Supplementary

Data-independent Acquisition for the orbitrap. A tutorial.

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supplementary 1

Calculation of the actual overhead time A and actual transient time.

To calculate the actual overhead time A and the actual transient times, scans were performed in the mass range from *m/z* 400 to *m/z* 1650 (the same mass range as intended for the DIA runs). Ion injection times were measured to confirm that these were substantially lower than the transient.

Table S1: Measured scan times of MS1 at various resolutions.

Resolution	Measured ion injection time (ms)	Measured scan time (ms)**
15000	< 5	39.6
30000	< 5	70.5
60000	< 5	132.6
120000	< 5	256.2
240000	< 5	504

To calculate the actual transient time and actual overhead time A, the following assumptions were made:

1) doubling of the resolution leads to doubling of the transient time

2) the overhead time A is constant in all measurements

Given these assumptions, the overhead time A then could be found as the y-intercept in the linear regression after plotting the data from the table in a scatter plot:

*Figure S1: measured scan time in the MS1 at various resolutions. Equation: Y=0.0021X * 8.6375 Correlation: R2=1*

This y-intercept (8.6375 ms) can then be used to calculate the actual transient times by subtracting this value from the measured scan times.

supplementary 2

Can we use the same approach on the Fusion Tribrid mass spectrometer?

Since there is a difference in geometry between the Fusion Tribrid mass spectrometer and the Q Exactive HF mass spectrometer, the scan times of each of the MS actions will be different. Therefor the scan times and cycle times need to be measured on the Fusion Tribrid mass spectrometer to be able to determine the optimal parameters for a given number of points per peak.

An important difference between these systems is that the Q Exactive HF mass spectrometer is able to analyze the ions in the orbitrap (both in MS1 and in MS2) while in parallel carries out actions like isolation, fragmentation and so on. The Fusion Tribrid mass spectrometer carries out similar parallel actions in the MS2 mode (when the AGC is predicted). However, the MS1 scan starts with the determination of the AGC. This is carried out in the linear ion trap, which is situated after the C-trap. This impacts the scan apparently such that the ion injection time for the MS1 scan needs to be added to the transient time (see figure S3).

It was observed that the AGC target of 3e6 is not reached as quickly or as readily for the Fusion Tribrid mass spectrometer as in the Q Exactive HF mass spectrometer (see table 2a manuscript), the scan time of the full scan single MS increases substantially in an ion poor situation since it will, in most cases, reach the maximum ion injection time before the AGC target is reached. figure S3 shows the effect on the MS1 scan time at resolution 15 000 and 60 000. At both resolutions the scan time increases linearly (and with a slope of 1) with increasing ion injection time. For the 15 000 resolution the overhead time is approx. 35 ms while for the 60 000 resolution this is approx. 19 ms. Since all the actions in a single MS cycle appear sequential, setting long maximum ion injection times will have a substantial impact on the scan time of this full scan single MS in an ion poor situation.

