Technical Overview

Metabolomics and Life Science Research

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A Guide for Optimization of Data-Dependent Acquisition Settings in Metabolomics

Using the Agilent Revident LC/Q-TOF

Authors

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Abstract

Metabolite annotation remains one of the major bottlenecks in mass spectrometry-based metabolomics, where reliable identification involves high-quality MS/MS fragmentation spectra and accurate spectral library matching.[1,2] To address this challenge, we previously established a robust, untargeted LC/Q-TOF metabolomics workflow for discovery experiments using hydrophilic interaction liquid chromatography (HILIC) optimized for metal-sensitive metabolites on an Agilent Bio LC system.[3] An Agilent Revident LC/Q-TOF was used for effective, iterative data-dependent acquisition (DDA)—including Iterative Auto MS/MS—to generate high-quality MS/MS spectra for confident metabolite identification, including retention time information.[4]

This technical overview provides a practical guide for optimizing key DDA parameters, such as precursor selection thresholds, spectral acquisition rates, the number of precursors per cycle, and exclusion list strategies. The presented workflow enhances MS/MS coverage and supports both beginners and experienced users in developing high-performance metabolomics methods. Overall, the combination of Revident Q-TOF technology, HILIC separation, and optimized Auto and Directed MS/MS settings forms a powerful foundation for comprehensive, untargeted metabolomics and streamlined data interpretation.

Introduction

Untargeted sample profiling and compound annotation

High-resolution mass spectrometry (HRMS)-based experiments for metabolomics profiling and biomarker discovery require high-quality data for reliable compound annotation. Measurement of accurate *m/z*, isotope pattern, and meaningful complementary MS/MS spectra with overall high MS/MS coverage is desired. This work demonstrates the capabilities of the Agilent Revident LC/Q-TOF to meet those needs and provides guidance for the development of robust data-dependent acquisition (DDA) methods. The goal is to gain as much information as possible from a biological sample for statistical analysis and ultimately compound annotation or identification. DDA with Revident and other Agilent Q-TOFs running Agilent MassHunter Data Acquisition for TOF/Q-TOF LC/MS version 12.0 or later supports two acquisition modes: The established Auto MS/MS mode and the new Directed MS/MS mode. Auto MS/MS can optionally use preferred and exclusion lists. However, Directed MS/MS requires a dedicated precursor list. Directed MS/MS is therefore a "preferred list only" Auto MS/MS experiment on the Q-TOF. For Directed MS/MS, precursors are exclusively selected from the user-defined list, focusing on the most relevant compounds while retaining decision criteria for precursor selection from the Auto MS/MS decision engine. Thus, Directed MS/MS focuses MS2 acquisition on only the most relevant metabolites, such as those that are statistically significant for group discrimination. Both Auto and Directed MS/MS acquisition modes can additionally be run as iterative MS/MS, enabled through the worklist as an intelligent reflex workflow.

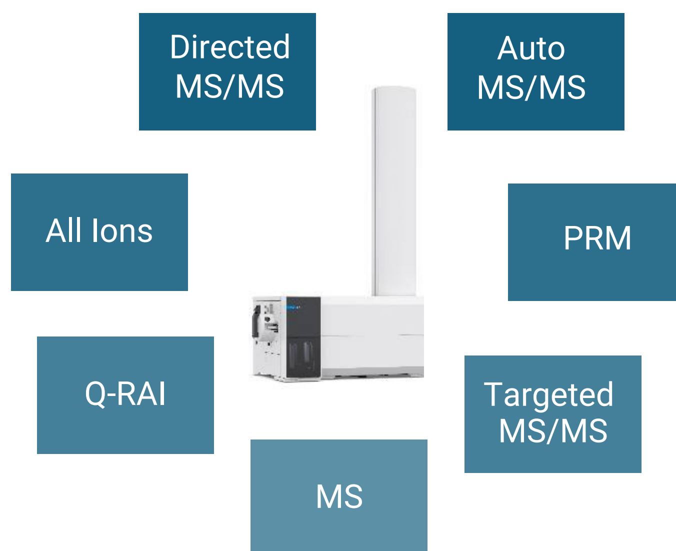

Figure 1. Acquisition modes available on the Agilent Revident LC/Q-TOF. Directed and Auto MS/MS acquisition modes were used for this work.

This technical overview describes various parameters for Auto MS/MS, including MS1 and MS/MS acquisition rate, precursor selection threshold, number of precursors per cycle, and variable MS/MS acquisition rate. These parameters were first optimized for Auto MS/MS data acquisition, followed by a comparison to Directed MS/MS. Additionally, different strategies for building up an exclusion list in Auto MS/MS were tested, and the value that an iterative injection logic adds to untargeted experiments was demonstrated.

Experimental

Captiva EMR SPE for metabolite extraction from plasma

Using the Agilent Captiva EMR workflow, 100 µL of plasma was extracted.[5] Proteins were precipitated by the addition of 450 µL MeOH/EtOH (1:1), followed by 350 µL of water. After centrifugation (10 minutes, 4 °C, 1,700 rcf), the supernatant was transferred to a Captiva EMR cartridge (3 mL, part number 5190-1003). The cartridge was washed with 800 µL water/MeOH/EtOH (2:1:1) and the eluate collected. After evaporating the eluate to dryness (N_2, 65 °C, 2 hours), the residue was reconstituted in 400 µL ACN/MeOH/water (8:1:1) and spiked with an in-house mixed standard. For preparation of the in-house standard, a physiological amino acid standard (A9906), adenosinetriphosphate (A2383), succinic acid (14079), adenosine (A9251), adenine hemisulfate salt (A9126), phospho(enol)pyruvic acid monopotassium salt (P7127), citric acid (251275), D-(+)-glucose (G7528), D-fructose-1,6-bisphosphate trisodium salt hydrate (6803), and D-glucose-6-phosphate disodium salt hydrate (G7250) were purchased from Merck KGaA, Darmstadt, Germany. Solid standards were dissolved and diluted in water. A 100 µM stock solution in pure water was prepared from liquid amino acid mix and diluted standards. The extracted plasma sample was spiked with 100 µM metabolite stock solution, yielding 6 µM in the final extract for injection.

Plant extract for demonstration of exclusion list strategies

For demonstration of exclusion list strategies, a plant extract was measured with a rapid RP-C18 method. This method used an Agilent ZORBAX Eclipse Plus C18 column (2.1 × 50 mm, 1.8 µm; part number 959757-902) and a 13-minute gradient of water and ACN containing 0.05% acetic acid for mobile phases A and B. Flow rate was set at 0.6 mL/min and the injection volume was 2 µL.

