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TKO6: A Peptide Standard to Assess Interference for Unit-Resolved Isobaric Labeling Platforms

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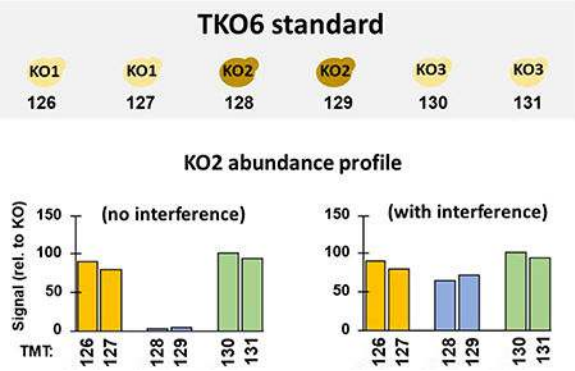
Abstract

Protein abundance profiling using isobaric labeling is a well-established quantitative mass spectrometry technique. However, ratio distortion resulting from co-isolated and co-fragmented ions - commonly referred to as interference - remains a drawback of this technique. Tribrid mass spectrometers, such as the Orbitrap Fusion and the Orbitrap Fusion Lumos with a triple mass analyzer configuration, facilitate methods (namely SPS-MS3) that can help alleviate interference. However, few standards are available to measure interference and thereby aid in method development. Here we introduce the TKO6 standard that assesses ion interference and is designed specifically for data acquired at low (unit) mass resolution. We use TKO6 to compare interference in MS2 versus MS3-based quantitation methods, data acquisition methods of different lengths, and ion trap-based TMT reporter ion analysis (IT-MS3) with conventional Orbitrap-based analysis (OT-MS3). We show that the TKO6 standard is a valuable tool for assessing quantification accuracy in isobaric tag-based analyses.

Graphical Abstract



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CONFLICT OF INTEREST DISCLOSURE

The authors declare no competing financial interest.

SUPPORTING INFORMATION

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Keywords

TKO; interference; TMT; iTRAQ; SPS-MS3

INTRODUCTION

Quantitative mass spectrometry-based proteomic strategies employing multiplexed isobaric labels are frequently used for proteome profiling (1–3). Sample multiplexing allows for high-throughput analyses across multiple samples in a single experiment. In MS3-based strategies that use synchronous precursor selection (SPS), isolated ions not originating from the spectrum-assigned peptide, but rather from co-eluting peptides, will often distort peptide quantification. Herein, we describe a versatile, yet simple isobaric tag-based peptide standard. This standard can be constructed with minimal effort yet affords high sensitivity for detecting interference from co-isolated ions in multiplexed strategies using either high- or low-resolution mass analyzers.

Previously, a yeast-human two proteome mixture has been used to evaluate ion interference and ratio compression (4). We sought to improve on this concept by simplifying the design and analysis in the form of a novel interference standard. Previously, we introduced a TMT9-plex standard consisting of digested yeast lysate arranged in three sets of triplicate channels, each of which was composed of a highly abundant *S. cerevisiae* yeast deletion strain (5). Thereby, we measured interference in a complex background by using a single deleted protein in each channel. Notably, the measured TMT signal should approach zero for the deleted protein in the appropriate strain. As such, signal measured in the designated "knockout" channels suggests instrumental noise and/or ion interference. However, the TKO9 standard required high resolution measurements and could not be used seamlessly to analyze unit-resolved reporter ions. Here, we present the TKO6 standard consisting of a TMT6-plex sample with three *S. cerevisiae* yeast deletion strains arranged in duplicate. Like the TKO9 standard, we used the highly abundant proteins: Met6, Pfk2, and Ura2 as our "TKO proteins." In doing so, we allow full compatibility at the peptide level for those using Skyline (6) or other software to track specific TKO peptides from either standard.