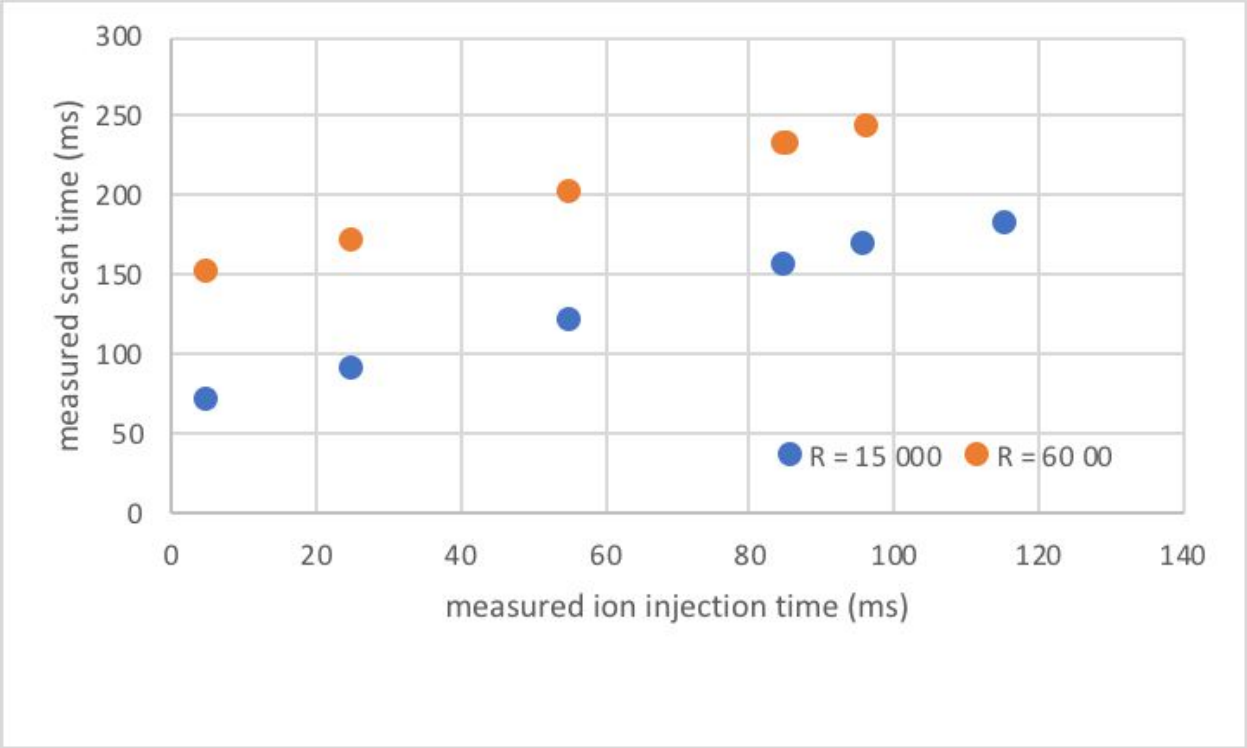


figure S3: influence of the ion injection time on the scan time of the full scan single MS (Fusion Tribrid mass spectrometer). For the ion injection times 85 ms and lower, the maximum ion injection time was reached before the AGC target. At a maximum ion injection time setting of 120 ms and 150 ms, the AGC target was reached before.

The optimal maximum ion injection time in the Fusion Tribrid mass spectrometer is approx. 10 ms more compared to the Q Exactive HF mass spectrometer, (see table S2). This has greater impact at the low resolutions and therewith higher number of isolation widths. At 15 000 resolution, the Fusion Tribrid mass spectrometer can accumulate ions for twice as long (assuming that the AGC is not reached) since the optimal ion injection time was 20 ms (Fusion) compared to 9 ms (Q Exactive). Another observation made was that the first DIA, regardless of the settings, always had an apparent scan time of approx. 11 ms. Although it is not understood why this is represented as such, the fact that it always has the same value makes it easier to predict the total cycle time.

*table S2: Scan times in the MS2. Both narrow isolation widths and wide isolation widths are measure*d.

	Resolution	maximum ion injection time (ms)	Measured scan time narrow isolation width (ms)	Measured scan time wide isolation width (ms)	Optimal maximum ion injection time (ms)	Calculated overhead (ms)
ion injection << transient time	15000	5	41.1	142.2	19.9	N/A
	30000	14	73	181.8	51.9	N/A
	60000	78	137.1	399.6	115.7	N/A
ion injection >> transient time	15000	50	71.2	214.2	N/A	21.2
	30000	80	101.1	301.2	N/A	21.1
	60000	150	171.3	505.8	N/A	21.4

To visualize the differences between the scan cycles of the Fusion Tribrid mass spectrometer and the Q Exactive HF mass spectrometer, compare a full scan cycle of Q Exactive HF mass spectrometer (figure 2) with a full scan cycle of the Fusion Tribrid mass spectrometer (figure S4).

