New Dynamic MRM Mode Improves Data Quality and Triple Quad Quantification in Complex Analyses

Technical Overview

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Abstract

Multiple Reaction Monitoring (MRM) mode has become the preferred method for the quantitative analysis of known or target compounds using triple quadrupole mass spectrometry. The current solution for MRM analysis uses time segmentation, where a method is divided into a series of time segments and predefined sets of MRM transitions are monitored for each segment. As sample complexity increases (e.g. quantifying very low levels of hundreds of pesticide residues in a wide variety of food matrices), very real practical limitations in the time-segmentation methodology become apparent. A better solution is required.

New dynamic MRM methods on the Agilent 6400 Series triple quad instruments create new capability to tackle large multi-analyte assays and to accurately quantify exceedingly narrow peaks from fast Agilent 1200 Series RRLC and 1290 Infinity UHPLC separations. Examples of pesticide analysis and rapid screening of drugs of abuse are highlighted. Dynamic MRM methods yield equivalent, or better, quality data and results as compared to traditional time segment based methods – plus easier method development and modification.

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Introduction

Utilizing multiple reaction monitoring (MRM) with a triple quadrupole tandem mass spectrometer enables extraordinary sensitivity for multi-analyte quantitative assays. The first quadrupole (Q1) selects and transmits a precursor ion with a specific *m/z*. This ion is then fragmented in the second quadrupole (Q2 collision cell), and a specific product ion with a defined *m/z* is selected and transmitted in the third quadrupole (Q3). See **Figure 1**. The combination of a specific precursor mass and a unique product ion is generally an unambiguous and sensitive method to selectively monitor and quantify a compound of interest. Since two stages of mass selection are utilized, MRM assays are particularly useful for the specific analysis of target compounds in complex mixtures and matrices. MRM mode has become the preferred method for the quantitative analysis of known or target compounds.

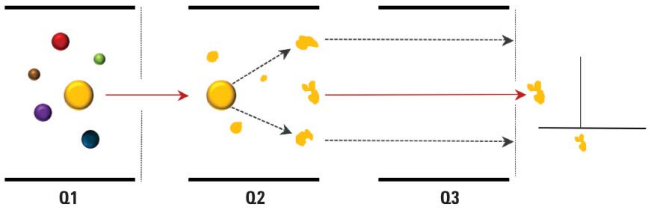

Figure 1: *A schematic diagram of MRM mode on a triple quadrupole instrument. The precursor ion is selected in Q1, fragmentation occurs in Q2, and the product ion is selected by Q3. Since two stages of mass selectivity are utilized, there is very little interference from background matrix resulting in excellent sensitivity.*

The Limitations of Time Segment Methods

The current solution to complex sample analysis is time segmentation. A method is developed with multiple predefined time segments and the triple quad MS is programmed to perform MRM assays for only those analytes that elute during each segment. **Figure 2** shows an example of a method with four time segments. One set of MRM transitions is analyzed during segment 1, another set during segment 2, etc. The benefit of such a method is that, rather than performing MRM scans for all analytes during the entire method, during any given segment the triple quad only monitors MRM transitions for the analytes that elute in that segment. The result is that there are fewer MRM transitions during each MS scan, allowing the mass spec method to use a longer dwell time and/or to reduce the overall cycle time for each MRM scan so that there are more data points per peak.

However, there are some limits to what can be accomplished with time segment methods. As the number of analytes in a method increases, so too will the number of concurrent MRM transitions in each segment. It will be necessary to either reduce the dwell times for these transitions or to increase the cycle time for each MS scan. Reducing dwell times (the amount of time required for the triple quad to analyze a single MRM transition) can compromise MS data integrity by introducing collision cell cross-talk (insufficient clearing of the collision cell between individual MRM experiments such that some product ions from a previous MRM may be detected in the subsequent MRM). Maintaining the same dwell time but increasing the overall MS cycle time may mean that not enough data points are collected during the elution of a very narrow LC peak to allow for reliable quantitation. Both of these factors can lead to compromises in data quality.

There is an additional challenge using time segments. In order not to compromise any data, the change from one segment to the next must occur during a time when no peaks are eluting from the LC column. In complex analyses such as pesticide analysis, where many co-eluting peaks are monitored at almost every time point during the chromatogram, this can be a formidable challenge as is highlighted in **Figure 2**. Furthermore, there is always the risk that adding analytes to a method may require complete redevelopment of a method to introduce these chromatographically quiet zones where segment changes can occur.

