Two approaches for MRM^3 data acquisition using the SCIEX 7500 system

Comparing the Q0 dissociation-enhanced method and QTRAP system-based MRM^3 method

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Analysis of small molecules in complex samples using mass spectrometry can often lead to quantitative and qualitative analytical challenges due to low analyte concentrations, presence of high background and interfering components of similar structure and mass. Generating a second-generation product ion for a target analyte reduces the potential for isobaric interference and elevated baseline signal by adding another layer of specificity to the assay for the target species. SCIEX QTRAP systems have long featured the ability to “trap” product ions in the linear ion trap, induce secondary fragmentation with excitation in the trap, then scan out second-generation product ions for quantification in an MRM^3 workflow. The SCIEX 7500 system[1] features updated front-end technology and software control relative to older models that allow for the generation of second-generation product ions in a different way, using the Q0 dissociation feature (Figure 1). This provides an effective alternative workflow to the trap-based MRM^3 workflow to maximize the quantitative selectivity of an assay.

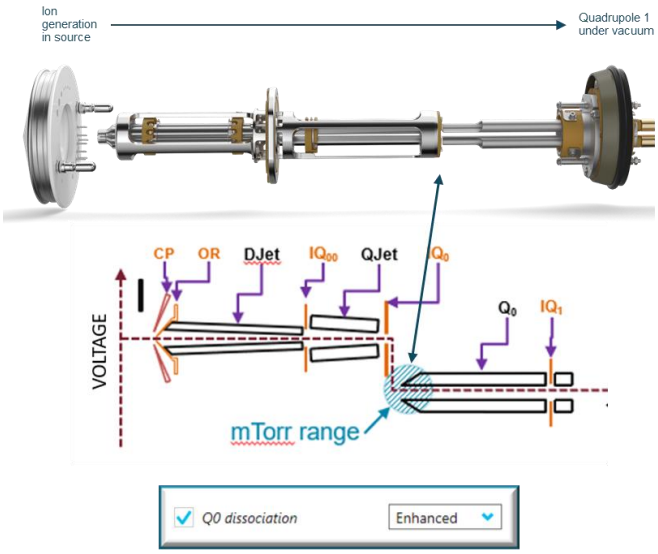

Figure 1. Q0 dissociation (Q0D) on the SCIEX 7500 system. Q0D is a single parameter in the SCIEX OS software that acts on all ions entering the vacuum region. In “Enhanced” mode (Q0DE), this parameter represents a voltage differential between the IQ0 and the Q0 lenses. It is a rampable or optimizable setting that can be tuned for a particular method or analyte.

This study aimed to compare 2 strategies for producing higher specificity MRM^3 assays using an example pharmaceutical compound, clenbuterol. Data were either generated using this alternative workflow, in which fragmentation was produced first before Q0 using the Q0 dissociation-enhanced feature and later in the collision cell, or using a conventional MRM^3 workflow, in which fragmentation was produced first in the collision cell and later in the linear ion trap. The data generated using these 2 workflows were compared based on quantitative analysis parameters including signal, sensitivity and linear response in a calibration curve.

Key features of the SCIEX 7500 system and the Q0 dissociation-enhanced method for targeted MRM^3 assays

- The Q0 dissociation-enhanced method for targeted MRM^3 analysis is easy to set up in SCIEX OS software, with the addition of the Q0 dissociation parameter to standard MRM analysis
- The Q0 dissociation-enhanced method for MRM^3 provides an additional tool to further improve method selectivity, especially when there are interferences in MRM transitions
- Data quality is comparable between the Q0 dissociation-enhanced workflow and a typical MRM workflow in terms of LLOQ, ULQ and %CV, demonstrating that it is a viable quantitative workflow
- Effectiveness depends on both analyte and interference properties and should be assessed case-by-case

Technology

MS/MS/MS methods use 2 sequential fragmentation steps to produce first- and second-generation product ions from a single precursor. One application of assessing subsequent fragment masses might be in structural elucidation, in which the structure of a compound can be determined by breaking it into parts that can be assigned structures based on fragment masses. More recently, the MS/MS/MS approach has been used in MRM^3 workflows for quantification to help distinguish analyte signal from interfering peaks from matrix, which confound peak integration at the lower end of the quantification range.[1]

