DOI 10.1002/pmic.201400096 *Proteomics* 2015, *15*, 10–15

RESEARCH ARTICLE

A rapid method for preparation of the cerebrospinal fluid proteome

Eivind Larssen[1,2], Cato Brede[3], Anne Bjørnstad Hjelle[2], Kjell Birger Øysæd[2], Anne Bolette Tjensvoll[4], Roald Omdal[5,6] and Peter Ruoff[7]

[1] Research Department, Stavanger University Hospital, Stavanger, Norway
[2] International Research Institute of Stavanger, IRIS Envrionment, Stavanger, Norway
[3] Department of Medical Biochemistry, Stavanger University Hospital, Stavanger, Norway
[4] Department of Neurology, Stavanger University Hospital, Stavanger, Norway
[5] Clinical Immunology Unit, Department of Internal Medicine, Stavanger University Hospital, Stavanger, Norway
[6] Department of Medical Science, Faculty of Medicine and Dentistry, University of Bergen, Bergen, Norway
[7] Center for Organelle Research (CORE), University of Stavanger, Stavanger, Norway

The cerebrospinal fluid (CSF) proteome is of great interest for investigation of diseases and conditions involving the CNS. However, the presence of high-abundance proteins (HAPs) can interfere with the detection of low-abundance proteins, potentially hindering the discovery of new biomarkers. Therefore, an assessment of the CSF subproteome composition requires depletion strategies. Existing methods are time consuming, often involving multistep protocols. Here, we present a rapid, accurate, and reproducible method for preparing the CSF proteome, which allows the identification of a high number of proteins. This method involves acetonitrile (ACN) precipitation for depleting HAPs, followed by immediate trypsination. As an example, we demonstrate that this method allows discrimination between multiple sclerosis patients and healthy subjects.

Received: March 17, 2014
Revised: July 18, 2014
Accepted: October 6, 2014

Keywords:
ACN-precipitation / Cerebrospinal fluid / LC-MS/MS / Sample preparation / Technology / Trypsination

Additional supporting information may be found in the online version of this article at the publisher's web-site

1 Introduction

When investigating diseases and conditions involving the CNS, cerebrospinal fluid (CSF) analysis is helpful, both for confirming the existence of a certain disease and for ruling out other diagnoses [1]. Although, a number of diagnostic, biochemical, and immunological tests are available for routine clinical use, identification of new biomarkers could increase the usefulness of CSF analysis [2].

More than 80% of the CSF proteome is represented by a handful of proteins, e.g. serum albumin, serotransferrin, Ig's, and apolipoproteins [3–6]. MS-based proteomics is heavily influenced by the presence of these high-abundance proteins (HAPs). To enable detection of biomarkers at low concentrations, several protein removal and fractionation strategies have been explored, including chromatographic techniques, immunoaffinity, and 2D PAGE [3, 7, 8]. Sensitivity of these techniques has increased in the last couple of years [9]. However, most existing procedures for HAP depletion are labor intensive, time consuming, and may introduce variation in the protein content.

Traditional 2D gel approach is particularly good for looking at proteins within the mass range of 20–250 kDa and p*I* values between 3 and 11. To be identified by this method, proteins need to be excised from the gel, subjected to in-gel proteolysis and analyzed by MS. In addition, 2D gel analysis has a low throughput.

LC is recognized as a useful tool in proteomics research since it provides high-speed, high-resolution, and

Correspondence: Eivind Larssen, International Research Institute of Stavanger – IRIS, Mekjarvik 12, 4070 Randaberg, Stavanger, Norway
E-mail: el@iris.no

Abbreviations: **CSF**, cerebrospinal fluid; **HAP**, high-abundance protein; **PSM**, peptide spectrum match

© 2014 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

high-sensitivity separation of macromolecules (e.g. proteins) [10]. However, sample preparation methods for LC require expensive instrumentation and will often have additional steps in order to increase the concentration of the collected protein fractions.

Finally, the immunoaffinity approach is particularly useful for targeted proteomics. Nevertheless, antibody stability may influence the reproducibility [11].

For CSF proteome preparation, a rapid and straightforward strategy is desirable. Varying degrees of success have been reported for HAP depletion using different protein precipitation agents (e.g. dithiothreitol and acetonitrile (ACN)) [4, 12–15]. The method outlined here for preparing the CSF proteome is based on ACN precipitation previously described by the Zhang et al. [16, 17] and Kay et al.[4]. Compared to these methods, the novelty of our approach involves immediate trypsination [5, 18] using ACN as the organic solvent for enhanced digestion. Herein, we have applied ACN precipitation at two different concentrations (75 and 80%) for HAP depletion allowing for direct trypsination (1-h).