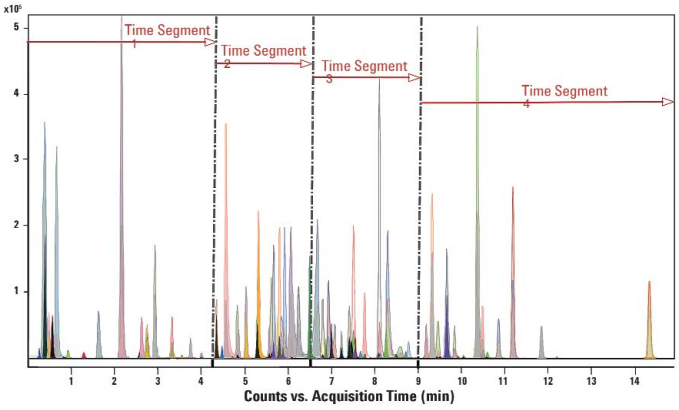

Figure 2: *Dividing the chromatogram into time segments. Detection of a complex pesticide mixture demonstrates the advantages and some of the limitations of time segment based MRM quantitation.*

Introducing Dynamic MRM Mode

Agilent's new and unique analytical method approach is now available on all 6400 Series Triple Quadrupole LC/MS systems. MassHunter acquisiton software allows the user to choose conventional MRM or dynamic MRM mode. Ion transitions and a retention time window for each analyte are stored in a method. MRM transition lists are then built dynamically throughout an LC/MS run, based on the retention time window for each analyte. In this way, analytes are only monitored while they are eluting from the LC and valuable MS duty cycle is not wasted by monitoring them when they are not expected. An added benefit of this approach is that MassHunter MS Optimizer software can readily determine and store optimal transition ions for each target analyte, greatly simplifying dynamic MRM method set up.

This approach addresses the limitations of the time segment methods for a large batch of compounds by replacing the group segmentation with individual time windows for every analyte transition and by dramatically reducing, on average, the number of individual MRM transitions that are monitored during each MS scan. This approach is demonstrated in **Figures 3-5**.

Dynamic MRM removes the requirement to resolve compounds to baseline and to create well-defined segments in the chromatogram where no compounds elute. This reduces the potential method impact of adding analytes and of retention time shifts. The inevitability of multiple co-eluting peaks is of lesser concern with dynamic MRM as long as the individual ion transitions are unique. **Figure 5** shows an expanded region of the analysis in **Figure 3**, with 22 compounds eluting between 5.58 and 6.51 min with substantially overlapped peaks. All analytes can be accurately quantified because their ion transitions are mutually exclusive, allowing total exclusion of background and interferences.

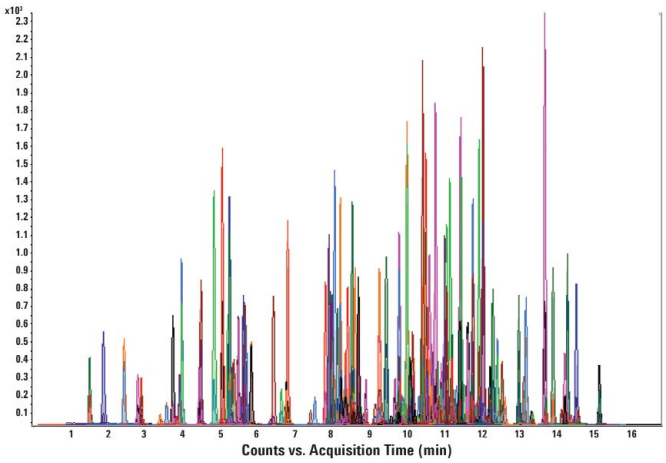

Figure 3: Dynamic MRM method does not require time segments. Extracted ion chromatogram of a 250 pesticide mix spiked into tap water (500 total transitions, 2.5 pg on-column) using a dynamic MRM method run on a 1290 Infinity LC and a 6460 Triple Quadrupole LC/MS system with Agilent Jet Stream technology.

