.

Parameter	MRM	MRM^3
Polarity	Positive	Positive
Ion spray voltage	3500 V	3500 V
Curtain gas	45 psi	50 psi
CAD gas	9	12
Temperature	475°C	400°C
Gas 1	40 psi	35 psi
Gas 2	70 psi	80 psi

Table 3. MRM compound-dependent parameters for AFG2 analysis.

ID	*m/z*	DP (V)	CE (V)	CXP (V)	Q0D (V)	Dwell time (ms)
AFG2_01	331.1 > 313.1	10	15	10	10	100
AFG2_02	331.1 > 245.1	10	45	14	10	100

Table 4: MRM^3 compound-dependent parameters for AFG2 analysis.

m/z	Mass range for scanning 2nd fragment*	CE (V)	AF2 (V)
331.1 > 313.1 > 245.1	100 – 300	35	0.128
331.1 > 245.2 > 217.0	180 – 230	41	0.170

* Smaller fragment mass acquisition range can be optimized to further improve specificity.

Data processing: Data acquisition and processing were performed using the SCIEX OS software, version 3.3.1. **Figure 3** shows the processing method parameters for data acquired in MRM^3 mode. The '**Experiment Index**' column enables the user to extract XIC data from each experiment corresponding to each MS^3 transition. Upon selection, the precursor mass (Q1), the first-generation fragment mass (Q3) and the mass range specified for the second-generation fragment are automatically populated in the processing method. Additional narrowing of the start-stop mass range of the second-generation fragment can help refine the XIC to obtain more specificity and lower noise.

Automated and guided optimization of MS^3 parameters

The SCIEX OS software offers an infusion-based guided workflow for automated MS^3 method development through the MS Method Editor workspace (**Figure 4**). The workflow consists of the automated determination of the Q1 and product ions for the mass range specified. Compound-dependent parameters such as collision energy (CE) and auxiliary frequency 2 energy (AF2) are also automatically optimized for the first- and second-generation product ions. The final tuned parameters are summarized in a report presented at the end of the workflow for user review (**Figure 5**). The software also provides a direct link to the MS Method Editor workspace where an MS^3 experiment with the optimized parameters is automatically created (**Figure 5**). This software feature streamlines the method development process with minimal user intervention and produces a baseline MS^3 method that can be further optimized.

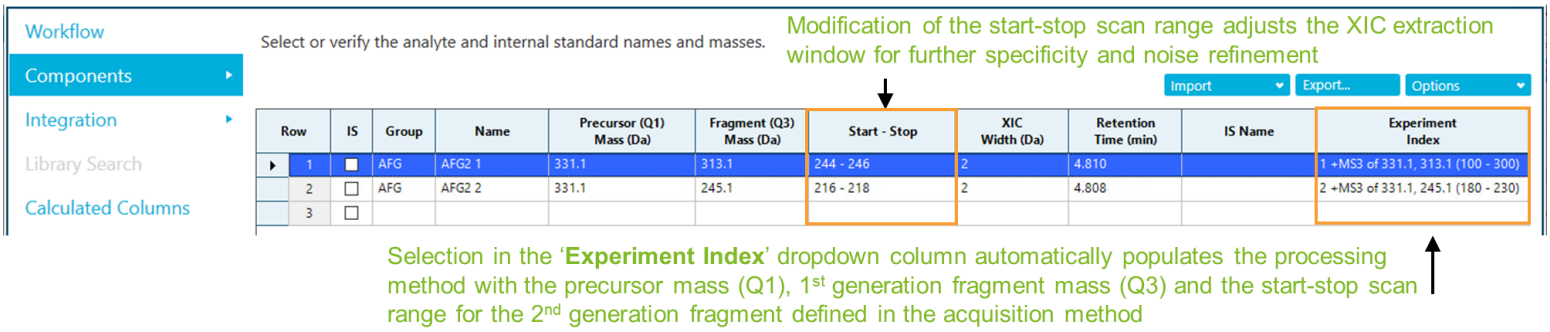

Figure 3. Processing method for MRM^3 data in SCIEX OS software. In the Analytics module of the SCIEX OS software, a processing method for MRM^3 data is easily created by selecting the corresponding experiment in the '**Experiment Index**' dropdown column. Each option in this column represents a MS^3 experiment with the corresponding precursor (Q1), first-generation fragment (Q3) and the start-stop scan range for the second-generation fragment displayed. Upon selection, this information is automatically populated in the components table.