The aim of the present work was to develop a rapid, accurate, and reproducible method for HAP depletion by comparing ACN precipitation at two different concentrations, which allowed an immediate solvent enhanced trypsination. A preliminary confirmation of the method's usefulness is presented here. Using this method, we are able to show that there are distinct differences in the CSF proteome between multiple sclerosis patients and healthy subjects.

2 Materials and methods

2.1 Sample collection

CSF was obtained from five patients with definite multiple sclerosis [19] at the Neurological Department of Stavanger University Hospital and from five healthy control subjects who had no neurological, immunological, or other diseases. Multiple sclerosis is a chronic autoimmune disease characterized by demyelination and loss of axons and neurons in the CNS [20]. Samples were preserved in 200 μL aliquots without additional proteinase inhibitors [21], and stored at −80°C until analysis.

This study was carried out in compliance with the Helsinki Declaration and approved by the regional ethics committee. All subjects gave informed consent to participate in this study.

2.2 Experimental design and sample preparation

CSF sample preparation was divided into three steps. First, we investigated whether the ACN (LiChrosolv®, Merck KGaA, Germany) precipitation could discriminate between samples from multiple sclerosis patients and healthy subjects (five individuals per group). To each 200 μL CSF aliquot, we added 600 μL of ACN. Samples were then centrifuged at 14 000 × *g* for 30 min, and the supernatants were used for further analysis. Samples were trypsinated adding 2 μL trypsin (0.1 μg/μL; Promega, Madison, USA) to the supernatant (trypsin-to-protein ratio 1:50), incubated for 1 h at 37°C, and speed vacuumed (Concentrator 5301, Eppendorf, Germany) for 1 h at 60°C to remove the ACN. Subsequently, 100 μL of 0.1% formic acid (mobile phase A) was added to the sample, and the entire content of the tube was transferred into a glass tube (Chromacol Ltd, UK) for MS/MS analysis.

Second, to test reproducibility, samples were precipitated with two different volumes of ACN, these volumes were chosen based on previous results by Kay et al. [4]. The soluble fraction was digested directly and the resulting peptide spectrum matches (PSMs) were compared using six CSF aliquots from one healthy individual. Strader et al. [18] reported that a 1 h digestion in 80% ACN yielded over 52% more peptides than an overnight digestion of 1 μg of a protein mixture in purely aqueous buffer. Thus, we added different amounts of ACN to 200 μL of CSF to achieve final ACN concentrations of 75 and 80%.

Finally, we compared two trypsination approaches: The 1 h ACN-mediated trypsin digestion versus the traditional ACN-free overnight (16 h) digestion in an aqueous buffer. Two aliquots (200 μL) from each of the five healthy subjects were used. To each sample of the overnight experiment, 600 μL of ammonium bicarbonate buffer (pH 8) was added and then vortexed. Trypsin was added as described above, and trypsination performed overnight at 37°C. Samples were speed vacuumed, 100 μL of 0.1% formic acid was added, and the tube contents transferred to glass tubes for MS/MS analysis. Samples incubated for 1 h were treated as described in the discrimination step.

In all of these three experiments, reduction of the HAP concentration was verified using PSM values. As an example, Table 1 shows the result of depletion efficiency for four samples of raw CSF versus four samples of ACN treated CSF.

2.3 LC-MS/MS analysis

LC-MS/MS analysis was performed using an UltiMate3000 dual-pump nanoflow HPLC system (Dionex Sunnyvale, USA)

Table 1. Depletion efficiency

Protein	Number of PSMs: raw CSF	Number of PSMs: ACN-depleted CSF
Serum albumin	949	89
Complement C3	79	11
Apolipoprotein A	60	13
Serotransferrin	42	0
α1-Antitrypsin	40	10
IgG	39	0

Comparison of four samples of raw CSF versus four samples of ACN depleted CSF. Six high-abundance proteins are reported as example of the depletion efficiency.