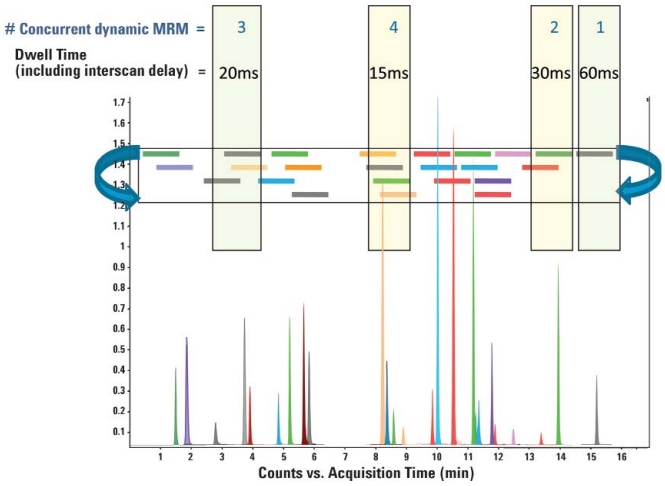

Figure 4: Dynamic MRM methods are based on individual retention time windows for each MRM transition. 24 pesticide transitions from the analysis in Figure 3 are highlighted and their retention time windows are shown. *Note that, on average, the number of transitions which are monitored at any point in the chromatogram is dramatically reduced relative to time segment methods, allowing much faster MS scan cycle times. Also note that this MS cycle time is held constant (60 ms in this case) in order to assure the highest possible data quality and quantitative result.*

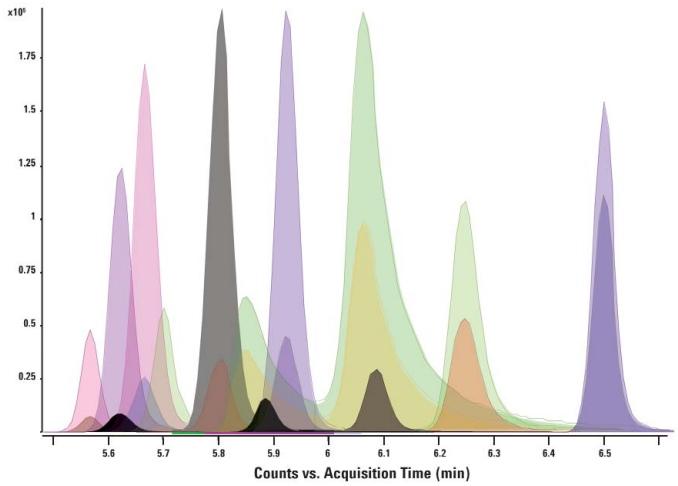

Figure 5: *Extracted ion chromatogram of 11 pesticides and 11 qualifier ions. In spite of significant co-elution, well-chosen MRM transitions allow for accurate quantification of all sample components.*

The Importance of Constant MS Scan Cycle Time in Dynamic MRM Methods

Agilent's dynamic MRM approach uses a constant sampling time across chromatographic peaks. Even data point spacing with adequate sampling across the peak provides the best and most precise representation of the peak. To maintain a constant cycle time, the individual MRM dwell time is also adjusted to keep a constant sampling rate across all peaks, even though the number of ion transitions being monitored will change dynamically and may vary cycle to cycle, dependent on elution time and the number of concurrent analytes. Because dynamic MRM yields generally fewer concurrent ion transitions per unit time than traditional time segments, MS cycle times can be reduced and individual transition dwell times are typically longer than traditional time segmented methods. While the Agilent 6460 and 6430 Triple Quadrupole LC/MS systems are capable of 1 ms dwell times, this is typically only required in the most extreme assays. Note: with the proprietary axial acceleration technology present on all Agilent triple quadrupole and Accurate Mass Q-TOF collision cells, all product ions are cleared from the collision cell in less than 600 µs so that there is no MRM cross-talk with the shortest dwell times (1).

Furthermore, by maintaining a constant dynamic MRM cycle time, MS methods can be matched to analyte peak widths to ensure that a statistically adequate number of data points is acquired for each analyte to yield excellent analytical accuracy and precision. This approach yields uniform data points across any given analyte peak and results in good peak symmetry — a distinct improvement over constant dwell time approaches.

Dynamic MRM Easily Accommodates Fast UHPLC

HPLC or UHPLC separations with the Agilent 1200 RRLC or 1290 Infinity LC systems can reduce method times dramatically without sacrificing peak capacity or chromatographic resolution. Individual peak widths may be reduced to just a few seconds. In the extreme, peak widths may be less than one second wide. Dynamic MRM methods require on average, fewer ion transitions to be monitored concurrently in a chromatogram. MS cycle times are much faster than with time segment methods and allow collection of many data points across narrow peaks, as is shown in **Figure 6**, for excellent quantitative results.

