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Parallel Notched Gas-Phase Enrichment for Improved Proteome Identification and Quantification with Fast Spectral Acquisition Rates

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Abstract

Gas-phase fractionation enables better quantitative accuracy, improves signal-to-noise ratios, and increases sensitivity in proteomic analyses. However, traditional gas-phase enrichment, which relies upon a large continuous bin, results in suboptimal enrichment, as most chromatographic

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Supporting Information

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Figure S1: Gas-phase fractionation schemes. Figure S2: Experimental optimization of notched isolation waveforms. Figure S3: Characterizing the yeast proteome with standard data-dependent and multiple gas-phase fractionation approaches. Figure S4: Optimizing notched gas-phase fractionation for label-free quantification. Figure S5: Implementing dynamic notched isolation waveforms for data-dependent analysis. Figure S6: Incorporating all sets of notched bins within a single injection (PDF)

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separations are not 100% orthogonal relative to the first MS dimension (MS^1 m/z). As such, ions with similar m/z values tend to elute at the same retention time, which prevents the partitioning of narrow precursor m/z distributions into a few large continuous gas-phase enrichment bins. To overcome this issue, we developed and tested the use of notched isolation waveforms, which simultaneously isolate multiple discrete m/z windows in parallel (e.g., 650–700 m/z and 800–850 m/z). By comparison to a canonical gas-phase fractionation method, notched waveforms do not require bin optimization via *in silico* digestion or wasteful sample injections to isolate multiple precursor windows. Importantly, the collection of all m/z bins simultaneously using the isolation waveform does not suffer from the sensitivity and duty cycle pitfalls inherent to sequential collection of multiple m/z bins. Applying a notched injection waveform provided consistent enrichment of precursor ions, which resulted in improved proteome depth with greater coverage of low-abundance proteins. Finally, using a reductive dimethyl labeling approach, we show that notched isolation waveforms increase the number of quantified peptides with improved accuracy and precision across a wider dynamic range.

Graphical Abstract

Keywords

multinotch; gas-phase fractionation; dynamic range; injection waveform; label-free quantification; reductive dimethylation; orbitrap

INTRODUCTION

Experimental, technical, and computational advances have enabled deep sampling of complex proteomes in relatively less time, yet the wide dynamic range found in a typical complex proteome ensures that low-abundance proteins remain difficult to routinely characterize. The dynamic range can be particularly troubling for precursor-level quantification workflows (label-free, SILAC, or reductive demethylation), which are dependent on the reproducible detection of low-intensity ions to produce accurate quantification. Due to a lack of intensity, the quantification of low-abundance proteins are often most perturbed, which results in lower accuracy and precision.¹ Several notable technical advancements have greatly expanded the dynamic range of MS^1 workflows and have enabled the broad adoption of precursor-based quantitative proteomics.^{2–4} While,

traditionally, this area fell under the purview of hardware-specific research, novel acquisition methods provide an alternative means of extending the dynamic range to enhance the depth and accuracy of precursor-level quantification of complex proteomes.

One straightforward experimental approach to extend the dynamic range of MS¹ acquisition is through the application of gas-phase fractionation (GPF).^{5–8} By reducing the analyzed mass range to a constrained portion, the GPF method limits the breadth of charges entering the analyzer, which in turn allows the mass spectrometer to commit more of its finite charge capacity toward analyzing the mass range of interest. The dynamic range within this constrained region is improved due to the increased investment in analyzed ions. In the context of a typical proteomics experiments, GPF enables the sampling of very low-abundance proteins by utilizing multiple sample injections, where each injection is limited to a portion of the total mass range, and together, all the injections cover the entire mass range of interest.⁹

The standard GPF approach utilizes a series of n continuous bins, each of which has a defined m/z range (e.g., bin 1: 300–600 m/z ; bin 2: 601–900 m/z ; bin 3: 901–1200 m/z) that is employed for n independent sample injections (Figure S1A). A primary drawback of the traditional GPF workflow is the necessity to layout useful bins that equally distribute the ion population across each injection. Accordingly, these GPF methods typically require careful selection of ideal m/z ranges, based on either prior analysis or *in silico* protein digestion. An alternative workflow, termed tiling, was previously published that limits the interrogation of precursors to specific discrete bins across the mass range, with n sets of discrete bins applied for n independent injections (Figure S1B). Although this method was shown to provide better proteome coverage when compared to traditional GPF acquisition, it did not provide an increase in the MS¹ dynamic range, as the full mass range is measured by the analyzer.⁶

Although GPF and the tiling approach have provided improvements in proteome depth, the incomplete orthogonality between the m/z values of the eluting molecules and their liquid chromatographic retention times (i.e., generally low- m/z peptides elute early while higher m/z peptides elute later) results in unequal enrichment across the MS¹ mass range and limited improvements in dynamic range. Furthermore, the need for *a priori* bin optimization and the sometimes limited gains in enrichment hamper the utility and application of continuous bin GPF.

