Journal of Chromatography B, 878 (2010) 1519–1530

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

# Assessment of the partitioning capacity of high abundant proteins in human cerebrospinal fluid using affinity and immunoaffinity subtraction spin columns

Magnus Wetterhall, Aida Zuberovic, Jörg Hanrieder, Jonas Bergquist*

*Department of Physical and Analytical Chemistry, Analytical Chemistry, Uppsala University, Uppsala, Sweden*

ARTICLE INFO

*Article history:*
Received 10 February 2010
Accepted 1 April 2010
Available online 13 April 2010

*Keywords:*
Cerebrospinal fluid (CSF)
Mass spectrometry (MS)
Proteomics
Immunoaffinity subtraction
Protein fractionation
iTRAQ quantification

ABSTRACT

The performance of three different affinity and immunoaffinity subtraction spin columns was investigated for the removal of the most abundant proteins in human cerebrospinal fluid (CSF). A pool of human CSF was processed with the spin columns and both the bound and flow through fractions were compared with each other and with intact CSF using 1D gel electrophoresis and nanoLC–MALDI-TOF/TOF-MS analysis. MASCOT MS/MS ionscores were compared before and after processing with the columns. The non-specific co-removal of proteins bound to the high abundant proteins, so called "sponge effect" was also examined for each spin column. The reproducibility of one of the spin columns, ProteomeLab IgY-12 proteome partitioning spin column, was further investigated by isobaric tags for relative and absolute quantification (iTRAQ) labeling and MS/MS analysis. Overall, 173 unique proteins were identified on a 95% MudPIT confidence scoring level. For all three spin columns, the number of proteins identified and their MASCOT scores were increased up to 10 times. The largest degree of non-specific protein removal was observed for a purely affinity based albumin removal column, where 28 other proteins also were present. The ProteomeLab IgY-12 proteome partitioning spin column showed very high reproducibility when combined with iTRAQ labeling and MS/MS analysis. The combined relative standard deviation (R.S.D.) for the high abundant protein removal, iTRAQ labeling and nanoLC–MALDI-TOF/TOF-MS analysis was less than 17.5%.



## 1. Introduction

In recent years, a lot of emphasis has been put on the proteomic analysis of cerebrospinal fluid (CSF), especially in the quest to find biomarkers for neurological disorders [1–9]. CSF is of great clinical interest due to the continuous contact and exchange with the central nervous system (CNS). The hypothesis is that changes in the CNS, caused by the disease, should be reflected in the CSF and therefore of high interest to measure. The greatest challenges in proteomic analysis of CSF are the complexity, diversity and large dynamic range of concentration of the proteins and peptides present in the sample. The total protein concentration in CSF is rather low (0.2–0.8 mg/mL) compared to plasma (50–80 mg/mL) and the majority of the proteins in CSF are believed to originate from plasma through the blood–brain barrier [5,10,11]. A few high abundant proteins constitute the greater part of the total protein concentration, thus limiting the sample loading and detection capabilities for low abundant proteins. For example, albumin represents approximately 60% of the total protein content in CSF and the 10 most common proteins in CSF constitute more than 80% of the total protein composition [5]. It is believed that potential biomarkers secreted in biofluids would be present at very low concentrations [12]. Therefore, there is a great need to remove the high abundant proteins to facilitate the detection of these low abundant potential biomarkers. Several protein removal, fractionation and concentration strategies have been explored for CSF including solvent depletion schemes [2,13], ultrafiltration [14–16], in-solution isoelectric focusing (IEF) [17–19], reversed phase solid phase extraction [20], peptide binding ligands [21], affinity [22–26] and antibody based chromatography [15,23,27–35]. All these techniques have shown an improvement in the number of proteins identified in CSF compared to analyzing the intact sample. Of the above mentioned techniques, the immunoaffinity based ones have shown the best specificity in the removal of targeted high abundant proteins. However, one should be aware that the subtraction of transport proteins, such as albumin and transferrin, can lead to co-removal of low abundant proteins bound to the carrier proteins [36,37]. The aim of this study was to compare the performance

*Abbreviations:* ACN, acetonitrile; B, bound fraction; CSF, cerebrospinal fluid; DTT, dithiothreitol; HAc, acetic acid; FT, flow through fraction; IAA, iodoacetamide; iTRAQ, isobaric tags for relative and absolute quantification; MALDI, matrix assisted laser desorption ionization; MS, mass spectrometry; TFA, trifluoroacetic acid; TOF, time-of-flight.

* Corresponding author at: Department of Physical and Analytical Chemistry, Analytical Chemistry, Uppsala University, P.O. Box 599, SE-751 24 Uppsala, Sweden. Tel.: +46 18 4713675; fax: +46 18 4713692.
*E-mail address:* jonas.bergquist@kemi.uu.se (J. Bergquist).

1570-0232/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2010.04.003

for three different affinity and immunoaffinity based spin columns for the partitioning of the most abundant proteins in human CSF by analyzing both the bound and flow through protein fractions. These spin columns were designed for human plasma applications, as there is still today no kit primarily intended for human CSF fractionation. However, since the protein composition is very similar in both body fluids, the columns can readily be used for the processing of CSF. The protein partitioning performance of the spin columns was evaluated by 1D gel electrophoresis and liquid chromatography in combination with matrix assisted laser desorption ionization time-of-flight tandem mass spectrometry (LC–MALDI-TOF/TOF-MS). Furthermore, the reproducibility for one of the columns was more thoroughly investigated in combination with the popular iTRAQ™ labeling technique [38] for quantitative mass spectrometry (MS) analysis of complex protein samples. Stable isotopic labeling, such as isobaric tags for relative and absolute quantification (iTRAQ), has virtually boomed the application of quantitative MS in proteomic research. The major reasons for this are the multiplexing possibilities which in turn imply comparative quantification under identical conditions and a higher sample throughput. Multiplexing is a very attractive feature in biomarker screening studies and differential diagnosis where healthy and disease states are quantitatively compared. The use of affinity and immunoaffinity fractionation in combination with stable isotopic labeling and MS detection is today the method of choice for such proteomic studies.

## 2. Experimental

### 2.1. Chemicals and reagents

Acetonitrile (ACN), acetic acid (HAc) and ammonium-dihydrogen-phosphate ($NH_4H_2PO_4$) were obtained from Merck (Darmstadt, Germany). Acetone and trifluoroacetic acid (TFA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). For tryptic digestion, urea, ammonium bicarbonate ($NH_4HCO_3$), iodoacetamide (IAA) and dithiothreitol (DTT) were obtained from Sigma and trypsin (sequence-grade from bovine pancreas (1418475); Roche diagnostics, Basel, Switzerland) was used. The water was purified with a Milli-Q (Millipore, Bedford, MA, USA) purification system.

### 2.2. Samples

Human cerebrospinal fluid used was taken from a pool consisting of >200 individual CSF samples drawn from patients in the age of 16–65 years. The majority of the samples were collected by lumbar puncture during epidural anesthetics procedures and none of the patients showed signs of neurological or psychiatric disorders. Routine CSF analysis revealed no signs of inflammation or damage to the blood–brain barrier function. The study was approved by the local Human Ethics Committee. The pooled CSF sample was stored at −80 °C until preparation and analysis.

### 2.3. Sample preparation

A schematic overview of the experimental set-up is shown in Fig. 1. Multiple aliquots of 100 μL, 250 μL, 500 μL and 1000 μL of the pooled CSF were centrifuged to dryness using a Speedvac system ISS110 (Thermo Scientific, Waltham, MA, USA). For high abundant protein fractionation, three different affinity/antibody spin column kits were used. All three kits are designed to process 10–15 μL of human plasma samples. The Montage Albumin Depletion Kit (Millipore) removes more than 50% of albumin and has less than 14% non-specific removal of proteins bound to albumin. The ProteomeLab™ IgY-12 proteome partitioning kit (Beckman Coulter, Fullerton, CA, USA) removes 12 of the most common plasma proteins and the ProteoPrep® 20 Plasma Immunodepletion Kit (Sigma) is designed for removal of 20 of the most abundant proteins. The targeted proteins for each kit are listed in Table 1. The dry CSF pellets were redissolved in 500 μL sample buffer (supplied with the kit) and treated according to the protocols provided by the manufacturers. Both the flow through (FT) and bound (B) fractions of CSF from each kit were collected. The FT fractions from each kit were centrifuged to dryness prior to 1D gel electrophoresis (Section 2.6) or enzymatic digestion (Section 2.5) followed by nanoLC–MALDI-TOF/TOF-MS analysis (Section 2.7). The bound fractions from each kit contained different detergents, which are not compatible with the downstream LC–MS analysis. Thus, these fractions were first precipitated with acetone and then analyzed by 1D gel electrophoresis (Section 2.6) or enzymatic digestion and nanoLC–MALDI-TOF/TOF-MS (Sections 2.5 and 2.7). The acetone precipitation was conducted by first adding six sample volumes of ice cold acetone. The samples were left in −20 °C over night and then spun at 10 000 × *g* for 30 min at 4 °C using a Sigma 2K15 centrifuge. The supernatant was removed and six sample volumes of ice cold 50% acetone were added. The samples were briefly vortexed and spun again at 10 000 × *g* for 30 min at 4 °C. The supernatant was discarded and the protein pellets were dried at ambient temperature prior to further processing and analyses. Finally, aliquots of 100 μL, 250 μL, 500 μL and 1000 μL of non-depleted CSF were also dried and analyzed with 1D gel electrophoresis (Section 2.6) and nanoLC–MALDI-TOF/TOF-MS (Sections 2.5 and 2.7) as control comparison.

### 2.4. Bradford protein assay

The total protein content was estimated on the start CSF and aliquots taken throughout the sample preparation with Bradford Coomassie® Brilliant Blue G-250 protein assay using bovine serum albumin (BSA) as standard (Bio-Rad, Hercules CA, USA). The absorbance was measured using a Bio-Rad Model 680 microplate reader at 595 nm.