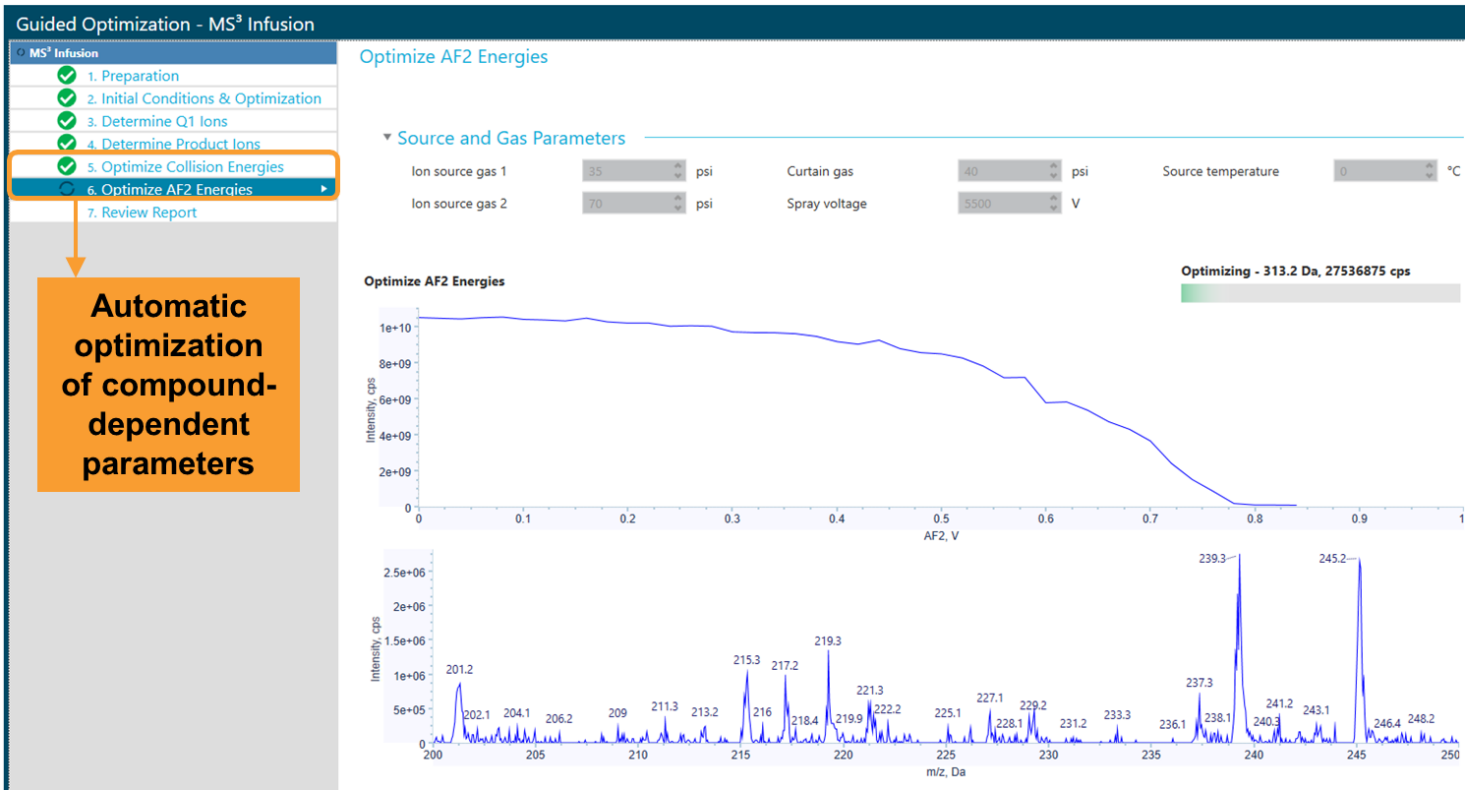

Figure 4. Guided optimization of MS³ parameters by infusion in SCIEX OS software. The SCIEX OS software provides a user-friendly and guided workflow for automated determination of Q1 and product ions and compound-dependent parameters such as CE and AF2 during MS³ method development.