We showcase the TKO6 standard by investigating interference in three applications. First, we compare MS2- and MS3-based reporter ion quantification methods. This comparison has been performed previously (7), but not with the TKO6 interference standard. Then, we assess interference with respect to different data acquisition times. Essentially, we examine if interference observed using the TKO6 standard is affected by gradient length. Finally, we evaluate the use of ion trap MS3 (IT-MS3) versus the more commonly used Orbitrap MS3 method for reporter ion quantification. Isobaric tags that are separated by approximately one Dalton (or more) can be analyzed in an ion trap, permitting faster scan speeds and thus shorter duty cycles. Building upon previous studies (8, 9), we showcase the utility of IT-MS3 versus conventional Orbitrap-based SPS-MS3 (OT-MS3) analysis with fractionated TKO6 standard. Overall, we show that the TKO6 standard can assess data quality and provide guidance in method development for isobaric tag-based quantitative analyses.

EXPERIMENTAL SECTION

Materials.

Tandem mass tag (TMT) isobaric reagents were from ThermoFisher Scientific (Waltham, MA). Water and organic solvents were from J.T. Baker (Center Valley, PA).

Media and growth.

Saccharomyces cerevisiae strains were obtained from the haploid MATalpha collection (BY4742 MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0). Cultures were grown in standard yeast-peptone-dextrose (YPD) media to an optical density (OD) of 0.8/mL and then harvested.

Cell lysis and protein digestion.

Cell lysis, protein digestion, and tandem mass tag (TMT) labeling of the yeast cultures were performed as described previously using the Streamlined-TMT (SL-TMT) method (10). Briefly, yeast pellets were resuspended at 4°C in a buffer containing 200 mM EPPS pH 8.5, 8 M urea, and protease (complete mini, EDTA-free) inhibitors (Roche, Basel, Switzerland). Cells were lysed via bead beating. Proteins from cleared lysates were subjected to disulfide reduction with 5 mM tris (2-carboxyethyl)phosphine and alkylation with 10 mM which was quenched with 10 mM dithiotreitol. Methanol-chloroform precipitation was performed prior to protease digestion. Samples were resuspended in 200 mM EPPS, pH 8.5 and digested at room temperature for 16 h with LysC protease at a 100:1 protein-to-protease ratio. Trypsin was then added at a 100:1 protein-to-protease ratio and the reaction was incubated 6 h at 37°C.

Tandem mass tag labeling.

TMT reagents (200 μg) were added to the peptides (100 μg) along with a final acetonitrile concentration of approximately 30% (v/v). Following incubation at room temperature for 1 h, the reaction was quenched with hydroxylamine to a final concentration of 0.3% (v/v). The TMT-labeled samples were pooled at a 1:1:1:1:1:1 ratio, vacuum centrifuged to near dryness, and subjected to C18 solid-phase extraction.

Basic pH reversed-phase (BPRP) fractionation allowed for deep proteome analysis.

For fractionation of the TKO6 standard, 600 μg of the mixed standard was fractionated with basic pH reversed-phase (BPRP) chromatography. Following desalting, peptides were resuspended in buffer A (10 mM ammonium bicarbonate, 5% ACN, pH 8) and loaded onto an Agilent 300Extend C18 column (5 μm particles, 4.6 mm ID and 220 mm in length). The peptide mixture was fractionated with a 60 min linear gradient from 0% to 42% buffer B (10 mM ammonium bicarbonate, 90% ACN, pH 8). A total of 96 fractions were collected and concatenated so that every 24th fraction was pooled (i.e., samples in wells A1, C1, E1, and G1 were combined) and only alternating pooled fractions (a total of 12) were analyzed using SPS-MS3 (11).

Liquid chromatography and tandem mass spectrometry.

The samples were reconstituted in 5% acetonitrile and 5% formic acid. For each analysis, we loaded 0.5 μg of the TKO standard onto the C18 capillary column using a Proxeon NanoLC-1200 UHPLC. Peptides were separated on a 35 cm long, 100 μm inner diameter microcapillary column packed with Accucore (2.6 μm, 150Å) resin (ThermoFisher Scientific) and separated in-line with the mass spectrometer using gradients of 6 to 26% acetonitrile in 0.125% formic acid at a flow rate of 500 nL/min. Table 1 summarizes the parameters for each mass spectrometry method used. The automatic gain control (AGC) setting for IT-MS3 was chosen according to the previously published optimized value that balanced accuracy and precision (9).