### 2.5. Protein digestion and desalting

The dry pellets from non-processed CSF and the corresponding dried FT fractions and B fractions from each affinity/antibody kit were redissolved in 100 μL 8 M urea, 0.4 M $NH_4HCO_3$ after which 10 μL of 45 mM DTT was added and the samples were incubated at 50 °C for 15 min to reduce the disulfide bridges between the cysteines. After cooling to room temperature, 10 μL of 100 mM IAA was added and the samples were incubated for 15 min at room temperature in darkness to irreversibly carbamidomethylate the cysteines. Finally, trypsin 100 μg dissolved in 1 mL 50 mM $NH_4HCO_3$ was added to the samples to yield a 2% (w/w) trypsin/protein concentration and the samples were digested over night at 37 °C. A volume of 20 μL of the tryptically digested sample was desalted on a ZipTip® C18 column (Millipore) using a procedure described by Bergquist et al. [39]. The tip was first wetted in 5 × 10 μL of 100% ACN and equilibrated with 5 × 10 μL 1% HAc. The samples were acidified to a concentration of 2.5% HAc, after which the peptides were adsorbed on the media using 20 repeated cycles of sample loading. The tip was washed using 5 × 10 μL of 1% HAc, and the peptides were eluted in 2 × 10 μL of 50% ACN, 1% HAc. This procedure was repeated twice for each sample. After the desalting, the eluate was vacuum centrifuged to dryness. The peptides were redissolved in 20 μL of 0.1% TFA prior to nanoLC–MALDI-TOF/TOF-MS analysis.

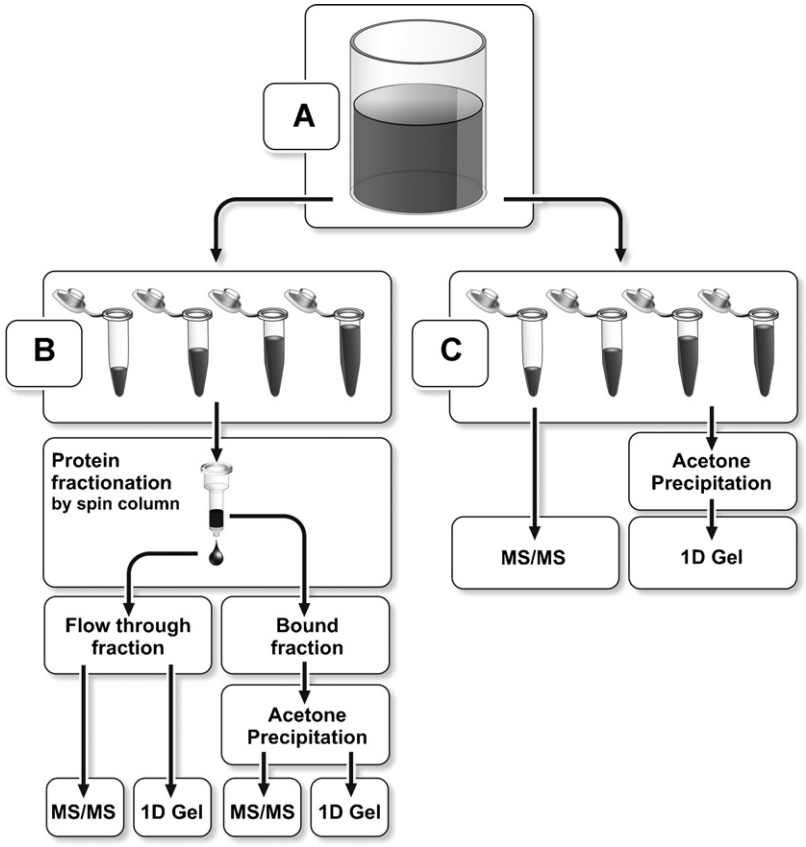


**Fig. 1.** (A) Schematic overview of the experimental set-up for comparing the protein partitioning efficiency for the different subtraction spin columns and non-processed human cerebrospinal fluid (CSF). A large pool of CSF (A) was split into multiple aliquots of 100 μL, 250 μL, 500 μL and 1000 μL for processing with the protein subtraction spin columns (B) or for use as non-processed control CSF (C). The subtraction spin columns yielded a flow through and bound fraction. The flow through fractions were either digested and analyzed by nanoLC–MALDI-TOF/TOF-MS or separated on a 1D gel. The bound fractions were first precipitated by acetone and then either digested and analyzed by nanoLC–MALDI-TOF/TOF-MS or separated on a 1D gel. The non-processed control CSF aliquots were either digested and analyzed by nanoLC–MALDI-TOF/TOF-MS or acetone precipitated and then separated on a 1D gel.

### 2.6. 1D gel electrophoresis

1D gel electrophoresis was performed on the non-depleted CSF, FT fractions and B fractions for each kit to visually examine the depletion efficiency and capacity. The 1D gel electrophoresis was performed with a Criterion XT$^{TM}$ system using precast Criterion XT 26 well 4–12% Bis–Tris gels with XT MOPS running buffer (Bio-Rad). The samples were redissolved in 25 μL XT sample buffer, 55 μL MQ water and 10 μL 45 mM DTT. The samples were heated to 95 °C for 5 min, cooled to room temperature and 10 μL 100 mM IAA was added. The gels were run at 200 V constant for 60 min (starting current 165–175 mA/gel, final current 60–70 mA/gel). Finally, the gels were visualized by either Coomassie Blue R-250 or Silver Stain Plus$^{TM}$ (Bio-Rad) according to the manufacturer's instructions.

### 2.7. LC–MALDI-TOF/TOF-MS analysis

The reversed phase liquid chromatography separation was performed with a 1100 nanoflow LC system (Agilent Technologies, Waldbronn, Germany), equipped with a fraction collector for direct fractionations onto a MALDI target plate [40]. A volume of 10 μL digestion products was injected into a 10 μL sample loop. For separating the peptides, a 15 cm × 180 μm, C18 column (Thermo) with 5 μm particle size and an $H_2O$:ACN:TFA solvent system ($H_2O$, 0.1% TFA mobile phase [A]; ACN, 0.1% TFA mobile phase [B]) was used. A flow rate of 2 μL/min was applied, starting with isocratic elution at 2% B during 20 min, followed by gradient elution from 2% to 8% B during 5 min, then from 8% to 32% B within 86 min, then from 32% to 40% B during 5 min and finally from 40% to 80% B during 1 min. The on-line fractionation onto an MALDI target was performed with four fractions per minute for 96 min within the elution period from 20 min (2% B) and 116 min (40% B) resulting in 384 fractions. For optimal MS results, disposable pre-spotted anchorchip targets (PAC-targets, Bruker Daltonics, Bremen, Germany) were chosen. The targets were washed with 10 mM $NH_4H_2PO_4$/0.1% TFA prior to MALDI-TOF/TOF-MS analysis. Mass data were acquired with an Ultraflex II MALDI-TOF/TOF-MS (Bruker Daltonics) in reflector positive mode. A mass range of 700–4000 Da was analyzed with a sum of 300 shots/spot and 50 shots/position, respectively, in a hexagonal pattern. The laser frequency was set to 100 Hz.

**Table 1**
Targeted high abundant plasma protein for each spin column kit used in the study.

| Montage | ProteomeLab IgY-12 | ProteoPrep 20 |
|---|---|---|
| Albumin | Albumin, IgA, IgG, IgM, α1-antitrypsin, transferrin, haptoglobin, α1-acid glycoprotein (orosmucoid), α2-macroglobulin, Apolipoprotein A-I, Apolipoprotein A-II and Fibrinogen | Albumin, IgA, IgD, IgG, IgM, α1-antitrypsin, transferrin, haptoglobin, α1-acid glycoprotein (orosmucoid), α2-macroglobulin, Apolipoprotein A-I, Apolipoprotein A-II, Fibrinogen, ceruloplasmin, Apolipoprotein B, complement C1q, complement C3, complement C4, plasminogen and prealbumin |

MALDI-TOF/TOF tandem MS analysis was performed in post source decay MS/MS mode with 30% increased laser energy to give the fragmentation spectra. Post fragmentation mother ion suppression was applied to deflect the precursor and elevate fragment ion intensity. Peptide monoisotopic signals were analyzed using the FlexAnalysis software provided with the instrument (Bruker Daltonics). The spectra were calibrated externally using the prespotted calibrants adjacent to the sample spots. For final protein identification, all collected MS/MS data were run in a comprehensive MS/MS ion search using the MASCOT search engine version 2.2.2 (Matrix Science, London, UK). Acquired MS/MS-spectra were evaluated with the Matrix Science MASCOT database SwissProt version 51.6. The search parameters were set to Taxonomy: Homo sapiens, Enzyme: Trypsin, Fixed modifications: Carbamidomethyl (C), Variable modifications: Oxidation (M), Peptide mass tolerance: ±50 ppm, Fragment mass tolerance: ±0.8 Da and maximum 1 missed cleavage site. Proteins were considered to be positively matched if at least one MS/MS spectrum fulfilled an individual MASCOT MS/MS MudPIT Ionscore > 27 (significance threshold set to 95% ($p \leq 0.05$)).

### 2.8. Sample preparation and iTRAQ labeling

Four 1 mL aliquots of the pooled CSF sample were centrifuged to dryness for processing with the ProteomeLab™ IgY-12 proteome partitioning kit. The dry CSF pellets were redissolved in 500 μL modified sample buffer containing 10 mM $NaH_2PO_4/Na_2HPO_4$ (pH 7.4) and 150 mM NaCl. A phosphate buffer was used instead of the tris-buffer supplied by the manufacturer since any added primary amines or ammonium salts will affect the following iTRAQ™ labeling efficiency. The redissolved CSF samples were then treated according to the protocol provided by the manufacturer with the exception of using the phosphate buffer instead of the tris-buffer for sample loading and washing. The four obtained FT fractions were centrifuged to dryness and then labeled with the iTRAQ™ 4-plex kit (Applied Biosystems, Foster City, CA, USA) following a slightly changed protocol concerning the denaturating agent. The samples were redissolved in 20 μL dissolution buffer, 1 μL 1 M urea solution (instead of SDS as suggested in the standard protocol) for protein denaturation. After iTRAQ labeling, equal volumes (90 μL) of each of the iTRAQ labeled samples were mixed and dried down under vacuum to remove the added ethanol. The dried sample was redissolved in 2.5% HAc and desalted on a ZipTip® C18 column. The eluate was centrifuged to dryness and then separated by nanoLC, fractionated onto a PAC MALDI target and analyzed by MALDI-TOF/TOF-MS as described previously in Section 2.7. The MASCOT search criteria were changed to: Taxonomy: Homo sapiens, Enzyme: Trypsin, Variable modifications: Oxidation (M), Peptide mass tolerance: ±50 ppm, Fragment mass tolerance: ±0.8 Da, maximum 1 missed cleavage site and Quantitation: iTRAQ 4-plex.