Recent work has extended the potential of GPF using multiplex MS (MSX)-based methods.¹⁰ MSX methods allow for the serial selection of ions from several m/z ranges. Multiple discrete m/z bins are injected, trapped, and ultimately stored together in an ion trapping device. Following injection and trapping of all the m/z bins, the entire ensemble of ions is then concurrently analyzed. In this way, multiple ion populations from different m/z ranges can be simultaneously analyzed to enrich for different MS¹ populations. Examples of MSX methods include BoxCar⁹ and MSX-DIA.¹¹ In the case of BoxCar, the method evenly distributes the desired total ion population into multiple segments, and via sequential quadrupole isolation, ions within each m/z segment are accumulated before the combined ion population is injected into the Orbitrap for a single-transient analysis. BoxCar alleviates the unequal enrichment of ion currents in different m/z windows and enables up to a 10-fold

gain in dynamic range. However, sequential quadrupole isolation naturally causes a concomitant increase in the total injection time, which increases MS¹ overhead and reduces the instrument duty cycle.

Herein, we describe a novel gas-phase enrichment-based approach that increases the detection of low-abundance peptides using injection waveforms with multiple discrete notches. As opposed to sequential quadrupole isolations, utilized by BoxCar and other MSX scan schemes, notched waveforms allow parallel isolation and accumulation of the desired *m/z* bins (Figure S1C). Parallel accumulation of all the *m/z* bins of interest helps limit the total MS¹ injection time and limits any impacts on duty cycle. We compare and contrast this novel discrete bin GPF approach against standard GPF for both yeast proteome characterization and quantification. The notching is analogous to the notched MS3 methods that have been extensively used for TMT quantification.¹² By distributing the discrete notches across the *m/z* range and by alternating between orthogonal sets of notches, we can efficiently normalize the degree of enrichment across the MS¹ spectra and across the chromatogram without any prior knowledge of the sample. We observed that the notched-bin GPF approach improves the depth of yeast proteome characterization, including more identifications of low copy number proteins, when compared to traditional data-dependent acquisition or GPF. Additionally, we observed an increase in MS¹ dynamic range that improves the accuracy and sensitivity of precursor-level quantification across a broad range of protein abundance.

METHODS

Yeast Preparation

Saccharomyces cerevisiae cells were grown to an optical density (OD) of 1.0, washed with ice cold PBS, and snap frozen in liquid N₂ until further use. Yeast cells were mechanically lysed with a homogenizer in a SDS lysis buffer [2.0% SDS w/v, 250 mM NaCl, PhosSTOP (Roche, Madison, WI) phosphatase inhibitors, EDTA free protease inhibitor cocktail (Promega, Madison, WI), and 50 mM HEPES, pH 8.5]. Lysates were reduced with 5 mM DTT, and cysteine residues were alkylated with iodoacetamide (14 mM) in the dark (30 min). Protein content was extracted by methanol-chloroform precipitation and subsequent ice-cold acetone washes. Protein pellets were dried and resuspended in 8 M urea that contained 50 mM HEPES (pH 8.5). Protein concentrations were measured by BCA assay (Thermo Scientific, Rockford, IL) prior to protease digestion. Protein lysates were diluted to 4 M urea and digested with LysC (Wako, Japan) in a 1/200 enzyme/protein ratio overnight. Protein extracts were diluted further to a 1.5 M urea concentration, and trypsin (Promega, Madison, WI) was added to a final 1/250 enzyme/protein ratio for 6 h at 37 °C. Digests were acidified with 200 μ L of 20% formic acid (FA) to a pH ~2 and subjected to C18 solid-phase extraction (SPE) (Sep-Pak, Waters, Milford, MA).