Data analysis.

Mass spectra were processed using a SEQUEST-based software pipeline (12). Database searching included all entries from the yeast SGD (*Saccharomyces* Genome Database) (downloaded March 20, 2015). The database contains 6717 entries with all possible ORFs, including hypothetical ORFs. This database was concatenated with one composed of all protein sequences in the reversed order. Searches were performed using a 50 ppm precursor ion tolerance. The product ion tolerance was set to 0.9 Da. TMT tags on lysine residues and peptide N termini (+229.163 Da) and carbamidomethylation of cysteine residues (+57.021 Da) were set as static modifications, while oxidation of methionine residues (+15.995 Da) was set as a variable modification. Peptide-spectrum matches (PSMs) were adjusted to a 1% false discovery rate (FDR) using a linear discriminant analysis, as described previously (12). PSMs with poor quality and/or isolation specificity of < 0.8 (i.e., peptide purity <80%) were excluded from quantitation (13). Peptide lists with associated TMT values were analyzed further in Microsoft Excel and BoxPlotR (14). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (15) partner repository with the dataset identifier PXD011653.

RESULTS AND DISCUSSION

The TKO6 standard can diagnose ion interference in isobaric tag-based studies.

The TKO standard was constructed as outlined in Figure 1A. We used TMT6-plex reagents in a 3×2 experimental design, in which 3 different yeast deletion strains were labeled in duplicate. To minimize analysis time, we chose abundant proteins for which several peptides could be identified routinely (5). Standard protein extraction methodology and TMT labeling techniques were used to construct the TKO6 standard (10). Preparing this standard did not require expensive synthetic peptides or the often-cumbersome mixing and analysis of multiple proteomes. We selected high abundance proteins to ensure that associated peptides can be consistently measured even with short gradients. When analyzing TKO6 data, the TMT signal should approach zero for the knocked-out proteins in the corresponding deletion strain (Figure 1B). The interference-free index (IFI) was calculated to obtain a quantitative value for interference, with “1” representing no interference (Figure 1C). Below we showcased the TKO6 standard for benchmarking SPS-MS3 performance.

The TKO6 standard illustrated that MS2-based TMT quantification yielded greater proteome depth, although average interference values were lower using SPS-MS3-based methods.

A major caveat of SPS-MS3 analyses is the increased duty cycle relative to MS2-only analysis, which we term OT-MS2. Here, we compared the number of quantified TKO peptides, as well as the interference, in MS2- and MS3-based TMT quantification strategies. We performed the analysis in triplicate using 0.5 μg TKO6 standard with 45 minutes of data acquisition. As anticipated, approximately 20% more quantified TKO peptides were measured when using OT-MS2 compared to OT-MS3 (referred to henceforth as OT-MS3) (Figure S1A). However, these gains are offset by an increase in interference of over 25% (Figure S1B). In addition, the larger deviation for IFI measurements using OT-MS2 compared to OT-MS3 indicated decreased measurement precision, likely due to varying degrees of interference associated with different peptides assigned to that protein. These results agreed with a previously-published study using the TKO9 standard for which reporter ions were analyzed at a higher resolution (50,000 at 200 m/z), as opposed to the resolution used here (7,500 at 200 m/z) (5). We next sought to determine if data acquisition time had a similar effect on proteome depth and interference.

Data acquisition time did not considerably impact interference.

