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A Novel MS³ Experiment for Quantifying Ions with a Linear Ion Trap

Running Title: Scan-free MS³

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Abstract

Liquid chromatography coupled with tandem mass spectrometry has long been employed for the quantitation of molecules. With judicious selection of precursor and fragment ions, multiple-reaction monitoring assays can be developed rapidly for these experiments. However, there are cases where analyses struggle due to high background signals caused by matrix effects that interfere with the analytical signal. An alternative to MRMs involves using two stages of tandem mass spectrometry - an MS³ experiment. While this technique can provide greater selectivity than MS/MS experiments, cycle times for MS³ experiments are typically longer than MRM-type experiments.

Here, we present a quantitation technique employing an MS³ method with shorter cycle times than traditional linear ion trap MS³ scans. Termed “scan-free” MS³, this technique performs “mass analysis” by isolating the ions of interest in the linear ion trap, then emptying the trap of these ions. The signal will be due only to those preselected ions, resulting in an MS³ experiment with up to a ~35% reduction in cycle times relative to standard MS³ experiments without loss of sensitivity. We compare the analytical performance of this method with MRMs, as well as standard MS³ experiments, finding equivalent or better performance from the scan-free MS³ method.

Keywords: mass spectrometry; linear ion trap; tandem mass spectrometry; MS³; quantitation

Introduction

Tandem mass spectrometry experiments (such as multiple-reaction monitoring, or MRM) [1] are routinely employed to perform quantitation assays on small molecule pharmaceuticals,[2, 3] environmental contaminants,[4, 5] and biological molecules.[6, 7] The general workflow (Figure 1a), which provides detection with excellent sensitivity and selectivity, proceeds through the following steps: (1) chromatographic separation of the analyte molecules from the matrix and other analytes, (2) electrospray ionization (ESI) of the separated components as they elute from the chromatographic column, (3) mass filtering for precursor ions in the first mass analysis stage (Q1 in the case of triple quadrupole instruments), (4) fragmentation of the precursor ion in a collision cell (in this case, q2), (5) mass filtering of one of the selected fragment ions (in this case, by Q3), and (6) subsequent detection. The intent is to filter out any ions that do not share the common m/z values with the analyte precursor and fragment ions, thereby increasing the selectivity of the analysis.

Unfortunately, the presence of chemical noise in MRM channels can introduce unwanted and unintended signals, thereby reducing the sensitivity and selectivity of these experiments. In order to compensate for these circumstances, multiple MRM channels are often monitored concurrently for each analyte. Signals from multiple channels can be summed to enhance the sensitivity of the overall analysis.[8] In addition, multiple fragment ions can be used to confirm the presence and identity of the precursor ion.[9] This methodology becomes vital as the size and complexity of the precursor ion's structure increases, as is the case with large biological molecules like proteins and peptides.[10, 11]

One alternative to an MRM-based strategy (Figure 1a) is to utilize a second stage of tandem mass spectrometry, or MS^3 experiments. In this scenario, a mass-selected precursor ion is subjected to collision-induced dissociation (CID) in a similar fashion as in an MRM experiment. This fragment ion is then contained in a linear ion trap (LIT) and isolated using an rf/dc pulse. Then, the ions are fragmented by kinetically exciting them at their resonant frequency using dipolar excitation (i.e., waveform applied across two opposing rods) inside of the LIT in the presence of inert collision gas (e.g., nitrogen).[12] These secondary fragment ions can then be used to quantitate the original analyte (Figure 1b). In cases where one or more MRM channels suffer from high background levels due to chemical noise or matrix effects, performing MS^3 for quantitation can provide a more sensitive and selective assay. In fact, there are examples of MS^3 scans used for quantitation [13,14] but experiment cycle times were several seconds long and not amenable to most liquid chromatography (LC) elution times. A recent quantitative workflow [15] has been shown to provide low ng/mL detection levels for peptides in plasma samples. This technique relies on the acquisition of a full-scan MS^3 mass spectrum, which provides several secondary fragment ions; the extracted ion chromatograms from these fragment ions can be co-added and used for quantitation. However, the process of acquiring these data requires a few hundred milliseconds per cycle, and this can potentially be detrimental if fast liquid chromatography, with peak widths of a few seconds or less, is employed.

Figure 1. The three tandem mass spectrometry experiments employed in this work, described using the symbolism of Lourié and coworkers [16]. (a) an MS/MS-based selected-reaction monitoring (MRM) experiment, (b) a “standard” MS³ experiment, and (c) the “scan-free” MS³ experiment.

