Investigation into the Qualitative and Quantitative Capabilities of a Database Search Algorithm for Alternate Scanning Multiplexed LC-MS Proteomics Data

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INTRODUCTION

A variety of database search algorithms are available for the qualitative analysis of MALDI and ESI product ion MS/MS spectra. In most instances, these fragmentation spectra are acquired with data dependant acquisition methods and the algorithms tailored to accommodate the nature and characteristics of the data. Multiplexed, data independent acquisition fragmentation spectra can therefore pose a problem and are typically penalized because of their unexpected nature. The quantitative analysis of a (sub) proteome is often not adequately addressed but is becoming increasingly important. Label-free LC-MS experiments were conducted to investigate the quality of the results of a search algorithm – including absolute and relative quantitative analysis – for multiplexed LC-MS data sets.

Previously characterized serum samples from four patients, prior to treatment, were depleted by removal of the six or twelve most abundant proteins. The samples were digested and analyzed by nanoscale LC-MS using a nano electrospray interface configured on a hybrid quadrupole/oa TOF mass spectrometer. Triplicate injections were performed for each sample and parallel qualitative and quantitative analysis of the obtained MS and multiplexed fragmentation spectra performed. Validation of the qualitative search results was conducted by means of the detailed analysis of a recombinant protein marker utilizing mass accuracy and time-alignment of the identified precursor and fragment ions. The quantitative aspects were validated by complementary analysis such as nanoscale LC - triple quadrupole MS/MS analysis and biochemical ELISA techniques.

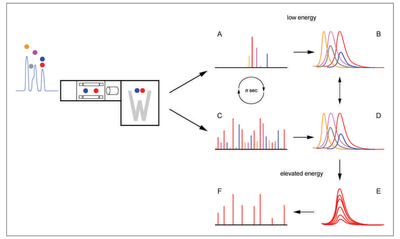

Figure 1. The principle of multiplexed, alternate scanning (LC-MS^E)

METHODS

Sample preparation

Pre treatment patient serum samples were diluted and solubilized prior to depletion with a affinity removal system (Agilent Technologies, Palo Alto, CA, USA or Beckman Coultar, Fullerton, CA, USA), which removes the six or twelve most abundant serum proteins, respectively. The depleted serum samples were subsequently denatured with RapiGest™ SF surfactant (0.1%) (Waters Corp.), reduced (10 mM DTT), alkylated (10 mM IAA) and enzymatically digested with trypsin, 1:50 (w/w) enzyme:protein ratio.

LC-MS conditions

Qualitative and quantitative LC-MS experiments were conducted using either a 1.5 or 0.5 hr reversed phase gradient from 5 to 40% acetonitrile (0.1% formic acid) at 250 nL/min on a nanoACQUITY UPLC™ System (Waters Corp.). An Atlantis® 3µm C18 75 µm x 15 cm nanoscale LC column (Waters Corp.) was used, with all samples run in triplicate. Typical on-column sample loads were 0.5 µg protein digest.

Multiplexed, alternate scanning (LC-MS^E) experiments — Figure 1 — were performed with a Q-Tof Premier™ mass spectrometer (Waters Corp.), which was programmed to step between normal (5 eV) and elevated (15-40 eV) collision energies applied to the gas cell, using a scan time of 1.5 seconds per function over the m/z range 50-1990.

Triple quadrupole MS/MS experiments were conducted with a Quattro Premier XE mass spectrometer (Waters Corp.) in MRM mode of analysis. The transmission window of both mass analyzers was typically 1 Da, the dwell time 25 ms and the collision energy approximately 20 eV.

ELISA

Chitinase-1 activity was measured with 4-methylumbelliferyl β-D-N,N′,N″-triacetylchitotriose as substrate at pH 5.2

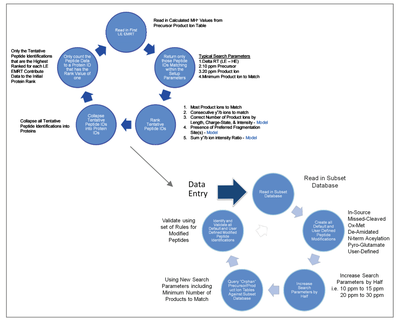

Figure 2. Workflow of the Identity^E search algorithm — see poster WPQ–275 for algorithm details

Search Algorithm

Protein identifications and quantitative information were generated by the use of dedicated algorithms (Identity^E, Waters Corp.), and searching against a human species-specific database. The principle and workflow of the search algorithm is illustrated in Figure 2

RESULTS

Precursor and fragment ion maps

Chitinase-1 is a marker protein for deficiency in the human glycosphingolipid catabolism pathway. The protein was recombinantly expressed and analyzed in its tryptic digested form to create a so-called ion map of all precursor and fragment ions that can be identified to this protein — Figure 3.