## 3. Results and discussion

### 3.1. 1D gel electrophoresis of fractionated CSF

All three investigated affinity/antibody kits are designed for the processing of 10–15 μL of human plasma. Although the protein concentration in human CSF is at least 100 times less than that for plasma, the major proteins and their relative abundance found in both fluids are very similar [5]. This means that comparatively large CSF volumes (at least 1 mL) can be processed with the plasma spin columns with retained protein removal capacity. Volumes of up to 1 mL of CSF were processed with each spin column and separated by 1D gel electrophoresis to visually examine the working range for CSF volumes. Fig. 2 shows the 1D gel electrophoresis separation of intact CSF and the bound and flow through fractions of 1 mL CSF using the different columns. It can clearly be seen that even though albumin is not completely removed, the sample loading of the less abundant proteins has dramatically increased. For all three spin columns, the number of protein bands in the FT fractions (lane D, F and H) are substantially greater than for intact CSF (lane B). The so called "albumin sponge effect" can also be observed in lane C; Montage bound fraction, showing a rather large co-removal of other proteins than the targeted albumin. This unspecific protein removal has also been reported by the manufacturer. Yet, it is important to be aware of this when any comparative or differential proteomic studies are performed, as the results may be biased. The results from the 1D gel shows that volumes of at least up to 1 mL of CSF readily can be processed with spin columns and still obtain satisfactory fractionation results.

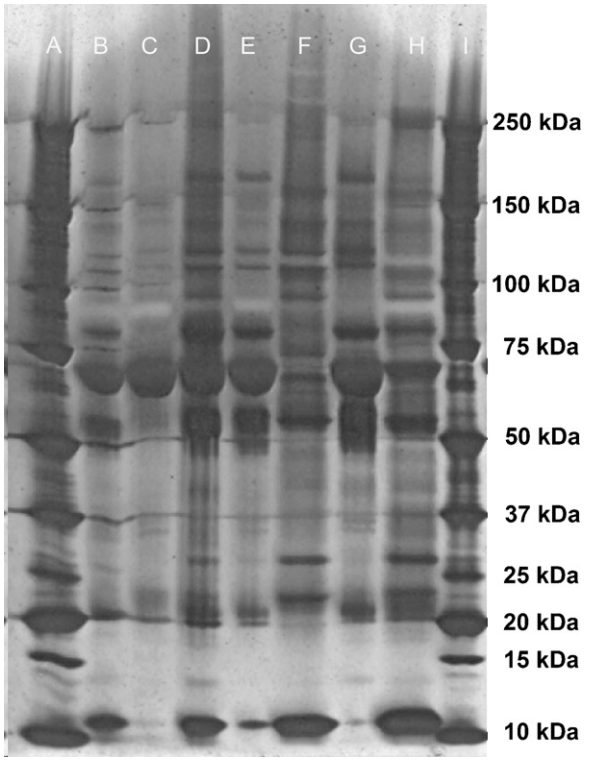


**Fig. 2.** 1D gel of 1 mL intact CSF and the bound and flow through fractions of 1 mL processed CSF from each spin column. The lanes are: A and I: molecular weight markers, B: 1 mL intact CSF, C: Montage bound fraction of 1 mL CSF, D: Montage flow through fraction of 1 mL CSF, E: ProteomeLab IgY-12 bound fraction of 1 mL CSF, F: ProteomeLab IgY-12 flow through fraction of 1 mL CSF, G: ProteoPrep 20 bound fraction of 1 mL CSF and H: ProteoPrep 20 flow through fraction of 1 mL CSF.

### 3.2. nanoLC–MALDI-TOF/TOF-MS analysis

To further evaluate the fractionation efficiency of the target proteins, the sample loading increase of the remaining medium–low abundant proteins and the effect of the observed albumin sponge effect, nanoLC–MALDI-TOF/TOF-MS analysis was performed on 1 mL intact CSF and the FT and B fractions of 1 mL CSF for each spin column. Overall, 173 unique proteins were identified in the CSF as listed in Table 2. For the intact CSF, 91 unique proteins were identified, 128 proteins for the Montage spin column, 123 proteins for the ProteomeLab IgY-12 spin column and 104 proteins for the ProteoPrep 20 spin column. The MASCOT scores before and after sample preparation for the matched proteins are visualized in Fig. 3. Again, in all three cases the MASCOT scores for the medium and low

**Table 2**
Proteins identified on 95% MudPIT confidence level in 1 mL intact CSF and the flow through (FT) and bound (B) fractions of 1 mL CSF for each spin column by nanoLC–MALDI-TOF/TOF-MS analysis. The protein classification, molecular weight (MW) and protein function are as given by the Uniprot database.

| Protein name | Uniprot entry | Uniprot Acc. | MW | Function | Intact CSF | | Montage FT | | Montage B | | IgY-12 FT | | IgY-12 B | | PP-20 FT | | PP-20 B | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Score | Pep. | Score | Pep. | Score | Pep. | Score | Pep. | Score | Pep. | Score | Pep. | Score | Pep. |
| *Transport/binding* | | | | | | | | | | | | | | | | | | |
| Serum albumin | ALBU_HUMAN | P02768 | 71317 | Transport | 4043 | 59 | 3625 | 59 | 3255 | 70 | 292 | 9 | 3378 | 61 | 3314 | 59 | 4171 | 69 |
| Serotransferrin | TRFE_HUMAN | P02787 | 79280 | Iron transport | 2105 | 35 | 2260 | 38 | 30 | 2 | | | 1187 | 23 | 1167 | 23 | 1775 | 29 |
| Hemopexin (Beta-1B-glycoprotein) | HEMO_HUMAN | P02790 | 52385 | Transport | 491 | 10 | 633 | 11 | 74 | 3 | 293 | 5 | 167 | 4 | 312 | 5 | 32 | 1 |
| Vitamin D-binding protein | VTDB_HUMAN | P02774 | 52964 | Transport/cell communication | 469 | 5 | 712 | 10 | | | 835 | 13 | | | 452 | 6 | | |
| Apolipoprotein A-I | APOA1_HUMAN | P02647 | 30759 | Cholesterol transport | 462 | 8 | 422 | 8 | 124 | 6 | 28 | 1 | 177 | 4 | 373 | 9 | 333 | 7 |
| Haptoglobin | HPT_HUMAN | P00738 | 45861 | Iron homeostasis, metabolism | 310 | 8 | 261 | 7 | | | | | 98 | 4 | 37 | 1 | 193 | 6 |
| Insulin-like growth factor-binding protein 6 | IBP6_HUMAN | P24592 | 25322 | IGF binding protein | 109 | 2 | 105 | 2 | | | 276 | 5 | | | 142 | 3 | | |
| Apolipoprotein H | APOH_HUMAN | P02749 | 38298 | Binding neg. charged subst. | 98 | 2 | 172 | 2 | | | 303 | 5 | | | 155 | 3 | | |
| Transthyretin | TTHY_HUMAN | P02766 | 15991 | Hormone transport | 46 | 2 | 138 | 5 | | | 124 | 2 | 198 | 3 | 216 | 3 | 149 | 2 |
| Hemoglobin beta chain | HBB_HUMAN | P68871 | 15998 | Oxygen transport | 44 | 1 | | | | | | | | | | | | |
| SPARC | SPRC_HUMAN | P09486 | 34632 | Cell growth | 37 | 1 | | | | | 93 | 3 | | | 52 | 1 | | |
| Collagen alpha-1(VI) chain | CO6A1_HUMAN | P12109 | 108529 | Cell binding | 37 | 1 | 40 | 1 | | | 60 | 2 | | | 87 | 3 | | |
| Insulin-like growth factor-binding protein 7 | IBP7_HUMAN | Q16270 | 29130 | IGF binding protein | 37 | 1 | 119 | 2 | | | 122 | 3 | | | 59 | 1 | | |
| Tetranectin | TETN_HUMAN | P05452 | 22567 | Binding/transport | 27 | 1 | 68 | 2 | | | 170 | 4 | | | 124 | 2 | | |
| Plasma retinol-binding protein | RETBP_HUMAN | P02753 | 23010 | Transport/binding | | | 209 | 4 | | | 278 | 5 | | | 47 | 1 | | |
| Hemoglobin subunit alpha | HBA_HUMAN | P69905 | 15258 | Oxygen transport | | | 115 | 1 | | | | | | | | | | |
| Insulin-like growth factor-binding protein 2 | IBP2_HUMAN | P18065 | 35138 | IGF binding protein | | | 88 | 2 | | | 149 | 4 | | | 30 | 1 | | |
| Lumican | LUM_HUMAN | P51884 | 38429 | Binds to Laminin | | | 83 | 2 | | | | | | | | | | |
| Protein kinase C-binding protein NELL2 | NELL2_HUMAN | Q99435 | 91346 | Binding | | | 61 | 2 | | | 137 | 3 | | | 39 | 1 | | |
| Opioid-binding protein/cell adhesion molecule | OPCM_HUMAN | Q14982 | 38008 | Opioid binding | | | 56 | 1 | | | 53 | 1 | | | | | | |
| Apolipoprotein C-I | APOC1_HUMAN | P02654 | 9332 | Transport | | | 25 | 1 | | | | | | | | | | |
| Huntingtin-interacting protein 1-related protein | HIP1R_HUMAN | O75146 | 119388 | Binding, stabilizing | | | | | 31 | 2 | | | | | | | | |
| Apolipoprotein D | APOD_HUMAN | P05090 | 21276 | Transport | | | | | | | 68 | 2 | | | 83 | 1 | | |
| Afamin | AFAM_HUMAN | P43652 | 69069 | Transport | | | | | | | 50 | 1 | | | 54 | 1 | | |
| *Immune response/defense* | | | | | | | | | | | | | | | | | | |
| Ig gamma-1 chain C region | IGHG1_HUMAN | P01857 | 36596 | Immune response | 480 | 5 | 397 | 9 | | | | | 241 | 4 | | | 491 | 7 |
| Alpha-1-acid glycoprotein 1 | A1AG1_HUMAN | P02763 | 23512 | Acute phase response | 335 | 4 | 196 | 4 | | | 41 | 1 | 94 | 2 | 119 | 2 | 240 | 3 |
| Alpha-1-acid glycoprotein 2 | A1AG2_HUMAN | P19652 | 23603 | Acute phase response | 204 | 3 | | | | | | | 79 | 2 | | | 223 | 3 |
| Ig gamma-2 chain C region | IGHG2_HUMAN | P01859 | 35885 | Immune response | 192 | 2 | 281 | 9 | | | | | | | | | | |
| Ig gamma-4 chain C region | IGHG4_HUMAN | P01861 | 35941 | Immune response | 190 | 3 | 267 | 5 | | | | | 180 | 3 | | | 375 | 5 |
| Ig kappa chain C region | KAC_HUMAN | P01834 | 11773 | Immune response | 185 | 2 | 412 | 5 | | | | | 208 | 3 | 58 | 1 | 215 | 4 |
| Ig alpha-1 chain C region | IGHA1_HUMAN | P01876 | 38486 | Immune response | 155 | 3 | 160 | 3 | | | | | 117 | 3 | | | 105 | 3 |