Notched Injection Waveforms

All methods utilizing notched injection waveforms for MS¹ gas-phase enrichment were enabled through modifications of the instrument control language. Although the functionality to calculate and generate MS¹ injection waveforms exist within the instrument

control language, these functions are not currently user exposed through the method editor. The simultaneous retention of multiple discrete m/z ranges, using isolation waveforms with discrete bins of frequency space, has been previously described and is now widely available on Orbitrap Fusion, Orbitrap Fusion Lumos, and Orbitrap Eclipse (Thermo Scientific, San Jose, CA) mass spectrometers.^{3,12,13} Similarly, the usage of isolation waveforms during ion injection to increase the MS dynamic range is now common place.^{12,14} This is the first study to apply and investigate the usage of notched isolation waveforms during ion injection for the sake of improving proteome characterization and quantification.

Initial efforts focused on generating notched waveforms that efficiently retained the ion current within the desired notches while minimizing the retention of the ion current outside the isolation notch. Waveform parameters were optimized through controlled variation of scan parameters related to notched isolation (Figure S2A). As an example, Figure S2B shows the optimization of the isolation waveform RF amplitude by scaling the amplitude from 50% to 210% of the nominally applied value. The violin plots display the degree of intensity of the GP enrichment (increased signal-to-noise ratio compared to standard MS¹ spectrum) with respect to the scaling of RF and the m/z of the isolation notch. As the main RF is scaled from 50% to 210%, we observed that the degree of intensity enrichment increased, relative to the standard spectrum, and resulted in uniform enrichment within the isolation window. As the scaling of the RF increases beyond 130%, the degree of the observed enrichment increased, but the distribution of enrichment became much broader. The shape of the notched isolation was assessed via a contour plot displayed in Figure S2C. As before, the degree of intensity enrichment relative to a standard spectrum was determined and plotted for each scaling factor. As before, when the main RF amplitude was increased, the observed intensity enrichment increased. Additionally, we observed that the shape of the isolation was more highly defined with increasing amplitude (Figure S2D). Following these experiments, a main RF amplitude scaling of 130% was chosen for a combination of intensity enrichment and isolation width shape.

The number and width of the notches was optimized to provide a balance between high-fidelity enrichment and the number of independent injections (data not shown). Wider notches (>100 m/z width) exhibited a subtle decrease in enrichment efficiency, and narrower notches (<50 m/z) impacted the shape and resolution of the isolation. Furthermore, the degree of enrichment was reduced when a greater proportion of the total mass range was included within each injection, independent of the width of the notches (e.g., two injections that include 600 m/z each versus three injections that include 400 m/z each). It was determined that (8×) 50 m/z wide notches (total of 400 m/z per injection) and three independent injections provided the most optimal balance between enrichment efficiency and the number of injections.

Orbitrap Elite Parameters

For the purpose of characterization and optimization of the notched injection waveforms, a series of 10 high-resolution Orbitrap scan events were implemented, which consisted of FTMS1 AGC targets of 1×10^6 , a FTMS resolution of 60000, a mass range between 300–1500 m/z , and max injection times of 10 ms. The first scan event utilized standard full scan

MS¹ parameters, while the proceeding nine scans (Figure S2A) would vary some scan parameter (e.g., the isolation waveform RF amplitude scaling factor or the isolation window width).

For experiments comparing GPF methodologies, the mass spectrometer was operated in a data-dependent mode. For each set of experiments, the 20 most intense precursors were selected ($z > 1$) for collisional induced dissociation using a normalized collision energy of 35. Additional MS² parameters included the following: an isolation width of 2.0, an AGC of 5000, a max injection time of 125 ms, and a dynamic exclusion of 55 s (± 10 ppm).

Data Processing and Spectra Assignment

A compilation of in-house software was used to convert mass spectrometric data (Thermo “.raw” files) to an mzXML format as well as to correct monoisotopic m/z measurements and erroneous peptide charge state assignments. The assignment of MS/MS spectra was performed using the SEQUEST algorithm utilizing the *S. cerevisiae* ORF database. In each case, reversed protein sequences were appended as well as known contaminants (e.g., human keratins). SEQUEST searches were performed using a 50 ppm precursor ion tolerance while requiring each peptide’s N/C terminus to have trypsin protease specificity and allowing up to two missed cleavages. Carbamidomethylation of cysteine residues (+57.02146 Da) were set as static modifications, while methionine oxidation (+15.99492 Da) was set as a variable modification. A MS² spectra assignment false discovery rate (FDR) of less than 1% was achieved by applying the target-decoy database search strategy. Filtering was performed using an in-house linear discrimination analysis algorithm to create one combined filter parameter from the following precursor ion and MS² spectra metrics: XCorr, Cn-difference score, peptide ion mass accuracy, charge state, peptide length, and missed-cleavages.¹¹ Linear discrimination scores were used to assign probabilities to each MS² spectrum for being assigned correctly, and these probabilities were further used to filter the data set to a 1% protein-level false discovery rate. Redundant peptides were assigned to proteins according to parsimony.

