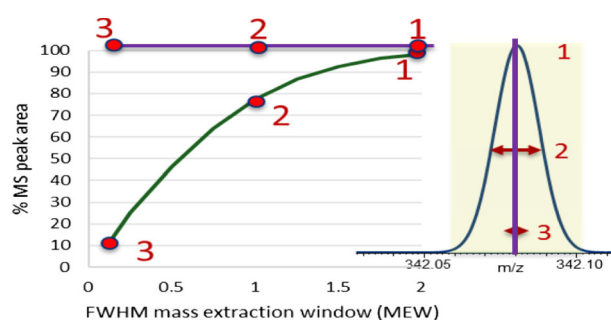


High-Resolution Mass Spectrometry Quantification: Impact of Differences in Data Processing of Centroid and Continuum Data

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Abstract. High-resolution mass spectrometry (HRMS) in full scan mode acquires all ions present in the sample of interest offering a lot of qualitative information. This, in combination with the improved performance of the new generation HRMS systems, triggers more (bio) analysts to switch from triple quad MS systems to HRMS for quantitative analysis. Quantitative processing of HRMS data is performed based on narrow mass extraction windows rather than on nominal mass

product ion chromatograms (SRM or MRM). Optimal processing of HRMS data requires different considerations and software tools and can have an impact on data processing and final results. The selection of centroid versus continuum/profile data for processing, selection of the optimal narrow mass extraction window, using theoretical versus measured accurate mass for the extraction of the ion chromatograms as well as differences in calculations and data handling residing in the different vendor software packages are tackled in the presented manuscript. These differences are illustrated on HRMS data acquired for the same plasma samples on three different platforms, i.e., a Sciex QToF, a Waters QToF, and a Thermo Orbitrap system, and processed in four different software packages, i.e., Sciex Analyst® TF, Waters Masslynx, Waters Unifi, and Thermo Xcalibur. The impact of these differences on quantitative HRMS performance was evaluated on calibration curves of eight small molecule compounds in plasma using four different ways of processing. Simple guidelines are provided for the selection of an optimal mass extraction window for continuum and centroided data.

Keywords: High-Resolution Mass Spectrometry, Mass Extraction Window, Profile data, Continuum data, Centroid data, Quantification, Q-ToF, Orbitrap

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Introduction

Quantitative analysis of small molecules plays a significant role in many research disciplines. In the pharmaceutical industry, liquid chromatography (LC) coupled to a triple quad mass spectrometer (MS) is in general the standard approach for quantitative analysis because excellent robustness, selectivity, and sensitivity can be achieved. Nevertheless, high-resolution mass spectrometry (HRMS) has been piloted as alternative plat-

form for quantitative analysis [1, 2]. On a triple quad mass spectrometer, quantitation is realized in selected or multiple reaction monitoring mode (SRM/MRM) while in HRMS, the extracted chromatograms are only generated post-acquisition. HRMS allows both full scan and product ion scan workflows for quantitative analysis. These instruments have the potential to become of greater interest in quantitative analysis in different fields of pharmaceutical research, e.g., peptide analyses and quan-qual approaches [3–5]. The newest generation high-resolution MS instrumentation offers not only higher resolution and better mass accuracy, but also better sensitivity and a larger dynamic range. In HRMS full scan mode, information on metabolites and/or biomarkers can be simultaneously obtained with the quantitative data [5–9]. This qualitative information can be extracted at a later stage

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through retrospective analysis of the data. Also, background monitoring (e.g., phospholipids) to prevent matrix effects during method development is simplified. In protein and peptide analysis, selection of the most optimal proteotypic peptide following digestion and mass extractions based on the isotope pattern of multiple charged ions are other examples of applications that are facilitated on these instruments [10].

An additional advantage of HRMS full scan mode is the reduced mass spec method development time, and particularly, the enhanced sensitivity when fragmentation efficiency is poor. HRMS quantitation has also been introduced in the validated arena to support concentration analysis in preclinical and clinical programs [11–14].

A key component for quantitative analysis of HRMS data is selection of the mass extraction window (MEW) to construct the extracted mass chromatogram. The narrow MEW (nMEW) defines the selectivity of the assay and is dependent on the mass resolving power of the instrument and the stability of the accurate mass measurements (both scan-to-scan and chromatogram-to-chromatogram stability) which has significantly improved on the latest generation HRMS instruments. Kaufman et al. have described that HRMS in full scan mode outperforms triple quad MRM methodology in terms of selectivity only if $res > 50,000$ is achieved [15].

For all the described HRMS approaches, whether full scan or MRM, justification of the selection of the MEW is critical in avoiding false positive results. In current bioanalytical method validation guidelines, specific guidance for critical parameters when using HRMS is lacking and uncertainty on the acceptance of regulators currently prevails [16]. A manuscript by Sturm et al. provides some guidance to implement HRMS analysis on the Q Exactive™ series MS instruments in regulated quantitative bioanalysis [17]. Roachat et al. and Glauser et al. have presented a proposal to investigate key parameters such as mass accuracy (MA) and mass extraction window [18, 19]. Based on an overall MA analysis, rational MEWs are calculated. These investigators also show that based on resolution and narrow MEWs, HRMS can be more selective than triple quad MS analysis. The recommendations proposed in these previous studies are well substantiated, but—at least in our perception—complex and laborious for widespread application. Therefore, we provide a lean strategy to select an optimal MEW for both continuum and centroid data, based on the resolution and mass accuracy of the instrument.

These guidelines will allow new HRMS scientists to select in an easy way the optimal mass extraction windows on the acquired high-resolution MS data. In this study, midazolam has been analyzed on three HRMS platforms of three different vendors. Extracted ion chromatograms with variable MEWs in centroid and continuum mode are compared. Based on the findings, in combination with a theoretical exercise on interference impact, recommendations are provided on the use of nMEW when high-resolution MS data are processed.