Table 2 (*Continued*)

| Protein name | Uniprot entry | Uniprot Acc. | MW | Function | Intact CSF | | Montage FT | | Montage B | | IgY-12 FT | | IgY-12 B | | PP-20 FT | | PP-20 B | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Score | Pep. | Score | Pep. | Score | Pep. | Score | Pep. | Score | Pep. | Score | Pep. | Score | Pep. |
| Alpha-1B-glycoprotein | A1BG_HUMAN | P04217 | 54809 | Defense response | 119 | 2 | 216 | 4 | | | 569 | 13 | | | 267 | 8 | | |
| Ig gamma-3 chain C region | IGHG3_HUMAN | P01860 | 32331 | Immune response | 86 | 1 | 180 | 5 | | | | | | | | | 125 | 2 |
| Ig heavy chain V-III region BRO | HV305_HUMAN | P01766 | 13332 | Immune response | 71 | 1 | | | | | | | 82 | 1 | | | 172 | 2 |
| Ig lambda chain C regions | LAC_HUMAN | P01842 | 11237 | Immune response | 33 | 1 | 55 | 1 | | | | | | | 27 | 1 | | |
| Ig alpha-2 chain C region | IGHA2_HUMAN | P01877 | 36508 | Immune response | | | 195 | 3 | | | | | | | | | | |
| Myeloid cell-spec. leu-rich glycoprotein | CD14_HUMAN | P08571 | 40076 | Inflammatory response | | | 101 | 2 | | | 82 | 1 | | | 72 | 3 | | |
| Ig heavy chain V-I region HG3 | HV102_HUMAN | P01743 | 12946 | Immune response | | | 63 | 1 | | | | | | | | | | |
| Semaphorin-7A | SEM7A_HUMAN | O75326 | 74824 | CNS immune functions | | | 53 | 1 | | | 100 | 2 | | | | | | |
| Ig kappa chain V-III region SIE | KV302_HUMAN | P01620 | 11775 | Immune response | | | 49 | 1 | | | | | | | | | 33 | 1 |
| Ig heavy chain V-III region WEA | HV302_HUMAN | P01763 | 12256 | Immune response | | | 48 | 1 | | | | | 89 | 1 | | | | |
| Ig kappa chain V-III region NG9 [Fragment] | KV303_HUMAN | P01621 | 10729 | Immune response | | | 44 | 1 | | | | | | | | | | |
| Coagulation factor XII | FA12_HUMAN | P00748 | 67818 | Coagulation factor | | | | | | | 54 | 2 | | | | | | |
| Ribonuclease K6 | RNAS6_HUMAN | Q93091 | 17196 | Defense response | | | | | | | 30 | 1 | | | | | | |
| Ig heavy chain V-III region HIL | HV310_HUMAN | P01771 | 13566 | Immune response | | | | | | | | | 43 | 1 | | | | |
| *Complement factors* | | | | | | | | | | | | | | | | | | |
| Complement C3 | CO3_HUMAN | P01024 | 188569 | Immune response | 805 | 14 | 857 | 22 | 38 | 3 | 549 | 13 | 235 | 7 | 99 | 3 | 827 | 17 |
| Complement C4-A | CO4A_HUMAN | P0C0L4 | 194247 | Complement activation | 443 | 10 | 802 | 17 | | | 526 | 12 | | | 193 | 5 | 368 | 9 |
| Complement factor H | CFAH_HUMAN | P08603 | 139070 | Complement activation | 177 | 5 | 140 | 4 | | | 29 | 1 | | | | | | |
| Complement factor B | CFAB_HUMAN | P00751 | 85533 | Complement activation | 135 | 2 | 285 | 5 | | | 500 | 9 | | | 127 | 2 | | |
| Complement component C7 | CO7_HUMAN | P10643 | 93518 | Complement activation/defense | 97 | 1 | 216 | 4 | | | 273 | 4 | | | 25 | 1 | | |
| Complement component C9 | CO9_HUMAN | P02748 | 63173 | Complement activation | 57 | 1 | 34 | 1 | 43 | 2 | 158 | 2 | | | 57 | 1 | | |
| Complement C1r | C1R_HUMAN | P00736 | 80174 | Complement factor C1 activity | 51 | 1 | 246 | 6 | | | 172 | 4 | | | | | | |
| Complement C1q subcomponent subunit B | C1QB_HUMAN | P02746 | 26459 | Complement activation | 46 | 1 | | | | | | | | | | | | |
| Complement C1q subcomponent subunit C | C1QC_HUMAN | P02747 | 25774 | Complement activation | 34 | 1 | | | | | | | | | | | | |
| Complement C2 | CO2_HUMAN | P06681 | 83268 | Complement activation | 33 | 1 | | | | | | | | | | | | |
| Complement C1s subcomponent | C1S_HUMAN | P09871 | 76684 | Complement activation | | | | | | | 49 | 2 | | | | | | |
| Complement C5 | CO5_HUMAN | P01031 | 188331 | Complement activation | | | | | | | 43 | 1 | | | | | | |
| *Metabolism/enzymes and inhibitors* | | | | | | | | | | | | | | | | | | |
| Apolipoprotein E | APOE_HUMAN | P02649 | 36154 | Lipid metabolism | 778 | 13 | 777 | 15 | 461 | 17 | 663 | 12 | 328 | 7 | 873 | 19 | 296 | 7 |
| Cystatin C | CYTC_HUMAN | P01034 | 15799 | Enzyme regulator | 726 | 12 | 601 | 13 | 166 | 9 | 950 | 22 | 131 | 3 | 930 | 19 | | |
| Alpha-1-antitrypsin | A1AT_HUMAN | P01009 | 46878 | Inhibitor/immune response | 608 | 10 | 567 | 9 | | | | | 344 | 7 | 79 | 2 | 526 | 9 |
| Alpha-2-macroglobulin | A2MG_HUMAN | P01023 | 164600 | Inhibitor | 501 | 10 | 635 | 14 | | | | | 186 | 6 | 62 | 2 | 369 | 9 |
| Dickkopf-related protein 3 | DKK3_HUMAN | Q9UBP4 | 38291 | Inhibitor of Wnt signaling pathway | 335 | 5 | 369 | 7 | 29 | 1 | 405 | 7 | | | 265 | 4 | | |
| Prostaglandin-H2 D-isomerase | PTGDS_HUMAN | P41222 | 21029 | Enzyme activity | 329 | 7 | 350 | 8 | 311 | 13 | 729 | 17 | 101 | 4 | 505 | 15 | 69 | 2 |
| Ceruloplasmin (Ferroxidase) | CERU_HUMAN | P00450 | 188569 | Iron transport, enzyme activity | 329 | 7 | 713 | 14 | | | 362 | 9 | | | 52 | 2 | 256 | 6 |
| Ectonucl. pyrophosphat./phosphodiest. 2 | ENPP2_HUMAN | Q13822 | 99004 | Lipid metabolism | 294 | 8 | 340 | 8 | | | 445 | 11 | | | 195 | 5 | | |
| Poly-N-acetyllactosamine extension enzyme | B3GN1_HUMAN | O43505 | 47119 | Enzyme | 272 | 4 | 283 | 6 | 40 | 1 | 251 | 7 | | | 115 | 3 | | |

| | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Kininogen | KNG1_HUMAN | P01042 | 72996 | Inhibitor/inflammatory response | 262 | 4 | 267 | 4 | | | 375 | 6 | | | 292 | 5 | | | |
| Antithrombin-III | ANT3_HUMAN | P01008 | 52602 | Serine protease inhibitor | 208 | 5 | 102 | 3 | | | 301 | 7 | | | 151 | 4 | | | |
| Apolipoprotein A-IV | APOA4_HUMAN | P06727 | 45399 | Lipid metabolism | 195 | 5 | 142 | 5 | | | 341 | 11 | | | 226 | 7 | | | |
| Plasminogen | PLMN_HUMAN | P00747 | 90569 | Enzyme/immune response | 144 | 5 | 304 | 7 | | | | | | | | | 57 | 2 | |
| Ribonuclease pancreatic | RNAS1_HUMAN | P07998 | 17644 | RNA cleavage | 139 | 1 | 251 | 4 | | | 437 | 7 | | | 148 | 4 | | | |
| CD59 glycoprotein | CD59_HUMAN | P13987 | 14177 | Inhibitor/defense | 127 | 2 | 121 | 2 | | | 184 | 3 | | | 117 | 2 | | | |
| Superoxide dismutase | SODC_HUMAN | P00441 | 15936 | Radical deactivation | 93 | 1 | 111 | 1 | | | 80 | 1 | | | 84 | 1 | | | |
| Phospholipid transfer protein | PLTP_HUMAN | P55058 | 54739 | Lipid metabolism/transport | 72 | 1 | 90 | 1 | | | | | 27 | 1 | 58 | 2 | | | |
| Zinc-alpha-2-glycoprotein | ZA2G_HUMAN | P25311 | 33872 | Lipid metabolism | 66 | 1 | 156 | 2 | | | | | | | 171 | 2 | | | |
| ADAM 22 | ADA22_HUMAN | Q9P0K1 | 100433 | Enzyme activity | 58 | 1 | 73 | 1 | | | 52 | 1 | | | | | | | |
| Ecto-ADP-ribosyltransferase 4 | NAR4_HUMAN | Q93070 | 35878 | Enzyme activity | 37 | 1 | 30 | 1 | 29 | 1 | | | | | | | | | |
| Angiotensinogen | ANGT_HUMAN | P01019 | 53154 | Metabolism | 36 | 1 | 140 | 2 | | | 108 | 1 | | | 65 | 1 | | | |
| Beta-Ala-His dipeptidase | CNDP1_HUMAN | Q96KN2 | 56692 | Enzyme activity | | | 76 | 3 | | | | | | | | | | | |
| Cathepsin D | CATD_HUMAN | P07339 | 44552 | Enzyme activity | | | 70 | 2 | | | | | | | | | | | |
| Procollagen C-endopeptidase enhancer 1 | PCOC1_HUMAN | Q15113 | 47972 | Enzyme activity | | | 47 | 2 | | | 48 | 2 | | | 58 | 2 | | | |
| Insulin-like growth factor II | IGF2_HUMAN | P01344 | 20140 | Growth promoting activity | | | 45 | 1 | 33 | 1 | 81 | 2 | | | 43 | 1 | | | |
| Glutathione S-transferase P | GSTP1_HUMAN | P09211 | 23356 | Enzyme activity | | | 34 | 1 | | | | | | | | | | | |
| Inter-alpha-trypsin inhibitor heavy chain H1 | ITIH1_HUMAN | P19827 | 101389 | Cell regulation | | | 29 | 1 | | | | | | | 32 | 1 | | | |
| DNA-binding protein SATB1 | SATB1_HUMAN | Q01826 | 85957 | Transcription regulation | | | | | 52 | 3 | | | | | | | | | |
| Nuclear factor erythroid 2-related factor 1 | NF2L1_HUMAN | Q14494 | 84704 | Transcription regulation | | | | | 31 | 1 | | | | | | | | | |
| TBC1 domain family member 8 | TBCD8_HUMAN | O95759 | 130835 | Enzyme activity | | | | | 30 | 2 | | | | | | | | | |
| Chromodomain-helicase-DNA-binding protein 8 | CHD8_HUMAN | Q9HCK8 | 290518 | Enzyme activity | | | | | 30 | 1 | | | | | | | | | |
| PAP-associated domain-containing protein 5 | PAPD5_HUMAN | Q8NDF8 | 63267 | Enzyme activity | | | | | 27 | 1 | | | | | | | | | |
| ProSAAS | PCSK1_HUMAN | Q9UHG2 | 27372 | Inhibitor | | | | | | | 67 | 2 | | | 34 | 1 | | | |
| Metalloproteinase inhibitor 1 | TIMP1_HUMAN | P01033 | 23171 | Inhibitor | | | | | | | 52 | 2 | | | 29 | 1 | | | |
| Peptidyl-glycine alpha-amidating monooxygenase | AMD_HUMAN | P19021 | 108332 | Enzyme activity | | | | | | | 50 | 1 | | | | | | | |
| Carboxypeptidase E | CBPE_HUMAN | P16870 | 53151 | Enzyme activity | | | | | | | 49 | 1 | | | | | | | |
| Extracellular superoxide dismutase | SODE_HUMAN | P08294 | 25851 | Radical deactivation | | | | | | | 48 | 1 | | | 52 | 2 | | | |
| Cathepsin L | CATL_HUMAN | P07711 | 37564 | Protein degradation | | | | | | | 41 | 1 | | | 44 | 1 | | | |
| Epididymal secretory protein E1 | NPC2_HUMAN | P61916 | 16570 | Lipid metabolism | | | | | | | 34 | 1 | | | | | | | |
| Plasma protease C1 inhibitor | IC1_HUMAN | P05155 | 55154 | Inhibitor | | | | | | | 30 | 1 | | | | | | | |
| Iduronate 2-sulfatase | IDS_HUMAN | P22304 | 61873 | Enzyme activity | | | | | | | | | | | 55 | 1 | | | |
| Calpain-11 | CAN11_HUMAN | Q9UMQ6 | 80583 | Enzyme activity | | | | | | | | | | | 34 | 1 | | | |
| Ribonuclease 4 | RNAS4_HUMAN | P34096 | 16840 | Nucleotide specificity | | | | | | | | | | | 34 | 1 | | | |
| Isocitrate dehydrogenase [NAD] subunit alpha | IDH3A_HUMAN | P50213 | 39592 | Enzyme activity | | | | | | | | | | | | | 32 | 1 | |
| Inter-alpha-trypsin inhibitor heavy chain H2 | ITIH2_HUMAN | P19823 | 106436 | Transport/binding | | | | | | | | | | | 32 | 1 | | | |
| *Cell proliferation/communication/signal transduction* | | | | | | | | | | | | | | | | | | | |
| Calsyntenin-1 | CSTN1_HUMAN | O94985 | 109793 | Signal transduction | 215 | 4 | 274 | 6 | | | 254 | 6 | | | 161 | 3 | | | |
| Amyloid-like protein 1 | APLP1_HUMAN | P51693 | 72176 | Signal transduction | 210 | 4 | 141 | 4 | 73 | 10 | 695 | 15 | | | 487 | 11 | | | |

Table 2 (*Continued*)

| Protein name | Uniprot entry | Uniprot Acc. | MW | Function | Intact CSF | | Montage FT | | Montage B | | IgY-12 FT | | IgY-12 B | | PP-20 FT | | PP-20 B | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Score | Pep. | Score | Pep. | Score | Pep. | Score | Pep. | Score | Pep. | Score | Pep. | Score | Pep. |
| Gelsolin | GELS_HUMAN | P06396 | 86043 | Actin modulation | 163 | 2 | 282 | 4 | | | 397 | 8 | | | 297 | 5 | 32 | 1 |
| Pigment epithelium-derived factor | PEDF_HUMAN | P36955 | 46484 | Differentiation/Inhibitor | 121 | 2 | 93 | 1 | 31 | 2 | 357 | 6 | | | 201 | 3 | | |
| Fibrinogen alpha chain | FIBA_HUMAN | P02671 | 95656 | Coagulation | 100 | 2 | | | | | | | | | | | 39 | 1 |
| Alpha-2-HS-glycoprotein | FETUA_HUMAN | P02765 | 39325 | Promotes endocytosis | 91 | 2 | 40 | 1 | | | | | | | | | | |
| Fibrinogen gamma chain | FIBG_HUMAN | P02679 | 52106 | Coagulation | 72 | 1 | 90 | 2 | | | | | | | | | | |
| Fibrinogen beta chain | FIBB_HUMAN | P02675 | 56577 | Coagulation | 63 | 1 | 140 | 4 | | | | | | | | | | |
| Amyloid beta A4 protein | A4_HUMAN | P05067 | 86943 | Signal/neurite outgrowth | 62 | 2 | 122 | 3 | | | 463 | 12 | | | 147 | 5 | | |
| Beta-2-microglobulin | B2MG_HUMAN | P61769 | 13715 | Signal transduction | 50 | 1 | 144 | 3 | | | 159 | 3 | | | 66 | 2 | | |
| CD44 antigen | CD44_HUMAN | P16070 | 81554 | Mediates cell–cell interactions | 46 | 1 | 37 | 1 | | | 180 | 4 | | | 87 | 2 | | |
| Tyrosine-protein phosphatase non-receptor type substrate 1 | SHPS1_HUMAN | P78324 | 54813 | Cell surface receptor/adhesion | 45 | 1 | 70 | 1 | | | 49 | 2 | | | 98 | 2 | | |
| Ephrin type-A receptor 4 | EPHA4_HUMAN | P54764 | 109860 | Signal transduction | 34 | 1 | 72 | 3 | | | | | | | | | | |
| Neurosecretory protein VGF | VGF_HUMAN | O15240 | 67287 | Growth factor/signal transduction | | | 38 | 1 | | | 73 | 1 | | | 40 | 1 | | |
| Guanine nucleotide-binding protein subunit alpha-13 | GNA13_HUMAN | Q14344 | 44050 | Signal transduction | | | | | 29 | 1 | | | | | | | | |
| Low-density lipoprotein receptor-related protein 4 | LRP4_HUMAN | O75096 | 212045 | Surface receptor | | | | | 28 | 1 | | | | | | | | |
| *Structural/membrane associated/extracellular matrix* | | | | | | | | | | | | | | | | | | |
| Fibronectin | FINC_HUMAN | P02751 | 266034 | Cell structure/communication | 206 | 5 | 411 | 11 | | | 130 | 3 | | | 52 | 2 | | |
| Fibulin-1 | FBLN1_HUMAN | P23142 | 77261 | Extracellular matrix protein | 176 | 5 | 183 | 6 | | | 333 | 7 | | | 260 | 6 | | |
| Neuronal cell adhesion molecule | NRCAM_HUMAN | Q92823 | 143894 | Cell adhesion | 150 | 3 | 248 | 6 | | | 284 | 7 | | | 200 | 4 | | |
| Neural cell adhesion mol. L1-like protein | CHL1_HUMAN | O00533 | 135027 | Extracellular matrix protein | 126 | 3 | 297 | 7 | | | 426 | 9 | | | 296 | 5 | | |
| EGF-containing fibulin-like extracellular matrix protein 1 | FBLN3_HUMAN | Q12805 | 54641 | Extracellular matrix protein | 116 | 2 | 217 | 5 | | | 316 | 8 | | | | | | |
| Thy-1 membrane glycoprotein | THY1_HUMAN | P04216 | 17935 | Cell–cell interaction | 80 | 1 | 128 | 2 | | | 124 | 2 | | | 67 | 2 | | |
| Extracellular matrix protein 1 | ECM1_HUMAN | Q16610 | 60674 | Extracellular matrix protein | 79 | 2 | 45 | 1 | | | 167 | 3 | | | 32 | 1 | | |
| Brevican core protein | PGCB_HUMAN | Q96GW7 | 99118 | Nervous system development | 77 | 2 | 105 | 3 | | | 176 | 4 | | | 98 | 2 | | |
| Vitronectin | VTNC_HUMAN | P04004 | 55069 | Cell adhesion/communication | 73 | 2 | 312 | 6 | | | 87 | 2 | | | 99 | 3 | | |
| Limbic system-associated membrane protein | LSAMP_HUMAN | Q13449 | 37393 | Neuronal growth | 69 | 2 | 128 | 2 | | | 200 | 3 | | | 109 | 2 | | |
| Dystroglycan | DAG1_HUMAN | Q14118 | 97581 | Cytoskeleton receptor | 63 | 1 | 56 | 1 | | | 61 | 2 | | | | | | |
| Mimecan | MIME_HUMAN | P20774 | 33922 | Bone formation | 57 | 1 | 45 | 1 | | | 70 | 2 | | | 80 | 2 | | |
| Neurocan core protein | CSPG3_HUMAN | O14594 | 142973 | Neuronal adhesion neurite growth | 51 | 1 | 55 | 2 | | | 108 | 3 | | | 54 | 1 | | |
| SPARC-like protein 1 | SPRL1_HUMAN | Q14515 | 75216 | Extracellular matrix protein | 29 | 1 | 156 | 4 | | | 290 | 5 | | | 116 | 3 | | |
| Charged multivesicular body protein 1b | CHM1B_HUMAN | Q7LBR1 | 22109 | Multivesicular body formation | 29 | 1 | | | | | | | 32 | 1 | 28 | 1 | 28 | 1 |
| Contactin-1 | CNTN1_HUMAN | Q12860 | 113320 | Nervous system development | | | 149 | 4 | | | 176 | 5 | | | 88 | 3 | | |
| Galectin-3-binding protein | LG3BP_HUMAN | Q08380 | 65331 | Cell adhesion | | | 138 | 5 | | | 43 | 1 | | | 118 | 3 | | |
| Neuronal growth regulator 1 | NEGR1_HUMAN | Q7Z3B1 | 38719 | Cell adhesion/neuron growth | | | 91 | 2 | | | 80 | 2 | | | | | | |

| | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Neural cell adhesion molecule 1, 140 kDa isoform | NCA11_HUMAN | P13591 | 93361 | Cell adhesion | | | 81 | 2 | | | 75 | 1 | | | 55 | 2 | | | |
| Neurotrimin | NTRI_HUMAN | Q9P121 | 37971 | Cell adhesion | | | 68 | 1 | | | 133 | 2 | | | 44 | 1 | | | |
| Collagen alpha-1(I) chain | CO1A1_HUMAN | P02452 | 138911 | Fibril forming | | | 67 | 2 | | | 47 | 1 | | | 44 | 1 | | | |
| IgGFc-binding protein | FCGBP_HUMAN | Q9Y6R7 | 572068 | Mucosal structure maintenance | | | 41 | 2 | | | | | | | | | | | |
| Macrophage colony-stimulating factor 1 receptor | CSF1R_HUMAN | P07333 | 107984 | CSF-1 receptor | | | 40 | 1 | | | 32 | 1 | | | 39 | 1 | | | |
| Contactin-2 | CNTN2_HUMAN | Q02246 | 113393 | Axon growth/cell adhesion | | | 29 | 1 | | | | | | | | | | | |
| Bullous pemphigoid antigen 1, isoforms 6/9/10 | BPAEA_HUMAN | O94833 | 590993 | Cytoskeleton linker | | | 27 | 1 | | | | | | | | | | | |
| Transmembrane protein 169 | TM169_HUMAN | Q96HH4 | 33611 | Membrane protein | | | | | 32 | 1 | | | | | | | | | |
| Spectrin alpha chain, erythrocyte | SPTA1_HUMAN | P02549 | 279998 | Cytoskeleton protein | | | | | 27 | 1 | | | | | | | | | |
| Neurofascin | NFASC_HUMAN | O94856 | 150027 | Cell adhesion | | | | | | | 92 | 4 | | | | | | | |
| Myelin-oligodendrocyte glycoprotein | MOG_HUMAN | Q16653 | 28179 | Myelin sheath component | | | | | | | 58 | 2 | | | | | | | |
| Oligodendrocyte-myelin glycoprotein | OMGP_HUMAN | P23515 | 49608 | Cell adhesion/myelination | | | | | | | 54 | 1 | | | | | | | |
| Protein NOV homolog | NOV_HUMAN | P48745 | 39162 | Cell growth regulation | | | | | | | 47 | 1 | | | | | | | |
| Cell adhesion molecule 3 | CADM3_HUMAN | Q8N126 | 43300 | Cell–cell adhesion | | | | | | | 46 | 1 | | | | | | | |
| Neural cell adhesion molecule 2 | NCAM2_HUMAN | O15394 | 92932 | Cell adhesion | | | | | | | 39 | 1 | | | | | | | |
| Cadherin-13 | CAD13_HUMAN | P55290 | 78287 | Cell adhesion/cell cycle | | | | | | | 39 | 1 | | | | | | | |
| Lethal(2) giant larvae protein homolog 1 | L2GL1_HUMAN | Q15334 | 115042 | Cytoskeleton protein | | | | | | | 32 | 1 | | | | | | | |
| Cell surface glycoprotein MUC18 | MUC18_HUMAN | P43121 | 71607 | Cell adhesion | | | | | | | 31 | 1 | | | | | | | |
| Collagen alpha-1(XVIII) chain | COIA1_HUMAN | P39060 | 178160 | Retinal/neural tube structure | | | | | | | | | | | 37 | 1 | | | |
| Membrane frizzled-related protein | MFRP_HUMAN | Q9BY79 | 62212 | Eye development | | | | | | | | | | | 27 | 1 | | | |
| *Miscellaneous* | Miscellaneous | | | | | | | | | | | | | | | | | | |
| Clusterin | CLUS_HUMAN | P10909 | 53031 | Apoptosis | 627 | 10 | 489 | 9 | 102 | 8 | 638 | 12 | 213 | 4 | 626 | 10 | 327 | 5 | |
| Prothrombin | THRB_HUMAN | P00734 | 71475 | Acute phase response | 143 | 4 | 82 | 2 | | | 272 | 7 | | | 105 | 3 | | | |
| Kallikrein-6 | KLK6_HUMAN | Q92876 | 26856 | Enzyme activity | 143 | 3 | 370 | 6 | | | 512 | 9 | | | 299 | 6 | | | |
| Alpha-1-antichymotrypsin | AACT_HUMAN | P01011 | 47651 | Probable inhibitor | 58 | 1 | 71 | 2 | | | | | | | | | | | |
| Histidine-rich glycoprotein | HRG_HUMAN | P04196 | 60510 | Not listed | 42 | 1 | 39 | 1 | 38 | 2 | 59 | 2 | | | 123 | 4 | | | |
| Secretogranin I (Chromogranin B) | SCG1_HUMAN | P05060 | 78246 | Precursor for active peptides | 42 | 1 | 56 | 1 | | | 93 | 2 | | | | | | | |
| Major prion protein | PRIO_HUMAN | P04156 | 27661 | Not listed | 31 | 1 | | | | | 55 | 2 | | | 36 | 1 | | | |
| AMBP protein | AMBP_HUMAN | P02760 | 38999 | Complex forming | | | 57 | 1 | | | 77 | 2 | | | | | | | |
| Leucine-rich alpha-2-glycoprotein | A2GL_HUMAN | P02750 | 38178 | Not listed | | | 54 | 2 | | | | | | | | | | | |
| Voltage-dependent calcium channel subunit alpha-2/delta-1 | CAC2D_HUMAN | P54289 | 123183 | Calcium channel protein | | | 47 | 1 | | | 62 | 3 | | | | | | | |
| Immunoglobulin superfamily member 8 | IGSF8_HUMAN | Q969P0 | 65034 | Multiple enzymatic functions | | | 41 | 1 | | | 27 | 1 | | | 25 | 1 | | | |
| Secretogranin-3 | SCG3_HUMAN | Q8WXD2 | 53005 | Not listed | | | 31 | 1 | | | | | | | | | | | |
| THUMP domain-containing protein 1 | THUM1_HUMAN | Q9NXG2 | 39315 | Unambiguous | | | | | 34 | 1 | | | | | | | | | |
| Protein FAM3C | FAM3C_HUMAN | Q92520 | 24680 | Not listed | | | | | | | 70 | 3 | | | 34 | 1 | | | |
| Retinoic acid receptor responder protein 2 | RARR2_HUMAN | Q99969 | 18618 | Calcium channel protein | | | | | | | 61 | 2 | | | | | | | |
| Cartilage acidic protein 1 | CRAC1_HUMAN | Q9NQ79 | 71421 | Not listed | | | | | | | 50 | 1 | | | | | | | |
| Ribonuclease T2 | RNT2_HUMAN | O00584 | 29481 | Not listed | | | | | | | 35 | 1 | | | | | | | |