Robust HILIC workflow for metabolite analysis

Plasma samples were analyzed in negative electrospray ionization (ESI) mode with the 23-minute robust hydrophilic interaction liquid chromatography (HILIC) metabolomics method (Table 1), providing reproducible retention times and peak shapes.[4,6] An Agilent 1290 Infinity II Bio LC was used to avoid discrimination of metal-sensitive metabolites. This workflow is also fully compatible with the Agilent Infinity III Bio LC and will achieve identical results. More details on the Agilent HILIC metabolomics workflow, including standardized protocols and supporting documentation, are available from Agilent upon request.

Table 1. LC parameters for HILIC analysis of metabolites using Agilent 1290 Infinity II/III Bio LC.

Parameter	Value
Stationary Phase	Agilent InfinityLab Poroshell 120 HILIC-Z, 2.1 × 150 mm, 2.7 µm (p/n 683775-924)
Temperature	15 °C
Mobile Phase A	20 mM ammonium acetate, pH 9.3, 5 µM medronic acid in H_2O
Mobile Phase B	Pure acetonitrile (ACN)
Autosampler Temperature	5 °C
Gradient	Time (min) / %B / Flow rate (mL/min) 0.0 / 90 / 0.4 1.0 / 90 8.0 / 78 12.0 / 60 15.0 / 10 18.0 / 10 19.0 / 90 / 0.5* 23.0 / 90 / 0.4 * The flow was increased during equilibration of the column
Injection Volume	3 µL
Needle Wash	Standard wash, 10 sec, IPA:ACN:H_2O, 1:1:1

Raw data were collected with a Revident LC/Q-TOF running MassHunter Data Acquisition for TOF/Q-TOF LC/MS version 12.1. MS parameters are given in Table 2. For data evaluation, MassHunter Qualitative Analysis software version 12.0 and the updated version 13.0 were used.

Table 2. MS source parameters for Directed and Auto MS/MS measurements in negative ESI mode.

Parameter	Value
Gas Temperature	225 °C
Drying Gas	9 L/min
Nebulizer Pressure	30 psi
Sheath Gas Temperature	375 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	3,000 V
Nozzle Voltage	500 V
Fragmentor	100 V
Skimmer	45 V
Instrument Mode	1,700 fragile
Reference Ions	Purine *m/z* 119.03632 HP-921 + acetate *m/z* 980.016375

Data analysis of MS/MS spectra

For evaluation of MS/MS spectral quality, acquired spectra were matched against the Agilent HILIC spectral library[3] containing retention time and MS/MS spectra for three collision energies (10, 20, and 40 V) acquired from metabolite standards. The resulting library score from matching the three collision energies was used to compare spectral quality across Auto MS/MS acquisition methods. Within the Identification Workflow tab (see Figure 2) in MassHunter Qualitative Analysis software, three different scores for a spectral library match are calculated: Forward (fwd) Score, Reverse (rev) Score, and Library (Lib) Score. When multiple collision energies are used, the Library Score is calculated for each collision energy separately, as well as for the combined match of all collision energies. If not indicated otherwise in this document, the term Library Score refers to the overall Library Score resulting from matching all collision energy spectra to the library.

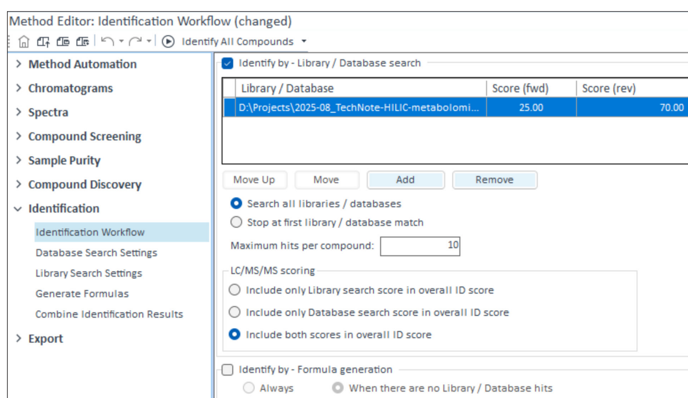

Figure 2. Identification workflow settings in Agilent MassHunter Qualitative Analysis software version 13.0. Forward and Reverse score numbers define the cutoff for matching an acquired MS/MS spectrum with the spectral library. By default, the cutoff values are 25 and 70 for the Forward and Reverse Scores, respectively.

The Forward Search Score is a result of comparing the acquired spectrum with the library spectrum, considering all peaks in both spectra, their *m/z* values, and relative intensities. In a reverse search, all peaks in the library spectrum are compared to the acquired spectrum. Any peaks in the acquired spectrum that do not match the library spectrum within the defined tolerances (Figure 3) are not considered for calculation of the Reverse Score.

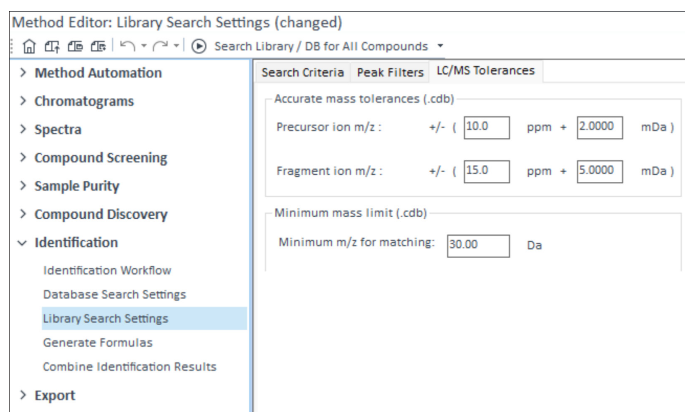

Figure 3. Library Search Settings tab for defining mass tolerances applied to the spectral library for matching the acquired data. The settings shown were used in this project.

For this work, the cutoff values for Forward and Reverse Scores (Figure 2) were set to zero to always enable spectral matching across all three collision energies. With these settings, an overall Library Score of 75 was considered indicative of a reasonable match after visual inspection of acquired MS/MS and library spectra. If different cutoff values for the Forward and Reverse Scores are used (for example, 25 and 70 as per default), the overall Library Score threshold for calling a match should be reconsidered, and 75 may not be appropriate. When working with multiple collision energies, the overall Library Score can be easily altered by the Forward and Reverse score matching cutoffs as shown in Figure 4 for the example of anserine.