To attempt to circumvent such challenges, a new analytical experiment has been developed for performing quantitative MS^3 analyses. In these “scan-free” MS^3 experiments, a precursor ion is mass selected in Q1, fragmented via beam-type CID in q2, trapped in Q3, and then isolated. This first-generation fragment ion is then excited in Q3 via dipolar excitation,[12] collided with nitrogen gas pulsed into this quadrupole, and a second-generation fragment ion is formed and isolated. This second isolation step serves as a form of “mass selection” and is followed by letting the ions be dumped from the LIT by applying a simple dc gradient to the LIT’s exit lens (Figure 1c). These ion handling steps require shorter ion cooling times than do standard MS^3 experiments, which need cooling to provide LIT-based full scan mass spectra. The “scan-free” MS^3 experiment’s signal is attributed to only the previously isolated ion, similar to MRM experiments. Similar “mass selection via isolation” experiments have been demonstrated with rectilinear ion traps.[17]

In this article, we compare the analytical performance of “scan-free” MS^3 with MRMs, as well as standard MS^3 experiments, and demonstrate how it obtains similar or better sensitivity as MRM and MS^3 quantitation experiments for five different compounds present in a complex matrix, with shorter experimental cycle times compared to MS^3 experiments.

Experimental

Reagents and Standards. All analytes (buspirone, clenbuterol, angiotensin I, arg-vasopressin, and exenatide) were obtained from Sigma-Aldrich (St. Louis, MO) and were used as obtained. HPLC-grade methanol and acetonitrile were purchased from Mallinckrodt Baker (Phillipsburg, NJ, USA) and deionized water (18 M Ω) was produced in-house (MilliQ Plus, Millipore, Billerica, MA). Calibration curve samples were prepared by spiking known quantities of analyte into protein-precipitated horse plasma (1:2 plasma:acetonitrile, then diluted with 1 part water). This matrix was intended to provide a source of chemical background noise consistent with many bioanalytical analyses. No internal standards were employed in the sample sets. Replicate injections (3) were performed for each analyte and concentration. Limits of quantitation (LOQs) were determined based upon FDA-established criteria accepted by the bioanalytical community (i.e., the lowest sampled concentration with replicate measurement statistics yielding CV $\leq$ 15% and Accuracy $\pm$ 20%).[18]

Liquid Chromatography. Samples were analyzed using a Prominence XL HPLC (Shimadzu Scientific Instruments, Columbia, MD), which included two solvent pumps, a cooled autosampler (5 °C), and a column oven (40 °C). A Phenomenex Luna C18(2)-HST column (50 x 2.0 mm; 2.5 μ m particle size) (Phenomenex, Torrance, CA) was used to separate the target analytes from background components in the plasma. A reverse-phase elution gradient (mobile phase A: H₂O / 5% ACN / 0.2% formic acid; mobile phase B: ACN / 5% H₂O / 0.2% formic acid) was employed wherein LC solvents were delivered with a flow rate of 400 μ L/min, with 5% B maintained for 0.80 min, followed by

a linear gradient from 5-90% B over 2 min, followed by high organic (90% B) for 2 min, and a further 4 min of re-equilibration at 5% B. Each experiment used a 10- μ L injection volume and was performed in triplicate.

Mass Spectrometry. All experiments were performed using a hybrid triple quadrupole/linear ion trap mass spectrometer [19] (SCIEX, Concord, ON, Canada) equipped with an electrospray ion source. Instrument control and data acquisition were performed using a modified version of standard control software, while data analysis was performed using research-grade PeakView software (SCIEX). The following ion source conditions were employed: ion spray voltage of +5500 V; nebulizer and auxiliary gas pressures set at 60 and 50 psi, respectively; source temperature of 450 °C; and curtain gas pressure of 15 psi. The collision gas pressure was set to high (arbitrary setting, $\sim$ 3 mTorr N₂). During MS³ experiments, a pulse of collision gas (N₂) was delivered to the Q3 LIT to enhance the efficiency of the fragmentation process [20]. Both Q1 and Q3 were held at unit resolution (0.7 units wide) for the experiments. During the MS³ experiments ("standard" and "scan-free", vide infra), ions were isolated at $q = 0.705$ prior to the in-trap fragmentation step; fragmentation was performed at $q = 0.233$. Lastly, fill times for all of the MS³ experiments were determined by dynamic fill time (DFT) [21] and could range from 0.05 – 150 ms, with the longer times being employed for lower signal levels.