Having shown that data acquisition using the OT-MS3 method resulted in less interference (Figure S1), we aimed to demonstrate further the utility of the TKO6 standard by investigating the effect of data acquisition time (i.e., gradient length) on interference. The same sample (0.5 μg TKO6 standard) was analyzed in triplicate with seven different data acquisition times: 30, 60, 90, 120, 150, 180, and 240 minutes. First, we examined the number of total peptides identified and note, as expected, a steady increase in peptide number with respect to data acquisition time (Figure 2A). These data show that short gradients (i.e., total data acquisition time of 30 min) were adequate to measure sufficient numbers of TKO peptides, thereby enabling an average IFI to be calculated. While the number of TKO peptides increased from ~20 to ~40 between the 30- and 60-min data acquisition time points, respectively, that value leveled off at ~50 TKO peptides from 60 min onward (Figure 2C). We used the IFI calculated from these TKO peptides to assess interference with respect to data acquisition time. Surprisingly, we observed that the IFI remained steady with increased data acquisition time (Figure 2D). Although true for yeast, further analysis may be required if more complex peptide mixtures, such as human lysates, are to be analyzed. For subsequent analyses, we chose an acquisition time of 45 min, which was used when analyzing the TKO9 standard (5).

The TKO6 standard can be used to assess interference in ion trap quantitation-based TMT methods.

A previous study has elegantly introduced the concept of ion trap-based TMT reporter ion quantification in Tribrid mass spectrometers (9). More recently, a separate group investigated IT-MS3 quantification further with additional focus on quantitative precision (8). The first group assessed interference using a yeast dilution standard (9), while the second included synthetic peptides spiked into a constant human whole cell lysate

background, mimicking a two-proteome mixture (8). We build upon these efforts using the TKO6 standard and associated interference-free index (IFI) to provide a platform to readily evaluate ion interference in unit-resolution isobaric labeling experiments.

We compared our traditional Orbitrap-based SPS-MS3 (OT-MS3) strategy (10) with ion trap-based SPS-MS3 (IT-MS3) using the TKO6 standard as our interference and quality control metric. First, we explored the use of different ion trap scan modes: enhanced (IT-MS3e), normal (IT-MS3n), rapid (IT-MS3r), and turbo (IT-MS3t) compared to traditional OT-MS3. An unfractionated TKO6 standard (0.5 μg) was analyzed over 45 minutes of data acquisition. We assessed the number of peptides, proteins, and the IFI values for each of these methods. Our data showed that for this unfractionated TKO6, over 25% more quantified peptides (Figure S2A) and proteins (Figure S2B) were identified using any of the four IT-MS3 strategies (with diverse scan rates) compared to OT-MS3. Coincidently, the number of TKO peptides (Figure S2C) and IFI values (Figure S2D) deviated little for each of the five methods tested. As no substantial differences were observed among scan rates, we chose to use the default “rapid” setting for subsequent analyses. Next, we fractionated the TKO standard into 12 fractions as detailed by our standard SL-TMT methodology (10) to investigate the effectiveness of fractionated TKO6 to assess SPS-MS3 strategies.

Fractionated TKO6 standard shows similar proteome depth and interference when using either OT-MS3 or IT-MS3.

Fractionated TKO standard was analyzed with OT-MS3 and ITMS3 strategies with identical liquid chromatography gradients (2.5 hr), as well as MS1 and MS2 settings. We quantified similar numbers of non-redundant peptides (over 30,000) using each method, of which approximately one-third (over 20,000) were in common (Figure 3A). Our findings were similar at the protein level as approximately 4,200 proteins were quantified with each method, over 3,900 of which were common to both (Figure 3B). Overall, in the fractionated sample, IT-MS3 methods quantified only ~ 5% more peptides and ~ 2% more proteins than OT-MS3. We also tallied the number of TKO peptides quantified per method in each of the 12 fractions (Figure 3C). At least 15 TKO peptides were quantified in each sample, with some fractions measuring over 40 peptides. In total, 365 TKO peptides were identified using IT-MS3, while a similar number, 379, was identified with OT-MS3. When examining the IFI values for IT-MS3 and OT-MS3, we observed similar distributions. However, this distribution was slightly narrower for OT-MS3. Moreover, the median IFI for the OT-MS3 analysis was 0.92, while that for IT-MS3 was negligibly lower at 0.90, yet the distribution had a longer tail towards lower IFIs (Figure 3D). In general, like the unfractionated standard, the fractionated TKO6 revealed similar performance in both proteome depth and TMT measurement accuracy when using either OT-MS3 or IT-MS3 for unit mass separated isobaric labels. However, the trend of slightly more identifications with IT-MS3 analysis was offset with less quantitative accuracy compared to OT-MS3. The data revealed marginally more quantified peptides when using IT-MS3 than conventional OT-MS3 analyses, while the IFI distributions were similar between the two methods, but trended lower for IT-MS3. As the catalog of isobaric tags expands with different labeling reagents and greater multiplexing capabilities, novel methods will be developed (16). As such, the TKO6 standard will be valuable for benchmarking improvements in these methods.

