LC-MS/MS-analysis.

Protein spots were excised from gels and tryptically digested according to the method by Shevchenko et al. [1]. Peptide extracts were dissolved in 0.1% formic acid and separated on a nano-HPLC system (Ultimate 3000™, LC Packings, Amsterdam, Netherlands). 70µl samples were injected and concentrated on the loading column (LC Packings C18 Pep- Map™, 5 µm, 100 Å, 300µm inner diameter x 1mm) for 5 min using 0.1% formic acid as isocratic solvent at a flow rate of 20 µl/min. The column was then switched into the nanoflow circuit, and the sample was loaded on the nanocolumn (LC-Packings C18 PepMap™, 75 µm inner diameter x 150 mm) at a flow rate of 300 nl/min and separated using the following gradient: solvent A: water, 0.3% formic acid, solvent B: acetonitrile/water 80/20 (v/v), 0.3% formic acid; 0 to 5 minutes: 4% B, after 40 minutes 55% B, then for 5 minutes 90% B and 47 minutes reequilibration at 4% B. The sample was ionized in a Finnigan nano-ESI source equipped with NanoSpray tips (PicoTip™ Emitter, New Objective, Woburn, MA, USA) and analysed in a Thermo-Finnigan LTQ linear iontrap mass-spectrometer (Thermo, San Jose, CA, USA). The MS/MS data were analyzed by searching the NCBI non redundant public database with SpectrumMill Rev. 03.03.078 (Agilent, Darmstadt, GER) software (for more details see supplementary data). Acceptance parameters were three or more identified distinct peptides according to Carr et al. [2].

[1] Shevchenko, A., Wilm, M., Vorm, O., Mann, M., Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal. Chem. 1996, 68, 850-858.

[2] Carr, S., Aebersold, R., Baldwin, M., Burlingame, A., Clauser, K., and Nesvizhskii, A. 2004. The need for guidelines in publication of peptide and protein identification data. Mol.Cell Proteomics. 3: 531-533.

Bezugsquellen

NH4HCO3	Sigma A-6141
CaCl2.2 H2O	Merck 102382
Acetonitrile HPLC grade	Merck UN 1648
DTT	Sigma D-0632
IAA	Sigma I-1149
Modified Trypsin sequencing grade	Promega V5111 (20 µg in 200 µl 1 mM HCl gelöst)

Supplementary data

General MS/MS Search Parameters

Data Extraction

"Peak List Files" from RAW-data were created using the "Data Extractor" of the "Agilent - Spectrum Mill" Software (Rev. A. 03.03.078). The following settings were used:

N-terminus: Hydrogen
C-terminus : Free acid
Cys modification: Carbamidomethylation
Sequence tag length > 1
MH+: 350 – 4500 Da
Scan time: 0 – 60 min
Merge Scans +/- 1 sec, +/- 1 m/z
Find charge
Maximum z = 4
Minimum S/N : 3
Find 12C

MS/MS Search

The database used was the "Mammals"- subset of the "nr.gz", downloaded from "ftp://ftp.ncbi.nih.gov/blast/db/FASTA/" on 18.6.2006 (3717264 total entries)

Database searching was performed with the "MS/MS Search" feature of the "Agilent - Spectrum Mill" Software (Rev. 03.03.078) and the following settings were used:

Enzyme: Trypsin
N-terminus: Hydrogen
C-terminus : free acid
Allowed missed cleavages: 2
Cys modification: carbamidomethylation
Sequence tag length > 3
Minimum detected. Peaks: 4
Minimum matched peak intensity: 50%
Precursor mass tolerance: +/- 2.5 Da
Product mass tolerance: +/- 0.7 Da

Homology search, possible multiple oxidised methionine and N-terminal pyro-glutamicacid.

Acceptance paramters:

Cut-off score value for accepting individual MS/MS spectra. 10

According to Spectrum Mill manual (Ref. See below) a combination of a peptide score of 10 (default 6) and a %SPI threshold of 70 /default 60) leads to an interpretation of MS/MS spectra that is likely to be valid.

Combined with the acceptance of proteins with at least 3 identified peptides we are quite sure to exclude false-positive hits.

Agilent G2721AA
Spectrum Mill MS
Proteomics Workbench
Application Guide

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Santa Clara, CA USA 95052

Filter peptides by

Score: Set a filter for peptide score. For ion trap data, peptide scores greater than 13 almost always represent valid results. Scores below 6 generally represent poor results. For more details, see Table 1 on page 37

%SPI Set a filter for scored peak intensity. This is the percentage of the MS/MS peak-detected ion current explained by theoretical fragments from the database hit. For ion trap data, values greater than 70 (or 60% for doubly-charged precursor ions) generally represent high-quality results.

Table 1 Guidelines for validating **ion trap** results based on MS/MS Search scores

Peptide score	Quality	Peptide fragmentation	Likelihood of valid interpretation
13-25	Excellent	Extensive	When combined with % **SPI** of 70 or greater, very likely to be valid
9-13	Good	Substantial	When combined with % **SPI** of 70 or greater, likely to be valid
6-9	Mediocre	Moderate	Review results to determine whether interpretation is valid
3-6	Weak	Sparse	Not likely to be valid