In addition, this manuscript reveals the differences in processing HRMS data by software packages of the different vendors. The effect of resolution and the selection of the MEW on sensitivity (lower limit of quantification) and

precision was evaluated by analyzing calibration curves of 8 small molecules spanning a mass range of m/z 200–800 on a Synapt G2-S instrument. Post-acquisition, different MEWs, and processing approaches were evaluated, and the accuracy and precision parameters were calculated.

Experimental Section

Chemicals and Materials

Mouse plasma was purchased at Bioreclamation (Hicksville, NY, USA). Loperamide, tolbutamide, abiraterone, and simeprevir were synthesized internally at Janssen Pharmaceutica (Beerse, Belgium). The midazolam standard was obtained from Sequoia Research Products (Pangbourne, UK). Acetaminophen and norethindrone were obtained from Sigma (Steinheim, Germany). Prednisone was purchased from Upjohn (Kalamazoo, Michigan, USA). Acetonitrile ULC/MS was purchased from Biosolve (Valkenswaard, The Netherlands); methanol spectroscopic grade, dimethylsulfoxide (DMSO) analytical grade, formic acid 98–100%, and ammonium acetate fractopur were obtained from Merck (Darmstadt, Germany). High purity water was obtained with a Milli-Q Reference Ultrapure Water Purification System (Merck Millipore, Billerica, MA, USA).

Preparation of Stock Solutions and Samples

A stock solution of acetaminophen, tolbutamide, norethindrone, midazolam, abiraterone, prednisone, loperamide, and simeprevir was prepared in DMSO at a concentration of 1 mg/mL; afterwards, combined dilutions were made in DMSO in a range of 0.05–20,000 ng/mL and spiked to blank mouse EDTA plasma. A protein precipitation was performed with four volumes of acetonitrile. The supernatant of each calibration level was injected 5 times on each instrument. For quantitative evaluation, each analytical run was processed and linear regression was applied following log-log transformation. Calibration points were accepted when % relative error (RE) of the back-calculated value was $\leq 20\%$ and % coefficient of variation (CV) per calibration point was $\leq 20\%$.

Liquid Chromatography–High Resolution Mass Spectrometry

All instruments were coupled to an Acquity UPLC system (Waters, Millford, Boston, USA). Single injections of 5 μ L were made on an Acquity UPLC BEH C18 2.1 \times 50 mm (1.7 μ m particles) column held at 55 °C at a flow rate of 600 μ L/min. The gradient consisted of an isocratic start at 2% solvent B (acetonitrile/methanol v/v ; 80:20) and 98% solvent A (ammonium acetate 0.01 M) for 0.5 min followed by a gradient to 95% B in 3 min and an isocratic hold for 0.8 min with a total run time of 4.5 min. Full calibration curves were injected using the same parameters but with a slightly different gradient, e.g., an isocratic start at 5% solvent B (acetonitrile) and 95% solvent A (ammonium acetate 0.01 M) for 0.6 min followed by a gradient to 95% B in 1.4 min and an isocratic hold for 0.5 min with a total run time of 3 min.

All systems were equipped with electrospray ionization (ESI) operated in the positive ion mode. Full scan analysis was performed within the mass range of m/z 100–1200. On the Synapt G2-S (Waters, Wilmslow, UK), external lock mass correction (at 30 s time interval) was performed with Leucine-Enkephaline at m/z 556.2771. Continuum data were acquired in resolution mode (resolution 20,000 at m/z 956) at a scan time of 0.2 s.

On the Q Exactive™ (Thermo, Bremen, Germany), an internal lock mass correction was performed using polydimethylsiloxane (m/z 445.1200) and diisooctyl phthalate (m/z 391.2843) background ions. Continuum data were acquired with a resolution of 35,000 (at m/z 200). The maximum injection time was 0.2 s with automatic gain control at $1e^6$.

Samples were also analyzed on a TripleTOF® 6600 (ABSciex, Toronto, Canada) using the DuoSpray™ source. The atmospheric pressure chemical ionization (APCI) probe was used for the Calibrant Delivery System (CDS) for external calibration every five injections in an analytical batch. Continuum data were acquired in the time of flight (TOF)-MS mode at a resolution of 35,000 (at m/z 956) and accumulation time of 0.2 s.

Data Processing

MassLynx v4.1 SCN 916 (Waters) was used for data acquisition on the Synapt G2-S. Within the software, an option for post-acquisition transformation to centroid data via automatic peak detection is present. However, the exact parameters used for the transformation of the entire chromatographic run are not clear. The transformed file can be stored and used afterwards. The MassLynx acquired data were also imported in Unifi 1.8.1 (Waters) and TargetLynx v4.1 (Waters) for further processing. For the TripleTOF® 6600, data acquisition was performed with Analyst® TF 1.7 (Sciex) and data processing in PeakView®2.1 (Sciex). Within the software, only a single spectrum can be transformed into centroid data, instead of an entire data file. Therefore, a separate scripted tool was programmed to perform the conversion. This tool is not commercially available and was only provided for these purposes. The algorithm used to calculate the peak intensity was peak height above baseline. Data acquisition and processing for the Q Exactive™ orbitrap instrument were all captured in Xcalibur 2.2SP1.48 (Thermo). Data acquired in continuum mode is automatically processed as centroid data by the QualQuant browser in Xcalibur.