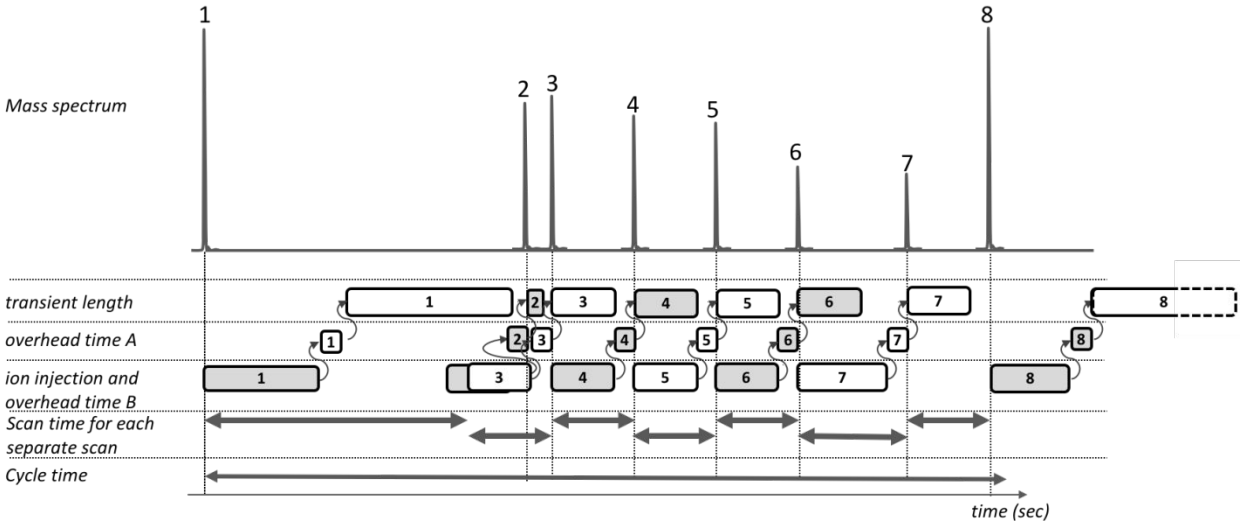


figure S4: Chromatographic representation of a full cycle from a full scan single MS (scan 1) to a next full scan single MS (scan 8) in the Fusion Tribrid mass spectrometer. The cycle consists of a full scan single MS (scan 1) and six sequential isolation windows (scans 1-7). Besides the transient length (performance of the actual scan), the overhead times A and B and the ion injection time determine the length of the scan. Overhead time A is time needed for ions to leave and enter the orbitrap. Overhead time B is the time needed for ion isolation in the quadrupole, ion transport and/or

fragmentation in the HCD except for full scan single MS (scan 1 and scan 8). In these cases the overhead time B consists of AGC determination. It shows that these actions are not parallelized.
Each number belongs to a certain set of ions going through all separate steps. The time point of each scan given in the chromatogram is the time at the start of the transient. The scan times are the time difference between the scans: the full scan single MS time is the difference in time between scan 2 and scan 1. The average scan time for the narrow isolation window is calculated from scan 3 - 6. The time for scan 2 was, regardless of the setting, always constant.The time needed around the wide isolation window is the difference between scan 8 (start of the next cycle) and scan 6.

Constructing a DIA method for the Fusion Tribrid mass spectrometer can be carried out using the same strategy as used for the Q Exactive HF mass spectrometer now by using data from figure S3, table S2 and figure S4. For a method with resolution of 60 000 in the full scan MS, and a resolution of 15 000 and the optimal maximum injection time (19.9 ms) in the MS/MS (narrow isolation windows in the *m/z* range 400 to 900, one wide isolation window from *m/z* 900 to 1650 - isolation width) and 2 sec/point.
Since approx. 180 ms is used by the full scan MS (at a resolution of 60 000, 118 ms ion injection time), 11 ms for the first DIA and approx. 400 ms is used for the wide isolation window (that is the total time of the scan preceding this isolation window and the scan time of the isolation window itself), 1409 ms is left for narrow isolation windows. If the optimal ion injection time of 19.9 ms in the MS2 is used (this means a scan time of 41.1 ms) that method will consist of 36 narrow isolation widths and 1 wide isolation width.