RESULTS AND DISCUSSION

Constructing Discrete Gas-Phase Fractionation Bins

Due to the correlation between peptide m/z values and liquid-chromatographic (LC) retention times, traditional continuous bin gas-phased fractionation (GPF) frequently results in nonuniform intensity enrichment across the gradient.⁶ Conversely, the discrete sampling of multiple defined mass ranges throughout the gradient overcomes this limitation and results in more uniform intensity enrichment.

The use of gas-phase enrichment enables the identification of low-abundance precursors that would remain undetected with standard survey scans.^{5,6} These methods typically involve dividing the MS¹ mass range into continuous segments that are then analyzed individually. Figure 1A depicts the m/z distribution of yeast tryptic peptides that were divided into three continuous bins. For each of the three injections, the m/z range is restricted to that particular bin. During the LC elution, the complexity of the sample at a given time varies. For

example, the red bin is restricted to an m/z range of 300–500. Early in the chromatographic elution, the majority of the eluting peptides are between 300 and 500 m/z , resulting in limited gas-phase enrichment. As the gradient and acquisition continues, the eluting peptide population transitions to higher m/z values, resulting in a more uniform distribution of precursor ions between the gas-phased fractions. However, toward the end of the gradient, the distribution of peptides are once again nonuniformly distributed, representing the inherent limitation of GPF with continuous bins.

As an alternative to previous GPF approaches, our approach replaces the continuous bins and instead implements smaller discrete bins spaced across the complete mass range. Our approach eliminates the need to predict the ideal gas-phase fractions while still enriching for ions in the MS¹ survey scans. By dividing the MS¹ survey scan mass range into several discrete slices, we can evenly distribute the precursor population without any prior knowledge of the precursor ion distribution (Figure 1B). We simply need to ensure that the bin size is small enough to sample the ion distribution multiple times irrespective of the chromatographic timing. By way of example, in our preliminary experiments we evenly distributed six 35 m/z -wide notches across the typical MS¹ mass range of 300–1500 m/z . As illustrated in Figure 1B, the same distribution of yeast tryptic peptides can be divided into three separate injections. Within each injection, the mass range is divided into six discrete bins (notches). When overlaid onto a chromatographic time scale, it is apparent that the sampling across the mass range is much more uniform. This results in greater enrichment across the entire chromatographic gradient.

The discrete gas-phased fractionation bins were generated by the application of an injection waveform, which included discrete frequency notches. For each MS¹ ion injection event, a waveform was computed that included notches in the frequency space that allowed the retention of ions within that notch and exclusion of all ions outside of the notches (Figure 1C). An example spectrum (Figure 1D) displays three MS¹ spectra collected consecutively utilizing either a standard MS¹ survey scan, continuous bin GPF window, or a series of discrete GPF bins. The standard method displays an abundant peak at ~500 m/z . For each of the GPF methodologies, the abundant peak at ~500 m/z is excluded, resulting in an enrichment of other ions. Across the mass range, especially at higher m/z values, ion intensities are increased by the notched waveform-based gas-phase enrichment, which resulted in a higher dynamic range and a greater sensitivity.

In order to optimize the response of the discrete bin GPF approach, we created a unique experimental scheme that varied relevant waveform parameters (described above in the methods, Figure S2A). For each set of parameter optimization experiments, we collected an MS¹ scan utilizing a standard approach. Subsequent scan events would vary a parameter, resulting in the collection of pairs of control and experimental spectra. One example parameter was the isolation waveform RF scaling and its impact on ion populations and the shape of the multitude of isolation windows. To optimize the waveform amplitude, we assessed the intensity of ion features (via signal-to-noise ratios, Figure S2B) and the shape and density of ions (Figure S2C–D). Utilizing this approach allowed us to optimize the notched GPF approach.