Theoretical Simulations of Peak Interference

HRMS can also be advantageous when there are interfering peaks present in the sample. In standard triple quad analysis, these peaks are also detected and will lead to false positive results or lead to higher limits of quantitation, due to lower resolving power (resolution < 10,000) of the instruments. To visualize the possible impact of the co-eluting peaks, an oxidized midazolam metabolite ($C_{18}H_{13}ClFN_3O$; m/z 342.08039) and the +2 isotope of a co-eluting dehydro-oxidized metabolite ($C_{18}H_{11}ClFN_3O$; m/z 342.06238 for +2 isotope) were considered in a theoretical exercise. The calculations of the % spectral peak area as function of a mass extraction window and the % spectral peak area of a co-

eluting unresolved MS peak were based on simulations in XCalibur Qual Browser 3.0.63 (Figure 5). Spectral peaks are visualized in Figure 1. Based on these simulations, % peak area losses as a function of resolution were also calculated when a mass accuracy shift happens (Figure 6).

Results and Discussion

Mass Extraction Window

Unlike dedicated SRM methods on a triple quadrupole instrument, full scan data acquisition on a high-resolution instrument will provide information on all ions present within a selected mass range. Post-acquisition, chromatograms of the compound(s) of interest are extracted based on a window around the exact mass, called the mass extraction window. The resulting chromatogram is referred to as the extracted ion chromatogram (XIC). The MEW determines the fraction of the MS peak area contributing to the peak area and height of the chromatographic peak for continuum data.

Figure 1 shows a MS peak in continuum mode. In this mode, data of all incoming ions are detected and visualized. All information is collected within the defined mass range. Continuum data contain more complete information compared to centroid data allowing better discrimination of real peaks from noise and, consequently, allow more advanced data processing (e.g., noise reduction, deconvolution) but can result in (much) larger data files. Also, the centroid stick plot is visualized in Figure 1.

With respect to quantitative analysis, the key question for processing continuum data relates to the part of the surface area of the spectral peak contributing to the quantitative signal, which is dependent on the mass extraction window. Thus, a compromise between sensitivity (including the complete surface area of the spectral peak) and selectivity (narrower extraction windows offer more selectivity) is required. However, in centroid mode, spectral data are just represented by a stick, where the height of the stick is responsible for the quantitative aspect but how the height is generated during the centroiding process can differ between vendors and is not always disclosed for the user.

Xia et al. [20] proposed a MEW of two times the full width at half maximum (FWHM) for extracting continuum data since this covers about 95–100% of the mass peak area and provides the highest signals for quantitative analysis (Figure 1). Based on the resolution of the instrument, the theoretical maximum mass extraction window can be calculated as follows:

$$\text{maximum MEW} = 2 \times FWHM = 2 \times \frac{M}{R}$$

with

FWHM: full width half maximum, the width of the spectral peak at half maximum (see Figure 1)

M: the nominal mass m/z

R: MS resolution

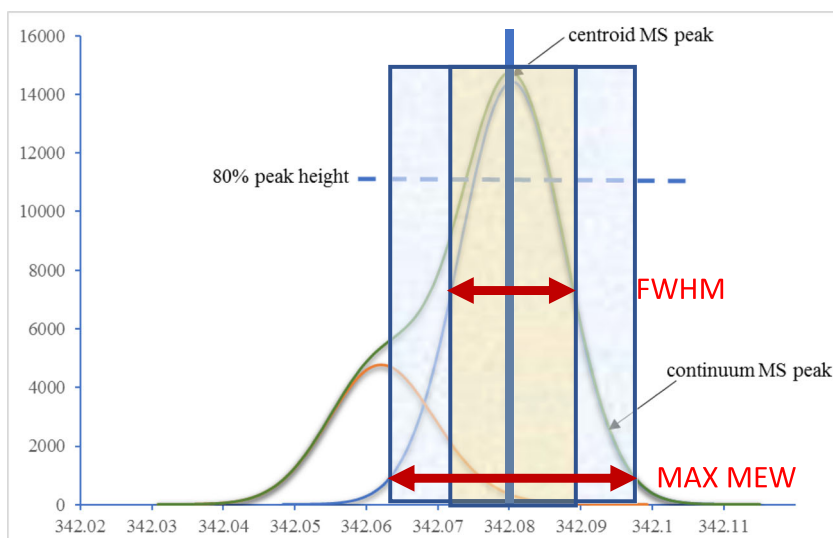


Figure 1. Maximum mass extraction window (maxMEW) and full width half maximum (FWHM) illustrated on continuum (at 20,000 MS resolving power) spectral peaks of an oxidized (blue line) and co-eluting interfering dehydro-oxidized (orange line) midazolam metabolite. The green line represents the resulting (combined) spectral peak at 20,000 resolution

The selected resolution of the systems applied in this work and their maximum MEW calculated based on the measured resolution is summarized in supplementary Table 1.

Besides MS resolution, mass accuracy and precision are important parameters. Any deviation (mass error) from the center of the peak used for the extraction of the XICs can result in a different loss of MS peak area at one side of the MS peak in continuum mode. In other words, the precision of the accurate mass measurement (scan-to-scan reproducibility) will determine the variability of the reported peak area.

Continuum Versus Centroid Data

First, the differences in applying smaller or larger MEWs to centroid or continuum data are depicted in Figure 1, the yellow area shows the peak area covered with MEW=FWHM, and the blue area shows the coverage with max MEW for continuum data. In Figure 2, different extraction windows, i.e. 2, 5, 10 mDa and the maximum MEW calculated from the measured values (supplementary Table 1) were applied to construct the XIC of midazolam. More background noise is filtered when applying smaller mass extraction windows which should result in better selectivity and, ultimately, better signal-to-noise ratios and sensitivity. However, MEWs smaller than the maximum MEW will also lead to loss of MS peak signal in continuum data while centroid data are not impacted (Figures 1 and 2c). This can be clearly noticed for QTOF continuum data illustrated in Figure 2a where both the midazolam peak height and area gradually decrease with decreasing MEWs. With small MEWs applied on continuum data, the reduction in signal can be similar or even higher than the reduction in background, negatively affecting signal-to-noise and sensitivity. This is also illustrated in Figure 3, expressing the signal-to-noise ratio normalized to that obtained at the maximum MEW. It can be noticed that smaller MEWs often lead to a decrease in signal-to-noise ratios for the continuum data. The small difference in

signal-to-noise profile between continuum data of the two QTOF instruments might originate from differences in data processing by the different vendor software packages. This is discussed in more detail in a separate paragraph below.

