Establishing A Comprehensive Workflow for Extracting MS1 Isotope Distributions in LC-MS/MS Proteomics

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Abstract

The continuous advancements in LC-MS/MS proteomics over the past decades have paved the way for transformative changes in the field of medicine, particularly in the realms of preventive and personalized healthcare. Many new algorithms are evaluated on unknown proteomes and using databases with annotated MS2-spectra. When the research is focused on MS1-spectra, such databases are not available yet. Specifically, we propose a comprehensive workflow to extract MS1 isotope distributions from spectra, which we validated using a proteomics standard kit comprising known proteins at varying concentrations in duplicate. Our workflow incorporated a database search utilizing a state-of-the-art algorithm at 1% FDR. Through this approach, we investigated the impact of protein concentration on the probability of protein identification. Confidently identified PSMs were used to extract the MS1 isotope distributions through the proposed workflow. A total of 138.111 MS1 isotope distributions were extracted. Isotope distributions with 2 or more peaks were compared with their theoretical isotope distributions using the spectral angle. A median spectral angle of 0,101 and 0,0992 was observed in both samples indicating a high similarity. The findings from this study were compiled into a dataset which can potentially facilitate the development of novel tools with a focus on MS1 data.

Title

Establishing A Comprehensive Workflow for Extracting MS[1] Isotope Distributions in LC-MS/MS Proteomics

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Abbreviations

DDA = Data Dependent Acquisition

DL = Deep Learning

ML = Machine Learning

ppm = parts per million

PSM = peptide spectral match

PRIDE = Proteomics Identifications Database

UPS = Universal Proteomics Standard

XIC = Extracted ion chromatograms

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Abstract

The continuous advancements in LC-MS/MS proteomics over the past decades have paved the way for transformative changes in the field of medicine, particularly in the realms of preventive and personalized healthcare. Many new algorithms are evaluated on unknown proteomes and using databases with annotated MS^2-spectra. When the research is focused on MS^1-spectra, such databases are not available yet. Specifically, we propose a comprehensive workflow to extract MS^1 isotope distributions from spectra, which we validated using a proteomics standard kit comprising known proteins at varying concentrations in duplicate. Our workflow incorporated a database search utilizing a state-of-the-art algorithm at 1% FDR. Through this approach, we investigated the impact of protein concentration on the probability of protein identification. Confidently identified PSMs were used to extract the MS^1 isotope distributions through the proposed workflow. A total of 138.111 MS^1 isotope distributions were extracted. Isotope distributions with 2 or more peaks were compared with their theoretical isotope distributions using the spectral angle. A median spectral angle of 0,101 and 0,0992 was observed in both samples indicating a high similarity. The findings from this study

were compiled into a dataset which can potentially facilitate the development of novel tools with a focus on MS^1 data.

Statement of Significance

LC-MS/MS-based proteomics is continuously advancing, allowing redefinition of disease at the molecular scale, transforming curative medicine to preventive and personalized medicine. While there are numerous large, annotated spectral databases available for the development of new bioinformatic tools focused on MS^2data, the same cannot be said for research focused on MS^1 data. In the MS^1 setting, each spectrum contains multiple peptides, and the primary interest often lies in their isotope distributions. However, extracting this information is not a straightforward task. Therefore, we propose a method to extract these isotope distributions combined with other important MS^1 features in a PSM data-driven manner and summarize them in a standardized format, creating an MS^1 isotope distribution benchmark dataset. We applied this workflow on a proteomics standard and demonstrated the results, showing a high similarity between the extracted and theoretical isotope distributions. The workflow can be applied in the future to further extend the benchmark dataset. The dataset itself can act as the foundation to develop new bioinformatic tools. The availability of an extensive MS^1 isotope distribution benchmark dataset will foster the development of innovative bioinformatic tools, enabling researchers to unlock new insights and further advance our understanding of molecular underpinnings of disease pathology.