SCIEX QTRAP systems have been used for this MRM^3 workflow for quantitative analysis in a variety of applications and laboratory types,[1] however, the method can be difficult to establish for non-expert users and requires in-depth knowledge of method development and optimization. For example, in this linear ion trap-based MRM^3 method, the compound-specific excitation parameter (AF2) to fragment the first-generation product ion must be tuned, which adds a step or series of steps to the method development and optimization. Other parameters that should be considered for optimal method design include:

- Linear ion trap fill time, which can be assigned to Dynamic Fill Time or Fixed Fill Time
- Q0 trapping, which can be toggled on or off
- Mass range window width assigned for MS^3 ions
- Which product ions and second-generation product ions to use for analysis

All these methodological parameters allow the user to make highly optimized and customized MRM^3 methods to maximize sensitivity and selectivity and are available on all QTRAP systems.

The SCIEX 7500 system, operating as a QTRAP system, has a new feature that utilizes Q0 dissociation settings to provide an alternative way to remove interference or increase the signal-to-noise ratio. Within the Q0 dissociation setting, either “Simple” or “Enhanced” can be selected. Q0 dissociation-simple has very strong de-clustering potential, which can help break up clusters. The Q0-enhanced option produces conditions that are similar to the use of collision energy in the Q2 collision cell and is intended for the collision-induced dissociation (CID) of precursor ions upstream before Q0, instead of Q2, as shown in Figure 1.

Using the Q0 dissociation-enhanced feature, a voltage differential is created on the Q0 side of the IQ0 lens, which accelerates ions from the IQ0 lens to Q0 in a much lower pressure region, allowing the fragmentation of precursor ions in Q0 (Figure 1). A fragment ion that is specific to the precursor ion

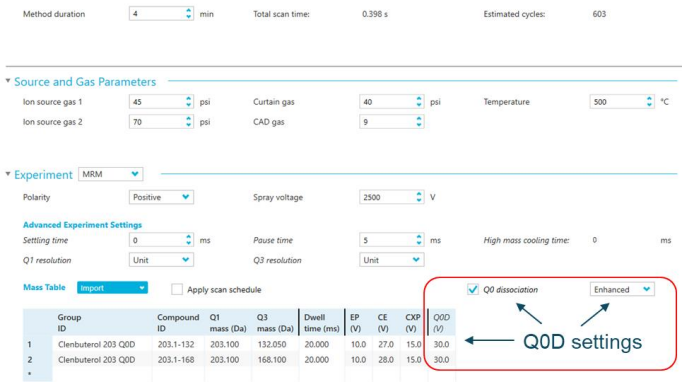

Figure 2. SCIEX 7500 system acquisition method for the Q0DE-MRM^3 workflow.

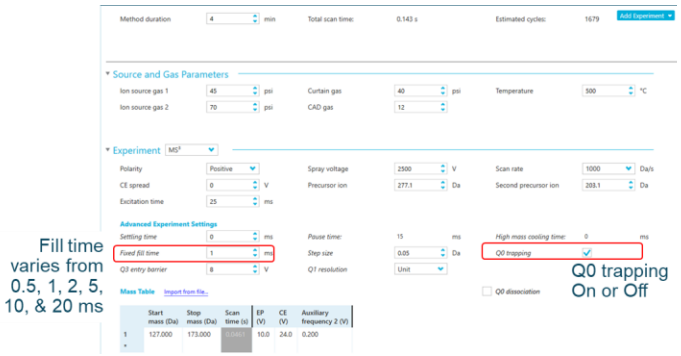

Figure 3. SCIEX 7500 system acquisition method for the LIT-MRM^3 workflow.

of interest can then be selected in Q1 and this fragment ion can undergo further fragmentation in Q2 where second-generation fragment ions are created by CID. One of these fragment ions can then be selected in Q3 and be detected. This results in 2 sequential fragmentation steps and gives the user another way to achieve MRM^3 data without using the linear ion trap.

Methods

Sample preparation: Clenbuterol was spiked in artificial urine matrix to prepare a standard calibration curve.