The same data used for Figure 2a were centroided post-acquisition. Centroiding was performed with the peak height above baseline algorithm in a research tool developed by Sciex. The extracted ion chromatograms generated with the same MEWs on these centroid data are illustrated in Figure 2b. In contrast to the continuum data, the peak height and area are not affected applying smaller extraction windows. Since the centroid peak falls either within or outside the MEW, the entire MS peak is always included in the XIC or completely lost (data point loss in the chromatographic peak). At the chromatogram level, this will result in a decrease of data points, which will be apparent in unsmoothed data. When the accurate mass measurements are too much off this can also lead to the complete loss of the chromatographic peak (see supplementary Figure 1). However, smaller MEWs will substantially reduce the background, while the peak height and area of interest are unaffected (Figure 2b and c). Therefore, signal-to-noise (S/N) is expected to improve with smaller MEWs.

This can be derived from Figure 3, i.e., the signal-to-noise ratios (normalized to max MEW) are gradually improving with decreasing MEWs and exceeding those obtained for the same data in continuum mode (up to 3–4-fold better S/N).

Differences in Processing Acquired Data

During this study, differences between vendor software packages were observed, which are often not noticed and difficult to get acquainted with since data manipulations are often performed automatically without user intervention. Masslynx, for instance, does not automatically apply online background subtraction, while this is the case in Analyst® TF 1.7 and Xcalibur (QualBrowser). Which algorithms are used for the

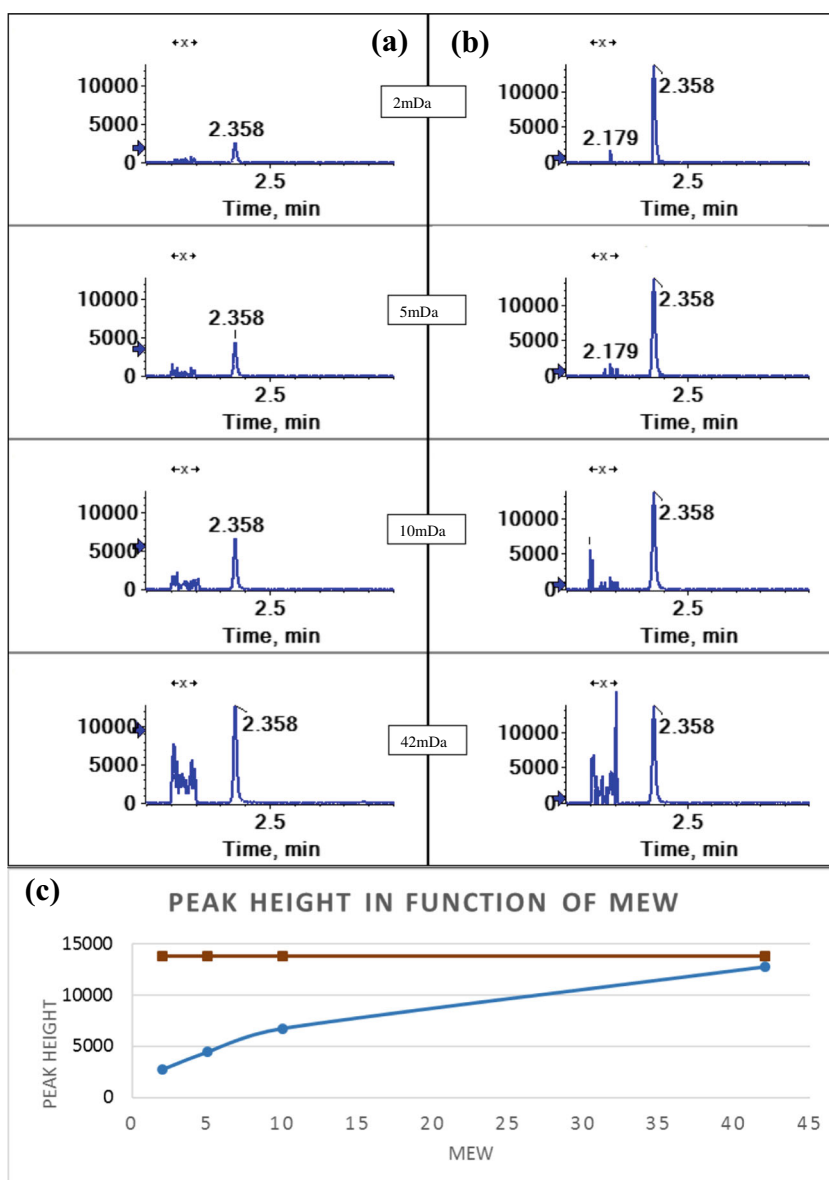


Figure 2. Comparison of TripleTOF® 6600 data in continuum (a) and centroid mode (b) using 2, 5, 10 mDa and max MEWs, based on the midazolam measured mass; Y-axes are regionally expanded 50 times ($\times 50$) to visualize the differences in noise and set at a fixed value to visualize the effect of smaller MEWs on centroid and continuum data obtained for midazolam. Figure 2c illustrates the effect of MEW on the peak height of centroid data (■) and continuum data (●)

background subtraction is not disclosed. Figure 4a shows the midazolam XICs on a Thermo orbitrap system after acquisition in continuum mode of the previously mentioned midazolam plasma sample. When applying the same MEWs, it can be noticed that, in contrast to the QToF data (Figure 2), no background was obtained. Therefore, a different, more profound background subtraction seems to be applied.