MRM experiments. MRM parameters were optimized by infusing solutions ($\sim 1 \mu\text{M}$ in 50/50 water/methanol with 0.1% formic acid) of each target analyte to observe the $[\text{M} + \text{H}]^+$ for each molecule, the optimal (i.e., the most abundant) fragment ions, and the voltages required for optimum sensitivity for each transition. At least two MRM channels were monitored for each target analyte ion (Table 1) with dwell times of 5 ms and 5-ms pause times between transitions. The analytical signal for each analyte was the peak area of an extracted ion chromatogram of the final products of interest and the sum of related MRM channels (where appropriate).

Table 1. MRM parameters employed for the analysis of five analytes examined in this study.

Analyte	Q1	Q3	Collision Voltage (V)
Buspirone	386.2	222.2	38.5
	386.2	122.0	41.0
Clenbuterol	277.2	203.0	23.0
	277.2	132.0	45.0
Exenatide	838.2	396.3	30.0
	838.2	299.2	45.0
	838.2	70.0	145.0
	838.2	202.1	60.0
Arg-vasopressin	542.8	120.0	69.0
	542.8	328.3	29.0
Angiotensin I	433.0	255.2	46.5
	433.0	110.0	46.5

“Standard” MS³ experiments. “Standard” MS³ parameters were optimized by infusing solutions of each target analyte to observe the molecular ion, as well as the best choices for the first- (MS/MS) and second-generation (MS³) fragment ions (Figure 2, Table 2). Several parameters were optimized, including relevant trapping and excitation parameters. After the MS³ fragmentation event, ions were scanned out of the LIT by mass-selective axial ejection (MSAE) [19,22] at 10,000 mass units / second. The final MS³ analytical signal was obtained by performing an extracted ion chromatogram of the final products of interest (up to 4 different fragment ions) and summing the peak areas from one or more of these fragment ions.

Table 2. MS³ parameters employed for the analysis of five analytes examined in this study. The signal from more than one MS³ fragment ion could be included in the quantitation of the analytes.

Analyte	Precursor	MS/MS Fragment	MS3 Fragments	q2 Collision Voltage (V)	LIT Collision Voltage (mV)
Buspirone	386.2	265.1	222.2, 180.1, 152.0	39.2	70.0
Clenbuterol	277.2	203.0	185.2, 167.1, 132.0	25.5	90.0
Exenatide	838.2	396.3	299.0, 202.1, 185.0	25.6	70.0
Arg-vasopressin	542.8	328.3	254.1, 212.2, 194.1	27.5	90.0
Angiotensin I	433.0	110.0	93.0, 83.0	46.5	70.0

Figure 2. MS³ spectra for the five analytes measured in this study. Each spectrum contains an inset image that specifies the precursor and first fragment ions utilized in each MS³ experiment; the ions formed from the second fragmentation event that could be used as analytical signals are highlighted in red boxes.

“Scan-free” MS³ experiments. The “scan-free” MS³ experiments are much like the MS³ experiments with a few key exceptions. After the MS³ fragmentation event in Q3, a second rf/dc isolation step was performed to isolate the targeted fragment ion (i.e., the most abundant fragment ion formed). After this isolation step, only the ions isolated by the last rf/dc pulse remain in the LIT. These ions are detected by simply lowering the dc trapping potential (EXB) applied to a lens on the detector side of the LIT and allowing the mass-selected ions to escape from the trap and strike the detector. The final analytical signal was obtained by recording the total ion chromatogram from these ions for each analyte (as noted in Table 3). “Scan-free” MS³ experiments required substantial changes to the underlying instrument controls (i.e., firmware and control software) and, as a result, are available only for the proprietary, research-grade version of the control software.

Table 3. Scan-free MS³ parameters employed for the analysis of five analytes examined in this study.

Analyte	Precursor	MS/MS Fragment	Scan Free MS3 Fragments	q2 Collision Voltage (V)	LIT Collision Voltage (mV)
Buspirone	386.2	265.1	222.2	39.2	70.0
Clenbuterol	277.2	203.0	132.0	25.5	90.0
Exenatide	838.2	396.3	202.1	25.6	70.0
Arg-vasopressin	542.8	328.3	254.1	27.5	90.0
Angiotensin I	433.0	110.0	93.0	46.5	110.0

Results and Discussion

MS³ scans can overcome limitations caused by high background noise levels that hinder MRM analyses

The use of MRMs to detect molecules in complex matrices (e.g., plasma, urine) using LC-MS can sometimes adversely suffer if one or more of the ion fragmentation channels – optimized under clean and controlled conditions – are shown to be of low sensitivity or to contain a significant level of chemical noise. Such a case is displayed in Figure 3, wherein the primary MRM channel for the drug molecule buspirone (chosen as the primary channel due to its highest intensity during optimization) is severely impacted by the presence of chemical background ions in the horse plasma sample that generate ions with the same MRM fragmentation values. This scenario is an unfortunate, but all-too-common occurrence in many bioanalytical workflows where many different small molecules and ionizing agents (e.g., alkali metal salts) are present.