Introduction

Continuous advancements from a technical point-of-view have made MS an appealing technique in different research fields, for example proteomics. In proteomics, researchers often rely on bottom-up proteomics, cleaving the proteins and peptides in a sample using digestion enzymes, e.g. trypsin, followed by LC-MS/MS. Different subfields of research within proteomics have emerged, including biomarker discovery , drug discovery , PTM research such as phosphorylation , immunopeptidomics , quantitative proteomics , and many more. The capability of MS to rapidly sequence peptides and proteins, and to detect mutations and modifications with an incredible high sensitivity makes it an appealing analytical tool to apply within a clinical setting.

Coupled with quantitative proteomics, MS-based proteomics has the potential redefine disease definitions at the molecular level and help shift the current curative medicine towards personalized medicine . However, current workflows are prone to experimental errors. Because of these experimental errors, it is essential to make a formal comparison of different proteomics techniques when creating a proteomics workflow. In the laboratory, different techniques may easily be compared by comparing the results from different laboratory techniques. From a bioinformatics point-of-view, this is less straightforward. Different algorithms, albeit for peptide identification, quantification, or different purposes, are usually compared on available experimental datasets. However, the comparison of algorithms on these experimental datasets may not be truly justified. Griss et al. found in a large-scale study done on the Proteomics Identifications Database (PRIDE) that on average 75% of the spectra analyzed in a MS experiment remained unidentified . Unidentified could mean three things: incorrectly identified, correctly identified but below scoring thresholds and truly unidentified. Hence, relying on public datasets with unknown proteomes proposes challenges when comparing different bioinformatic tools.

Additionally, machine learning (ML) and deep learning (DL) algorithms are becoming more popular in MS-based proteomics due to advancements in the computational field and the availability of large amounts of (training) data. As a consequence, these algorithms are now commonly used in every processing step of mass spectrometry data. When performing spectral clustering prior to analyzing the data, GLEAMS is a novel algorithm that relies on neural networks . For the identification of spectra, Ionbot and Casanovo are recent machine learning and deep learning applications . Lastly, as a part of post-processing, the scores from

PSMs are almost always rescored using algorithms to increase the amount peptide identifications. Commonly used ML and DL algorithms for this purpose are Percolator , Prosit , MS^2Rescore and MSBooster . Other applications include, but are not limited to, the prediction of MS^2 peak intensities from peptide sequences, e.g. using Prosit, MS^2PIP or AlphaPeptDeep , or retention time prediction, e.g. using AlphaPeptDeep or DeepLC . All mentioned ML and DL applications have been developed using publicly available datasets using annotated MS^2 spectra. Their usage in improving the identification of MS^2 spectra and PTMs has been extensively shown in literature.

Contrary to MS^2-based research, MS^1spectra contain information on multiple peptides with a corresponding isotope distribution. This requires researchers to extract the isotope distribution from specific regions of interest before analysis. Little research has been done on extracting these isotope distributions, causing a lack of MS^1 standardized benchmark isotope distribution datasets . In this work, we aim to develop a workflow to extract the isotope distribution in a PSM data-driven manner and we present the results in a standardized way. Our objective is to create a database with annotated MS^1 isotope distributions and other relevant features, which can be used as a foundation to develop new ML and DL applications in the future. To evaluate our workflow, we analyzed the Universal Proteomics Standard 2 (UPS2) from Sigma-Aldrich with state-of-the-art software and applied the workflow, presenting it as a first MS^1 benchmark dataset.

Materials and Methods

Data

A publicly available LC-MS/MS experiment using the UPS2-kit was used. The Universal Proteomics Standard 2 contains 48 human proteins with a molecular weight ranging from 6.000 to 83.000 Daltons. The proteins have a dynamic range of concentrations between 0,5 to 50.000 femtomole. The data is publicly available on the PRIDE repository with identifier PXD000331 . The dataset contains raw data exclusively from the UPS2-kit, but also the UPS2-kit in combination with micro-organisms such as*Mycoplasma pneumoniae* , *Drosophila melanogaster* and*Leptospira interrogans* . For the purpose of the manuscript, only the raw data on the UPS2-kit was selected. In the experiment, the proteins in the UPS2-kit were enzymatically cleaved into peptides using trypsin. The peptide-mixture was separated using LC for 120 minutes prior to performing MS/MS with the LTQ Orbitrap Velos. The UPS2-kit was measured in duplicate, A11-12042.raw and A11-12043.raw. For more specific information about the experiment, we refer to the original article of Ahrné et al. .