Linearity with dynamic MRM methods is at least as good as traditional time segment approaches. Typically, linear correlation coefficients are excellent and assay linearity exceeds three orders of dynamic range. **Figure 7** shows a calibration curve for the pesticide compound oxamyl, with excellent sensitivity, linearity, and dynamic range. Triplicate injections of a 25 pg sample on-column yielded a peak area %RSD of only 1.08.

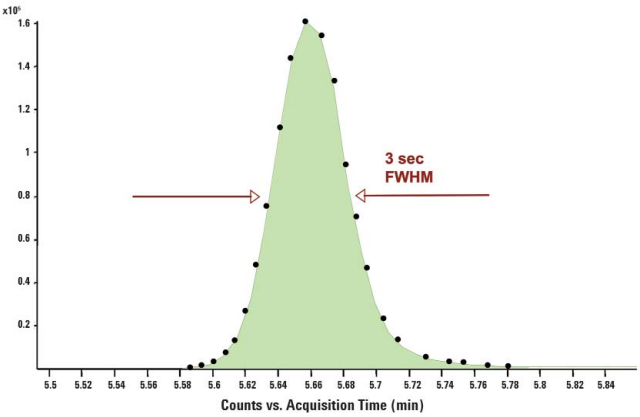

Figure 6: Dynamic MRM allows accurate quantification of narrow LC peaks. *A pesticide analysis gave this 6-sec wide peak for atrazine (5pg on-column). A dynamic MRM method allowed for collection of sufficient data points to assure an excellent quantitative result. The MS scan cycle time was 350 ms and remained constant across the peak. Quantitative precision showed a peak area %RSD < 3.5 for this compound.*

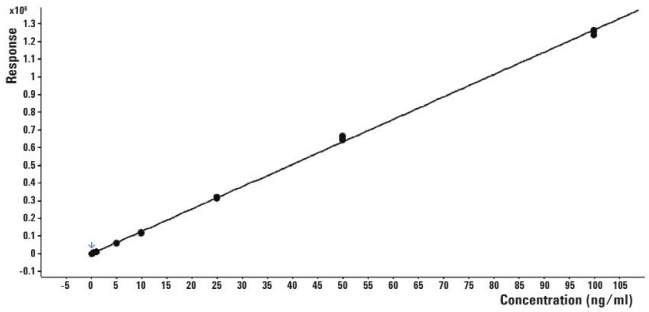

Figure 7: *Dynamic MRM methods provide excellent quantitative data. Linearity of oxamyl from 0.1 pg to 100 pg on-column, R2 = 0.9992.*

Application of dynamic MRM

Pesticide Screening

A challenging real world application for dynamic MRM is the quantitative analysis of a very complex sample run at ultra high pressure using a high resolution column and a fast gradient. 300 pesticides with internal standards were run on a sub-two micron column with a 15 min gradient at pressures exceeding 800 bar, or 11600 psi. A dynamic MRM method with 600 transitions was created using retention time windows of only 0.5 min. Results of this analysis are shown in **Figure 8**.

Comparison of dynamic MRM with conventional time segment methods reveals an excellent correlation. Eight pesticides were injected in 20 replicates at the 10 pg level and both average area and relative standard deviation were calculated. As shown in **Figure 9**, the correlation in peak areas derived with both the dynamic and time-based MRM methods was outstanding with $R^2 = 0.99992$. The peak area relative standard deviations for the time segment based method was less than 6% and less than 4% for the dynamic MRM method.

Rapid Screening for Drugs of Abuse

A further example of dynamic MRM capability is the fast screening for 100 drugs of abuse in oral fluids over a 5 minute gradient — a typical work-place drug test.

This is a particularly challenging analysis given the timescale of the assay relative to the number of analytes in the target screen. Further, since qualifier ions and quantifier ions were necessary for confirmatory purposes, a total of 200 dynamic MRM transitions were employed during the analysis, covering classes of analytes such as opiates, amphetamines, cannabinoids and benzodiazepines, among others.

In this example, peak widths were approximately 2 sec. A dynamic MRM method using retention time windows of only 12 sec was used. The maximum number of concurrent dynamic MRM transitions was never more than 52. The assay showed excellent sensitivity, (LOD = 23 fg on-column) and linearity. External calibration linearity: ($R^2 = 0.9987$) for one of the spiked analytes (Prazepam) is described in **Figures 11** and **12**, respectively.