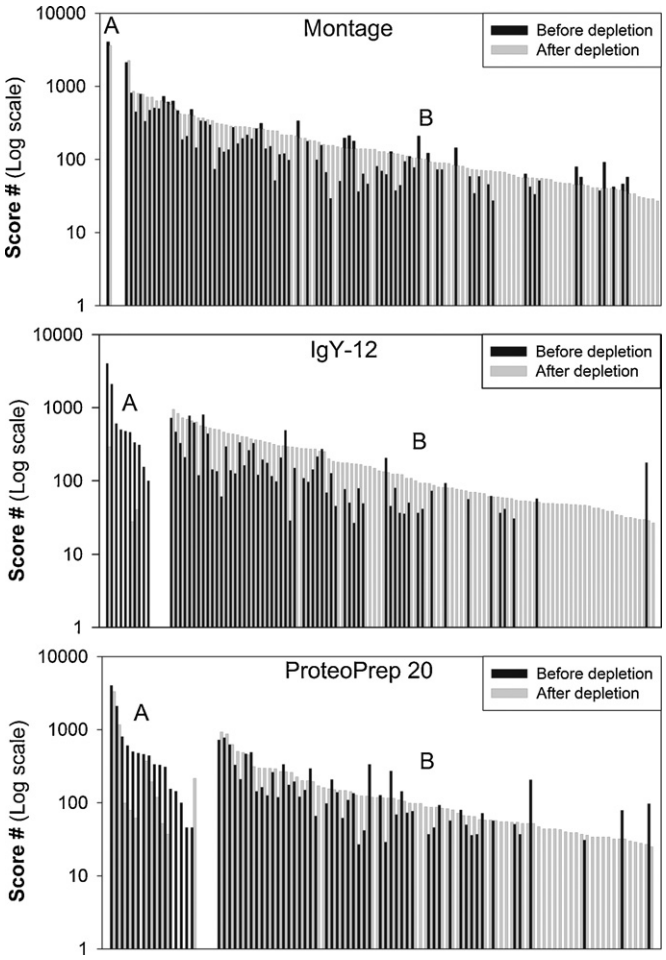


**Fig. 3.** MASCOT scores (log10) for significantly matched (MudPIT 95% ($p \leq 0.05$) confidence) proteins before (black bars) and after (grey bars) sample preparation of 1 mL CSF by the three different spin column kits. A: High abundant proteins targeted for removal, B: remaining medium–low abundant proteins.

abundant proteins are dramatically increased even though the targeted subtraction proteins are not removed but, as for the Montage column, merely reduced in concentration. The greatest reduction of the high abundant proteins was observed for the ProteomeLab IgY-12 spin column were the MASCOT scores for the majority of the proteins were completely diminished and for the remaining reduced by up to 17 times. Additionally, MASCOT scores for the medium and low abundant proteins were increased by more than 10 times or, for most low abundant proteins, only identified after the removal of the high abundant proteins. Although the removal or reduction of the high abundant proteins in CSF leads to an increased number of medium–low abundant proteins identified with increased MASCOT scores, it is also important to be aware of the possibilities of co-removal of low abundant proteins. Proteins identified in each corresponding bound fraction are listed in Table 2. Both the ProteomeLab IgY-12 and ProteoPrep 20 spin columns show very high specificity and low non-specific co-removal, while the entirely affinity based Montage column has a higher degree of non-specific protein subtraction. The ProteomeLab IgY-12 bound fraction contains 10 out of the 12 targeted proteins, only Apolipoprotein A-II and Fibrinogen are not detected. However, human CSF should normally contain moderate concentrations of Fibrinogen, which explains the lack of identification. The subtraction column has primarily been developed for human plasma samples and is therefore not completely optimal for human CSF. The bound fraction from the ProteomeLab IgY-12 column contains 9 non-targeted proteins. Nonetheless, most of these proteins, such as Prostaglandin-H2 D-isomerase (beta-trace) and Transthyretin (prealbumin) are considered high abundant in CSF and are therefore likely to be present in the bound fraction due to non-specific binding. The presence of these non-targeted proteins in the bound fraction will, however, have implications on any quantitative studies performed on the flow through fractions. These proteins should not be quantitatively evaluated in the FT fraction, since their concentrations are questionable. Thus, it is clearly of interest to analyze the protein content in the bound fractions when fractionating the sample and exclude, or at least be very cautious in including the identified proteins in any comparative studies. The bound fraction for the ProteoPrep 20 spin column contained 17 out of the 20 targeted proteins and 6 non-targeted. The targeted proteins missing were Apolipoprotein A-II, Apolipoprotein B and Complement factor C1q. Again, this column was designed for the preparation of human plasma. Apolipoprotein B is not high abundant in human CSF. In fact, it can be used as an indicator for blood contamination in CSF [2] and the absence of Apolipoprotein B in the bound fraction is therefore not surprising. The bound fraction for the Montage spin column contained 28 proteins, which clearly demonstrates the non-specific co-removal of proteins due to the albumin sponge effect. It can also be explained by the fact that this column is purely affinity based and therefore has a tendency to bind other non-targeted proteins. Again, this non-specific removal of the proteins will have implications on any quantitative analysis on the remaining protein fractions in the corresponding FT sample.

### 3.3. Column reproducibility and iTRAQ labeling

Based on the number of proteins identified, in combination with the specificity of protein removal, the ProteomeLab IgY-12 spin column was further evaluated regarding reproducibility and compatibility for LC–MS/MS quantification using stable isotopic labeling. Differential diagnosis based on comparative proteomic studies with quantitative mass spectrometry has seen a major breakthrough in recent years and the use of isotope coded tags is specifically popular. One of the most accepted approaches is the isobaric tags for relative and absolute quantification [38] that enables multiplexed quantitative analysis of up to eight samples simultaneously. The reproducibility and use of the ProteomeLab IgY-12 spin column on human CSF was evaluated iTRAQ labeling and quantification. This approach will estimate the reproducibility of the combination of the high abundant protein removal, the iTRAQ labeling and the MS/MS detection and thereby mimic the experimental conditions for a screening study of clinically relevant samples. The obtained iTRAQ ratios are plotted in Fig. 4A (99% MudPIT confidence scoring) and Fig. 4B (95% MudPIT confidence scoring). In the ideal case, the expected iTRAQ ratios would be 1:1:1:1. The iTRAQ manufacturer (Applied) states an estimated labeling and MS/MS variation of roughly 20%. On normalized values this would imply that ratios between 1.2 and 0.83 are considered as "no change" or within the expected experimental variation. Abdi et al. [2] have previously stated that iTRAQ ratios greater than 20% but less than 50% to have an unlikely and uncertain significance in a quantitative study of human CSF. Therefore, changes in the protein expression in the CSF have to yield iTRAQ ratios increased or decreased by 50% to be considered significant. Consequently, a normalized ratio greater than 1.5 should be considered as a significantly up-regulated protein and a normalized value smaller than 0.67 should be considered as a significantly down-regulated protein. Almost all iTRAQ ratios plotted in Fig. 4A and B are within the boundaries to be considered "no change". Since all ratios are expected to be 1:1:1:1, the average ratio values and relative standard deviation (R.S.D.) can be calculated for the entire method. For the 99% MudPIT MASCOT scoring the average 115/114, 116/114

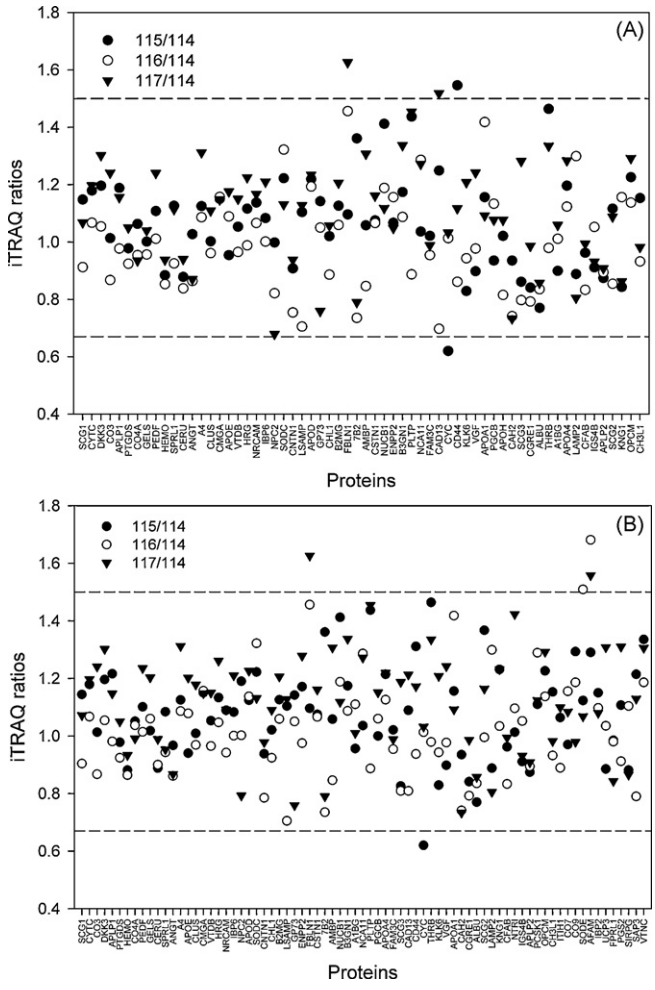


**Fig. 4.** (A and B) iTRAQ 4-plex ratios for the significantly matched proteins in CSF after ProteomeLab IgY-12 processing with A: 99% MudPIT confidence scoring and B: 95% MudPIT confidence scoring. All iTRAQ ratios are expected to be 1:1:1:1. The dashed lines represent iTRAQ ratios of 1.5 and 0.67, which corresponds to significant up and down-regulation respectively according to Abdi et al. [2].

and 117/114 ratios were found to be 1.07, 0.99 and 1.11 with R.S.D. values of 16.1%, 16.9% and 16.9%, respectively. On a 95% MudPIT MASCOT scoring level the average 115/114, 116/114 and 117/114 ratios were found to be 1.08, 1.02 and 1.12 with R.S.D. values of 14.9%, 17.5% and 16.0%, respectively. The obtained average and R.S.D. values are well within the 20% variation stated for the labeling alone. This indicates that the high abundant protein subtraction with the ProteomeLab IgY-12 spin column followed by iTRAQ labeling, nanoLC separation and MALDI-TOF/TOF-MS detection yields very reproducible results. However, looking further on individual protein levels one can notice that a few proteins actually show up- and down-regulated levels both on a 99% and 95% MudPIT MASCOT scoring level. On the 99% level, Fibulin-1 and Cadherin-13 had iTRAQ 117/114 ratios greater than 1.5 and the 115/114 ratios for Cytochrome C and CD 44 antigen were less than 0.67 and greater than 1.5, respectively. On the 95% level, the same results were obtained for Fibulin-1 and Cytochrome C but not the other proteins. Meanwhile, the 116/114 ratio for Extracellular superoxide dismutase was greater than 1.5, which was also the case for both the 116/114 and 117/114 ratios for Afamin. Statistically, a few outlier results are to be expected. However, these outliers accentuate the need to have an experimental design that includes numerous biological and technical replicates when performing biomarker screening studies. Trends of up- and down-regulations must be consistent throughout all biological and technical replicates to be considered as potential biomarkers. For instance, both Cytochrome C and Extracellular superoxide dismutase that by chance and wrongfully showed significant changes have extensively been reported to be altered in numerous diseases. Finally, as stated previously, it is believed that many potential biomarkers secreted in biofluids would be present at very low concentrations. This will have implications on any quantitative study. These proteins are likely to be identified by only 1–2 peptides at low MS intensities, which in turn give very poor data for any statistics or quantification.