Conclusions.

Consistent instrument performance and reliable benchmarking are key to highquality datasets. We introduced the TKO6 standard as a tool for assessing ion interference in unit resolution isobaric tag-based multiplexed proteomic experiments. Like other standards, the TKO6 can measure and track informative figures of merit, such as the number of quantified peptides, success rate (MS2 spectra identified/ collected), ion injection times, signal-to-noise measurements for reporter ions, and peak width/shape. However, the TKO6 can also evaluate ion interference in isobaric tag-based experiments. In addition to the applications discussed herein, further parameter optimizations are possible using the TKO6 standard, such as determining the appropriate isolation window widths, ion times, and signal-to-noise thresholds. The TKO6 standard is unique compared to TKO9 as it is composed only of peptides labeled with unit-resolved TMT reagents and not ^{13}C and ^{15}N isotopologues. This feature permits method optimization and development of unit resolution reporter ion-based strategies, such as IT-MS3 as investigated here. However, like the previous iteration of the standard, the deleted proteins used in the TKO are among the most highly abundant in yeast. This was by design as our goal was to quantify these peptides with very short gradients and/or following fractionation, as we did here. Future iterations of this standard may replace the current high abundant TKO proteins with those of low and moderate abundance to enable interference assessment across the range of protein abundance. However, regardless of the selected TKO proteins, the TKO6 standard can effectively measure interference and thereby guide parameter optimization and method development in efforts to alleviate interference afflicting isobaric tag-based quantification strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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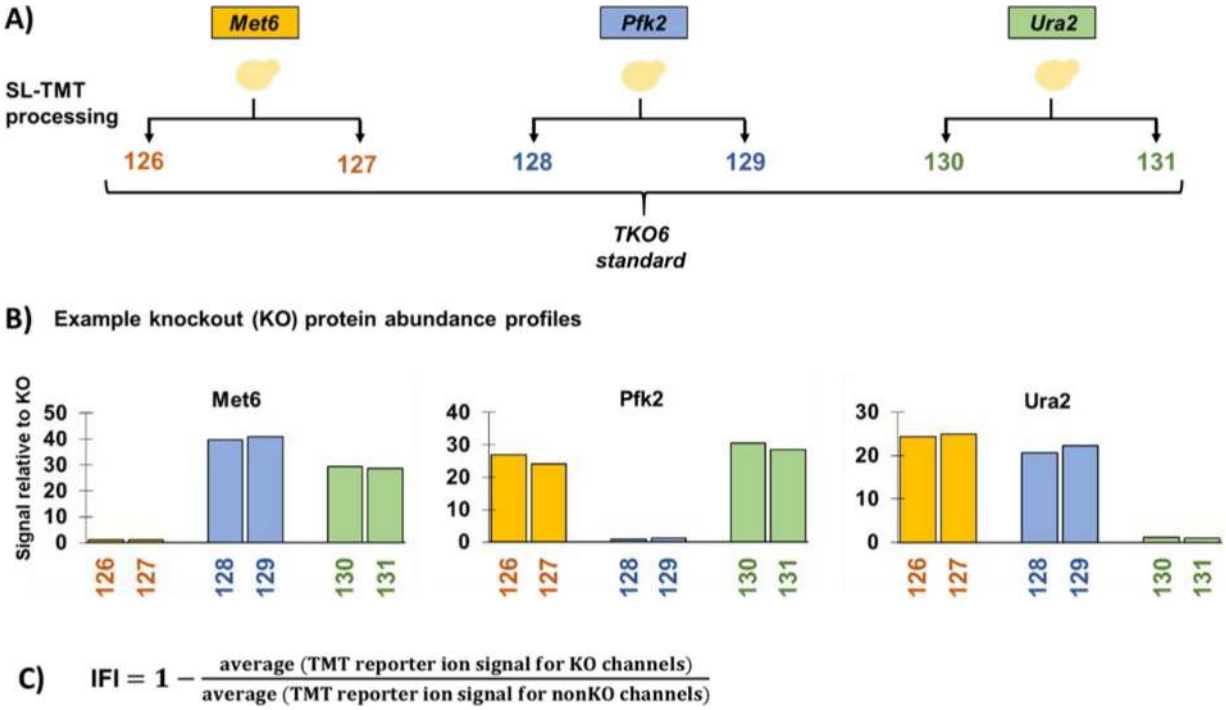