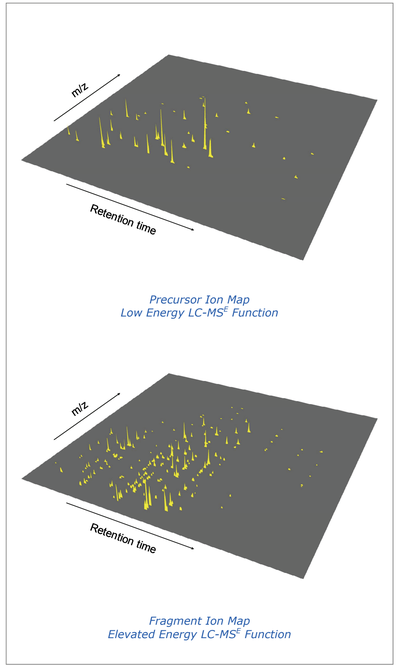

Figure 3. Ion map precursor (top) and fragment ions (bottom) of recombinant Chitinase-1

Chitinase-1 was positively identified in the depleted serum of four pre-treatment patients. The observed ion maps — precursor and fragments distributions — were compared with the results obtained for recombinant Chitinase-1 — Figures 4 and 5

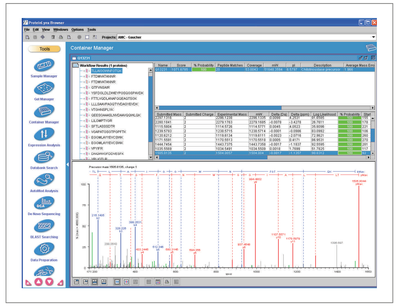

Figure 4. Alternate scanning multiplexed LC-MS^E identification of Chitinase-1

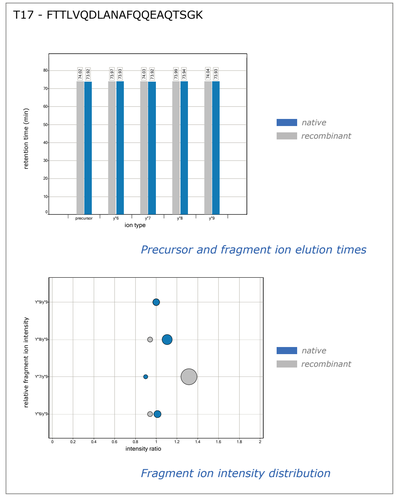

Figure 5. Time and relative intensity distribution maps T17 peptide recombinant Chitinase-1 and native pretreatment human serum Chitinase-1

Absolute quantification

The concentration and on-column amounts of Chitinase-1 for the LC-MS^E experiments was determined by means of the following formula for all four investigated patients:

$$\frac{\sum_{i=1}^{n}\text{normalized peptide intensity protein x}}{\sum_{i=1}^{n}\text{normalized peptide intensity internal standard}}\cdot[\text{internal standard}]$$

The results of the alternate scanning multiplexed LC-MS^E experiments were also used to develop and optimize quantitative triple quadrupole LC-MS/MS analysis — see Figure 6.

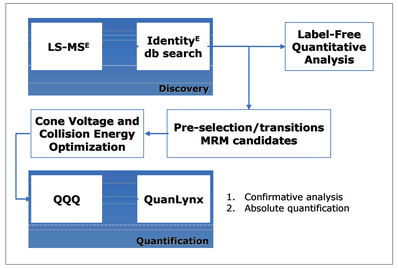

Figure 6. Identity^E discovery/MRM quantification workflow — see poster MPZ6-499 for detail on peptide MRM optimization strategies

Table 1 summarizes the ELISA activity measurements and the quantitative LC-MS analysis by means of nanoscale LC-MS^E alternate scanning and triple quadrupole LC-MS/MS MRM — 2 peptides; 2 transitions/peptide — analysis.

Figure 7 illustrates a comparison of the 3 applied techniques to determine Chitinase-1 activity of pretreatment serum samples of glucocerebrosidase deficiency patients. The LC-MS determined concentration levels were converted to activity units.

patient	*activity[1] (nmol/mL h)**	*concentration[2] (fmol/µL)***	*concentration[3] (fmol/µL)***
A	31,800 ± 1590	1.59 ± 0.31	NA
B	15,900 ± 800	0.99 ± 0.16	2.8 ± 0.2
C	62,100 ± 3100	1.59 ± 0.18	10.4 ±0.4
D	20,400 ± 1020	1.01 ± 0.05	4.4 ± 0.1

*Table 1. Chitinase-1 activity (ELISA[1]) and LC-MS determined concentrations (LC-MS^{E,2} and MRM[3]); * n = 1 (5% method error); ** different original sample dilutions; n = 3*

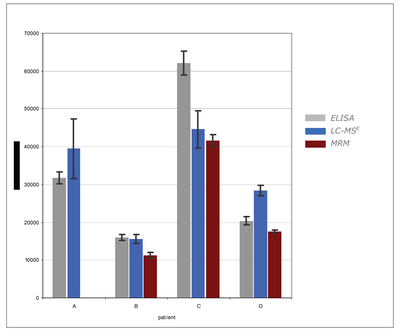

Figure 7. Chitinase-1 activity determined by ELISA (n = 1; 5% method error), LC-MS^E (n = 3) and MRM LC-MS/MS (n = 3) for four independent glucocerebrosidase deficiency patients

CONCLUSION

- **A specific glucocerebrosidase deficiency biomarker — Chitinase 1 — has been positively identified, quantified and its enzyme activity determined**
- **Ion maps — including retention time and relative intensities of both precursor and fragment ions — allowed for the search validation and confirmation of sub fmol levels of Chitinase-1 in the presence of 0.5 µg depleted serum**
- **Independent quantitative methods — ELISA and triple quadrupole MRM LC-MS/MS analysis — confirmed the determined amounts, concentrations and enzyme activities for all investigated patient pretreatment samples**

References

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