Robust, high-throughput, fast polarity switching quantitation of 530 mycotoxins, masked mycotoxins and other metabolites

Using the SCIEX Triple Quad™ 5500+ LC-MS/MS System – QTRAP® Ready

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Mycotoxins are toxic fungal metabolites, which are derived from certain molds and fungi. The growth of mold can occur before crops are harvested or under inappropriate storage conditions such as warm and humid conditions. Consumption of food products containing mycotoxins can have serious health implications. According to the World Health Organization (WHO), the effects of some foodborne mycotoxins are acute, with symptoms of severe illness appearing quickly after consumption.[1] Others have been linked to long term human health effects, such as cancers or immune deficiency.

The most important classes of mycotoxins including the highly carcinogenic Aflatoxins (e.g. AFB1), trichothecenes (e.g. DON), Fumonisins (e.g.FB1), Ochratoxins (OTA) and Zearalenone (ZEN) and several others are regulated in many countries. In China, GB 2761 regulates mycotoxin limits in certain products; in the EU, mycotoxins in foodstuffs are regulated by the EC1881/2006.[2,3,4]

A living plant can change the chemical structure of toxins and produce so-called “masked mycotoxins”. The plant might modify the chemical structure of the toxin with a glucose or sulfate moeity, which reduces its toxicity to the plant. The plant itself may now contain only the conjugated form of the toxin, but the original mycotoxin may emerge during human or animal digestion if the conjugate functional groups are cleaved, thus exposing the consumer to the dangers of the toxin.[5] The term 'masked mycotoxins' was coined to refer to this group of conjugated or otherwise transformed mycotoxins which become undetectable by targeted methods for the original compounds. Current knowledge of these “emerging mycotoxins” (e.g: NX-Toxins), as well as masked or other modified forms of mycotoxins, is limited but the number of compounds that need to be analyzed is increasing rapidly, requiring more comprehensive analytical LC-MS methods.[6]

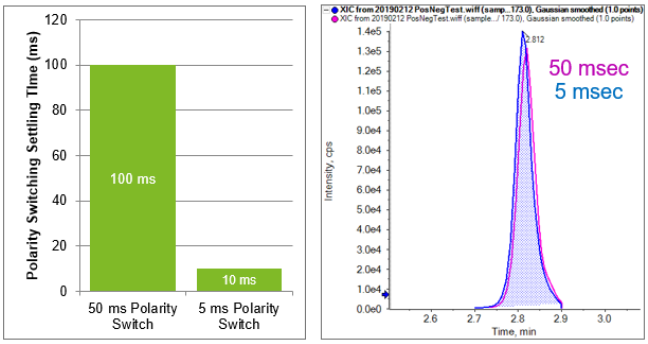

Figure 1. Advantages of fast polarity switching. Reducing the polarity switching time from 50 to 5 msec means a gain of 90 msec per cycle (Left). This time can be used to add more compounds to each scan, or dwell longer on existing MRMs to improve ion statistics. Similar peak areas are observed in the data collected for these mycotoxins between the 50 and 5 msec tests.

Mycotoxin analysis needs to be comprehensive and able to deliver accurate and consistent results across a wide range of matrices. This application note introduces an improved approach to testing Mycotoxins, their metabolites and emerging masked mycotoxin compounds using LC-MS/MS with fast polarity switching.

Key features of high-throughput mycotoxin analysis

- Workflow which incorporates 530 mycotoxins, their metabolites and emerging masked mycotoxins in one comprehensive method using Scheduled MRM™ Algorithm.
  - Demonstrated barley and corn extracts
- Utilizes the rapid 5 msec positive and negative polarity switching of the SCIEX Triple Quad 5500+ System to condense the standard two injection method into a single 22 min analysis.
- Reduced analysis time means results can be delivered quicker, and a cost reduction can be realized by the minimizing of consumables, such as mobile phase solvents, used in the analysis.

Methods

Sample preparation: Barley and corn samples were extracted with 4mL of Acetonitrile/Water/Acetic Acid at a ratio of 79/20/1 per gram of sample. Following the extraction, the samples are diluted to a 1:1 with Acetonitrile/Water/Acetic Acid at a ratio of 20/79/1.