supplementary 3

The time the quadrupole needs to select ions for further mass spectrometric analysis contributes to the overhead time B. This quadrupole selection time increases (discontinuously) when the isolation width of a window increases. This becomes clear after measuring the scan times (with ion injection times lower than the transient) of MS/MS scans with a center at *m/z* 500 and a center at *m/z* 1200. The figure below shows a plot of the measured scan times with varying isolation widths. Having the center at *m/z* 500 (red line), the scan times are approx. 41 ms having the isolation width below *m/z* 140 while they were approx 89 ms having the isolation width above *m/z* 145. Having the center at *m/z* 1200 (blue line), the scan times are approx. 43 ms having the isolation width below *m/z* 339 while they were approx 92 ms having the isolation width above *m/z* 340.

These data suggest that all the isolation windows (widest isolation width in this range < *m/z* 85) with an isolation center in the lower mass range (< *m/z* 900) as described in the paper should be treated as narrow isolation windows. It also suggests that the isolation window with the isolation center in the higher mass range (*m/z* 1275) and an isolation width of *m/z* 751 should be treated as wide isolation window.

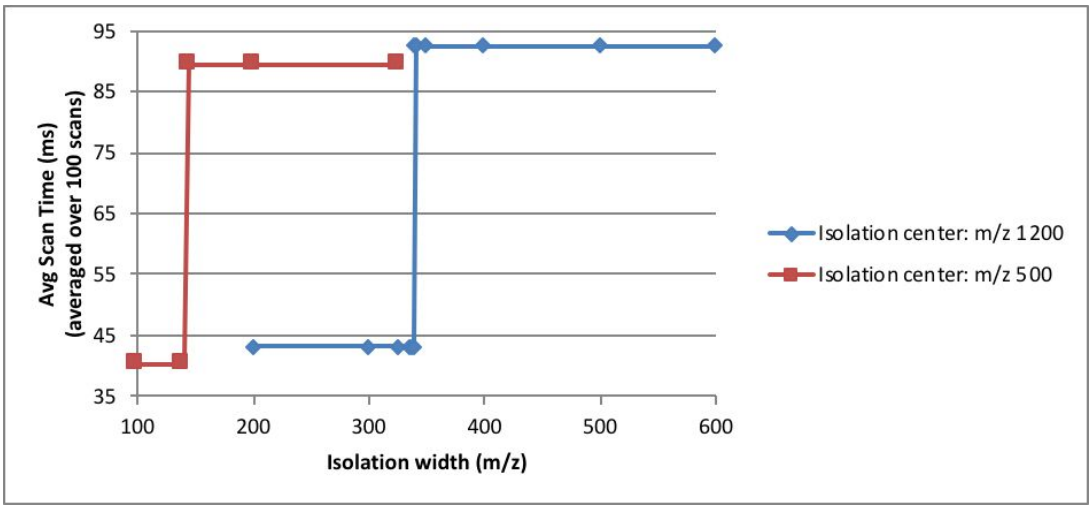


Figure S2: average scan time of an MS/MS scan for two isolation windows (at m/z 500 and m/z 1200) and varying isolation widths

supplementary 4

Description of the model-free method for calculating number of SWATHs and maximum injection times

The alternative method described in this supplementary is based on measurement of whole cycle times. So, in contrast to the strategy described in the main paper, the time of the narrow or wide SWATHs is determined by measuring timepoints always at the beginning of the cycle (in this case the timepoint for the MS1) whereas in the strategy described in the main paper is based on the measurements of the separate scan events within a cycle.