Differences in processing continuum data can also be observed between different software packages as illustrated in Figure 4. The peak intensities of the Thermo data (acquired on a Q Exactive™) shown in Figure 4a are identical at different MEWs, although these data were acquired in continuum mode. Thermo Xcalibur (Qual and Quan Browser) software converts the data into centroid while they are still visualized as

continuum data. This is an elegant way of data handling since it combines the higher spectral quality of continuum data with more selective XIC generation using centroid data. The method of centroiding is, however, not visible or adaptable by the user (e.g., % of the peak top, centroid bar length based on area or height of the peak, minimum peak width, apex track, etc.).

When continuum data (acquired on a Synapt G2-S) are visualized in MassLynx/TargetLynx (Figure 4b), a decrease in peak height is only observed between the 42 mDa MEW (maximum MEW) and the 10 mDa MEW. No further decrease was detected with the smaller MEWs (5 and 2 mDa). In MassLynx/TargetLynx software, instead of processing a part of the spectral continuum peak area, the sum of the intensities of the individual data points of the spectral peak is used. If the number of MS data

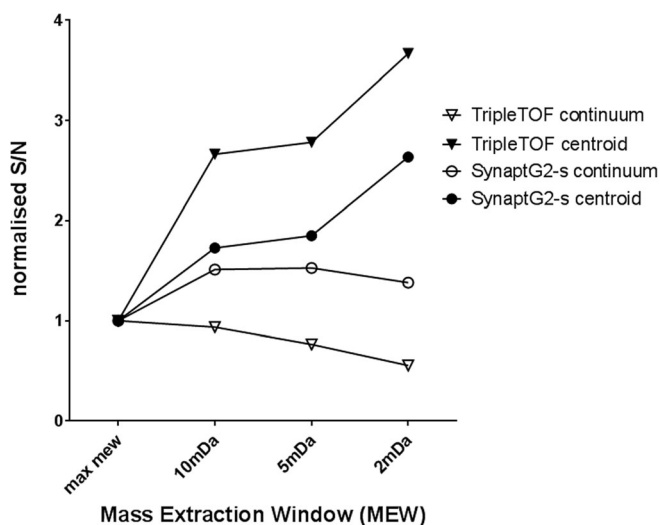


Figure 3. Normalized signal-to-noise ratios ($n = 5$) for different modes and extraction windows on both QTOF instruments, S/N was determined for midazolam at a concentration of 1 ng/mL.

points within different MEWs is constant, the signal intensity and area will not change. In the case of midazolam, the number of spectral data points present in the 2, 5, and 10 mDa MEW are exactly the same and as a result identical XICs are generated. This differs with the data processing in Waters Unifi, Sciex PeakView®, and Thermo Tracefinder where a part of the spectral peak area proportional to the MEW applied is taken for the generation of the XICs (Figure 4c).

Selection of the Optimal Mass Extraction Window

In case the analyte of interest is not fully MS resolved from an interfering co-eluting ion (as visualized in Figure 1 with the example of the oxidized and dehydro-oxidized metabolites of midazolam), a narrower MEW will result in better selectivity for continuum data although never completely excluding contribution of the overlapping ions. This is illustrated in Figure 5 (■) showing a theoretical calculation of the % area contribution of an overlapping MS peak at different MEW relative to the overlap observed at max MEW ($2 \times \text{FWHM}$), also discussed in the “experimental section”.

A narrower MEW will on the other hand result in the integration of a smaller part of the continuum peak of the analyte of interest and, therefore, in a gradual decrease in peak area (Figure 5 (●)). From these two graphs, it can be derived that FWHM should be chosen for optimal MEW selection. The FWHM MEW provides the best compromise between maximum selectivity with minimal loss of peak area as can be noted from the two graphs that are the furthest apart at FWHM (see arrow Figure 5). With increasing mass resolution, one additional parameter needs to be considered. This is shown in Figure 6 plotting the loss in MS peak area relative to the MEW applied at three different MS resolutions if the measured mass deviates 1 mDa from the accurate mass used for extracted ion chromatogram generation. At 20,000 resolution, this peak area loss is limited and for all MEWs applied less than 1%. At

increasing MS resolution, as illustrated for 40,000 and 100,000 resolutions in Figure 6, the peak area lost due to experimental mass error can be substantial. Similarly, this will negatively affect precision due to small scan-to-scan mass accuracy variation which will become higher with higher MS resolution. Therefore, scan-to-scan mass accuracy (not taken as an average over the LC peak) is another parameter that should be considered for MEW selection. In agreement with all these considerations, we can propose the following widely applicable optimal MEW selection rule for continuum data:

Rule A: Optimal MEW Continuum Data = MS Peak FWHM + Mass Accuracy Window (All Expressed in mDa)

This rule of thumb can also be applied relatively easy. The FWHM value is easily obtained from most instrumentation software where FWHM (or MS resolution) can be plotted in the MS spectrum. The FWHM was observed to be narrower at higher concentrations in the calibration curve (see Figure 7), so determination is preferred at lower concentrations. The mass accuracy window can be derived from the instrument specifications (e.g., $1 \times$ the expected maximum accurate mass error for unsmoothed data, $2 \times$ the expected maximum accurate mass error for smoothed data) or experimentally obtained. The MEW selection rule might be further simplified for systems with relatively low MS resolution ($< 30,000$) where the optimal MEW selection primarily depends on FWHM and the contribution of mass accuracy error and variation will be negligible and can be ignored or for ultra high-resolution systems ($> 200,000$ MS resolution) where the optimal MEW primarily depends on the mass accuracy and the FWHM contribution is negligible. The latter is also true for centroid data where a narrower MEW will not reside in loss of MS peak area and, therefore, the following optimal widely applicable MEW selection rule can be applied:

Rule B: Optimal MEW Centroid Data = Mass Accuracy Window (All Expressed in mDa)

One can also select a MEW optimized per analysis since this is much easier for centroid data. The smallest MEW value that provides XICs without data point losses offers the mass accuracy window and is the best MEW to select. It is of course critical to perform this assessment on unsmoothed data since otherwise the loss of data points will be masked by the smoothing process. This is illustrated in supplementary Figure 1 where the unsmoothed data show loss of data points for a MEW of 10, 20, and 33 mDa, while the zero data points are lost upon smoothing (supplementary Figure 1). A smaller MEW applied to centroid data has the advantage, besides the better qualitative selectivity discussed earlier, that a mass shift due to combined centroiding with a co-eluting peak will be more rapidly picked up. For continuum data, even with a large mass error, part of the continuum peak, i.e., from the tail of the peak, will still reside within the MEW. Since a chromatographic peak can still be observed, it can go unnoticed by the analyst that the data are processed in a suboptimal way.