Chromatography: Chromatographic separation was performed using the ExionLC™ AD System which was selected as it combines near zero carryover and full UHPLC capabilities to provide world-class performance under the most demanding analytical conditions. The column used was a Phenomenex Kinetex Polar C18 2.6µm, 100 x 2.1mm. Details are outlined in Table 1.

Table 1. Gradient profile and mobile phase composition.

Total Time (min)	Flow Rate (µL/min)	A%	B%
0.0	400	98	*2*
0.5	400	*98*	*2*
0.6	*400*	*80*	*20*
1.5	*400*	*80*	*20*
14	*400*	*20*	*80*
14.1	*400*	*2*	*98*
18	*400*	*2*	*98*
18.1	*400*	*98*	*2*
22	*400*	*98*	*2*

Mobile Phase A: H_2O +0.1% Acetic Acid + 5mM Ammonium Formate
Mobile Phase B: Acetonitrile +0.1% Acetic Acid + 5mM Ammonium Formate

Mass spectrometry: These experiments were performed using the SCIEX Triple Quad 5500+ LC-MS/MS System – QTRAP Ready. This highly sensitive instrument incorporates the Turbo V™ Ionization Source which has the power and efficiency to easily handle the dirty matrices associated with mycotoxin analysis. Data was acquired using Analyst® Software 1.7.1. Table 2 outlines the MS conditions used.

Data processing: Data was processed using SCIEX OS-Q Software.

Table 2. Source and key MS parameters.

Parameter	Positive mode	Negative mode
CAD	8	8
CUR	25 psi	*25 psi*
GS1	*60 psi*	*60 psi*
GS2	*70 psi*	*70 psi*
IS	*5500 V*	*-4500 V*
TEM	*400°C*	*400°C*
Pause Time	*3 msec*	
Target Scan Time	*0.5 sec*	
MRM detection window	*12 sec*	
Number of MRMs	*582*	*458*
Switching Time	*Tested 5, 10, 15, 50 msec (5msec used in final method)*	

Analytical targets

This study analyzed a large suite of mycotoxins, masked mycotoxins and their metabolites in corn and barley extracts. The test was to observe trace detection and quantitation and to evaluate how the SCIEX Triple Quad 5500+ System handles a sample batch representative of the high throughput demands of the food testing industry.

The crucial elements of high-throughput mycotoxin analysis to be assessed were (in no particular order) linearity, sensitivity, data points, reproducibility, carryover, and robustness.

An acquisition method of 1040 MRM transitions was created using the Scheduled MRM™ Algorithm in Analyst® Software 1.7 to ensure the best quality data was acquired. This acquisition method combined both positive and negative polarity experiments. Approximately 40% of the target analytes require negative ionization.

Transforming samples into results

To process such a large amount of data, SCIEX OS-Q Software was used. SCIEX OS-Q Software is an integrated platform which can process high-throughput screening of time scheduled quantitative MRM data. The software can be customized to relay the processed data to the operator in a format which is familiar and appropriate to all analytical requirements.