A **Cut-offs:** fwd 0 | rev 0 → Library Score 57.98

ID Techniques Applied: DBSearch-LibSearch-FBF

Best	Name	Formula	m/z	Mass	Score (Lib)	RT	Score (RT)	Score (DB)	Species	Flags	Notes
	L-Anserine	C10H16N4O3	239.1158	240.1232	57.98	10.878		0	(M-H)-		IUPAC Name: (2S)-2-(3-aminopropanoylamino)-3-(3-methyli

CE	Name	Forward Score	Flags	Species	Lib/DB	Notes	Num Peaks	m/z (Prec)	Reverse Score	Score (Lib)
10	L-Anserine	76.46		(M-H)-	D:\Projects\2025-08_...	IUPAC Name: (2S)-2-(3-aminopropanoylami...	9	239.115	86.16	86.16
20	L-Anserine	40.85		(M-H)-	D:\Projects\2025-08_...	IUPAC Name: (2S)-2-(3-aminopropanoylami...	15	239.115	75.5	75.5
40	L-Anserine	0.3		(M-H)-	D:\Projects\2025-08_...	IUPAC Name: (2S)-2-(3-aminopropanoylami...	10	239.115	6.33	6.33

B **Cut-offs:** fwd 25 | rev 70 → Library Score 79.5

ID Techniques Applied: DBSearch-LibSearch-FBF

Best	Name	Formula	m/z	Mass	Score (Lib)	RT	Score (RT)	Score (DB)	Species	Flags	Notes
	L-Anserine	C10H16N4O3	239.1158	240.1232	79.5	10.878		0	(M-H)-		IUPAC Name: (2S)-2-(3-aminopropanoylamino)-3-(3-methyli

CE	Name	Forward Score	Flags	Species	Lib/DB	Notes	Num Peaks	m/z (Prec)	Reverse Score	Score (Lib)
10	L-Anserine	76.46		(M-H)-	D:\Projects\2025-08_...	IUPAC Name: (2S)-2-(3-aminopropanoylami...	9	239.115	86.16	86.16
20	L-Anserine	40.85		(M-H)-	D:\Projects\2025-08_...	IUPAC Name: (2S)-2-(3-aminopropanoylami...	15	239.115	75.5	75.5

Figure 4. Library Scores for anserine depend on the cutoffs defined for Forward and Reverse Scores in the Identification Workflow tab.

Results and discussion

Auto MS/MS parameters were selected in the Method Editor tab of the acquisition software. A previous Agilent technical overview (5994-0322EN) provides detailed information on the basic principles, parameters, and setup of an Auto MS/MS method for the identification of unknowns in water samples.[7] This work therefore focuses on new features available in MassHunter Data Acquisition for TOF/Q-TOF LC/MS version 12 and on additional details not covered in 5994-0322EN.[7] The parameters for Auto MS/MS are specified in the Acquisition Parameters tab, which includes five subsections in the method editor of the acquisition software (Figure 5).

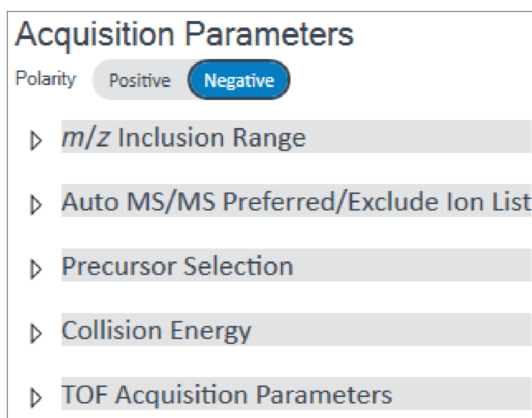

Figure 5. Overview of the Acquisition Parameters tab with subsections to select Auto MS/MS settings.

Acquisition Parameters tab: Inclusion Range and Exclusion List

In the Acquisition Parameters tab, the range for precursor ions to be selected for fragmentation is specified by the *m/z* inclusion range (Figure 6). Only ions within this spectral range in MS1 can be selected as precursor ions. This parameter allows focusing on a defined spectral region, typically covering the expected *m/z* range of the compound class(es) of interest, and avoids spending cycle time on redundant background ions outside the *m/z* region of interest.

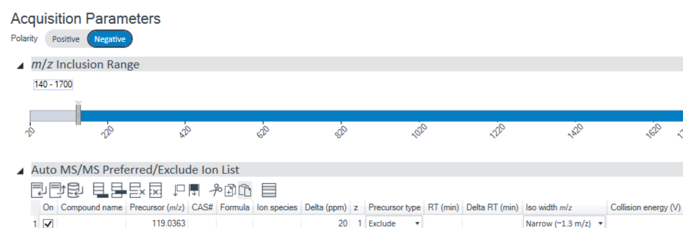

Figure 6. Acquisition Parameters tab in Agilent MassHunter Data Acquisition for TOF/Q-TOF LC/MS software. Selection of the *m/z* range is shown, including precursors for MS/MS and the table of preferred or excluded ions during precursor selection.

In addition to the *m/z* inclusion range, it is important to use a proper exclusion list to prevent unnecessary isolation and fragmentation of reference mass and background ions that originate from the system itself or the solvents.

Four common approaches for defining an exclusion list were tested to show how the exclusion list strategy affects instrument precursor selection. To ensure generation of a relevant exclusion list, knowledge of the system background is mandatory. The system background is also dependent on the application and buffers used. Thus, proper evaluation of a blank injection is needed. As a best practice, an exclusion list should at minimum include reference masses and high-intensity background ions. This simple exclusion list can then be expanded either manually or automatically by using iterative injection logic. Figure 7 summarizes the results of the four tested approaches. Each dot represents a found feature with MS/MS information. Regions appearing as lines are related to constant background ions originating, for example, from solvents or LC hardware.

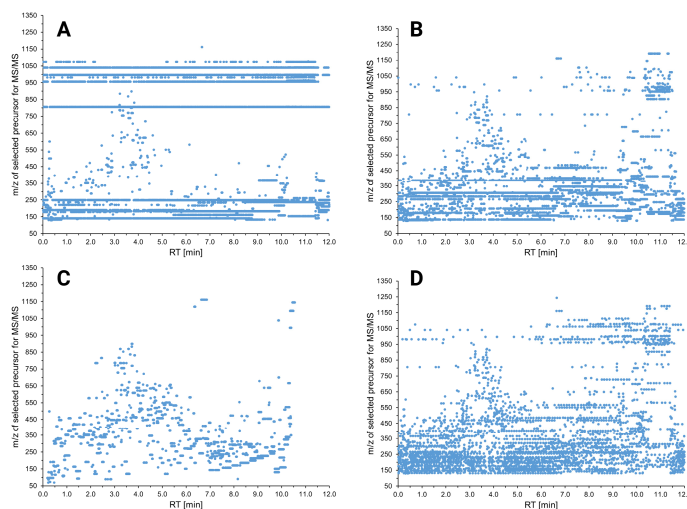

Figure 7. Illustration of four different strategies to set up an exclusion list for DDA experiments. (A) Fixed exclusion list with only the reference masses and high background, (B) static exclusion list from a blank injection, (C) Directed MS/MS with a preferred list of features found in the sample, and (D) dynamic exclusion using iterative injection.