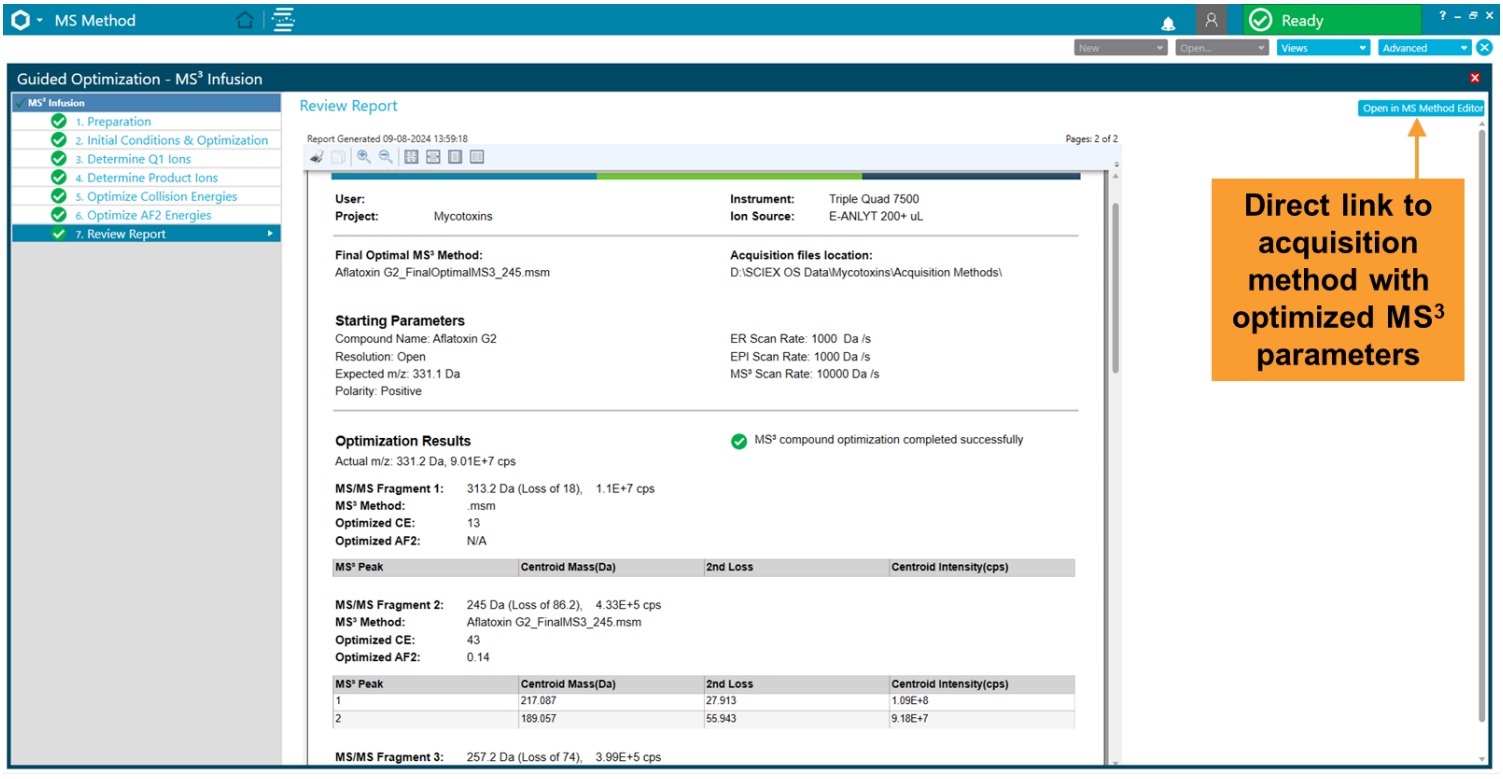

Figure 5. MS³ optimization report and easy creation of MS³ experiment in SCIEX OS software. Upon completing the guided optimization workflow, the software presents a report summarizing the optimized results for user review. The software also provides a direct link to the MS Method Editor workspace where an MS³ method is automatically created with the optimized parameters.