Database search

Both duplicates were analyzed with the FragPipe graphical user interface (version 19.1), using the Thermo Fisher .RAW files as input. FragPipe incorporates the MSFragger database search engine (version 3.7) . The default workflow was used to process the data except for the following adjustments. A precursor mass tolerance of ± 10 parts per million (ppm) and a fragment mass tolerance of ± 5 ppm was specified. Carbamidomethylation of Cysteine was set as a fixed modification and oxidation of Methionine as a variable modification. Trypsin was specified as the digestion enzyme with up to 2 missed cleavages. MSBooster and Percolator were used for rescoring the PSM with an FDR of 0.01 using a reverse target-decoy approach. An FDR of 0.01 was selected to ensure a high-quality benchmark dataset created from the MSFragger identifications. The results were further investigated using R (Version 4.3.0) and RStudio (Version 2023.3.0.386) .

Benchmark dataset construction

The general workflow is shown in Figure 1. The Thermo Fisher .RAW files were converted into mzML-format using MSConvert (version 3.0.23051) . MSConvert had vendor specific peak picking enabled to centroid the

spectra. The data was processed further using a custom written Python script (version 3.9) . The Python bindings of OpenMS (version 2.7.0) were used to process the mzML files , such as selecting the MS1 spectra, acquiring the peak information, retention times, etc. All PSMs from MSFragger were used to construct the dataset. The amount of possible isotopic peaks was set to the monoisotopic peak followed by up to 5 isotopic peaks. It should be noted that this was an arbitrary choice. To construct the extracted ion chromatogram (XIC), the error margin on the observed m/z for the PSM was set to 5ppm, and we opted for a 5 second window before the retention time of the PSM and 30 seconds after. The 5 second window before the retention time of the PSM was selected as 5 seconds was twice the maximum time between two MS^1spectra. A window of 30 seconds after the retention time of the PSM was selected as Ahrné et al. enabled a dynamic exclusion of 30 seconds after sampling a precursor ion. Hence, it was possible that the peptide was still present in the following MS^1 spectra for 30 seconds without being sampled again. The extracted isotope distributions with at least 2 peaks were compared with the theoretical isotope distributions acquired using BRAIN (version 1.44.0) by computing the spectral angle . The MS^1 isotope distribution dataset with additional metadata was stored as an Excel-file. The algorithms and code are available on https://github.com/VilenneFrederique/MS1IsotopeDistributionsDatasetWorkflow.

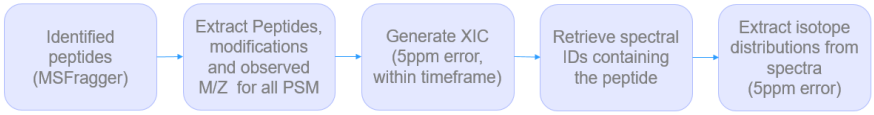

Figure 1 Workflow for extracting MS^1 isotope distributions.

Results

Database search

The results for the database search using MSFragger are summarized in Table 1. Sample A-1112042 had slightly more PSMs compared to sample A11-12043. The PSMs resulted in approximately the same number of identified peptides for both samples and the same number of identified proteins. All detailed results including p-values can be found in Supplementing Information 1.

Table 1 Summarized results of the MSFragger database search on duplicates of a UPS2-kit.