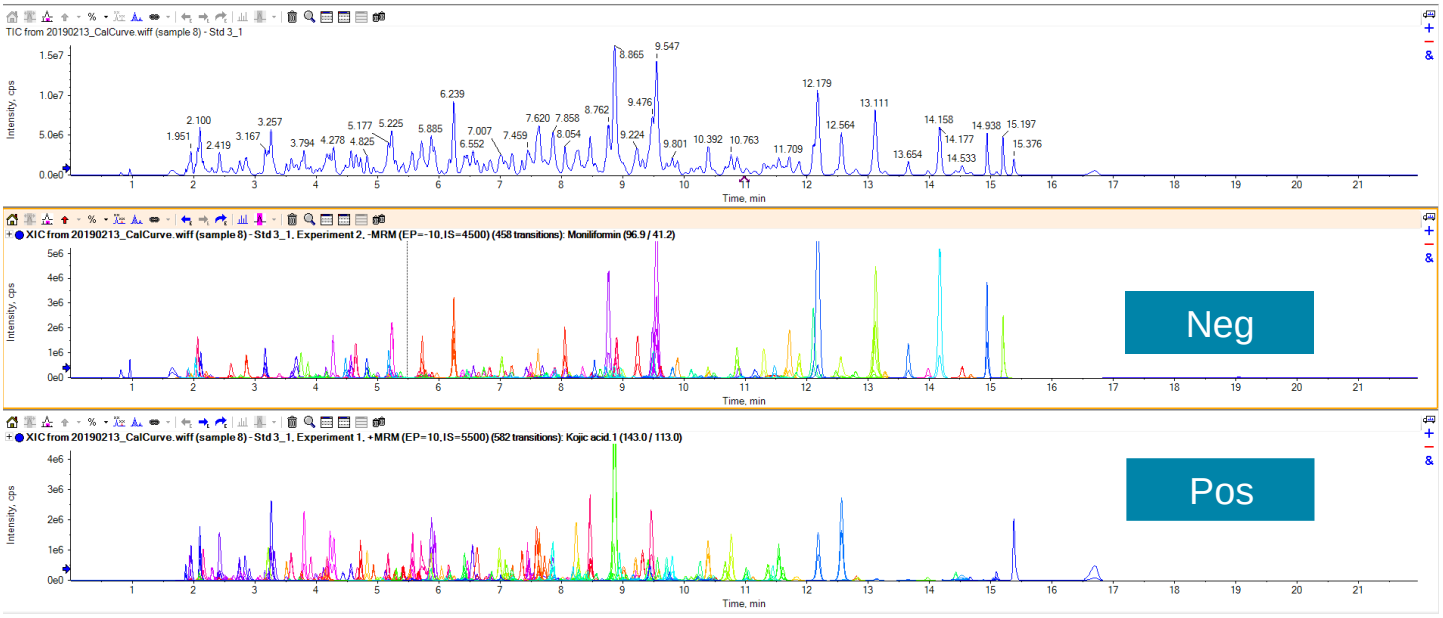

Figure 2. Chromatography for analyzing 530 mycotoxins. 1040 transitions for 530 mycotoxings were monitored using the Scheduled MRM Algorithm in Analyst Software 1.7. All analytes within the method are detected within a 16-minute window. The remaining time on the run is used to wash and equilibrate the system for the next injection.

Evaluation of switching time

Very good separation was achieved for the mycotoxins using the Phenomenex Kinetex Polar C18 2.6µm, 100 x 2.1mm (Figure 2). All analytes eluted within a 16 min window and the analytes were well spread out across the time window. There were 458 analytes run in negative ionization mode and 582 analytes requiring positive ionization mode.

In Figure 3, an example of different switching times for some Toxins on the SCIEX Triple Quad 5500+ System is shown. This extracted ion chromatogram (XIC) illustrates the compounds which use 50, 15, 10 and 5 msec polarity switching times. Here, extracted responses for Aflatoxin M1, Aflatoxin G1, Ochratoxin B, Aflatoxin B1, Ochratoxin A, and T-2 Toxin are shown. No significant differences either on signal intensity or signal/noise are observed. So, the method is using 5 msec switching time was selected for the final method.

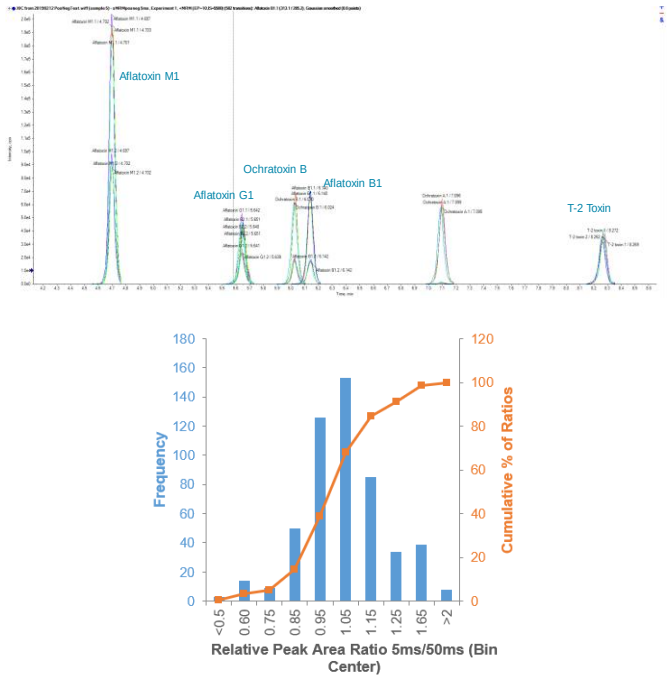

Figure 3. Minimal impact on peak area with fast polarity switching. (Top) XICs of select mycotoxins showing the peak areas at the various polarity switching times. (Bottom) Majority of peak area ratios between 5 and 50 msec showed equivalent or better data with 5 msec switching, with 85% of the analytes having 90% or better peak areas of that observed for 50 msec data.