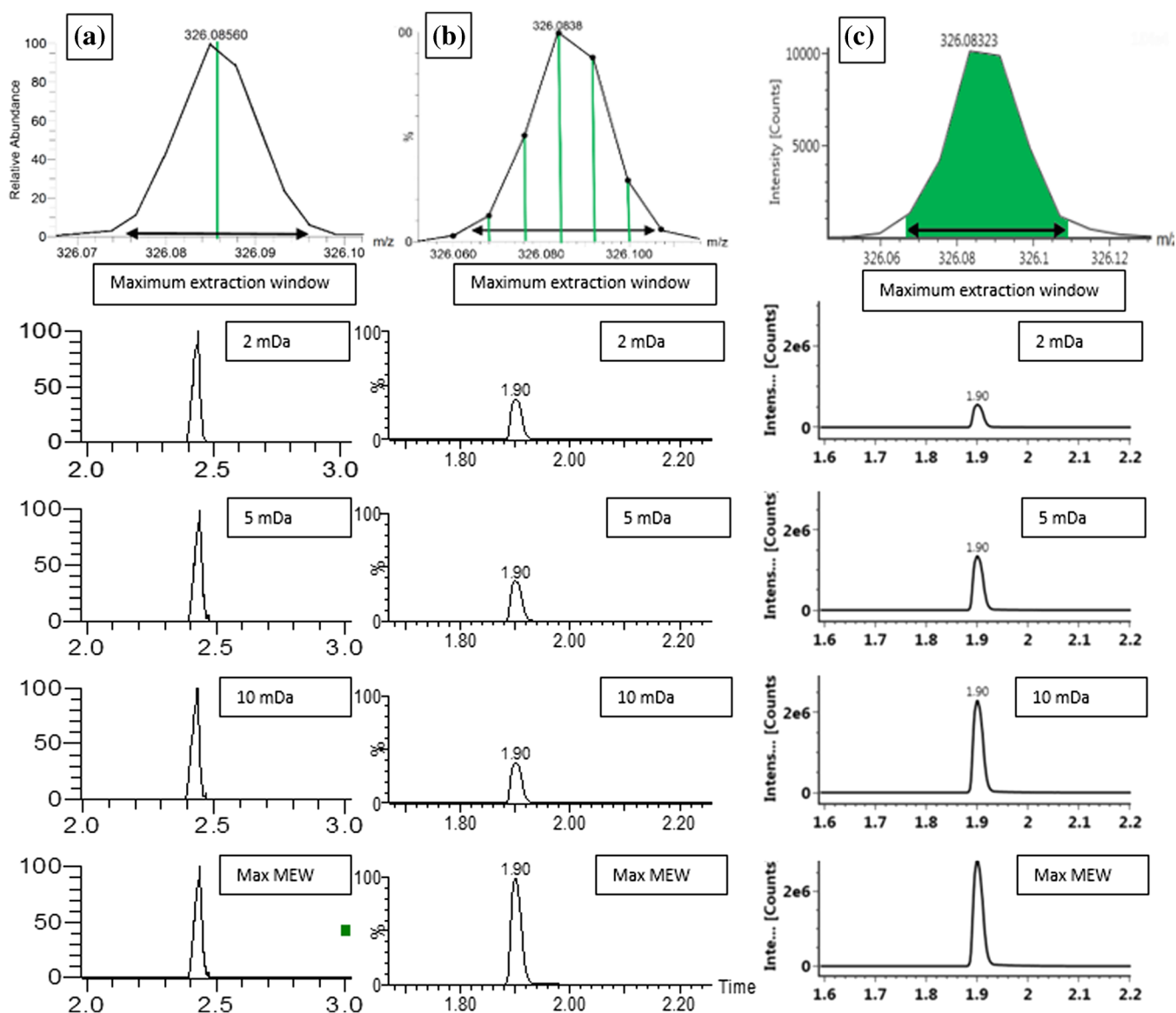


Figure 4. Differences in data handling: continuum data of midazolam processed as centroid data by Xcalibur (Thermo) (a), as summed individual spectral points in the MEW by MassLynx\TargetLynx (Waters) (b) and as peak area used in Unifi (Waters) (c). XIC are extracted with 2–5–10 mDa and max MEW from top to bottom

Effect on Quantitative LC/MS Performance

Calibration curves covering a wide concentration range of acetaminophen, tolbutamide, norethindrone, midazolam, abiraterone, prednisone, loperamide, and simeprevir in plasma were analyzed in five-fold on a Synapt G2-S QToF system. Data were acquired in continuum mode and processed in four different ways: (i) as centroid data in Targetlynx after post-acquisition centroiding of the continuum data, (ii) as continuum data using the sum of the intensities of the individual data points in Targetlynx and as continuum data using (iii) 2D and (iv) 3D processing in Unifi. The results are added in the supplementary data (Table 2). Here, the guidelines proposed in this manuscript were applied. After processing unsmoothed centroid data as discussed earlier, MEWs larger than the mass accuracy window (MA) or $2 \times$ MA were

considered to mitigate loss in data points. Although there are clear differences in selectivity of the XICs generated from centroid data compared to those generated from continuum data especially using smaller MEWs, this did not translate in clear differences in the limit of quantification and/or precision. For the simeprevir data, the LLOQ for centroid data with a 10 mDa MEW was 10 times higher than that at a larger MEW. This is a clear effect of a too narrow extraction window resulting in a loss of (too many) data points (supplementary Figure 1). For all other data, small differences in performance (accuracy and precision) were observed but these were rather scattered between the different processing techniques and, thus, no clear trend could be deduced. This can mainly be explained by a lack of mass spectrometric separation between the analyte of interest and co-eluting background ions. Therefore, a narrower MEW will not affect the lower