Chromatography: An ExionLC AD system was used with the analytical Kinetex C18 (50 x 2.1 mm, 2.6 mm) column. The injection volume used for all experiments was 2 µL and the column temperature was held at 30°C. The mobile phase A for LC separation was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile.

Mass spectrometry: Using the SCIEX 7500 system upgraded to a QTRAP system, both Q0 dissociation-enhanced (Q0DE-MRM^3) and linear ion trap-based MRM^3 (LIT-MRM^3) experiments were conducted and the quantitative performance metrics of the

2 methods were compared. A typical MRM workflow was also assessed as a baseline of comparison by which to assess the MRM^3 data. The acquisition methods used for the Q0DE-MRM^3 and LIT-MRM^3 workflows are shown in Figures 2 and 3, respectively.

The effect of Q0 trapping on method performance was tested. The LIT-MRM^3 method was run with Q0 toggled on and off for this comparison.

Data processing: All standard curve data were processed using the Analytics module of SCIEX OS software and the AutoPeak algorithm. The saturation correction was set at 8e7 and calibration curve regressions were calculated to fit a quadratic model with 1/x^2 weighting. All concentration units reported within this dataset are pg/mL. For data processing, 2 different second-generation product ions were monitored and for the assessment of the method performance, the data traces of the individual ions and the sum of the 2 ions were used.

MRM vs. Q0 dissociation-enhanced MRM^3 vs. linear ion trap-based MRM^3

Quantification performance metrics compared between the data acquisition types included lower limit of quantification (LLOQ), linear dynamic range (LDR) of the calibration curves and the %CV reproducibility of the calculated concentrations. These method performance indicators are typical metrics for any laboratory developing or validating a sensitive and robust analytical protocol. Table 1 shows a comparison of these metrics for each of the experiment types performed.

The data generated by the novel Q0DE-MRM^3 method was first compared to the data generated by a typical and widely accepted MRM method. For this analysis, 2 MRM transitions and 2 MRM^3 transitions were monitored. The LLOQ, LDR and %CV of the Q0DE-MRM^3 and MRM data were comparable (Table 1) in the absence of isobaric interference and elevated baseline complexity, demonstrating that there is minimal negative impact of this workflow.

Next, the data generated by the Q0DE-MRM^3 and LIT-MRM^3 approaches were compared. This comparison required additional experiments to consider the impact of Q0 trapping. As seen in the metrics summarized in Table 1, the LIT-MRM^3 results vary in LLOQ and LDR and are of lower quality compared to the MRM and Q0DE-MRM^3 data quality.

The results from the LIT-MRM^3 workflow indicate:

- Q0 trapping allows the maintenance of the same low-level LLOQ as the MRM method, but limits the LDR
- Without Q0 trapping, both LLOQ and LDR suffer compared to MRM and Q0DE-MRM^3 workflows

Table 1. Comparison of MRM, Q0DE-MRM^3 and LIT-MRM^3 methods on the SCIEX 7500 system.

Experiment Type	Transition	LLOQ (pg/mL)	ULOQ (pg/mL)	%CV
MRM	277-203	3	*100,000*	*0.52-2.76*
	277-168	*3*	*100,000*	*0.59-11.29*
	Sum	*3*	*20,000*	*0.57-6.32*
Q0DE-MRM^3	*277-203-168*	*3*	*100,000*	*0.20-15.53*
	277-203-132	*3*	*100,000*	*0.07-20.67*
	sum	*3*	*20,000*	*0.39-9.61*
LIT-MRM^3, Q0 trapping off	*277-203-168*	*20*	*10,000*	*3.29-21.02*
	277-203-132	*20*	*3,000*	*2.14-9.05*
	sum	*3*	*3,000*	*2.83-10.66*
LIT- MRM^3, Q0 trapping on	*277-203-168*	*3*	*3,000*	*1.49-25.29*
	277-203-132	*3*	*1,000*	*1.62-12.47*
	sum	*3*	*3,000*	*1.68-19.54*

Table 2. Effects of fixed fill time (FFT) and Q0 trapping on quantification performance for the LIT-MRM³ workflow.