Sample	A11-12042	A11-12043
PSMs	2.378	2.215
Peptides	532	538
Proteins	36 (75%)	36 (75%)

The database search results for both samples were investigated in-depth to gain more insight into the identifications and their quality. On the peptide level, both samples had very similar identifications. Specifically, 501 peptides were identified in both samples, and only 68 peptides were identified in only one of the samples (Figure 2 A). On the protein level, both samples managed to identify 35 common proteins and 1 unique protein in each sample (Figure 2 B). Cytochrome C was identified as a unique protein in sample A11-12042 and Ubiquitin-conjugating enzyme E2 C in sample A11-12043. Both proteins had a concentration of 0,005 pmol in the UPS2 standard. In general, it can be observed that the concentration of the proteins in the UPS2 samples influences the probability of identifying a protein. In both samples, the proteins with a concentration of 0,5, 5 and 50 pmol were all detected. As the concentrations of the proteins was reduced, the percentage of detected proteins also decreased up to a point that all proteins with a concentration of 0,0005

pmol remained undetected (Figure 2 C). The same phenomenon was observed when looking into the coverage of the proteins. While both samples had somewhat equal coverages, it is visible that as the concentration of the proteins decreases, so does the coverage (Figure 2 D). Proteins with a concentration of 50 pmol were almost always identified with a coverage above 90%, except for the protein Complement C5 being an outlier for both samples. Proteins with a concentration of 0,005 pmol almost always had a coverage well below 10% for both samples apart from GTPase HRas in sample A11-12043, having a coverage of 20,11%.

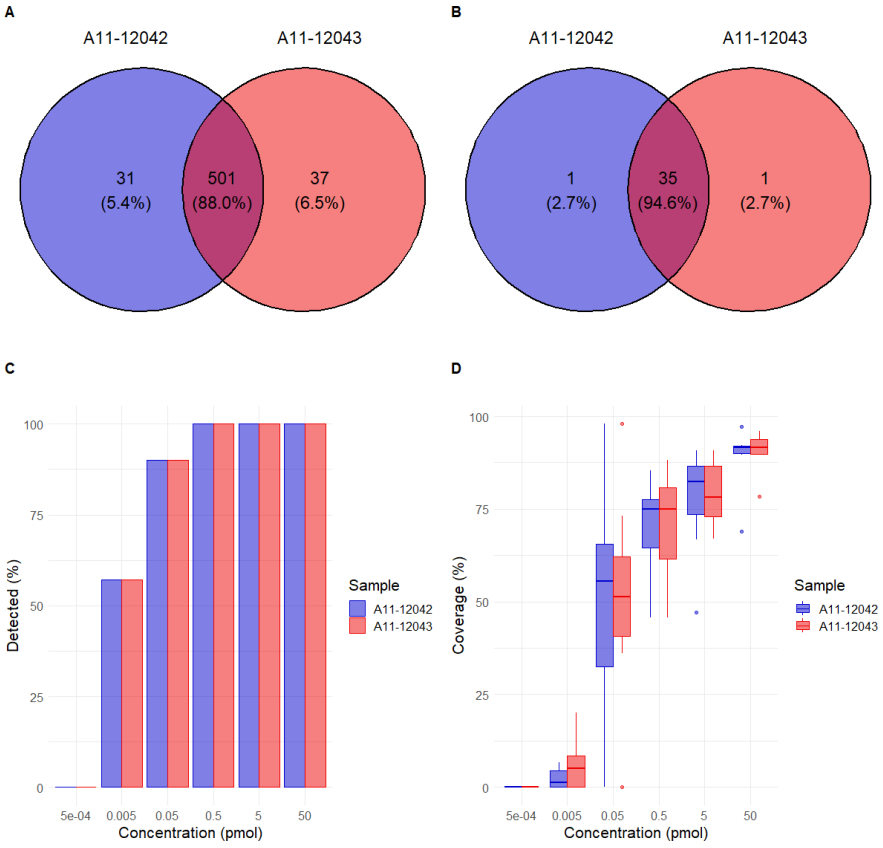

Figure 2 Results of the database search for the UPS2 standard kit using MSFragger.

Sample A11-12042 is always shown in blue and sample A11-12043 is always shown in red. **(A)** Venn-diagram with the number of identified peptides in both samples with percentages between brackets. **(B)**Venn-diagram with the number of identified proteins in both samples with percentages between brackets. **(C)** A bar plot with the percentage of proteins detected for both samples for all 6 concentrations present in the UPS2 standard kit. **(D)** Boxplots showing the coverage of the proteins in the UPS2 standard kit at each concentration for both samples.