Quantifying the Degree of Gas-Phase Enrichment

A robust gas-phase enrichment method requires evenly distributing the precursor population among all the MS¹ spectra.⁹ Otherwise, an overabundance of precursor ions in some spectra will result in a minimal increase in precursor ion intensities. We compared how precursor ion current varies as a function of retention time for both the standard gas-phase enrichment-based experiment and the notched waveform-based gas-phase enrichment experiment. The degree of gas-phase enrichment was quantified by devising an experimental scheme that included a series of MS¹ scan events, which consisted of a standard full mass range MS¹ scan, a large continuous bin GPF scan (300–510 *m/z*), and a notched GPF scan (six notches, 35 *m/z* per notch). This provided the ability to directly compare the intensity of features found in each of the three MS¹ methods. Figure 2 displays the layout of the GPF windows we employed to measure the degree of enrichment. The GPF windows were constructed such that the total amount of *m/z* space retained in the summed GPF mass range(s) was the same for each GPF method. For example, the “Notched GPF MS¹ scan” (Figure 2A) was composed of six notches, each containing 35 *m/z*, resulting in a total of 210 *m/z* analyzed throughout the experiment. Similarly, the “Standard GPF MS¹” experiment retained 210 *m/z* in its GPF MS¹ scan, but in the case of the latter, the entire GPF window was composed of a single continuous bin of 300–510 *m/z*. Following spectral acquisition, intensity comparisons among identified peptides were performed to identify the degree of intensity enrichment relative to the standard method. Highlighting the use of the notched GPF approach, Figure 2B displays the degree of enrichment observed.

For this experiment we are comparing one GPF range from the experiment with the continuous bin to one set of discrete GPF bins. The uneven distribution of ions in the standard GPF experiment is displayed in the plot of retention time versus fold-enrichment, wherein the degree of enrichment is non-linear as a function of time. During a typical LC-MS chromatogram, most low-*m/z* ions elute early (Figure 1). Correspondingly, the gains in ion enrichment with the standard GPF MS¹ survey spectra are minimal throughout the first half of the chromatogram and increase as the *m/z* distribution of ions transitions into higher *m/z* regions that fall outside of the continuous GPF bins (Figure 2B, gray line). Accounting for this retention time *m/z* bias by adjusting the size of the gas-phase enrichment bin requires careful synchronization of the bin width with the retention time and also detailed prior knowledge of when precursor ions appear as a function of retention time. By comparison, with the notched waveform-based gas-phase enrichment method, the precursor population is evenly spread out between all the MS¹ survey scans. As such, there are no biases in precursor intensity enrichment as a function of retention time and the degree of enrichment is approximately linear throughout the elution (Figure 2B, red line). Hence, this gas-phase enrichment method does not require any complicated scheduling or detailed *a priori* knowledge of the proteome and provides consistent enrichment of ion intensities throughout the gradient.

Notched Gas-Phase Fractionation Provides Greater Yeast Proteome Coverage

Gas-phase fractionation increases the dynamic range of the MS analyzer such that the sensitivity of the instrument aligns more closely with the wide dynamic range of protein abundance found in complex proteomes. This improvement in dynamic range ultimately

enables the characterization of proteins that are present at lower relative abundances in the proteome. In order to quantify how the various GPF methods affect the degree of proteome sampling depth, we implemented and employed several GPF approaches to characterize a yeast whole-cell lysate. For each of the three approaches compared below, the alternative sampling of features enabled by GPF should theoretically result in increased proteomic sampling depth by permitting the interrogation of peptides that span a wider range of abundances.

The methods compared included, (1) standard DDA (SDDA), (2) a tiled precursor selection approach (does not provide an increase in ion intensity), (3) a standard GPF, and (4) a notched GPF approach. For each method, triplicate injections of each sample were collected, and depending on the method, the precursor sampling range was adjusted.