Enhanced specificity and sensitivity in MRM^3

Compared to the single-level fragmentation in MRM, the MRM^3 workflow comprises 2 steps, starting with the fragmentation of the precursor ion to an initial fragment, followed by further fragmentation into secondary fragments. In this work, the precursor ion of interest for AFG2 at *m/z* 331.1 was first isolated in Q1, followed by fragmentation in the Q2 collision cell to produce a range of product ions, including *m/z* 313.1 and *m/z* 245.2. These product ions (or second precursor ions) were then trapped in the Q3 linear ion trap (LIT) before undergoing secondary fragmentation by AF2 excitation (**Figure 2**). Using this QTRAP functionality on the SCIEX 7500 system, two MS^3 transitions, *m/z* 331.1 > 245.1 > 217.0 and *m/z* 331.1 > 313.1 > 245.1, were optimized and compared against the corresponding MRM transitions, *m/z* 331.1 > 245.1 and *m/z* 331.1 > 313.1, that are commonly monitored for the analysis of AFG2 in plant-based meat.

The *m/z* 331.1 > 245.1 MRM transition is often selected for AFG2 monitoring due to its high intensity and specificity, as it corresponds to the loss of a $C_3H_2O_3$ fragment from the coumarin lactone ring structure common to the aflatoxins.[5]

This MRM transition exhibited good S/N responses (S/N >10) at concentrations as low as 0.01 ng/mL in solvent (**Figure 6**), which demonstrates the capability of the SCIEX 7500 system to achieve sub-ppb instrumental LOQs for AFG2.[6] However, the increased specificity of MRM^3 resulted in improved S/N responses in both the solvent standards and matrix spikes (**Figure 6**). Higher S/N values typically result in increased reproducibility, which can greatly facilitate the experimental determination of method LOQs in low-level matrix spikes.

Due to its high abundance, the *m/z* 331.1 > 313.1 MRM transition has been extensively used as the quantifier transition for AFG2.[5,7] However, this M-18 fragment is formed from the non-specific loss of water, which is observed in many compounds, rendering it prone to interferences. Using the *m/z* 331 > 245 and *m/z* 331 > 189 transitions have been shown to reach LOQs as low as 25 ppt for AFG2 on the SCIEX 7500 system,[6] and are generally recommended when maximum sensitivity is required. However, multiple transitions may not always be available for some analytes and even then, ion ratios may still fail in the presence of challenging matrix interferences. Here, the nonspecific *m/z* 331.1 > 313.1 MRM transition was used as a proof-of-concept to showcase the power of MRM^3 to obtain more specific XIC traces for more confident identification.