Figure 1: Overview of the TKO6 standard.

A) Deletion strains of three abundant yeast proteins were processed using the SL-TMT protocol, resulting in a pooled TMT6-plex in which each deleted protein was absent from two of the six channels. **B)** The protein abundance profiles should measure minimal signal for peptides of a given TKO protein in TMT channels corresponding to the appropriate deletion strain. **C)** Equation for calculating the interference-free index (IFI). TMT, tandem mass tag; KO, knock out.

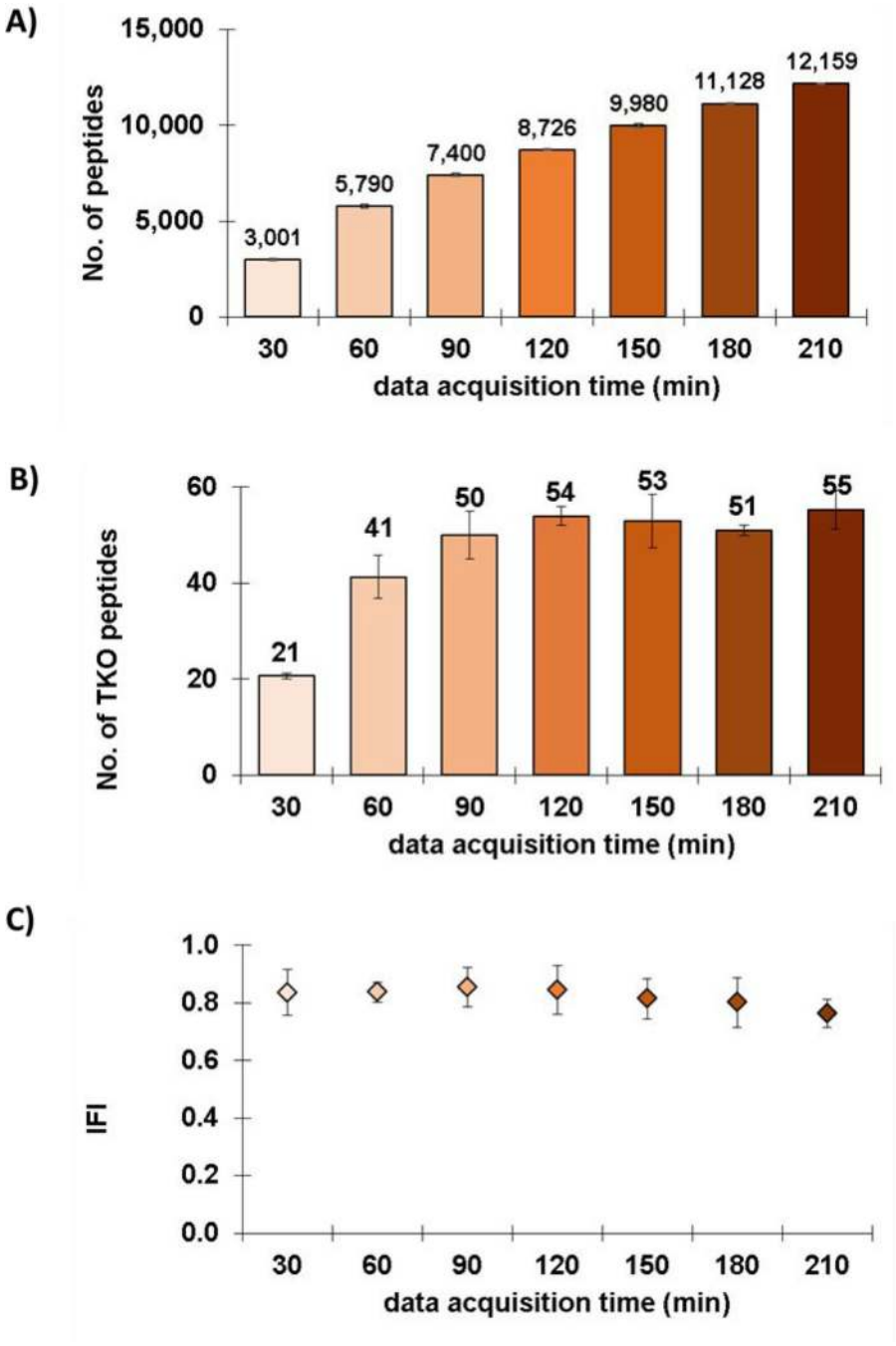

Figure 2: The effect of acquisition time investigated using the TKO6 standard.

A) The bar chart depicts the number of total peptides quantified for specific data acquisition times. **B)** The bar chart illustrates the number of total TKO peptides per data acquisition time point. **C)** The dot plot illustrates the effect of acquisition time on the interference-free index (IFI). Error bars represent the standard deviation (n=3), as these analyses were performed in triplicate.