First, a short, fixed exclusion list was used with only the reference masses and acetate anion from the buffer. This approach resulted in selection of redundant system background for MS/MS experiments and thus in a low MS/MS coverage of 12% (Figure 7A and Table 3). MS/MS coverage was calculated as a percentage of the number of features found in MS1 that have corresponding MS2 information. Second, a detailed, fixed exclusion list was used. This exclusion list contained the accurate *m/z* of the selected precursor and retention time (RT) of a blank injection (Figure 7B, Table 3). This approach was highly effective at removing the redundant triggers and improved MS/MS coverage. In the third approach, a preferred list, created from all features found in the sample with Directed MS/MS acquisition, was used for DDA. This again resulted in improved isolation of relevant precursors and thus better MS/MS coverage (Figure 7C, Table 3). The fourth approach employed fully untargeted DDA by Auto MS/MS and used a dynamic exclusion list generated by the iterative function. Here, a single blank injection was used to start building the exclusion list prior to the sample injection. This approach achieved the highest MS/MS coverage of 41% in a single-sample injection and required the lowest amount of manual work (Figure 7D, Table 3). Ultimately, by spending more cycle time on relevant compounds in the plant extract, the MS/MS coverage for a single-sample injection was increased from 12% to 41% in the measured plant extracts (see Table 3). Still, MS/MS coverage can be improved even further by optimizing the full set of DDA method parameters and by using an iterative injection logic implemented in the worklist through the intelligent reflex workflow.

Table 3. Summary of tested exclusion list approaches and their impact on MS/MS coverage for single-sample injections of plant extract.

Background Exclusion	Approach	Features Found	MS/MS Coverage (% of MS1 Features with Corresponding MS2)
Figure 7A	Reference mass exclusion only	1,471	12%
Figure 7B	Static exclusion from blank	1,353	29%
Figure 7C	Directed MS/MS	1,569	27%
Figure 7D	Dynamic iterative exclusion	1,533	41%

Ultimately, by selecting the proper acquisition mode, in combination with a list of preferred or excluded ions, the density of biologically relevant information is enriched, and more relevant MS/MS data is acquired for metabolite annotations and identification. To generate a preferred list of relevant precursors for the biological question, statistical methods such as fold-change analysis and analysis of variance (ANOVA), among others, as well as knowledge of compound classes of interest, are most valuable. In group comparison studies, a relevant preferred list can be created, for example, after running pooled condition samples in MS1 only and using ANOVA to extract relevant features for group discrimination. Alternatively, knowing what relevant compounds are (for example, polyphenols) can also inform preferred list creation.

Precursor Selection tab: Active exclusion and duty cycle

In the Acquisition Parameters tab, there is a section to specify the rules for precursor selection (Figure 8). The maximum number of precursors per cycle needs to be selected in consideration of MS1 and MS2 spectral acquisition rates, the number of collision energies, whether active exclusion is used, and the chromatographic peak width. These parameters need to be considered in parallel for optimization of the Auto MS/MS duty cycle on the Q-TOF. This is important to maximize the information obtained from a sample and to ensure sufficient data quality, for example, by providing sufficient data points across the chromatographic peak.

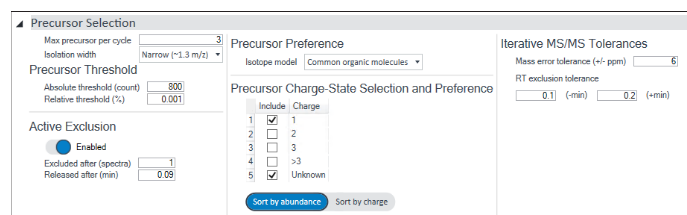

Figure 8. Precursor Selection tab showing the decision engine settings for precursor selection.

The DDA duty cycle (Figure 9) on the Q-TOF starts with acquiring an MS1 spectrum, from which the maximum number of precursors are selected according to the rules defined in the Precursor Selection tab. After this MS1 spectrum acquisition, the instrument switches to MS2 and acquires MS2 spectra for the defined number of collision energies for the first precursor. Thereafter, the instrument switches to the next precursor and the process is repeated. Switching from MS1 to MS2 and back requires a fixed, instrument-specific amount of time, referred to as overhead time. After acquiring all the MS2 spectra in the cycle, the instrument switches back to MS1, and the next cycle starts again with an MS1 spectrum acquisition.

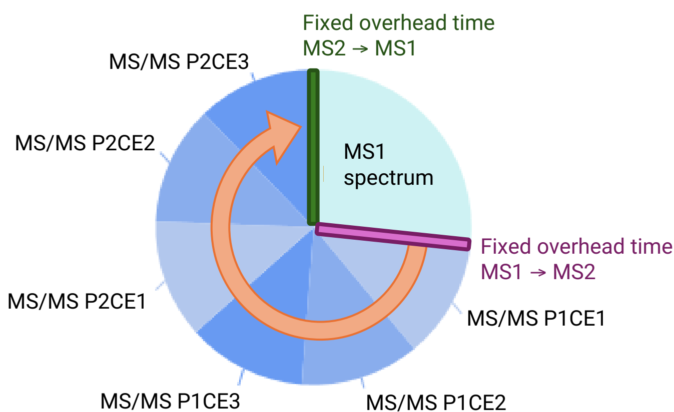

Figure 9. Illustration of a duty cycle with two precursors (P) per cycle and three different collision energies (CE).

For analysis of plasma metabolites, three collision energies (10, 20, and 40 V) were used. These collision energies were chosen to cover the range in which informative fragmentation of small-molecule metabolites is expected. These energies are also curated in the Agilent spectral library for metabolites. For DDA with HILIC chromatography, a good starting point was a maximum of three precursors per cycle, with an MS1 acquisition rate of 6 Hz and an MS2 rate of 10 Hz. The robust HILIC method provided an average baseline peak width of 10 s (standard deviation ±5 s, median 9 s), which resulted in an estimated number of 9 points across the LC peak. In MassHunter Data Acquisition version 12 or later, a cycle time calculator is available (Figure 10). This calculator assists in optimizing the duty cycle and ensuring enough data points across the chromatographic peak.