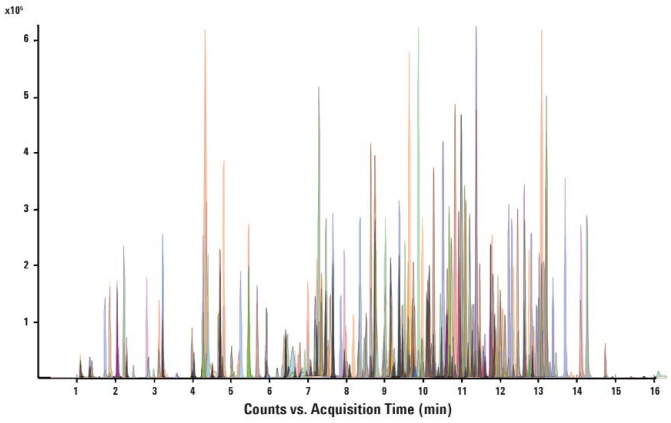

Figure 8: *Dynamic MRM analysis allows quantification of 300 pesticides using internal standards in a 15 min method. Data was generated with an Agilent 1200 Infinity LC and 6460 Triple Quadrupole LC/MS system with Agilent Jet Stream technology.*

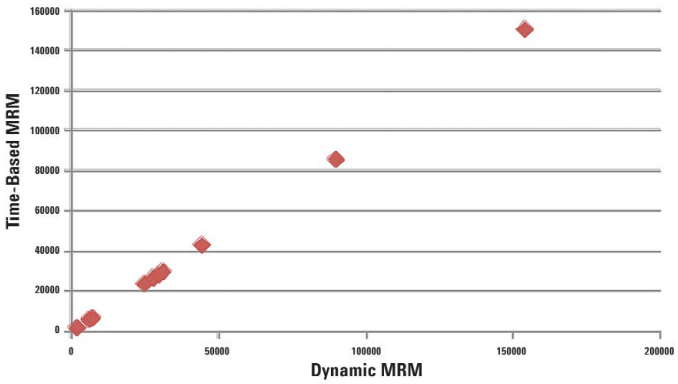

Figure 9: *Comparing pesticide peak areas with dynamic MRM and time segment based methods.*

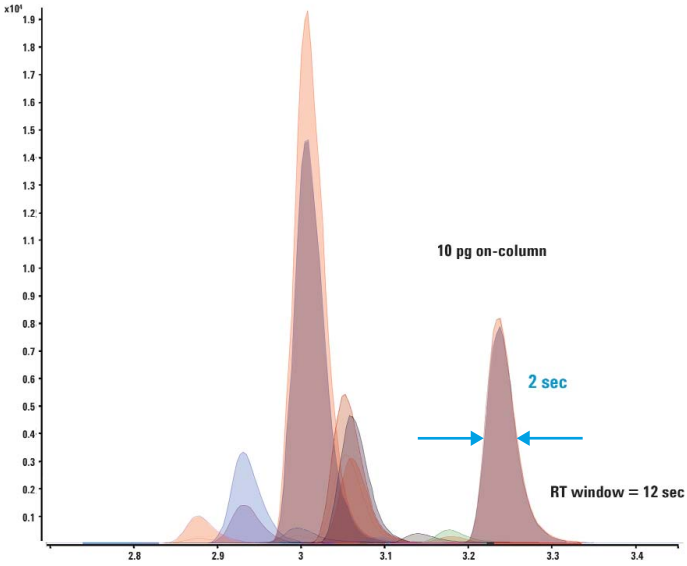

Figure 10. Detection of 10 spiked benzodiazepine drugs in an oral fluid extract using a dynamic MRM method with >200 MRM transitions and 12 sec retention time windows. *This study was performed on an Agilent 1290 Infinity LC and 6460 Triple Quadrupole LC/MS system with Agilent Jet Stream technology.*