Quantitative accuracy

Acceptance criteria will stipulate the minimum number of data points required for acquired results to be confirmed. Figure 4 illustrates the number of data points and Retention Time (RT) acquired across key positive compounds.

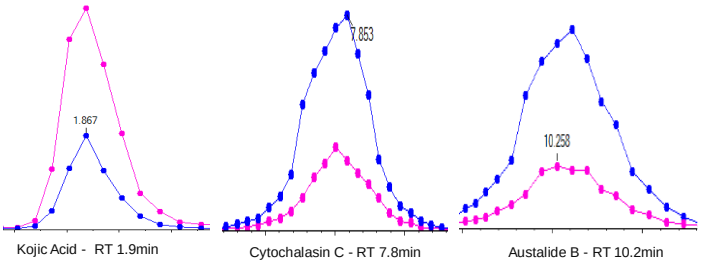

Figure 4. Good peak sampling for positive mode compounds. Kojic Acid, Cytochalasin C and Austalide B show sufficient data point coverage for confirmation reporting of results.

As detailed earlier in this application note, approximately 40% of the compounds in this acquisition list are ionized in negative mode. This method has 1040 sMRM transitions, so the 5 msec polarity switching time is critical to attaining comprehensive coverage and data quality in both positive and negative modes. The figure below (Figure 5) details the number of data points, and RT acquired for a selection of negative mode amenable mycotoxins.

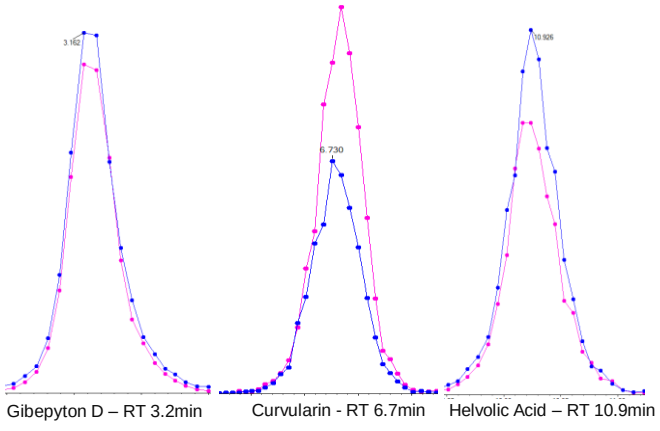

Figure 5. Good peak sampling for negative mode compounds. Gibepyton, Curvularin, and Helvolic Acid show sufficient data point coverage for confident result submission.

The SCIEX Triple Quad 5500+ ─ QTRAP Ready LC-MS/MS System has demonstrated that even with a very large number of target compounds, the accuracy and number of data points acquired is sufficient to enable confident and accurate quantitation of mycotoxins, masked mycotoxins, and their metabolites.

All other compounds across the batch also showed sufficient data points to ensure accurate quantitation of mycotoxins in complex matrices such as the corn and barley that have been tested in this study.

Sensitivity, linearity and ion ratios

The sensitivity and linearity of calibration standards is essential to any analysis. Using the AutoPeak algorithm delivers consistent integrations that reduce the need for manual intervention. The Automatic Outlier Removal Algorithm calculates linear regression and highlights any point that fails to meet user-specified rules. This reduces the need for hands-on analysis and minimizes the time taken to create calibration curves. These features are unique to the SCIEX OS Software platform.

The following are examples of calibration curves (Figure 6 and 7) which detail the range and various coefficients. Also in the report are the ion ratio qualification guides which are essential to delivering confirmed and approved positive results.