Figure 3. Chromatograms displaying the signal acquired while monitoring the primary buspirone-specific MRM channel (386.3 $\rightarrow$ 123.1) for (a) a blank horse plasma sample, (b) a horse plasma sample containing 0.1 ng/mL of buspirone, and (c) a horse plasma sample containing 1.0 ng/mL of buspirone. Note the high background levels that hinder detection of this drug molecule.

In some cases, the addition of signals from multiple MRM channels for a given analyte can improve the overall sensitivity (signal-to-noise) of bioanalytical assays.[8] However, this is not always the case, especially when one or more of the MRM channels to be added brings with it a high level of chemical background noise. For example, MRM data obtained during the analysis of exenatide in protein-precipitated horse plasma demonstrates this (Figure 4). During the analysis of the protein, four (4) MRM channels were monitored based upon the optimized MS/MS of the most intense precursor ions (m/z 838.2). While one of the MRM transitions (838.2→396.3, Figure 4a) provided a relatively noise-free signal for the 1-ng/mL solution, the other MRM transitions do not. The MRM transitions 838.2→299.2 and 838.2→202.1 produce analytical signals that extend above the background noise levels produced by the matrix-matched blank (Figure 4b and c, respectively). However, the most intense MRM transition for exenatide (as determined by infusion of standard) - 838.2→70.0 - contains a level of background (chemical) noise equivalent to the analyte signal (Figure 4d). However, since the background noise can vary and is dependent upon the particular matrix involved, predicting which MRMs will be interfered with *a priori* is extremely difficult as any of the four MRM channels could be subject to interference in a given experiment. Ultimately, such MRM data can translate into poorer performance and limits of quantitation (LOQs).

Figure 4. Extracted ion chromatograms for four different MRM channels monitored during the analysis of exenatide at 1 ng/mL in protein-precipitated horse plasma (plotted in blue) and during the analysis of a separate matrix-matched blank sample (plotted in magenta).

Table 4 displays the quantitative results of co-adding contaminated MRM channels on analyte quantitation, in this case for exenatide in protein-precipitated horse plasma. While the primary MRM channel (838.2 → 396.3) provides an LOQ of 0.1 ng/mL, addition of the MRM signal from the secondary MRM channel (838.2 → 299.2) results in a poorer overall LOQ of 1 ng/mL; given that this signal channel (Figure 4b) contains a much higher relative level of background noise compared to the primary MRM channel (Figure 4a), this outcome makes sense. As the other MRM channels are co-added (Figure 4c and d) to the analytical signal, the LOQ rises to 10 ng/mL, again as a result of the higher background noise and variability in these channels. The lack of chemical noise impacting the primary MRM channel is fortuitous for this workflow, since this scenario could just as easily be hampered by chemical noise present at the same *m/z* values of that channel.

Table 4. Quantitation of exenatide from horse plasma using selected MRM channels as the analytical signal. Numbers in red highlight the LOQs for each analytical signal.

Primary (Best) MRM Channel	Actual Concentration	Mean	Standard Deviation	CV (%)	Accuracy (%)
	0.01	N/A	N/A	N/A	N/A
	0.1	0.100	0.015	15.32	100.08
	1	0.980	0.049	5.01	98.01
	10	11.170	0.819	7.34	111.67
	100	106.000	4.157	3.92	106.00
	1000	842.400	24.730	2.94	84.24
Co-add Best 2 MRM Channels	Actual Concentration	Mean	Standard Deviation	CV (%)	Accuracy (%)
	0.01	N/A	N/A	N/A	N/A
	0.1	0.099	0.058	58.46	99.32
	1	1.060	0.059	5.58	106.02
	10	10.800	0.565	5.23	107.98
	100	101.600	1.790	1.76	101.58
	1000	851.000	24.690	2.90	85.10
Co-added Best 3 MRM Channels	Actual Concentration	Mean	Standard Deviation	CV (%)	Accuracy (%)
	0.01	N/A	N/A	N/A	N/A
	0.1	0.434	0.407	93.87	433.53
	1	1.648	0.494	29.99	164.80
	10	11.070	0.522	4.72	110.67
	100	114.400	2.904	2.54	114.36
	1000	812.700	25.040	3.08	81.27
Co-added Best 4 MRM Channels	Actual Concentration	Mean	Standard Deviation	CV (%)	Accuracy (%)
	0.01	N/A	N/A	N/A	N/A
	0.1	0.411	0.387	94.19	411.10
	1	1.605	0.437	27.23	160.54
	10	10.990	0.463	4.22	109.91
	100	113.900	3.001	2.63	113.92
	1000	822.600	26.440	3.21	82.26