Figure 3A displays the results of this comparison among the methods for several metrics. SDDA resulted in nearly 50% more MS² spectra and total peptides than all other methods collected. We attribute this result to the repeated sampling of high-intensity precursors (resulting from high-abundance proteins) that persist across the chromatographic elution. Notably, the disparity in the number of MS² spectra and total peptides among the SDDA and the GPF approaches does not provide a corresponding difference in the number of unique peptides. In fact, the notched GPF approach identified the most unique peptides, with the SDDA approach providing nearly an equivalent number of unique peptides. However, we do observe a difference in the number of identified proteins among the various methods. Each of the GPF approaches resulted in more identified proteins than the SDDA approach, with the notched GPF approach identifying the most proteins. The observed increase in the number of identified proteins among the GPF approaches, without a dramatic increase in the number of either the total or unique peptides, suggests that these methods are sampling an additional population of proteins. These additional proteins are most likely expressed at lower concentrations than the population that is routinely identified via SDDA analyses.

To quantify the improvements in low-abundance protein characterization, we compared the number of peptides of low copy number proteins from the SDDA and notched GPF approach (Figure 3B). The lowest copy number proteins from yeast and their corresponding peptides were identified in the SDDA and notched experiments. We observed that for the vast majority of identified low-abundance proteins, the notched method routinely identified more unique peptides (red bars). Furthermore, the notched GPF approach provided greater coverage of all proteins that span the bottom two orders of magnitude of the observed yeast protein expression distribution (Figure S3A). Finally, we observed that the notched method identified more distinct peptides relative to the three alternative sampling methods and to the SDDA method (Figure S3B). It is evident that the notched method provides the identification of more low copy number proteins, more unique peptides corresponding to lower abundance proteins, and more distinct peptides.

Assessing the Quantitative Sensitivity and Accuracy of Notched Gas-Phase Fractionation

The observed gains in signal intensity and identification of low-abundance proteins suggested that MS¹-based quantification could also benefit from notched MS¹ GPF, especially for low-abundance/high-dynamic-range proteins. To assess the potential

improvement in MS¹ quantitative accuracy and precision we labeled a yeast whole-cell lysate by reductive demethylation, and we prepared different mixtures with varying ratios (1:1, 5:1, 10:1, and 20:1, Figure S4A).¹⁵ The resulting samples were analyzed individually utilizing either a standard method or a notched MS¹ method (Figure S4B). Following the application of a minimum signal/noise threshold (Figure S4C), meant to limit ratio variation due to poor ion counts, the ratios for all proteins within each mixture were compared (Figure 4). For the 1:1 mixture, the standard DDA and the notched MS¹ GPF approach provided nearly identical distributions of ratios. However, for the 5:1, 10:1, and 20:1 mixtures, the notched MS¹ GPF approach quantified a larger number of peptides with ratios at the expected value.

To further illustrate the utility of notched MS¹ GPF for MS¹-level quantification, we generated extracted-ion chromatogram (XIC) examples from the ratio dilution experiments for peptides identified in both workflows (Figure 5). For the 20:1 mixture, two examples XICs of yeast peptides are displayed. These XICs highlight the improved dynamic range (improved signal-to-noise values) and overall improvement to the measurement accuracy following the use of GPF. For these peptides, we observed absolute percent errors of 11.1 and 5% and 25 and 20% for the notched MS¹ GPF and standard DDA, respectively (Figure 5A). In fact, we observed that the median percent error for the standard approach is at least two-fold greater than the notched approach (Figure 5B and 5C). One potential source of error, especially for low-abundance proteins, could be insufficient ion intensity, resulting in fewer measurements across the elution profile. As highlighted in Figure 5D, the notched GPF approach routinely provides more measurements across the peptide elution than the SDDA approach.

Outlook

Extensions of the notched GPF fractionation approach could include applications for dynamic or intelligent data acquisition. For example, the *m/z* and width of the isolation notches could be specifically determined, in real-time, to isolate and enrich multiple features selected through data-dependent or targeted acquisition. This contrasts directly with the previously described implementations and applications that utilized a static set of discrete notches. An example workflow for data-dependent acquisition with label-free quantification would include a standard survey scan for feature selection, the generation and execution of a dependent notched isolation waveform against those features, and, finally, MS² interrogation of each of the features (Figure S5A). This approach would provide an increase in the dynamic range as well as an improvement in the accuracy and precision of the label-free quantitative measurements. Preliminary experiments have shown that this approach is feasible and enhances quantitative performance (Figure S5B and S5C).

Instrument acquisition rates continue to improve due to advancements in hardware and software. These duty cycle improvements could eventually offset the reduction in acquisition rate with GPF and enable single-injection gas-phase fractionation (Figure S6). The feasibility of such an approach is improved with intelligent data acquisition, such as RTS-MS³, which dramatically improves the acquisition rates for sample multiplexed workflows.