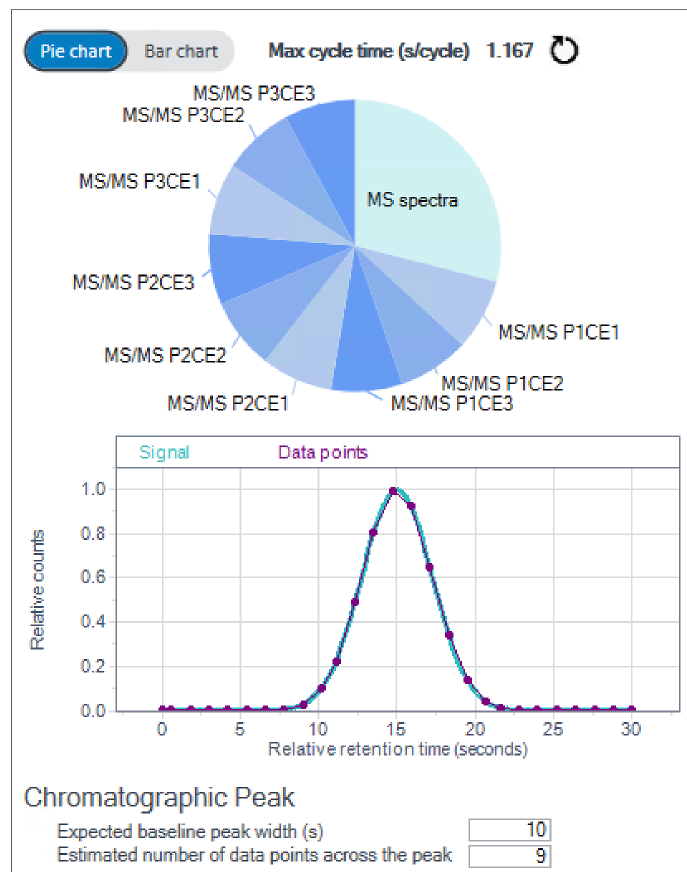

Figure 10. Cycle time calculator, including an estimated number of data points across the chromatographic peak.

Using this calculator, the number of points across the LC peak was calculated based on the MS2 rate, number of discrete collision energies, and maximum number of precursors per cycle. Table 4 summarizes the results. At a moderate MS2 acquisition rate of 25 Hz, increasing the number of precursors per cycle improves duty cycle efficiency by better using overhead time, while coverage of the chromatographic peak is still preserved. When the MS2 acquisition rate is increased to 25 Hz or higher, the number of precursors per cycle should also be increased. However, increasing the rate of spectral acquisition comes with a reduced number of transients per spectrum and thus lower signal intensity. If the number of ions is especially low, mass accuracy may also be limited due to poor ion statistics. It is therefore advisable to acquire data at a rate that is fast enough, but not faster than necessary. The trade-offs of this relationship are shown and discussed later in this technical overview.

Table 4. Optimization of the duty cycle by varying MS1 and MS2 spectral acquisition rates and the maximum number of precursors per cycle. A total of three fixed collision energies (10, 20, and 40 V) and a peak width (base-to-base) of 10 s were assumed for the calculation.

MS1 Rate (Hz)	MS2 Rate (Hz)	Maximum Number of Precursors per Cycle	Estimated Number of Data Points Across the Peak
6	10	3	9
		6	5
6	25	3	18
		6	12
6	50	3	25
		6	18
		9	13

Next to the maximum number of precursors per cycle, there is the option to enable active exclusion in the Precursor Selection tab (Figure 8). The precursors are sorted either by abundance or charge state (Figure 8), after which "n" precursors per cycle are selected based on this sorting. It is important to properly select charge states because charges that are not selected here are ignored for precursor selection. If no charge state can be determined, this analyte is also ignored unless unknown charge state is selected.

If active exclusion is turned off, the same set of precursors will be isolated in several consecutive cycles (Figure 11C), which reduces the overall MS2 coverage. A common strategy is to enable active exclusion and set it in a way that the MS1 peak is triggered at the beginning, the apex, and the end (Figures 11A, 11B, 11D, and 11G). Using active exclusion for Auto MS/MS ensures the collection of high-quality spectra while also increasing the diversity of precursors sampled within a given time window.

The precursor should be excluded after one or two spectra and released at the average or median baseline half-peak width. A common practice is to use an active exclusion time that is one-third to one-half of the total chromatographic peak width (Figures 11A, 11B, 11D, 11E, and 11G–I). It is also important to set the precursor threshold accordingly to enable early triggering of the peak (Figures 11G, 11H, and 11I). A good starting point is to set the precursor selection threshold (Figure 8) approximately a factor of three above the MS1 noise region.

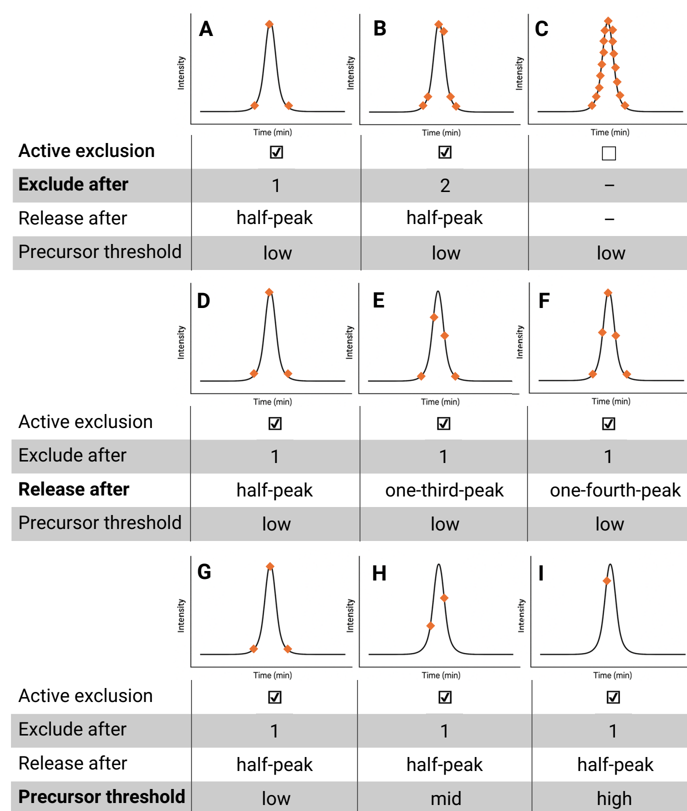

Figure 11. Simulation of different parameters for active exclusion in combination with the precursor threshold. Orange squares mark Auto MS/MS trigger events. Settings for active exclusion and precursor threshold are given below the chromatogram. Bold text indicates the parameters that were varied within each row.

Figure 12 illustrates trigger behavior when active exclusion is applied—excluding a precursor after a single MS/MS event and releasing it at the average baseline half-peak width. In a biological sample with hundreds to thousands of extracted features, active exclusion usually entails a compromise between sharp signals at narrow peak width and broader peaks. Figure 12 highlights examples of the typical trigger behavior for signals with different peak widths and heights. Narrow peaks with a baseline peak width below the application's average baseline peak width (Figure 12A)

will be triggered only once, while broad peaks above the average peak width (Figure 12C) will be triggered multiple times. Compounds with a baseline half-peak width near the average will show the desired trigger pattern as illustrated in Figure 12B.