Using MS³ experiments instead of MRMs can counteract the impact of background noise caused by complex matrices

By using MS³ experiments to quantitate an analyte in a complex matrix, an additional degree of fragmentation of the analyte ion can provide a unique feature to the analytical signal (beyond the simple precursor/fragment relationship established with MRMs). This extra stage of fragmentation greatly reduces the probability of another adventitious contaminant ion sharing all three common ions (one precursor ion and two subsequent fragment ions), bringing potentially higher signal-to-noise ratios to MS³ experiments over MRMs. An example of such an improvement can be observed in Figure 5, where the MS³ analysis of buspirone (one fragment ion extracted) is compared to the MRM signal (see Figure 3) for the same concentration of analyte (0.1 ng/mL).

Figure 5. Identical injections of 0.1-ng/mL buspirone analyzed by MRM (blue trace) or MS³ (pink trace), showing the reduction in the amount of chemical background contamination in the MS³ signal channel given its added degree of selectivity (i.e., third identifying ion).

However, the compromise that must be made to provide such an enhancement in selectivity is the longer time spent carrying out the MS³ experiments compared to MRMs. Generally, MS³ experiments can require hundreds of milliseconds to execute, compared to 5 – 10 milliseconds (or less) for MRMs. This can translate into fewer data points acquired across an eluting LC peak, which can lead to less precise integration of peak areas (the metric commonly used to quantify an analyte). However, when scenarios arise, like the buspirone example (Figure 5), where a complex matrix is challenging the use of an MRM-based workflow, MS³ analyses should be considered. Again, in contrast to MRM data, MS³ data (both the “standard” and the “scan-free” forms, *vide infra*) demonstrate lower levels of background chemical noise. This is due in part to the increased selectivity provided by the additional stage of mass selection and isolation performed within the LIT.

Rationale behind implementing “scan-free” MS³ experiments

The goal of the “scan-free” MS³ experiments was to perform MS³-based quantitation experiments with shorter cycle times than standard MS³-based experiments, like MS³ (Figure 6). In addition, the “scan-free” MS³ experiment is a simpler form of MS³ whose signal more resembles the MRM output from a linear pentaquadrupole mass spectrometer,[23] with two stages of CID layered between three stages of mass filtering. A full-scan mass spectrum is not acquired during the “scan-free” MS³ experiments – only the signal resulting from the third ion in the MS³ fragmentation pathway is detected and used for the quantification of the analyte.[24] This would also require the implementation of multiple “scan-free” MS³ experiments in order to monitor multiple MS³ transitions, possibly impacting overall cycle times.

Figure 6. Timing diagrams for (a) a standard MS³ experiment and (b) a “scan-free” MS³ experiment, with events and their relevant times indicated.

(a)	Event	Empty All Ions	Fill Precursor & Concomitant Beam-type MS/MS (q2) of Precursor 1	Cool	rf/dc Isolation of First Fragment Ion (Precursor Ion 2)	Cool	MS/MS of Precursor 2 (Resonant dipolar excitation in Q3 LIT)	Cool	Mass-selective Axial Ejection
	Duration (ms)	5	Variable*	20	1	10	20	50	~10**

(b)	Event	Empty All Ions	Fill Precursor & Concomitant Beam-type MS/MS (q2) of Precursor 1	Cool	rf/dc Isolation of First Fragment Ion (Precursor Ion 2)	Cool	MS/MS of Precursor 2 (Resonant dipolar excitation in Q3 LIT)	Cool	rf/dc Isolation of Second Fragment Ion (Precursor 3)	Cool	Lower Exit Lens Voltage (Detection)
	Duration (ms)	7	Variable*	20	1	12	20	10	1	5	1

While the overhead time of the scan-free MS³ experiment is reduced by ~35% over standard MS³ (77 ms versus 116 ms, respectively), these returns are diminished with increased ion fill (accumulation) times. By using dynamic fill time [21] in these standard and scan-free MS³ experiments (as it would normally be implemented by a user of this technology), longer fill times will be implemented for analytes present at lower concentrations. These longer fill times will reduce the differences in overhead time between the two MS³ experiments, although they will both still benefit from the gain in selectivity that the extra stage of mass fragmentation/selection brings over MRM.