## 4. Conclusions

In this study, the performance of three different affinity/antibody protein subtraction kits for the preparation of human CSF was compared. A rather large CSF volume, up to 1 mL, could be processed with all three columns with retained partitioning efficiency. All three columns also yielded an increased number of proteins identified as well as increased MASCOT ionscores for the remaining medium–low abundant proteins. The analysis of the protein content of the bound proteins showed varying degrees of non-targeted protein removal. It is of interest to analyze the bound fractions as non-targeted protein removal will influence any quantitative proteomic studies. These proteins should be excluded in the study, unless they exclusively can be quantified in the bound protein fractions. The reproducibility for the overall procedure of sample processing and quantitative MS/MS analysis was investigated for the ProteomeLab IgY-12 spin column in combination with iTRAQ labeling. The overall process of protein removal, isotopic labeling and MS/MS analysis was very reproducible and the overall variation was less than 17.5%, making this approach suitable for quantitative comparisons of CSF samples. However, the experimental data support previous statements that limits for up and down-regulation in CSF should be set to at least 50% change and be consistent in numerous biological replicates.

## Acknowledgements

This research was supported by Uppsala Berzelii Technology Center for Neurodiagnostics, with financing from the Swedish Governmental Agency for Innovation Systems and the Swedish Research Council Grant numbers P29797-1 and 621-2008-3562. Andreas Dahlin is acknowledged for assistance with Fig. 1.

## References

[1] J. Zhang, D.R. Goodlett, T.J. Montine, J. Alzheimers Dis. 8 (2005) 377.
[2] F. Abdi, J.F. Quinn, J. Jankovic, M. McIntosh, J.B. Leverenz, E. Peskind, R. Nixon, J. Nutt, K. Chung, C. Zabetian, A. Samii, M. Lin, S. Hattan, C. Pan, Y. Wang, J. Jin, D. Zhu, G.J. Li, Y. Liu, D. Waichunas, T.J. Montine, J. Zhang, J. Alzheimers Dis. 9 (2006) 293.
[3] W.M. Caudle, S. Pan, M. Shi, T. Quinn, J. Hoekstra, R.P. Beyer, T.J. Montine, J. Zhang, Proteomics Clin. Appl. 2 (2008) 1484.
[4] J. Zhang, C.D. Keene, C. Pan, K.S. Montine, T.J. Montine, J. Neuropathol. Exp. Neurol. 67 (2008) 923.
[5] S. Roche, A. Gabelle, S. Lehmann, Proteomics Clin. Appl. 2 (2008) 428.
[6] M. Shi, W.M. Caudle, J. Zhang, Neurobiol. Dis. 35 (2009) 157.
[7] S. Jesse, P. Steinacker, S. Lehnert, F. Gillardon, B. Hengerer, M. Otto, CNS Neurosci. Ther. 15 (2009) 157.
[8] R. Craig-Schapiro, A.M. Fagan, D.M. Holtzman, Neurobiol. Dis. 35 (2009) 128.
[9] A. Westman-Brinkmalm, U. Ruetschi, E. Portelius, U. Andreasson, G. Brinkmalm, G. Karlsson, S. Hansson, H. Zetterberg, K. Blennow, Front. Biosci. 14 (2009) 1793.
[10] H. Reiber, J.B. Peter, J. Neurol. Sci. 184 (2001) 101.
[11] S. Hu, J.A. Loo, D.T. Wong, Proteomics 6 (2006) 6326.
[12] S.M. Ahn, R.J. Simpson, Proteomics Clin. Appl. 1 (2007) 1004.
[13] J. Zhang, D.R. Goodlett, E.R. Peskind, J.F. Quinn, Z. Yong, W. Qin, C. Pan, E. Yi, J. Eng, R.H. Aebersold, T.J. Montine, Neurobiol. Aging 26 (2005) 207.
[14] J.P. Noben, D. Dumont, N. Kwasnikowska, P. Verhaert, V. Somers, R. Hupperts, P. Stinissen, J. Robben, J. Proteome Res. 5 (2006) 1647.
[15] K.S. Shores, D.R. Knapp, J. Proteome Res. 6 (2007) 3739.

[16] R. Siegmund, M. Kiehntopf, T. Deufel, Clin. Biochem. 42 (2009) 1136.
[17] P. Davidsson, L. Paulson, C. Hesse, K. Blennow, C.L. Nilsson, Proteomics 1 (2001) 444.
[18] P. Davidsson, S. Folkesson, M. Christiansson, M. Lindbjer, B. Dellheden, K. Blennow, A. Westman-Brinkmalm, Rapid Commun. Mass Spectrom. 16 (2002) 2083.
[19] L.N. Waller, K. Shores, D.R. Knapp, J. Proteome Res. 7 (2008) 4577.
[20] X.L. Yuan, D.M. Desiderio, Proteomics 5 (2005) 541.
[21] K.S. Shores, D.G. Udugamasooriya, T. Kodadek, D.R. Knapp, J. Proteome Res. 7 (2008) 1922.
[22] B.N. Hammack, G.P. Owens, M.P. Burgoon, D.H. Gilden, Mult. Scler. 9 (2003) 472.
[23] G. Maccarrone, D. Milfay, I. Birg, M. Rosenhagen, F. Holsboer, R. Grimm, J. Bailey, N. Zolotarjova, C.W. Turck, Electrophoresis 25 (2004) 2402.
[24] M. Ramstrom, C. Hagman, J.K. Mitchell, P.J. Derrick, P. Hakansson, J. Bergquist, J. Proteome Res. 4 (2005) 410.
[25] M. Ramstrom, A. Zuberovic, C. Gronwall, J. Hanrieder, J. Bergquist, S. Hober, Biotechnol. Appl. Biochem. 52 (2009) 159.
[26] E. Mouton-Barbosa, F. Roux-Dalvai, D. Bouyssié, F. Berger, E. Schmidt, P.G. Righetti, L. Guerrier, E. Boschetti, O. Burlet-Schiltz, B. Monsarrat, A. Gonzalez de Peredo, Mol. Cell. Proteomics, Published online January 21st, 2010, doi:10.1074/mcp.M900513-MCP200.
[27] B.R. Wenner, M.A. Lovell, B.C. Lynn, J. Proteome Res. 3 (2004) 97.
[28] Y. Hu, J.P. Malone, A.M. Fagan, R.R. Townsend, D.M. Holtzman, Mol. Cell. Proteomics 4 (2005) 2000.
[29] Y. Ogata, M.C. Charlesworth, D.C. Muddiman, J. Proteome Res. 4 (2005) 837.
[30] J.A. Burgess, P. Lescuyer, A. Hainard, P.R. Burkhard, N. Turck, P. Michel, J.S. Rossier, F. Reymond, D.F. Hochstrasser, J.C. Sanchez, J. Proteome Res. 5 (2006) 1674.
[31] Y. Hu, J.S.K. Kauwe, J. Gross, N.J. Cairns, A.M. Goate, A.M. Fagan, R.R. Townsend, D.M. Holtzman, Proteomics Clin. Appl. 1 (2007) 1373.
[32] Y. Ogata, M.C. Charlesworth, L. Higgins, B.M. Keegan, S. Vernino, D.C. Muddiman, Proteomics 7 (2007) 3726.
[33] L. Dayon, A. Hainard, V. Licker, N. Turck, K. Kuhn, D.F. Hochstrasser, P.R. Burkhard, J.C. Sanchez, Anal. Chem. 80 (2008) 2921.
[34] A. Zuberovic, J. Hanrieder, U. Hellman, J. Bergquist, M. Wetterhall, Eur. J. Mass Spectrom. 14 (2008) 249.
[35] E. Thouvenot, S. Urbach, C. Dantec, J. Poncet, M. Seveno, E. Demettre, P. Jouin, J. Touchon, J. Bockaert, P. Marin, J. Proteome Res. 7 (2008) 4409.
[36] J. Granger, J. Siddiqui, S. Copeland, D. Remick, Proteomics 5 (2005) 4713.
[37] R.L. Gundry, Q. Fu, C.A. Jelinek, J.E. Van Eyk, R.J. Cotter, Proteomics Clin. Appl. 1 (2007) 73.
[38] T. Hunt, Y. Huang, P. Ross, S. Pillai, S. Purkayastha, D. Pappin, Mol. Cell. Proteomics 3 (2004) S286.
[39] J. Bergquist, M. Palmblad, M. Wetterhall, P. Hakansson, K.E. Markides, Mass Spectrom. Rev. 21 (2002) 2.
[40] J. Hanrieder, M. Wetterhall, P. Enblad, L. Hillered, J. Bergquist, J. Neurosci. Methods 177 (2009) 469.