FFT (ms)	Without Q0 trapping								
	277-203-132			*277-203-168*			Sum		
	LLOQ (pg/mL)	ULOQ (pg/mL)	%CV	LLOQ (pg/mL)	ULOQ (pg/mL)	%CV	LLOQ (pg/mL)	ULOQ (pg/mL)	%CV
0.5	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
1	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
2	N/A	N/A	N/A	N/A	N/A	N/A	3,000	100,000	2.77-8.67
5	20	20,000	0.7-10.13	200	20,000	2.38-8.50	20	20,000	1.26-6.66
10	20	20,000	2.22-14.14	30	20,000	0.38-12.08	20	20,000	1.16-6.44
20	20	20,000	1.34-23.64	30	20,000	2.02-15.83	30	20,000	0.87-14.63
	With Q0 trapping								
FFT (ms)	277-203-132			277-203-168			Sum		
	LLOQ (pg/mL)	ULOQ (pg/mL)	%CV	LLOQ (pg/mL)	ULOQ (pg/mL)	%CV	LLOQ (pg/mL)	ULOQ (pg/mL)	%CV
0.5	200	100,000	2.62-23.52	200	100,000	1.28-20.79	30	20,000	1.37-19.56
1	30	20,000	1.08-20.13	200	100,000	1.86-12.44	30	20,000	1.53-7.15
2	20	10,000	1.14-13.80	200	20,000	0.92-6.36	20	20,000	0.38-8.51
5	20	3,000	0.82-3.54	20	3,000	2.08-13.23	20	3,000	0.82-5.19
10	20	3,000	1.33-7.68	20	3,000	0.90-11.71	20	3,000	1.24-8.79
20	20	3,000	0.47-7.59	20	3,000	0.99-15.33	20	3,000	0.29-3.82

Fixed fill time

The time for filling the ion trap with product ions for excitation must be considered when designing and optimizing MRM³ methods. The fixed fill time (FFT) is predominantly selected for methods used for quantification, to allow for comparability between standards and samples. Adjustments to the user-defined FFT, however, can affect the quantitative performance metrics of the overall method. To assess this effect, a series of FFTs was applied to the LIT-MRM³. The resulting LLOQs and LDRs were compared between the LIT-MRM³ quantification results (Figure 4).

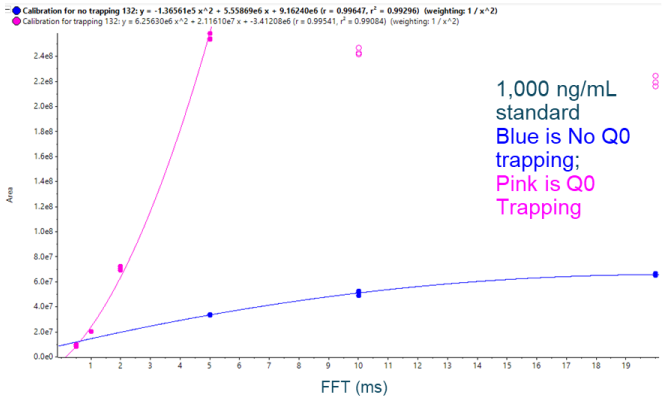

Figure 4. Impact of FFT and Q0 trapping on signal for the LIT-MRM³ workflow. Increasing FFT increased the signal intensity for the MRM³ data trace. Turning on Q0 trapping quickly saturated the detector and therefore limited the LDR. The same pattern was observed for both secondary product ions assessed.

The performance of the LIT-MRM³ workflow was further compared across different fixed fill times when the Q0 trapping was turned on or off. The results of this analysis are shown in Table 2.

As might be expected, increasing the FFT resulted in lower observed LLOQ values for both MRM³ transitions. The addition of Q0 trapping also facilitated the ability to detect lower levels of analyte at lower FFTs. However, the signal tended to become saturated with Q0 trapping on as FFT was increased (Figure 4). This assessment can help inform future method development of MRM³ methods using the linear ion trap on QTRAP systems.