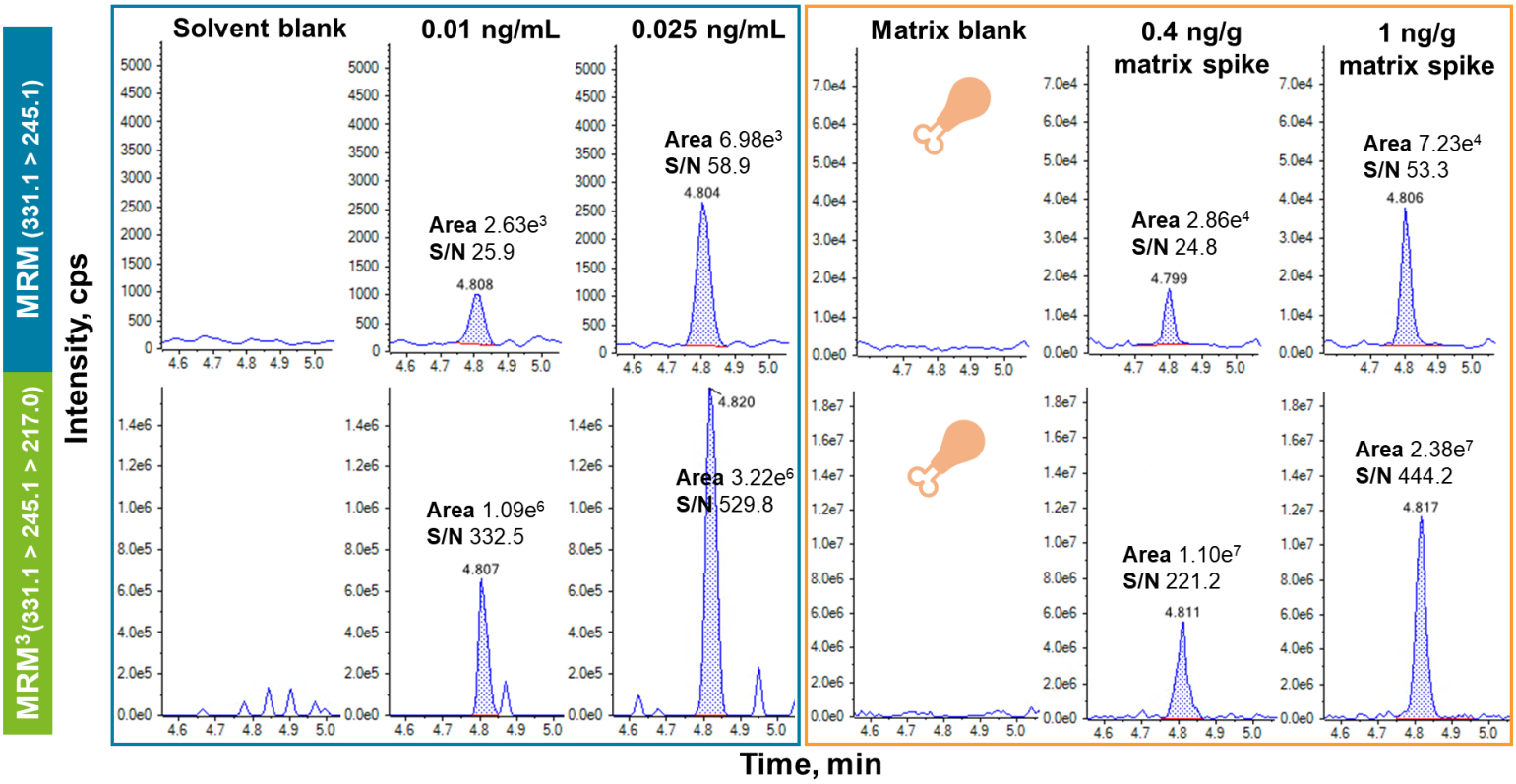

Figure 6. Comparison of the detection of AFG2 in solvent standards (blue) and plant-based chicken extracts (orange) between the *m/z* 331.1 > 245.1 transition in MRM and the *m/z* 331.1 > 245.1 > 217.0 transition in MRM^3 mode. The top panel shows the XICs of AFG2 in the solvent blank, solvent standards at 0.01 and 0.025 ng/mL, matrix blank and matrix extracts post-spiked at 0.4 and 1 ng/g acquired in MRM, while the bottom panel shows the XICs acquired in MRM^3. The increased specificity in MRM^3 improved the S/N response of AFG2 in both the solvent standards and the plant-based chicken extracts compared to standard MRM.