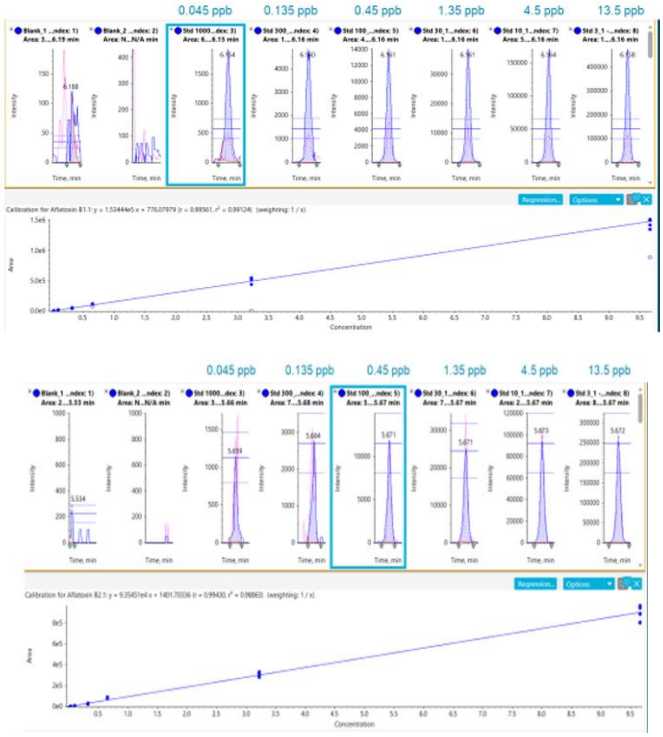

Figure 6. Standard concentration curves. The sensitivity and linearity achieved for Aflatoxin B1 (top) and Aflatoxin B2 (bottom) Aflatoxin B2 is shown.

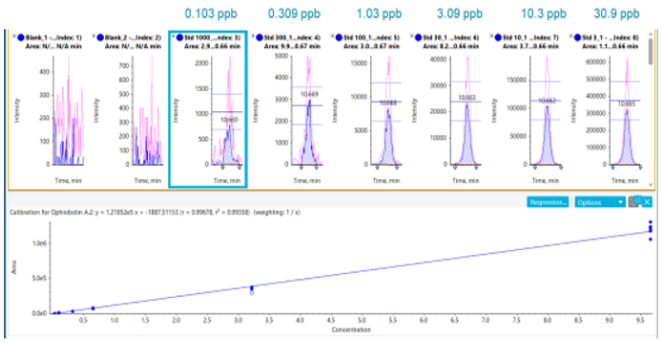

Figure 7. Standard concentration curve. The sensitivity and linearity achieved for Ophiobolin A is shown,

Repeatability

Having a method which consistently delivers the same quality and accuracy for every injection of every batch is imperative to delivering reliable analytical results. During this experiment, a series of replicate injections were run in order to evaluate the reproducibility of the method and SCIEX Triple Quad 5500+ System.

5 injections were made per standard and the Percent Coefficient of Variance (%CV) and the Accuracy for each concentration were calculated. Figure 8 highlights the results for two representative analytes.

		Mean	Standard Deviation	Percent CV	Accuracy
Aflatoxin B1.1	Std 1000_1	3.077e-2	3.281e-3	10.66	102.58
Aflatoxin B1.1	Std 300_1	1.058e-1	9.051e-3	8.55	105.80
Aflatoxin B1.1	Std 100_1	3.254e-1	2.886e-2	8.87	101.68
Aflatoxin B1.1	Std 30_1	7.472e-1	4.892e-2	6.55	114.95
Aflatoxin B1.1	Std 10_1	3.341e0	3.159e-1	9.46	103.74
Aflatoxin B1.1	Std 3_1	9.436e0	4.881e-1	5.17	97.68

		Mean	Standard Deviation	Percent CV	Accuracy
Pentoxyfylline.1	Std 1000_4	2.957e-2	2.531e-3	8.56	98.56
Pentoxyfylline.1	Std 300_4	9.488e-2	5.866e-3	6.18	94.88
Pentoxyfylline.1	Std 100_4	3.027e-1	3.005e-2	9.93	94.60
Pentoxyfylline.1	Std 30_4	7.341e-1	3.877e-2	5.28	112.94
Pentoxyfylline.1	Std 10_4	3.345e0	1.152e-1	3.44	103.88
Pentoxyfylline.1	Std 3_4	9.479e0	2.717e-1	2.87	98.13

Figure 8. Mycotoxin repeatability. Using SCIEX OS-Q Software, all necessary calculations are performed automatically. Here the %CV and accuracy across 5 replicates are computed for the standards 3x to 1000x. (Top) For Aflatoxin B1, all dilutions had %CV and accuracy well within the required limits. (Bottom) Pentoxifylline, a compound used in treating mycotoxin poisoning shows excellent %CV in this large multi- analyte method.