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CONCLUSIONS

The novel application of injection waveforms to isolate multiple populations of precursors simultaneously eluting from an analytical column improved LC-MS identification rates and quantitative accuracy. Importantly, the injection waveform-based isolation can be used in place of serial isolation and precursor collection (e.g., the MSX approach). The notched waveform method reduces the number of sample injections necessary to achieve GPF across a wide mass range and thereby results in improved efficiency in terms of instrument duty cycle. Reduced duty cycles, increased quantitative accuracy, and improved dynamic range have the potential to benefit both global and targeted instrument methods moving forward.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Standard gas-phase fractionation utilizes continuous bins that are often optimized via *in silico* prediction of peptide distributions or through extra injections of the sample. The mass-dependent elution of peptides during liquid chromatographic (LC) separation limits the degree of enrichment observed with continuous bins. Each bin (red, blue, and green) is employed within an independent, replicate sample injection. (B) A series of notches in an injection waveform results in discrete m/z slices or bins across the mass range. This approach is not reliant on *in silico* prediction or hindered by LC peptide elution. (C) The notched injection waveform is illustrated through discrete wells in the mass range permitting the retention of ions within wells and ejection of all other ions. (D) Example spectra from an LC-MS analysis of yeast whole-cell lysate. Consecutive MS¹ spectrum were collected via a standard full mass method, a binned GPF method, and a notched injection waveform.

Figure 2.

Yeast whole-cell lysate was analyzed via LC-MS. Back-to-back full scan MS¹ spectra were collected utilizing full mass range MS¹ scan, a standard GPF scan with a large continuous bin, and an MS¹ scan with a notched GPF. Features from the standard MS¹ scan were identified in the GPF spectra, and the degree of enrichment was calculated based on the reported signal-to-noise values for the feature in each spectra. (A) Continuous (Standard GPF) and discrete (Notched GPF) were compared. In each GPF scan, the total m/z space within the mass range stayed the same. (B) The mean fold enrichment was plotted against the chromatographic retention time. The notched injection waveform exhibits more uniform enrichment when compared to the standard GPF.

Figure 3.

Method comparison for enhanced proteome characterization. (A) Yeast whole-cell lysate (YWCL) was injected three times per method. For the GPF experiments (notched, tiling, and binned), the mass range for each of the injections was shifted to permit full coverage of the entire mass range. The standard experiment utilized triplicate injections of YWCL and collected MS¹ spectra across the full mass range. The large number of MS² for the standard method reflects the redundant sampling of peptides even at a dynamic exclusion of 55 ms. The notched and standard methods resulted in the largest numbers of unique peptides. However, due to the redundant sampling of peptides in the standard method, the total number of proteins is higher in each of the GPF experiments. (B) To assess the ability of the notched method to identify low-abundance yeast proteins, the unique peptides from the standard and notched method were compared. Low copy number yeast proteins (*x*-axis), which were identified in both methods, are plotted according to the expected number of molecules per cell (blue-dashed line, *y*-axis). For each protein, the number of unique peptides from the standard method was subtracted from the number of unique peptides from the notched method, and the difference was plotted (*y*-axis, black bars represent more protein coverage in the standard method and red bars represent more protein coverage in the notched method). Utilizing the notched injection waveforms resulted in greater sampling of unique peptides from low-abundance yeast proteins.¹⁶

Figure 4.

Yeast whole-cell lysate was split and labeled via reductive dimethylation. Light- and heavy-labeled peptides were mixed in 1:1, 5:1, 10:1, and 20:1 ratios and then analyzed through back-to-back collection of MS¹ via a standard method and by application of a notched injection waveform. For each mixture, the distribution of calculated ratios is plotted. The notched approach (red line) yielded more accurately quantified peptides at ratios of 5:1, 10:1, and 20:1.

Figure 5.

(A–C) Back-to-back comparisons of extracted ion chromatograms for peptides identified from yeast whole-cell lysate. For each mixture, a standard MS¹ and a notched MS¹ were collected consecutively. For the 20:1 sample (A), the notched method routinely resulted in higher signal-to-noise values and more accurate quantification. Across all ratios, the gain in signal-to-noise values from the notched method results in more accurate ratio quantification. (D) The notched method permits greater sampling of peptides across their elution, as was observed for the mean number of measurements across the peptide elution.