Benchmark dataset

The MS^1 isotope distribution benchmark dataset can be found in Supplementing Information 2. The complete workflow resulted in 138.111 possible MS^1 isotope distributions, 70.593 identifications in sample A11-12042 and 67.518 identifications in A11-12043. There were 127.646 peptide isotope distributions with 2 or more peaks and 10.465 peptide isotope distributions consisting only out of the monoisotopic peak. Isotope distributions with 2 isotope peaks (30.706 identifications) and isotope distributions with 3 isotope peaks (30.873) occurred the most (Table 2).

Table 2 Amount of extracted MS[1] identifications with their maximum number of isotope peaks per sample.

Amount of Isotope peaks	A11-12042	A11-12043	Total
1	5.595	4.870	10.465
2	15.772	14.934	30.706
3	15.605	15.268	30.873
4	12.138	12.153	24.291
5	9.927	9.062	18.989
6	11.556	11.231	22.787
Total	70.593	67.518	138.111

The 127.646 peptide isotope distributions consisting out of 2 or more peaks were compared to their theoretical isotope distributions using BRAIN by calculating the spectral angle. A similar distribution of spectral angles in both samples was observed with a median spectral angle of 0,101 in sample A11-12042 and 0,0992 in sample A11-12043 (Figure 3). A non-parametric 95% median percentile interval was constructed for both samples using 10.000 iterations with seed "1234". Sample A11-12042 had a slightly higher 95% median percentile interval of [0,1001468;0,1019028] compared to sample A11-12043 [0,0982923;0,1000837]. The complete benchmark dataset consists out of 965 unique combinations of peptide sequences, PTMs and charge states.

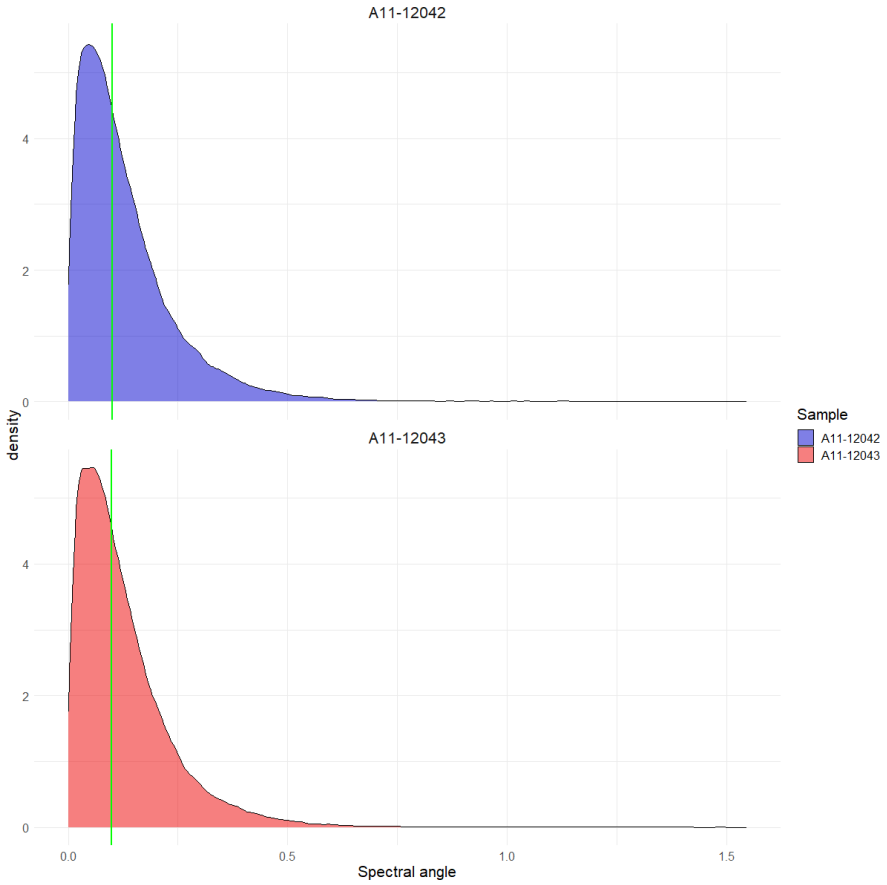

Figure 3 Distribution of spectral angle scores in both samples.