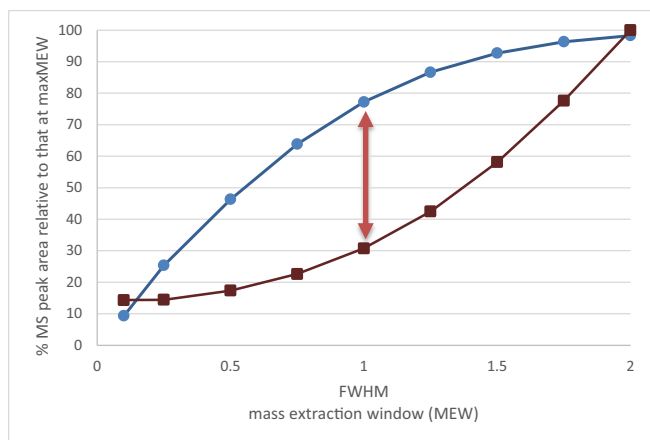


Figure 5. ● % area of a continuum peak integrated relative to the mass extraction window applied; ■ % area of a co-eluting MS peak (as visualized in Figure 1 (simulations calculated based on the m/z of the oxidized and dehydro-oxidized metabolite of midazolam)) co-integrated with the analyte peak of interest relative to the mass extraction window applied and normalized to the area obtained at max MEW (= $2 \times$ FWHM)

limits of quantitation (LLOQs) or precision since there is no improvement on selectivity. Table 2 also shows the same calibration curves processed with MEWs of 10 mDa, 20 mDa and the calculated max MEW (all smoothed data). The obtained LLOQ values are equal or differ by a factor of 2. Background compounds that are not chromatographically and mass spectrometrically separated from the ions of interest will be included in the centroiding. This will be especially true at LLOQ level where the number of potential MS unresolved peaks proportionally grow with the noise level. When the same co-eluting background ions would elute at a different retention time, i.e., away from the peak front or tail of the peak of interest, the correct centroiding of these ions can result in values outside the selected MEW which provides more selectivity and lower S/N values for the overall extracted ion chromatogram. This can

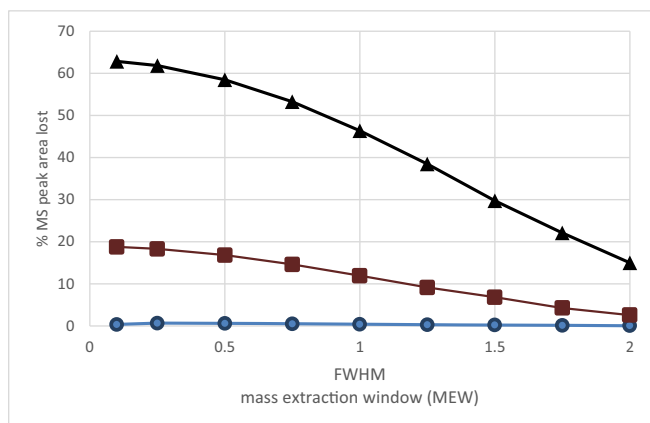


Figure 6. % of the MS peak area lost at different mass extraction windows in case of a 1 mDa experimental error relative to the accurate mass used for mass extraction at ● 20,000, ■ 40,000, and ▲ 100,000 mass resolving power (simulations calculated for an oxidized metabolite of midazolam)

be beneficial for qualitative analyses, reducing the number of false positive hits in a targeted or untargeted analysis. For quantitative analyses, however, there is no advantage since only the selectivity in the retention time region of the peak matters.

Concentration Dependent FWHM and Impact of Regression Model

As mentioned in the previous paragraph, the FWHM on QToF instruments can differ depending on the concentration of the analyte giving narrower MS peak widths at higher signal intensities. This will have an impact on the determination of the MEW but also on the portion of the MS peak that will be used to generate the XIC. This is shown in Figure 7 for a molecule with nominal mass 541. A gradual decrease of the FWHM can be noted from 26 mDa at 20 ng/mL to 17 mDa at 10,000 ng/mL (analysis on a TripleTOF® 6600). Consequently, applying smaller MEWs a relative larger peak area of the spectral peak surface will be included in the extracted mass chromatograms of the higher concentration levels. As a result, overestimation is observed at the higher concentration levels and is dependent on the MEW applied. Figure 8 illustrates that a 15 mDa MEW results in more pronounced overestimations than a 50 mDa MEW. Additionally, overestimation is also more pronounced with linear regression with $1/x^2$ weighing that is most frequently applied in the bioanalytical community compared to linear regression following log transformation of both concentrations and peak areas. Similar observations could also be noticed on Synapt G2-S data in different concentration ranges and with different compounds.