Testing the unknown

As part of this experiment, a series of extracts were performed on regular corn and barley extracts, with no known mycotoxins present in the samples. The same extraction process detailed earlier in this application note was used, and standards were then spiked into the matrix extracts at a low concentration around the regulatory limits. The extracts were run in this batch and processed in SCIEX OS-Q Software against the standard calibration curves. Shown in Figure 9, as examples, are the results for penecillic acid and xanthotoxin, detected at the regulated concentration level in both the corn and barley matrices. All positive results were confirmed in SCIEX OS-Q Software with the ion ratio along with ion ratio confidence tool which allows a rapid review of results to confirm positive identifications.

The MRM chromatograms for aspinonene clearly showing both the quantitative and confirmatory transitions, and the blank response (Figure 10). This increases user confidence in results by providing a fast, visual cross check of both positive and negative results as well as a good indication of analytical performance since there is no observed carry-over.

Sample Name	Component Group Na...	Accur...	Ion R...	Form...	Precu... Mass	Ion Ratio Confide...
Blank_3	Penicillic acid	N/A	N/A		171.200	■
Matrix Barley 1:10 300	Penicillic acid	N/A	0.3152		171.200	✓
Matrix Corn 1:10 300	Penicillic acid	N/A	0.3241		171.200	✓
Blank_3	Penicillic acid	N/A	N/A		171.200	■
Matrix Barley 1:10 300	Penicillic acid	N/A	0.3551		171.200	✓
Matrix Corn 1:10 300	Penicillic acid	N/A	0.3048		171.200	✓
Blank_3	Penicillic acid	N/A	N/A		171.200	■
Matrix Barley 1:10 300	Penicillic acid	N/A	0.3069		171.200	✓
Matrix Corn 1:10 300	Penicillic acid	N/A	0.3202		171.200	✓
Blank_3	Penicillic acid	N/A	N/A		171.200	■
Matrix Barley 1:10 300	Penicillic acid	N/A	0.2820		171.200	✓
Matrix Corn 1:10 300	Penicillic acid	N/A	0.3494		171.200	✓

Sample Name	Component Group Na...	Accur...	Ion R...	Form...	Precu... Mass	Ion Ratio Confide...
Blank_3	Xanthotoxin	N/A	N/A		217.00	■
Matrix Barley 1:10 300	Xanthotoxin	N/A	1.0361		217.00	✓
Matrix Corn 1:10 300	Xanthotoxin	N/A	0.9838		217.00	✓
Blank_3	Xanthotoxin	N/A	N/A		217.00	■
Matrix Barley 1:10 300	Xanthotoxin	N/A	0.7877		217.00	✓
Matrix Corn 1:10 300	Xanthotoxin	N/A	0.8568		217.00	✓
Blank_3	Xanthotoxin	N/A	N/A		217.00	■
Matrix Barley 1:10 300	Xanthotoxin	N/A	1.0623		217.00	✓
Matrix Corn 1:10 300	Xanthotoxin	N/A	0.9519		217.00	✓
Blank_3	Xanthotoxin	N/A	N/A		217.00	■
Matrix Barley 1:10 300	Xanthotoxin	N/A	1.2499		217.00	✓
Matrix Corn 1:10 300	Xanthotoxin	N/A	1.0897		217.00	✓

Figure 9. Ion ratios for confirmation of detection. (Top) Penicillic acid was positively confirmed using ion ratios at all of the concentrations in matrix tested. (Bottom) Similarly, the ion ratio confirms Xanthotoxin detection in the matrix.