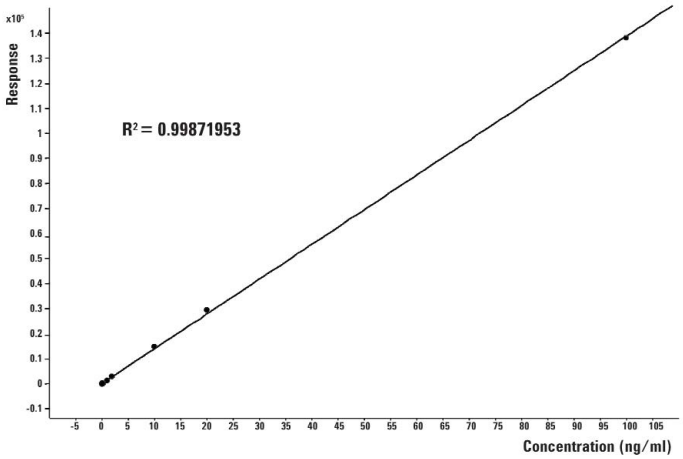

Figure 12. *Linearity of Prazepam from 23 fg to 100 pg on-column, R^2 = 0.9987.*

Compound	LOD (fg on-column)
Clobazam	126
Clonazepam	91.5
Flunitrazepam	47.5
Flurazepam	43.0
Lorazepam	186
Lormetazepam	45.9
Midazolam	1.2 pg
Oxazepam	145
Oxazolam	235
Prazepam	23.7
Temazepam	156

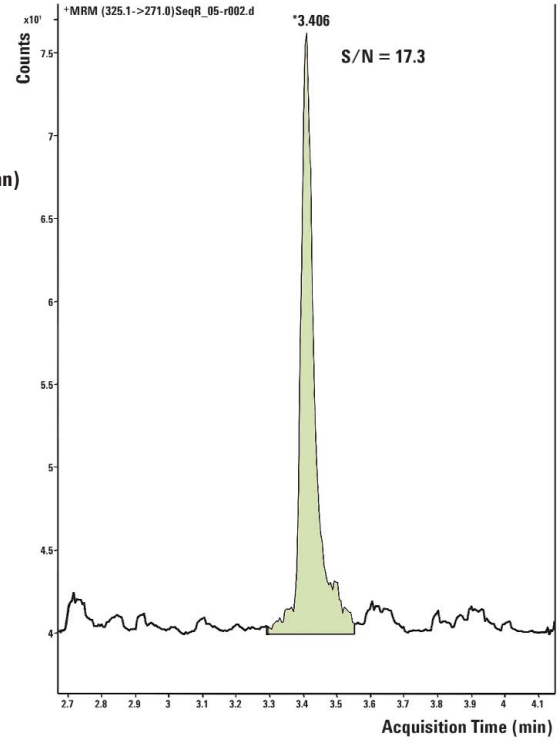

Figure 11. *Limit of detection (LOD) of Prazepam is 23.7 fg with a S/N of 17.3.*

Summary

New dynamic MRM methods on the Agilent 6400 Series triple quad instruments create new capability to tackle large multi-analyte assays and to accurately quantify exceedingly narrow peaks from fast Agilent 1200 Series RRLC and 1290 Infinity UHPLC separations. The number of MRM transitions is adjusted dynamically throughout the LC run, selecting only transitions with relevant retention time windows. This means that, on average, many fewer MRM transitions are monitored during a typical MS scan than would be the case with a time segment based method – with the added benefit that dynamic MRM methods are less demanding to develop and adapt.

Fewer transitions allow methods with shorter MS scan cycle times (more scans/second) and the ability to provide excellent quantification of very narrow (even sub-second) RRLC and UHPLC peaks. Importantly, this dramatically shortened MS scan cycle time is kept constant so optimized sampling and consistent accurate quantitation is ensured (the same cannot be said for methods that vary MS scan cycle time).

Practically, dynamic MRM methods can be used to accurately quantify hundreds of individual analytes, plus their internal standards and qualifier ions, in a relatively short LC run. Compared to benchmark time segment methods, dynamic MRM methods achieve similar sensitivity, linear dynamic range, and quantitative accuracy, with better precision.

Key Points

- Key enabling technology for fast, accurate LC/MS quantitation of complex samples
- Matches performance of Agilent 6400 series triple quad with separation power of 1200 Series RRLC and UHPLC with 1290 Infinity LC
- Many data points collected across very narrow peaks for accurate LC/MS quantitation
- Constant MS scan cycle time ensures accurate quantitation
- Equivalent and better quality data and results than traditional time segment based methods – plus easier method development and modification with MassHunter Optimizer software
- Up to 4,000 ion transitions per LC run
- Diverse applications: pesticide analysis, drug screening, targeted protein quantitation

References:
(1) Agilent publication 5989-7408EN: Ion optics innovations for increased sensitivity in hybrid MS systems

www.agilent.com/chem/qqq

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