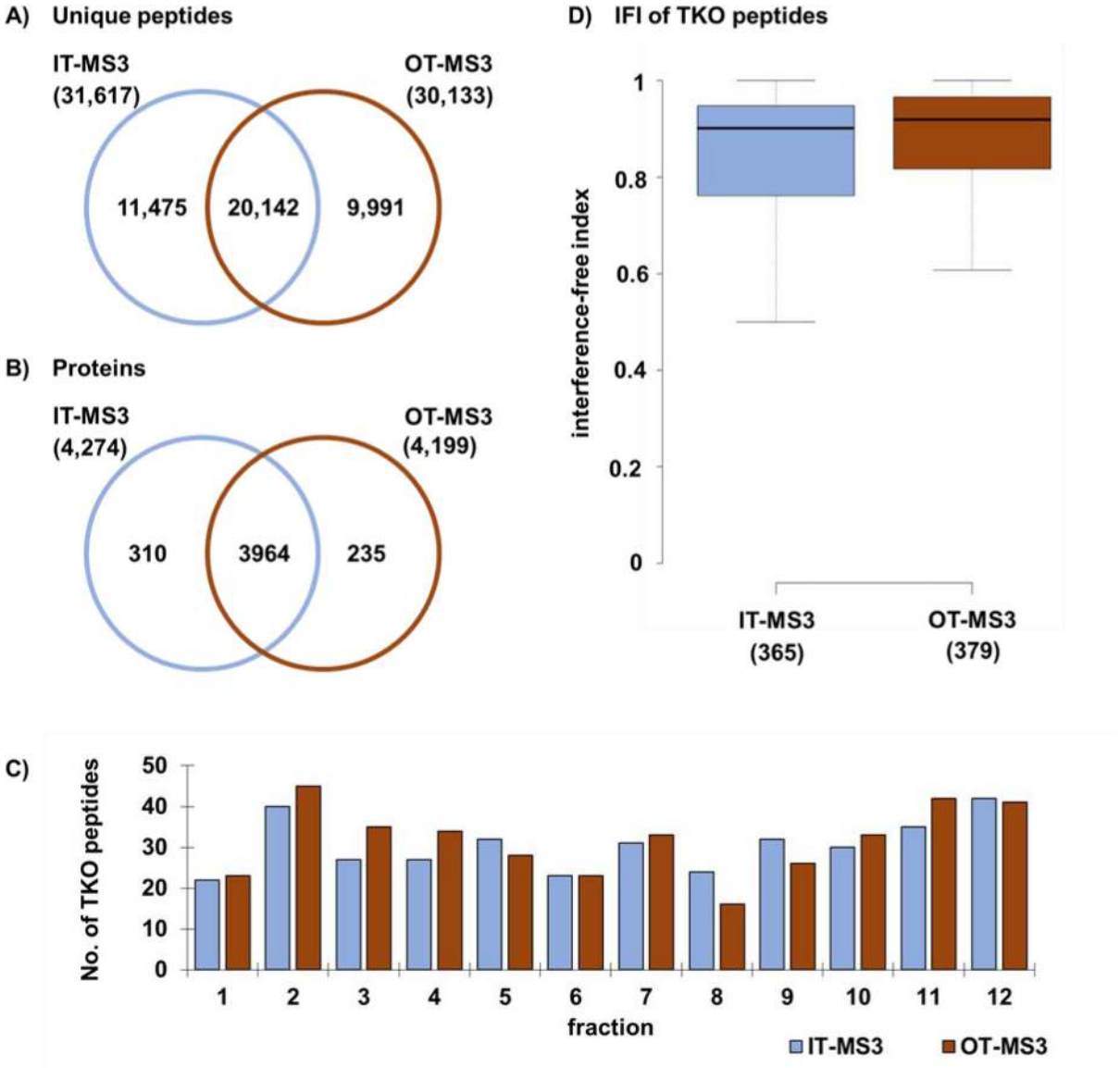

Figure 3: Assessment of fractionated TKO standard analyzed by IT-MS3 and OT-MS3 for SPS-MS3 analyses.

Venn diagrams illustrate quantified **A)** peptides and **B)** proteins that are common between and unique to IT-MS3 and OT-MS3 analyses. **C)** The number of total TKO peptides identified per fraction by IT-MS3 and OT-MS3 analysis. Redundant peptides are included across fractions. **D)** Distribution of interference-free index (IFI) for fractionated TKO6 analyzed by IT-MS3 and OT-MS3. On this box-and-whiskers plot, the center lines show the medians; box limits indicate the 25th and 75th percentiles; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles.

Table 1:

Methods summary

	OT-MS3	OT-MS2	IT-MS3e	IT-MS3n	IT-MS3r	IT-MS3t
MS1						
Mass analyzer	Orbitrap	Orbitrap	Orbitrap	Orbitrap	Orbitrap	Orbitrap
Resolution	120K	120K	120K	120K	120K	120K
Scan range	400–1400	400–1400	400–1400	400–1400	400–1400	400–1400