Comparing 2 MRM^3 strategies

In the comparison of quantitative method parameters, there was little observed difference in sensitivity and reproducibility between the 2 MRM^3 workflows. Both approaches achieved similar limits of quantification with similar %CVs (Table 1). To achieve this performance, however, the LIT-MRM^3 method required more involved optimization with additional parameters (Table 3), all of which take time to optimize any of which might influence LLOQ, %CV and LDR. In contrast, the Q0DE-MRM^3 workflow only requires the optimization of the Q0DE voltage to generate product ions and the CE to generate secondary product ions. The method development for the Q0DE-MRM^3 workflow is therefore considerably more straight-forward than that of the LIT-MRM^3 method.

One main difference between these techniques was the observed LDR. The LDR was more limited when using the LIT-MRM^3 approach relative to the Q0DE-MRM^3 workflow because the use of fixed fill time with Q0 trapping limited the upper limit of quantification.

The speed of the analysis varied between approaches. For the traditional MRM approach and the Q0DE-MRM^3 method, all method information is stored in a single MRM table, making it efficient to monitor multiple compounds with multiple transitions. Fast dwell times can be achieved, as the instrument operates with all elements continuously transmitting. With the LIT-MRM^3 approach, however, a separate MS/MS/MS experiment must be set up for each primary fragment ion to be monitored. Additionally, the time required for analysis includes both the excitation time and the time to scan the secondary product ions out of the ion trap. Thus, the acquisition time per compound is longer and fewer compounds can be multiplexed into a single assay with the LIT-MRM^3 approach.

For this study a single analyte, clenbuterol, and a single sample matrix, urine, were used to assess and compare each scan type. Analytes with different physico-chemical properties might have different fragmentation properties that could make one approach more desirable than another. Fragmentation of the primary fragment ion to a secondary fragment ion uses a different mechanism between the approaches, as CID is used in the Q0DE-MRM^3 workflow and resonant excitation is used in the LIT-MRM^3 workflow.

Table 3. Tunable parameters for the different targeted workflows.

Parameter	MRM	Q0DE-MRM^3	LIT-MRM^3
Collision energy (CE)	*Ramp*	*Ramp*	*Ramp*
Q0 dissociation (Q0D)	-	*Ramp*	-
AF2	-	-	*Ramp*
Fill time	-		*Set*
Q0 trapping	-		*On / off*

Note: Source conditions were optimized and held constant across these experiments.

Optimizing methods for real samples

When optimizing the LIT-MRM^3 workflow, it is recommended to perform a wide scan from the trap for all potential second-generation ions to select the highest intensity peak with the least background noise from the sample matrix. Once selected, the AF2 values for individual MS/MS/MS fragments should be optimized for the final assay.

Performing the selection and optimization of both the first- and second-generation fragments using real matrix samples will ensure selection of product ions that provide the best selectivity in matrix.

While linear ion trap-based scans are known to have lower LDR than MRM-type experiments, the LIT-MRM^3 workflow might be a valuable tool in some studies. If the analyte fragments well in the ion trap and provides a very good signal-to-noise ratio, it is possible to establish the analyte concentration range in matrix and determine whether it is sufficient on a case-by-case basis.

Conclusions

Here, the clenbuterol example was used to show proof-of-concept evidence that the Q0DE-MRM^3 workflow, available on the SCIEX 7500 system, has potential to be a sensitive, reproducible and easy to set up option for addressing analytical challenges of selectivity.

- Compared to the historically utilized LIT-MRM^3 workflow that leverages the QTRAP system, the Q0DE-MRM^3 requires much less development and optimization and can be higher multiplexed
- The Q0DE-MRM^3 method produced data with quality comparable to that of the MRM-based acquisition method in terms of LLOQ, ULOQ and %CV and outperformed the LIT-MRM^3 workflow
- Multiple targeted workflows are available on the SCIEX 7500 system with the QTRAP system upgrade that allow method developers to select the right tool for quantification studies when additional selectivity is needed.

References

1. MRM^3 Quantitation for Highest Selectivity in Complex Matrices. SCIEX technical note, RUO-MKT-02-2739-B.
2. Enabling new levels of quantification. SCIEX technical note, RUO-MKT-02-11886-B.

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