Overall comparison of MRMs, Standard MS³, and “Scan-free” MS³

Table 5 outlines the results of the three MS-based bioanalytical methods, and highlights some benefits of the two MS³ experiments over the MRM-based workflows. In general, the MS³-based methods matched or outperformed the MRM-based workflows in terms of lowest LOQ, a likely outcome of those methods ability to discriminate better between analytical ions and chemical background ions present at the same nominal m/z values (i.e., isobaric interferences). Exenatide serves as an exception to this outcome, although the primary MRM channel (Figure 4a) was the only one of four that did not suffer from substantial interference from the chemical background; this was fortuitous. The MRM-based method also performed as well as the MS³-based methods for the analysis of clenbuterol. Again, this protein-precipitated horse plasma (matrix) might not have provided high chemical background for this analyte, but other matrices (such as urine) are known to hamper clenbuterol detection.[254] Also, while the “scan-free” MS³ method provides additional data points

across an analyte's LC elution profile (Figure 7) compared to the standard MS³ variant, standard MS³ enjoys the benefit of being able to survey more than one fragment ion as the final analytical signal (i.e., "scan-free" MS³ is limited to only one).

An interesting comparison can be made between the standard and the "scan-free" MS³ quantitation results when including only the signal of the most intense fragment ion in each MS³ event. As documented in Table 6, when the LOQs achieved by each MS³ method using only the most intense fragment ion ("1 Frag"), "scan-free" MS³ meets or exceeds the LOQs achieved by the slightly slower standard MS³ experiment. This possible advantage provided by the somewhat faster acquisition rate of the "scan-free" MS³ method could be useful in certain scenarios (e.g., when very fast LC elution is employed); this will be the subject of further studies. However, given that the final mass analysis stage of a standard MS³ experiment will contain a plethora of fragments (and analyte signal channels as a result), the summation of multiple fragment ion channels ultimately equalizes the LOQs achieved by both MS³ methods.

Figure 7. LC elution profiles for the analysis of the 0.1-ng/mL sample of buspirone as measured by MRM (top), standard MS³ (middle), and “scan-free” MS³ (bottom). Note the larger number of points across the peaks for the MRM-based compared to the MS³ experiments; “scan-free” MS³ – with its shorter relative cycle time – acquires a few more points than standard MS³.

Table 5. Results of the three MS-based methods employed in this study to detect five different analytes in protein-precipitated horse plasma. These results include the number of MRM transitions or standard MS³ fragments co-added to yield the best analytical performance, with values in red highlighting LOQs for each analytical signal.