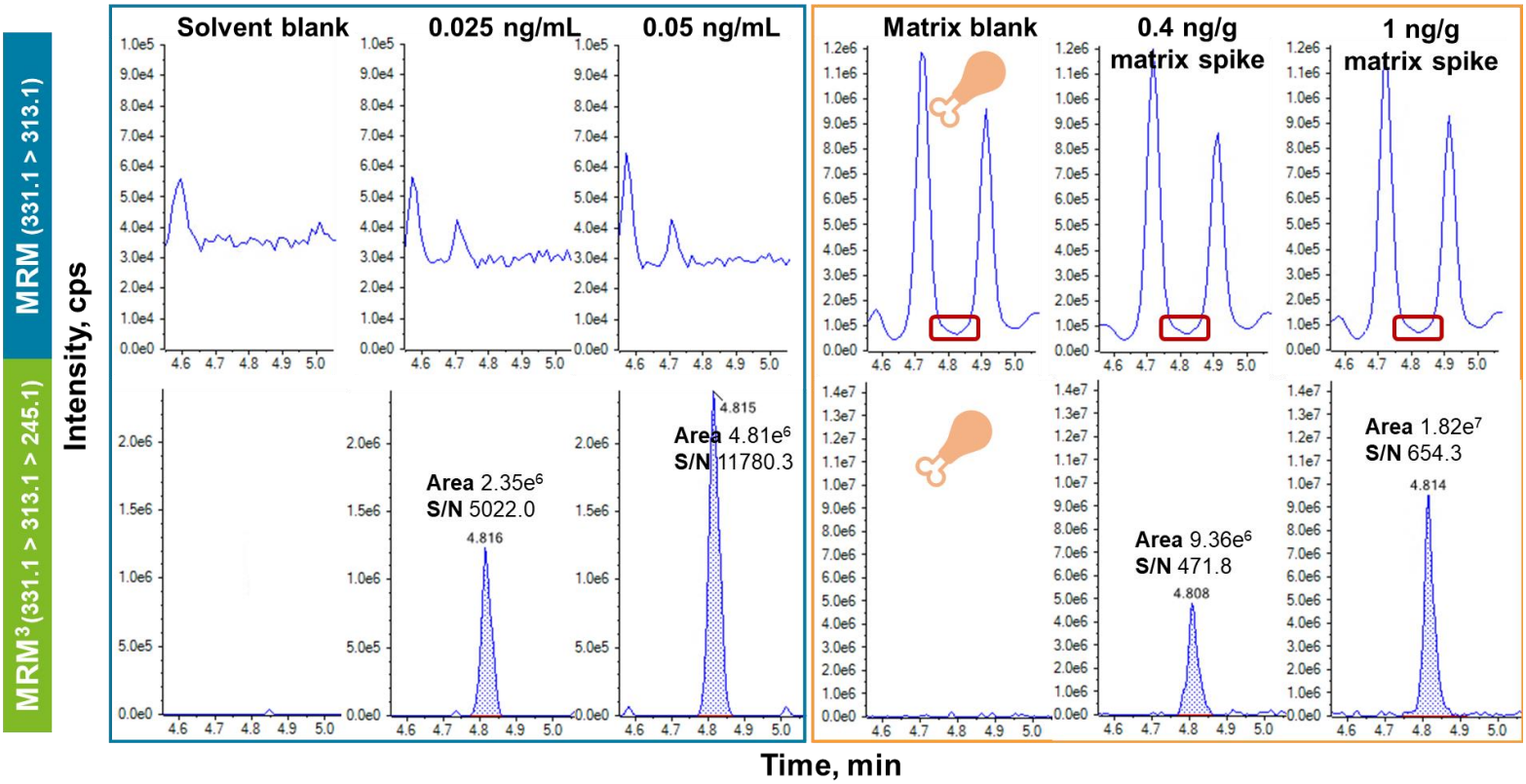

Figure 7. Comparison of the detection of AFG2 in solvent standards (blue) and plant-based chicken extracts (orange) between the *m/z* 331.1 > 313.1 transition in MRM and the *m/z* 331.1 > 313.1 > 245.1 transition in MRM³ mode. The top panel shows the XICs of AFG2 in the solvent blank, solvent standards at 0.025 and 0.05 ng/mL, matrix blank and matrix extracts post-spiked at 0.4 and 1 ng/g acquired in MRM, while the bottom panel shows the XICs acquired in MRM³. The S/N improvement from MRM³ specificity resulted in detection of AFG2 at sub-ppt levels in both the solvent standards and matrix spikes; while the analyte was obscured by high background and co-eluting interferences in standard MRM.

Figures 1 and **7** highlight significant interference of the *m/z* 331.1 > 313.1 MRM transition in the plant-based chicken matrix, obscuring the AFG2 detection, even at higher spiked levels. In contrast, MRM³ removed these interferences, which enabled the detection of AFG at concentrations as low as 0.4 ng/g against a significantly cleaner baseline in the matrix extracts.

Figure 7 also demonstrates the improved sensitivity in MRM³ for the *m/z* 331.1 > 313.1 > 245.1 transition in which reliable detection (S/N > 10) occurred in the 0.025 ng/mL solvent standard. While at the same concentration in solvent, the high MRM baseline for the *m/z* 331.1 > 313.1 transition suggests the non-specificity of this transition and the need to monitor for more compound-specific transitions. However, these data demonstrate that MRM³ may provide a viable alternative when MRM monitoring is challenged by the lack of stable and unique fragments, or complex matrices which show high background interferences.

Conclusions

- The increased specificity of the MRM³ workflow provided an alternative approach to MRM quantitation for analytes that suffer from high background or matrix interferences.
- S/N improvement of AFG2 in MRM³ enabled easier peak integration and potentially lower LOQs, especially for transitions encumbered by matrix interferences during conventional MRM acquisition.
- The guided optimization feature in SCIEX OS software streamlined the infusion-based tuning of MRM³ parameters and enabled the easy transfer of the optimized values to the final acquisition method.

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