The MS^1 isotope distributions with at least 2 peaks were compared to the theoretical isotope distributions acquired through BRAIN. The green line shows the median spectral angle score for both samples. A11-12042 is shown in blue and A11-12043 is shown in red.

Discussion

During our research, the objective was to establish a systematic workflow and generate a high-quality dataset of MS^1isotope distributions. To eliminate the inherent stochasticity associated with working on unknown proteomes, we utilized the UPS2 standard kit. As the UPS2 standard kit only contains known proteins, we know what proteins to search for, giving an increased reassurance in the identifications made by the database search algorithm. Additionally, the varying concentrations within the kit allows researchers to test the sensitivity of their newly developed tools.

The initial step in our research involved performing a database search on both UPS2 samples. To ensure the production of high-quality PSMs, we employed MSFragger with a reverse target-decoy approach, maintaining an FDR of 1%. An equal amount of PSMs was identified in both samples, and there was a high level of agreement between the peptide and protein identifications. Upon further investigation, it was found that the protein concentration is one of the most influential factors for protein identification. Specifically, proteins with lower concentrations in the UPS2 standard kit exhibited reduced coverage and overall detection probability. While this might seem like a logical finding, we do want to express the importance of it. When using an unknown proteome to evaluate different algorithms, the PSMs will be influenced by the concentrations of the peptides and proteins present in the sample. While there are many other factors influencing the probability of identifying proteins and peptides, such as the preprocessing of samples or the dynamic range of the LC-MS/MS device itself, it is an important point to consider and well described in literature .

Next, we used a workflow developed in-house to extract MS^1 isotope distributions for the PSMs acquired by the MSFragger database search. A total of 138.111 peptide isotope distributions were acquired combined over both samples with at least 127.646 peptide isotope distributions having 2 or more peaks. There were more MS^1 isotope distributions extracted from sample A11-12042 compared to sample A11-12043, which corresponds to sample A11-12042 having more PSMs in comparison to sample A11-12043. The spectral angle was used to check the similarity between the experimental isotope distributions and their expected theoretical isotope distributions computed by BRAIN. The spectral angle can take on values between 0 and 1.57, with values closer to 0 indicating a higher similarity between the experimental and theoretical isotope distributions . The bell shape of the distributions of the spectral angle scores in both samples lay close to 0, indicating a high similarity between theoretical and experimental isotope distributions (Figure 3). While the dataset still includes isotope distributions with a high spectral angle score, indicating a high dissimilarity between the theoretical and experimental isotope distributions, we opted to leave them in the dataset, as they may still serve as valuable input for training machine learning and deep learning models. There were 10.465 isotope distributions consisting of just the monoisotopic peak. There are currently no ways of validating these monoisotopic peaks MS^1 spectra, that we are aware of. Their only legitimacy is that they have been extracted at approximately the same time as confidently identified PSMs and within the specified mass window. Lastly, the complete MS^1 isotope distribution dataset consists out of 965 unique peptides based on their sequence, modifications and charge state. While the complete dataset is quite large, it is also limited to a set of unique UPS peptides. However, we believe that the workflow presented may be used in the future to extract more MS^1 isotope distributions from proteome standards such as the large-scale ProteomeTools dataset .

In this manuscript, we provided a data-driven approach to extract MS^1 isotope distributions of high-quality while presenting them in a standardized manner. The proposed workflow can be used in the future to further extend the benchmark dataset. The benchmark dataset itself provides an ideal foundation for the development of new bioinformatics tools in the future, such as new machine learning and deep learning model. These novel algorithms may further advance our understanding of the

molecular underpinnings of disease pathology. All code and algorithms have been made available https://github.com/VilenneFrederique/MS1IsotopeDistributionsDatasetWorkflow

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Conflicts of interest

The authors have declared no conflicts of interest.

Associated data

The data that support the findings of this study are openly available in the PRIDE repository at https://doi.org/10.1002/pmic.201300135, reference number PXD000331.