Analyte	MS Mode	Concentration (ng/mL)	0.01	0.1	1	10	100	1000
Buspirone	MRM (2 Channels)	Mean	0.010	0.11	1.2	7.4		
		Standard Deviation	0.005	0.01	0.0	0.1		
		CV (%)	55.7	8.3	3.8	1.0		
		Accuracy (%)	98.6	112.4	115.2	73.8		
	Standard MS ³ (2 Frags)	Mean	0.010	0.09	1.1	9.9		
		Standard Deviation	0.001	0.01	0.0	0.2		
		CV (%)	7.5	9.3	3.9	1.9		
		Accuracy (%)	101.0	89.3	111.1	98.7		
	Scan-free MS ³	Mean	0.010	0.11	1.1	8.7		
		Standard Deviation	0.001	0.01	0.1	0.5		
		CV (%)	13.1	7.1	6.0	5.5		
		Accuracy (%)	99.2	107.4	106.1	87.3		
Clenbuterol	MRM (1 Channel)	Mean	0.010	0.10	1.0	9.5		
		Standard Deviation	0.001	0.00	0.0	0.1		
		CV (%)	14.1	3.6	3.9	1.0		
		Accuracy (%)	99.8	102.0	103.5	94.8		
	Standard MS ³ (1 Frag)	Mean	0.010	0.11	1.1	8.1		
		Standard Deviation	0.001	0.01	0.0	0.2		
		CV (%)	13.8	5.0	0.3	2.8		
		Accuracy (%)	98.6	113.9	106.1	81.4		
	Scan-free MS ³	Mean	0.010	0.12	1.0	8.1		
		Standard Deviation	0.001	0.00	0.0	0.1		
		CV (%)	11.8	4.2	2.4	1.2		
		Accuracy (%)	98.4	116.2	104.8	80.7		
Angiotensin I	MRM (2 Channels)	Mean	N/D	0.10	1.0	10.8		
		Standard Deviation	N/D	0.02	0.0	0.3		
		CV (%)	N/D	19.7	3.5	3.0		
		Accuracy (%)	N/D	99.9	100.4	108.0		
	Standard MS ³ (2 Frags)	Mean	N/D	0.10	1.1	14.1		
		Standard Deviation	N/D	0.04	0.1	0.3		
		CV (%)	N/D	39.2	6.3	2.4		
		Accuracy (%)	N/D	98.9	106.8	141.4		
	Scan-free MS ³	Mean	N/D	0.10	0.9	9.9		
		Standard Deviation	N/D	0.01	0.0	0.5		
		CV (%)	N/D	6.5	2.7	5.3		
		Accuracy (%)	N/D	101.0	90.4	99.4		
Arg-Vasopressin	MRM (2 Channels)	Mean	N/D	N/D	1.0	11.3		
		Standard Deviation	N/D	N/D	0.2	1.4		
		CV (%)	N/D	N/D	17.4	12.8		
		Accuracy (%)	N/D	N/D	98.7	113.0		
	Standard MS ³ (4 Frags)	Mean	0.010	0.11	0.9	9.2		
		Standard Deviation	0.008	0.04	0.1	0.9		
		CV (%)	83.2	36.3	11.8	9.6		
		Accuracy (%)	99.5	106.4	88.0	92.4		
	Scan-free MS ³	Mean	N/D	0.07	1.0	10.5		
		Standard Deviation	N/D	0.06	0.0	0.6		
		CV (%)	N/D	86.5	4.5	5.8		
		Accuracy (%)	N/D	67.0	97.8	105.3		
Exenatide	MRM (1 Channel)	Mean	N/D	0.10	1.0	11.2	106.0	842.4
		Standard Deviation	N/D	0.02	0.0	0.8	4.2	24.7
		CV (%)	N/D	15.3	5.0	7.3	3.9	2.9
		Accuracy (%)	N/D	100.1	98.0	111.7	106.0	84.2
	Standard MS ³ (4 Frags)	Mean	N/D	0.10	0.9	10.1	108.5	972.3
		Standard Deviation	N/D	0.06	0.1	0.5	10.6	122.7
		CV (%)	N/D	58.6	15.9	4.5	9.8	12.6
		Accuracy (%)	N/D	100.7	92.5	101.0	108.5	97.2
	Scan-free MS ³	Mean	N/D	0.10	0.8	10.4	110.8	1023.0
		Standard Deviation	N/D	0.02	0.1	0.6	13.6	0.3
		CV (%)	N/D	18.9	6.4	5.5	12.3	0.0
		Accuracy (%)	N/D	101.9	80.7	104.3	110.8	102.3

Table 6. Comparing the LOQs achieved by both the “scan-free” and standard MS³ methods as a function of the number of fragment ion channels summed (for standard MS³).

Analyte	MS Mode	LOQ (ng/mL)
Buspirone	Standard MS ³ (1 Frag)	0.1
	Standard MS ³ (2 Frags)	0.01
	Scan-free MS ³	0.01
Clenbuterol	Standard MS ³ (1 Frag)	0.01
	Standard MS ³ (2 Frags)	0.01
	Scan-free MS ³	0.01
Angiotensin I	Standard MS ³ (1 Frag)	1
	Standard MS ³ (2 Frags)	1
	Scan-free MS ³	0.1
Arg-Vasopressin	Standard MS ³ (1 Frag)	10
	Standard MS ³ (2 Frags)	1
	Scan-free MS ³	1
Exenatide	Standard MS ³ (1 Frag)	10
	Standard MS ³ (3 Frags)	1
	Scan-free MS ³	1

Conclusions

In many cases where high levels of chemical background begin to hinder MRM-based bioanalyses, MS³-based analyses should be chosen as a viable alternative workflow. To help offset the increased cycle times of these experiments over their MRM alternatives, we have evaluated a “scan-free” form of MS³ to perform mass analysis by selectively isolating the fragment ions in the final stage of “mass analysis” in the linear ion trap, then simply emptying the trap of any isolated ions. The detected signal will be due only to those preselected ions in a manner very similar to standard MRM experiments. The result is an MS³ experiment with up to a ~35% reduction in overall experiment cycle times without any losses in sensitivity and equivalent analytical figures of merit compared to similar bioanalytical MRM- and MS³-based workflows for a range of analytes samples from a reasonably complex biological matrix. However, given that “scan-free” MS³ can only survey one fragment ion for its analytical signal (as opposed to the myriad fragment ions possibly available for many ions), the equivalent analytical performance of “scan-free” and standard MS³ workflows can be rationalized.