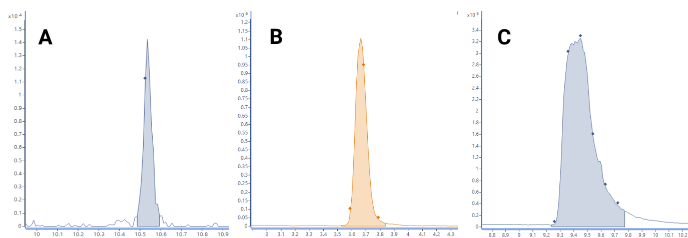

Figure 12. Extracted ion chromatogram (EIC) for different peaks in the HILIC application. (A) A sharp signal with a narrow peak width below the average is triggered only once. (B) A peak at average half-peak width shows the desired trigger pattern and is triggered three times. (C) Peaks above the average baseline peak width with a broad shape will be triggered multiple times. The diamond symbol indicates the cycle in which the precursor was isolated and fragmented for MS/MS.

TOF Acquisition Parameters tab: Acquisition rate

Finally, the spectral mass range for MS1 and MS2 are specified in the TOF Acquisition Parameters tab, along with the spectral acquisition rate (Figure 13).

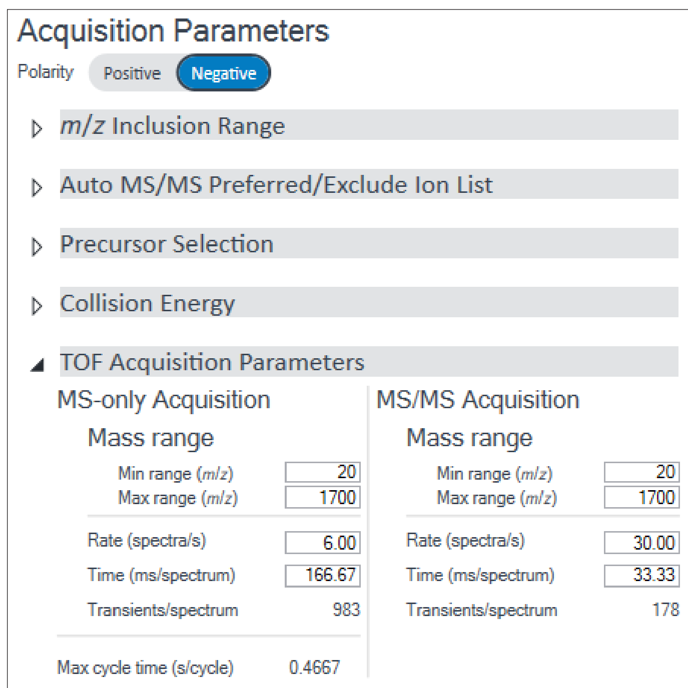

Figure 13. TOF Acquisition Parameters tab with settings for spectral mass range and acquisition rate for MS1 and MS2.

As is characteristic of time-of-flight mass analyzers, resolving power and mass accuracy of the Revident LC/Q-TOF are not diminished by increasing spectral acquisition rates. Thus, if there is sufficient initial precursor abundance, a DDA metabolomics experiment with three distinct collision energies benefits from a reduced cycle time by increasing the MS2 spectral acquisition rate without compromising spectral quality. It is still important to consider that, for low-intensity compounds, the lower-intensity fragment ions will disappear in the noise region when increasing the MS2 acquisition rate. Thus, for low-abundance features there is a trade-off between the MS2 acquisition rate and the spectral Library Score, which is demonstrated in Figure 14. Here, MS1 acquisition rate was fixed at 6 Hz and MS2 rate was 5 (Figure 14C), 25 (Figure 14B), or 50 Hz (Figure 14A). Overall, resolution and mass accuracy are stable, while the Library Score decreases due to a loss of low-intensity fragments (for example, at *m/z* 58.0298 and 87.0564 for anserine). Figure 15 summarizes the overall trend of Library Scores based on the MS2 spectral acquisition rate for all metabolites spiked into the extracted plasma. Overall, Library Scores decrease with increased spectral acquisition rate. Still, a high number of metabolites had a library score above 75, indicating a positive hit (Figure 15).

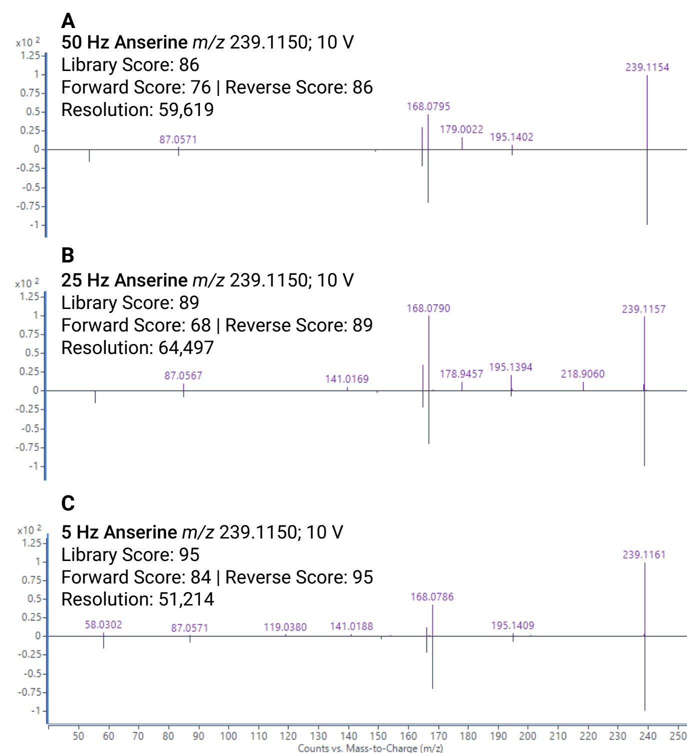

Figure 14. Resolution and library scores based on MS/MS acquisition rate for anserine. Mirror plots of the acquired spectrum (top, colored) against the library (bottom, black) for 10 V collision energy are shown.