AGC target	4.0E+05	4.0E+05	4.0E+05	4.0E+05	4.0E+05	4.0E+05
Max. inject time (ms)	50	50	50	50	50	50
RF lens (%)	30	30	30	30	30	30
MS2						
Charge states	2–6	2–6	2–6	2–6	2–6	2–6
Mass analyzer	ion trap	Orbitrap	ion trap	ion trap	ion trap	ion trap
Activation type	CID	HCD	CID	CID	CID	CID
Isolation window	0.4	0.4	0.4	0.4	0.4	0.4
Collision energy	35	35	35	35	35	35
Scan rate/resolution	turbo	7.5K	turbo	turbo	turbo	turbo
AGC target	1.0E+04	5.0E+04	1.0E+04	1.0E+04	1.0E+04	1.0E+04
Max. inject time (ms)	50	150	50	50	50	50
MS3						
Number of SPS ions	10		10	10	10	10
Isolation window	1.2		1.2	1.2	1.2	1.2
Activation type	HCD		HCD	HCD	HCD	HCD
Collison energy	65		65	65	65	65
Mass analyzer	Orbitrap		ion trap	ion trap	ion trap	ion trap
Scan rate/resolution	7.5K		enhanced	normal	rapid	turbo
Scan range (Th)	"normal"		116–141	116–141	116–141	116–141
AGC target	1.5E+05		5.0E+03	5.0E+03	5.0E+03	5.0E+03
Max. inject time (ms)	150		150	150	150	150