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Figure and Table Captions

Figure 1. The three tandem mass spectrometry experiments employed in this work, described using the symbolism of Louris and coworkers [16]. (a) an MS/MS-based selected-reaction monitoring (MRM) experiment, (b) a “standard” MS³ experiment, and (c) the “scan-free” MS³ experiment.

Figure 2. MS³ spectra for the five analytes measured in this study. Each spectrum contains an inset image that specifies the precursor and first fragment ions utilized in each MS³ experiment; the ions formed from the second fragmentation event that could be used as analytical signals are highlighted in red boxes.

Figure 3. Chromatograms displaying the signal acquired while monitoring the primary buspirone-specific MRM channel (386.3 → 123.1) for (a) a blank horse plasma sample, (b) a horse plasma sample containing 0.1 ng/mL of buspirone, and (c) a horse plasma sample containing 1.0 ng/mL of buspirone. Note the high background levels that hinder detection of this drug molecule.

Figure 4. Extracted ion chromatograms for four different MRM channels monitored during the analysis of exenatide at 1 ng/mL in protein-precipitated horse plasma (plotted in blue) and during the analysis of a separate matrix-matched blank sample (plotted in magenta).

Figure 5. Identical injections of 0.1-ng/mL buspirone analyzed by MRM (blue trace) or MS³ (pink trace), showing the reduction in the amount of chemical background contamination in the MS³ signal channel given its added degree of selectivity (i.e., third identifying ion).

Figure 6. Timing diagrams for (a) a standard MS³ experiment and (b) a “scan-free” MS³ experiment, with events and their relevant times indicated.

Figure 7. LC elution profiles for the analysis of the 0.1-ng/mL sample of buspirone as measured by MRM (top), standard MS³ (middle), and “scan-free” MS³ (bottom). Note the larger number of points across the peaks for the MRM-based compared to the MS³ experiments; “scan-free” MS³ – with its shorter relative cycle time – acquires a few more points than standard MS³.

Table 1. MRM parameters employed for the analysis of five analytes examined in this study.

Table 2. MS³ parameters employed for the analysis of five analytes examined in this study. The signal from more than one MS³ fragment ion could be included in the quantitation of the analytes.

Table 3. Scan-free MS³ parameters employed for the analysis of five analytes examined in this study.

Table 4. Quantitation of exenatide from horse plasma using selected MRM channels as the analytical signal. Numbers in red highlight the LOQs for each analytical signal.

Table 5. Results of the three MS-based methods employed in this study to detect five different analytes in protein-precipitated horse plasma. These results include the number of MRM transitions or standard MS³ fragments co-added to yield the best analytical performance, with values in red highlighting LOQs for each analytical signal.

Table 6. Comparing the LOQs achieved by both the “scan-free” and standard MS³ methods as a function of the number of fragment ion channels summed (for standard MS³).

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154x86mm (168 x 168 DPI)

602x514mm (168 x 168 DPI)

180x244mm (96 x 96 DPI)

60x97mm (168 x 168 DPI)

284x131mm (150 x 150 DPI)

Draft

(a)

Duration (ms)	Event
5	Empty All Ions
Variable*	Fill Precursor & Concomitant Beam-type MS/MS (q2) of Precursor 1
20	Cool
1	rf/dc Isolation of First Fragment Ion (Precursor Ion 2)
10	Cool
20	MS/MS of Precursor 2 (Resonant dipolar excitation in Q3 LIT)
50	Cool
~10**	Mass-selective Axial Ejection

(b)

Duration (ms)	Event
7	Empty All Ions
Variable*	Fill Precursor & Concomitant Beam-type MS/MS (q2) of Precursor 1
20	Cool
1	rf/dc Isolation of First Fragment Ion (Precursor Ion 2)
12	Cool
20	MS/MS of Precursor 2 (Resonant dipolar excitation in Q3 LIT)
10	Cool
1	rf/dc Isolation of Second fragment Ion (Precursor 3)
5	Cool
1	Lower Exit Lens Voltage (Detection)

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