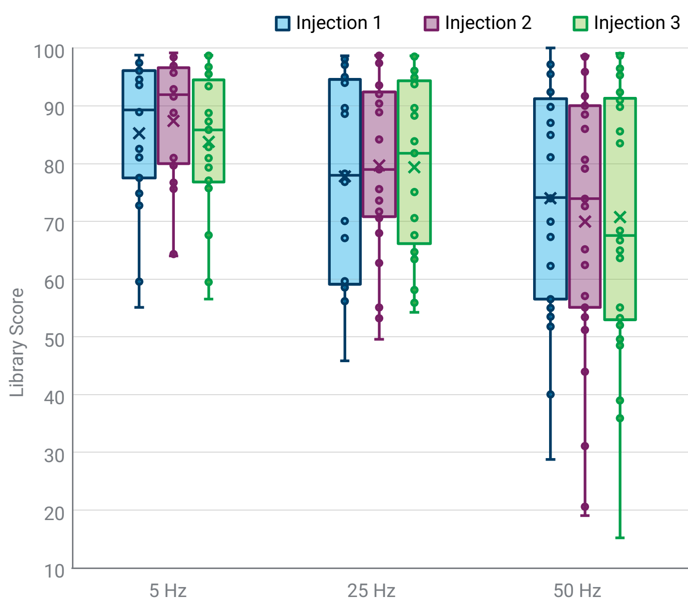

Figure 15. Boxplots showing Library Scores as a function of MS/MS acquisition rate for all spiked metabolite standards. MS1 acquisition rate was fixed at 6 Hz, and three technical replicates were measured for each acquisition method. In the boxplots, a cross symbol represents the mean, and a horizontal line represents the median of the distribution.

The Revident LC/Q-TOF supports acquisition rates up to 50 Hz for both MS1 and MS2. At faster rates, fewer TOF transients are summed per spectrum, resulting in fewer ions sampled and a corresponding decrease in absolute signal intensity per spectrum. Therefore, absolute precursor threshold, found in the Precursor Selection tab (Figure 8), should generally be decreased at faster MS1 acquisition rates and adapted to the new noise level. The relative precursor threshold is calculated based on the base peak intensity within each spectrum, making it a dynamic value that adjusts with signal variability. Depending on the application, either a static (absolute) threshold or a dynamic (relative) threshold may yield more reliable precursor selection. Choosing between them is dependent on the application and should be guided by expected signal stability and the complexity of the sample. Here, for negative HILIC with an acetate buffer, the acetate peak was the base peak and saturated in every spectrum. Thus, the absolute threshold was preferred, and the relative threshold was set as low as possible.

Advanced Parameters tab: Data storage and variable acquisition rate

Next to the Acquisition Parameters tab is the Advanced Parameters tab (Figure 16), which provides access to configuration options for data storage, variable acquisition rate, and precursor purity.

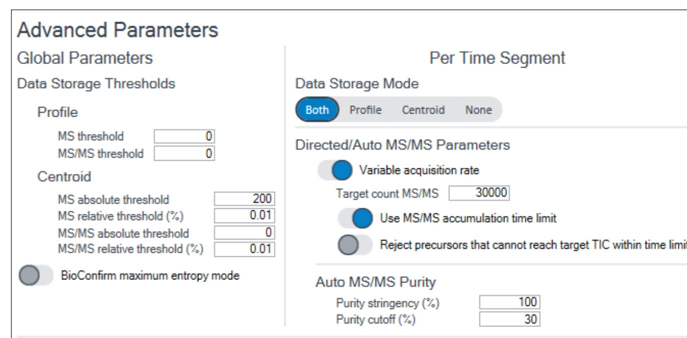

Figure 16. Advanced Parameters tab, with additional settings for data storage, variable acquisition rate, and purity for precursor selection in MS1.

Data storage thresholds help optimize file size by excluding low-intensity or noise-dominated signals. Thresholds should be set to balance data completeness and file size. The thresholds are absolute intensity counts below which data are either not saved (profile threshold) or converted into centroided data (centroid threshold). It is therefore recommended to set the profile threshold to zero, set the centroid thresholds sufficiently low, and select "both" as the data storage mode. For setting the centroid threshold, knowledge of the noise region in the profile data is mandatory. The noise region is assessed by extracting a profile spectrum and zooming in to the lower-intensity region. Figure 17 illustrates how to identify the noise region.

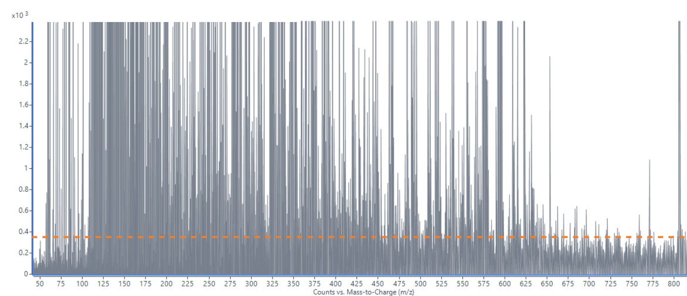

Figure 17. Extracted profile spectrum zoomed to *m/z* 50–800, with intensity below 2×10^3. The dotted line represents the estimated noise level for MS1 spectrum acquisition at 6 Hz.

In this work, the relative centroid threshold for MS1 and MS/MS was set at 1×10^{-4}%, and the absolute thresholds were set to 100 for MS1 and 5 for MS/MS data centroiding. When using Agilent SureMass conversion, it is important to set the profile data storage thresholds to zero, because any thresholding of profile data will compromise the SureMass algorithm.

Next to the data storage settings, there are two additional Auto MS/MS method parameters: variable acquisition rate and purity settings. Auto MS/MS purity settings are covered in another technical overview (5994-0322EN).[7] In brief, the purity stringency and cutoff help reduce unwanted chimeric MS2 spectra. The Auto MS/MS purity calculates a signal-to-noise ratio (S/N) within the isolation window of the quadrupole. The purity stringency (%) uses this S/N ratio to rank a precursor. When set to 100%, high-purity precursors tend to move higher on the precursor ranking, despite the "Sort by abundance/Sort by charge state" settings in the Precursor Selection tab (Figure 8). When set to 0%, purity ranking is disabled completely, but the purity scores are still calculated, and the purity cutoff is still in effect. The purity cutoff (%) is the minimum threshold for the purity stringency, below which a precursor candidate will be rejected. Lower purity stringency and cutoff permit more co-isolated ions, leading to more chimeric spectra. Using a narrow quadrupole isolation width (*m/z* ~1.3), a purity stringency of 100% and a 30% cutoff were chosen for this study.