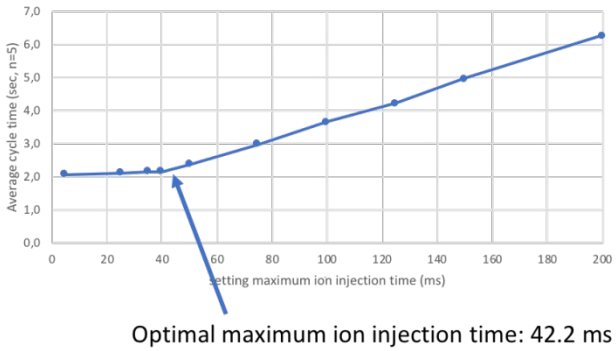
Determination of the number of SWATHs

After the cycle time and resolutions of the MS1 and MS2 spectra have been decided, one must then determine how many SWATHs can be performed within the target cycle time. To estimate this, two generic DIA methods are constructed, each with an MS1 full scan, one with 10 narrow MS2 and one with 11 narrow MS2, and each with a wide MS2. For each scan in these methods, the maximum injection time should be 5 ms. These methods should be run for a short duration in ion limited conditions (as described earlier), so that the cycle time (time between MS1 spectra) can be measured. In all measurements of cycle time, it is recommended that times be averaged over multiple cycles and that the ion injection times for every scan is at the maximum (5 ms). After these times have been measured, one can use regression to fit a simple linear model with a y-intercept of the cycle time based on the number of narrow MS2 spectra. It is then possible to calculate the number of narrow MS2 spectra that can be acquired in the target cycle time.

Determination of the optimal maximum injection time

Given all of the previous parameters plus the number of narrow MS2 spectra, one must then determine the maximum ion injection times. To this, the generic DIA method is updated with the calculated number of narrow MS2 spectra, and with maximum injection times still at 5 ms, a baseline cycle time is measured from a short duration run in ion limiting conditions. One can then increase MS1 maximum ion injection times until there is an increase in cycle times. If the ion injection times are plotted against the cycle time, a graph that similar to that of supp figure S5 should appear, where initial increases of MS1 ion injection times have little to no impact on cycle times, then there is a linear increase in cycle time. The maximum MS1 ion injection time can be found at the elbow in this graph, where the increase in cycle time goes from minimal to linear.

The same procedure can be repeated for the MS2 maximum ion injection times (see figure S5 for a typical graph and optimal maximum ion injection times). Note that while it is possible to optimize the narrow and wide ion injection times independently, we found that on the Q Exactive HF any increase in ion injection time for the wide MS2 spectrum resulted in an increase in total cycle time. We therefore chose to optimize the ion injection times simultaneously for both the narrow and wide MS2 maximum ion injection times.



	Strategy from main paper	Strategy from supplementary
15 000	9	9.1
30 000	41	42.2
60 000	106	105.9
120 000	Not measured	240.3

Figure S5: *Graph on the left hand side: Average cycle times measured in an ion poor situation plotted against the set maximum ion injection time at 30 K resolution (MS2). The elbow is situated at the optimal maximum ion injection time. In the table the optimal maximum ion injection times for both strategies are shown.*

supplementary 5

Acquisition sequence of the experiments.

Sequence order for analyses Table 3. Each run was performed in triplicate (technical triplicate, right after each other)		
Order	Resolution MS1/MS2	# isolation windows
1	60/15	43
2	60/30	24
3	60/60	12
4	60/15	12
5	60/30	12
6	60/15	6
7	60/30	6
8	60/60	6
9	60/15	24
10	60/120	6

Sequence order for analyses Figure 4. Each run was performed in triplicate (technical triplicate, right after each other)		
Order	DxA (time)	MS2 resolution / # isolation windows
1	DDA (60 min)	N/A
2	DIA (60 min)	15/24
3	DDA (30 min)	N/A
4	DIA (30 min)	15/24
5	DDA (15 min)	N/A
6	DIA (15 min)	15/24
7	DIA (60 min)	60/12
8	DIA (30 min)	60/12
9	DIA (15 min)	60/12