Theoretical or Experimentally Measured Accurate Mass

Considering the stability of the accurate mass measurements on modern HRMS systems (both scan-to-scan and chromatogram-to-chromatogram stability), the mass error will be minimal, but still needs to be evaluated properly after analysis of the analytical batch. Since ideally the MEW should be centered against the MS peak, it could be argued that the better practice is to use the measured accurate mass rather than the theoretical m/z commonly

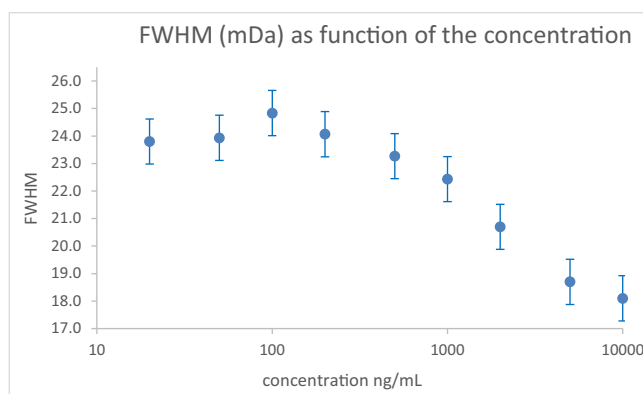


Figure 7. FWHM in mDa ($n = 3$) as function of the concentration, analyzed on a TripleTOF® 6600 for a compound with theoretical m/z 541.1283

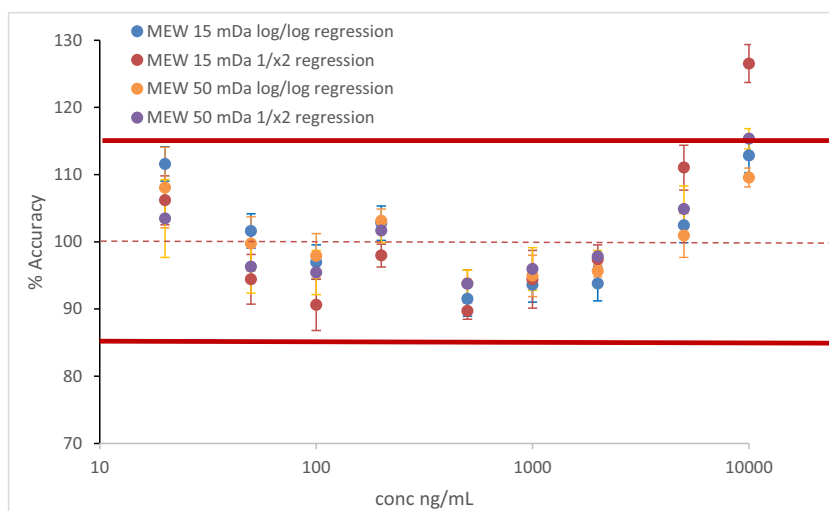


Figure 8. % accuracy as function of MEW and regression model with weighing applied. Red lines indicate the 100% accuracy (dotted line) and the 15% RE (full line) allowed in quantitative analysis

applied for the selection of the MEW. However, in quantitative analysis on a set of samples, it might be challenging to determine the overall measured mass. Ideally, software tools should be developed providing the best accurate mass and MEW settings automatically based on the calculation of MS resolution, the average accurate mass and precision of the measured data. Sturm et al. [17] also stated two approaches to define the accurate mass, where the *in silico* calculated mass would result in larger MEWs, compared to the smaller MEWs obtained after averaging the scans across the FWHM of a mid-level standard calibration point.

Accurate Mass Calculation

Another important aspect in the determination of the theoretical mass is the difference in calculation between the different software packages. For the $[M + H]^+$ ion of midazolam ($C_{18}H_{13}ClFN_3$), a value of 326.0860 was obtained in Masslynx (Waters), while 326.0855 was calculated as the accurate mass in Xcalibur (Thermo), Analyst®/PeakView® (ABSciex), and Unifi (Waters). The difference in these calculated masses can be attributed to the mass of one electron ($m_e^- = 0.00055$ u). When applying the same software for the calculation of the theoretical accurate mass and the MS analysis, the same mass difference will be applied for the calibration of the system and no error will be made if both the calibrant and the analyte of interest have the same charge state. However, when different software packages (e.g., chemical drawing software) are used, this small mass difference of one electron can already result in a noticeable additional error, e.g., 0.5 mDa (1.6 ppm) error for midazolam. Therefore, attention to the details of the calculation method used is warranted.

Conclusions

Centroid data in combination with smaller MEWs provide advanced selectivity resulting in cleaner XICs. While this can be beneficial for qualitative analyses, reducing the number of

false positive hits in a targeted or untargeted analysis, the effect on quantitative performance was shown to be minimal. This can be attributed to the lack of MS separation for (partially) co-eluting background ions with an accurate mass falling within the MS peak width of the analyte of interest.

The selectivity of the quantitative assay can be improved by the use of narrower MEWs. Therefore, the mass accuracy of the instrument defines the optimal MEW selection when centroid data are processed as defined in rule B. In case continuum data are being processed, the improved selectivity is counterbalanced by a loss in MS peak area affecting signal-to-noise. The full width half maximum (FWHM) of the peak in addition to the mass accuracy (rule A), therefore, defines the most widely useable optimal MEW selection for continuum data. These MEW selection guidelines for centroid and continuum data are easily applicable. More thorough validations and advanced calculations might be performed but will, in our opinion, generally not contribute to better quantitative data as was illustrated by the negligible impact on quantitative performance when comparing different MEWs.

The discussion on the selection of the optimal MEW mainly applies to 2D peak processing as most commonly used for peak integration. Recently, also 3D peak processing has been introduced (e.g., in Unifi) that takes away the decision on the selection of the optimal MEW from the user. 3D peak processing comes with different challenges. In the current software version used, it is for instance difficult or impossible to review and manually adjust peak integrations.

In this work, we focused on the impact of different software approaches on the extraction of MS data to generate XICs. It must be noticed that other data processing algorithms are involved, such as centroiding and background subtraction, which could not be evaluated since they are not fully disclosed but differ between the vendor software packages as well. Evaluation of the effect of differences in centroiding algorithms might be a valuable subject of future research highlighting how centroiding might be improved especially at the LLOQ level.

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