The variable acquisition rate adjusts the MS2 acquisition rate depending on the MS1 precursor abundance. If this option is not selected, the rate is fixed to the settings in the TOF Acquisition Parameters tab (Figure 13). If variable acquisition rate is enabled, then the MS2 acquisition rate is adjusted for each precursor separately, based on its MS1 abundance. Thus, for high-abundance precursors, MS2 is acquired faster, with a smaller number of transients than for precursors of lower abundance in MS1. If the MS/MS accumulation time limit is enabled, the instrument will not acquire MS2 more slowly than what is specified as the MS/MS acquisition rate in the Acquisition Parameters tab (Figure 13), even if the target MS/MS count (Figure 16) is not reached. Additionally, the option to reject precursors that cannot reach the target count within the time limit can be activated to avoid generating MS/MS spectra for low-abundance precursors, which are unlikely to generate a quality spectrum. The time limit in this case is the MS/MS acquisition rate specified in the Acquisition Parameters tab (Figure 13). If the variable acquisition rate is used to allow the Q-TOF system to speed up during MS2 acquisition, observed cycle times will often be shorter in comparison to using a fixed MS2 acquisition rate. The target count specifies the desired total ion count in the MS2 spectrum, which is reached by adjusting the MS2 acquisition rate. For generation of meaningful MS2 spectra for small molecule metabolomics, a target count of 30,000 or higher resulted in good spectral quality. However, it is important to note that when the variable acquisition rate is enabled, the instrument does not currently reach the highest possible MS2 acquisition rate with MassHunter Data Acquisition version 12.1. Instead, a minimum limit of 200 transients is used. Moreover, the variable MS2 acquisition rate will also affect the MS2 noise level, which needs to be considered when setting the data storage thresholds in the Advanced Parameters tab (Figure 16).

Auto MS/MS and Directed MS/MS in single and iterative injection workflows

After optimizing the Auto MS/MS method parameters for metabolite analysis in plasma using the established HILIC metabolomics workflow, performance was assessed in terms of MS2 coverage. MS2 coverage was calculated as the percentage of features found in MS1 that have corresponding MS2 spectra. Auto and Directed MS/MS acquisition modes were used in single and iterative injection workflows. Iterative MS/MS is a worklist-enabled feature of the MassHunter Data Acquisition software. To increase MS2 coverage, a sample is injected multiple times, and precursors previously selected for MS/MS fragmentation are excluded from subsequent injections on a rolling basis. The function and logic of iterative MS/MS are described in studies that also demonstrate its value for increasing MS2 coverage in peptides[8] and lipids[9].

For this technical overview, three different fixed MS2 acquisition rates were tested with variable acquisition rate disabled. For single-injection Auto MS/MS, the exclusion list was a detailed, static list generated from a blank injection (approach shown in Figure 7B). For Iterative Auto MS/MS injections, a blank injection at the beginning of the iteration was used for dynamic exclusion (as shown in Figure 7D). Figure 18 shows the resulting MS2 coverages. The highest coverage was achieved at maximum acquisition rate using Iterative Auto MS/MS after n = 3 iterative sample injections. For 5 and 25 Hz acquisition rates, single-injection Directed MS/MS resulted in higher MS2 coverage compared to single-injection Auto MS/MS.

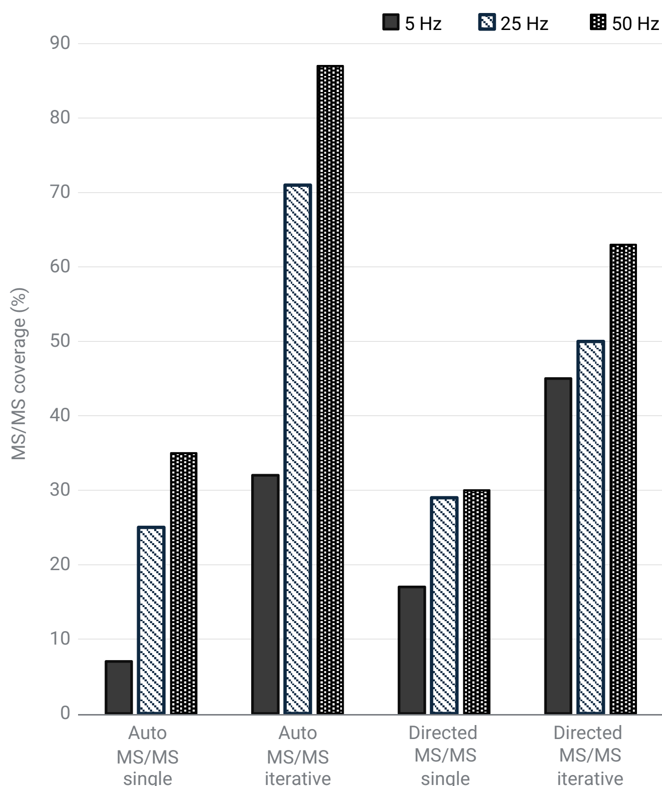

Figure 18. MS/MS coverage, calculated as the percentage of features detected in extracted plasma that have corresponding MS2 spectra, based on fixed MS/MS acquisition rates. Single-injection Auto MS/MS and Directed MS/MS measurements were compared to iterative injections (n = 3 iterations each).

To ensure generation of meaningful spectra, acquired spectra were matched against an in-house build library. The in-house HILIC metabolite library contains information for 558 metabolites, including MS2 spectra for 10, 20, and 40 V collision energies, as well as retention times for measurements using the HILIC workflow on an Agilent Bio LC system in multiomics configuration.[3] As the MS/MS acquisition rate decreased, average Library Scores for acquired Auto MS/MS spectra increased from 46 ± 2 (50 Hz) to 54 ± 1 (25 Hz) and 69 ± 1 (5 Hz). Similarly, the percentage of compounds with high Library Scores (> 85) increased from 13% for 50 Hz to 38% for 5 Hz.

For Directed MS/MS, the same trend was observed for the Library Scores: 43 ± 2 (50 Hz), 46 ± 2 (25 Hz), and 52 ± 2 (5 Hz) and the percentage of compounds with high Library Scores (> 85), which increased from 12% at 50 Hz to 25% at 5 Hz.

Conclusion

Auto and Directed MS/MS are powerful complementary data-dependent acquisition (DDA) modes for untargeted profiling experiments. For generation of high-quality data and to obtain the most information out of a sample, it is critical to understand how the decision engine for precursor selection works and which settings have the highest impact on the results. Any DDA method needs to be optimized according to the chromatographic conditions and overall purpose of the experiment. This technical overview highlights the most important method parameters and provides exemplary data from real sample measurements.

Depending on the purpose of the experiment, Directed MS/MS can be useful for obtaining MS2 information for compounds relevant to the biological question. For example, in semi-targeted discovery analysis, Directed MS/MS may be applied after feature finding and subsequent statistical analysis of an initial experimental batch. Directed MS/MS also demonstrated higher MS2 coverage for a single injection at low to medium MS2 acquisition rates. However, running optimized Auto MS/MS methods with iterative injection logic at the maximum MS2 acquisition rate of 50 Hz resulted in the highest MS2 coverage (~90%). Nevertheless, maximal MS2 coverage does not necessarily correspond to maximal information content, since a trade-off exists between the MS2 spectral acquisition rate and library scores.

The Agilent Revident LC/Q-TOF provides a range of features to assist users in method optimization for DDA. For example, the cycle time calculator assists in optimizing the duty cycle, thereby enabling straightforward collection of high-quality metabolomics data.

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