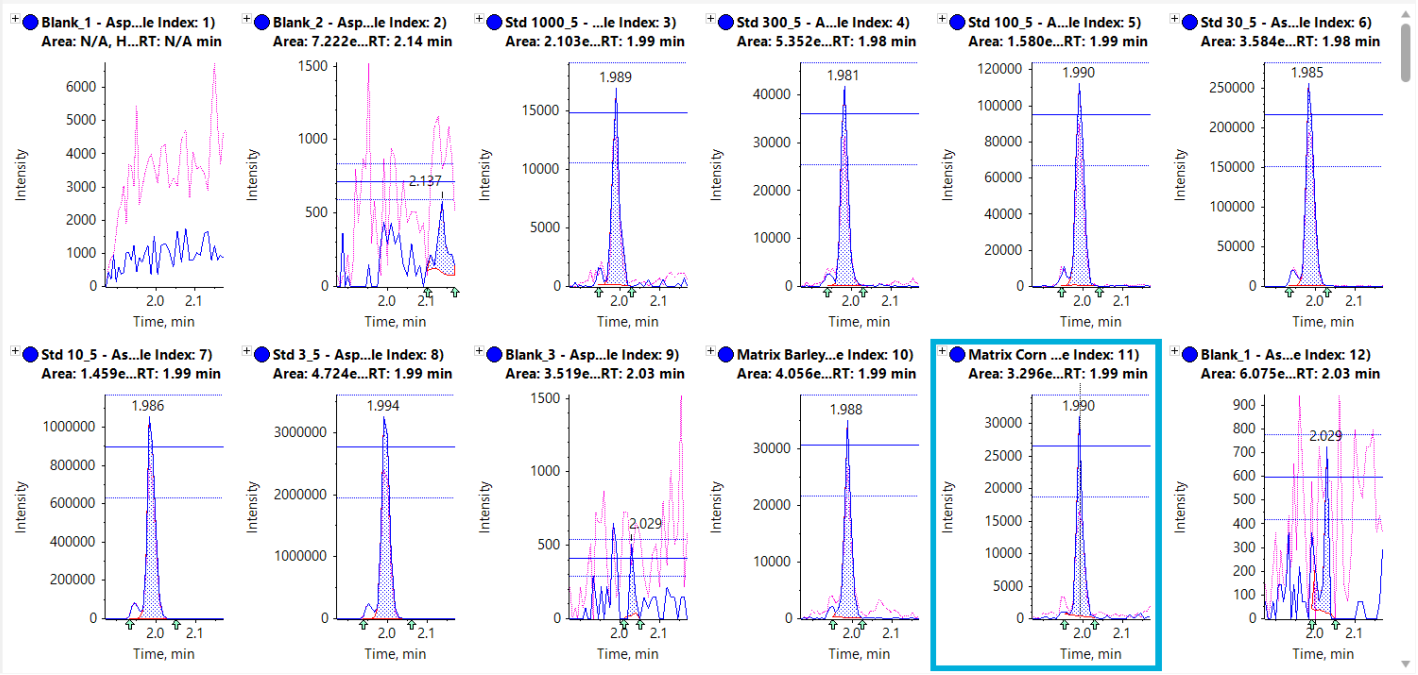

Figure 10. Visualization of results in SCIEX OS-Q Software. The AutoPeak algorithm has integrated the positively confirmed peaks for Aspinonene. Within SCIEX OS-Q Software, both transitions are overlaid along with the confirmatory ion ratio lines for quick review of data.

Injection order was: blank, blank, standards with 6 different concentrations, blank, barley as a matrix, corn as a matrix, blank.

This workflow demonstrates the key robustness and detection levels required for the screening of corn and barley for mycotoxins, masked mycotoxins, and their metabolites. With very little adjustment, this method can be adapted to the analysis of other matrices of interest such as milk, other grains, and ingredients.

Conclusions

This study has shown that the SCIEX Triple Quad 5500+ LC-MS/MS System ─ QTRAP Ready delivers high-quality quantitation of mycotoxins, masked mycotoxins and their metabolites. The method combines both positive and negative polarity electrospray ionization modes into one comprehensive quantitation/screening method which covers an unmatched range of analytes without any sacrifice or compromise to data quality and integrity.

Data processing has become more efficient with the adoption of SCIEX OS-Q Software. Using key automated features like AutoPeak and the Automatic Outlier Removal algorithms, which are clearly displayed in Figures 8 and 9, data review becomes a less manual task and more consistent peak integration is achieved.

Data quality is paramount to delivering reliable results. Assurances of data quality are found in the confirmatory ion ratio scoring and confidence reports. These reduce the risk of reporting false positive results which could have impact outside of the laboratory.

The scope for further enhancing data quality for this and many other methods on this instrument can be achieved with a simple license activation of the QTRAP System technology. By unlocking the power of the QTRAP System functionality, laboratories can incorporate additional scans such Enhanced Product Ion for spectral confirmation and MRM^3 which enables MS/MS/MS analysis to effectively negate the problems often associated with matrix suppression.

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