© 2014 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

connected to a linear ion trap-orbitrap mass spectrometer (LTQ-Orbitrap XL, Thermo Fisher Scientific, Germany). Two columns were used in series: a Nanoviper monolithic PS-DVB precolumn (PepSwift 200 μm × 5 mm; Thermo Fisher Scientific, Germany) and a monolithic PS-DVB PepSwift® analytical column (100 μm id, 25-cm length; Nano Viper Thermo Fisher Scientific, Germany). The sample loading mobile phase was 0.1% formic acid. The separation mobile phase A was 2.5% ACN in 0.1% formic acid, and the separation mobile phase B was 80% ACN and 0.1% formic acid. Loading flow through the precolumn was 2 μL/min during injection, analytical flow was set at 300 nL/min, and the injection volume was 5 μL. The optimized gradient used was a multistep linear gradient between the A and B phases: 0–10 min, 100% A; 10–175 min, 100–70% A; 175–185 min, 70–0% A; 185–215 min, 0–0% A; 215–220 min, 0–100% A; and 220–250 min, 100–100% A. The MS method was data dependent, using dynamic exclusion-based MS/MS analysis on peptides with two or more charges.

2.4 Bioinformatics

The raw data files were analyzed using Proteome Discoverer 1.3 and the Sequest algorithm (Thermo Fischer Scientific, Germany) to search against the *Homo sapiens* database (UNIPROT Tax.ID 9606; downloaded on November 19, 2012, containing 137 152 sequences). Trypsin was set as the digestion enzyme, allowing for two missed cleavages. Peptide mass tolerance was set at 10 ppm and fragment mass tolerance was 0.8 Da. Oxidation of methionine (M) was set as dynamic modification and protein grouping was applied. The peptide confidence filters were set with the following combinations of X correlation and charge (z): high significance, 1.9 ($z = 2$), 2.3 ($z = 3$), and 2.6 ($z \geq 4$); medium significance, 0.8 ($z = 2$), 1 ($z = 3$), and 1.2 ($z \geq 4$).

Protein quantification was performed using a label-free approach based on PSMs. This method counts the number of spectra identified for a given peptide in different samples, then combines the results for all measured peptides of the quantified protein(s) [22].

2.5 Statistics

PSM values were used to verify protein abundances and to evaluate HAP reduction [15]. PSMs for the identified peptides were extracted from Proteome Discoverer 1.3, imported into Excel and normalized according to the following equation:

$$PSM_{\mathrm{norm(i)}} = \left(\frac{PSM_{\mathrm{i}}}{\sum_{i=1}^{n} PSM_{\mathrm{i}}}\right) + 1 \quad (1)$$

The PSMs were then imported into SIRIUS 8.1 (Pattern Recognition System AS, Norway) [23]. PCA plots were based on a partial least square discrimination analysis.

3 Results

3.1 Depletion efficiency

Depletion efficiency of ACN precipitation in CSF has been tested comparing raw sample material with ACN treated samples. The presence of the six reported HAPs significantly decreased after the ACN precipitation step, Table 1.

3.2 Discrimination

Our analysis discriminated between the multiple sclerosis group and the healthy subjects. Results in the healthy group were more spread, especially according to the first PCA component (Fig. 1A). PSMs were used as a semiquantitative measure of protein abundance.

Protein precipitation in ACN (75%) and 1 h incubation time were applied to compare multiple sclerosis patient and healthy subject samples. In total, 443 proteins were identified. For the multivariate analysis, a cut-off of ≤3 was chosen (i.e. a specific protein should be present in at least three samples to be considered valid for the analysis). This criterion was met by 106 proteins; 101 were proteins shared by the two groups (Fig. 1B). The first component explained 49% of the total variance, and the second one 29% (total explained variance 78%). For details, see also Supporting Information Table 1.

3.3 Reproducibility

The 75 and 80% ACN groups separated according to the PCA plot (Fig. 2A). The first component explained 93% of the total variance and the second one 7%. With respect to the first component, both groups showed good reproducibility. In total, 344 proteins were identified for both groups. For the multivariate analysis, a cut-off of ≤2 was chosen; 154 proteins fulfilled this criterion and 98 were shared by the two groups (Fig. 2B). The total amounts of PSMs were 284 and 181 for the 75 and 80% ACN samples, respectively. See also Supporting Information Table 2.

3.4 Trypsination reaction time

The two trypsination protocols, 1 h incubation and overnight incubation, were well separated according to the PCA plot (Fig. 3A). The first component explained 99% of the total variance and the second one 1%. In total, 545 proteins were identified. For the multivariate analysis, a cut-off of ≤3 was chosen; 159 proteins fulfilled this criterion and 81 occurred in both groups (Fig. 3B). The total numbers of PSMs were 237 and 414 in the 1 h incubation and the overnight incubation groups, respectively. See also Supporting Information Table 3.

© 2014 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

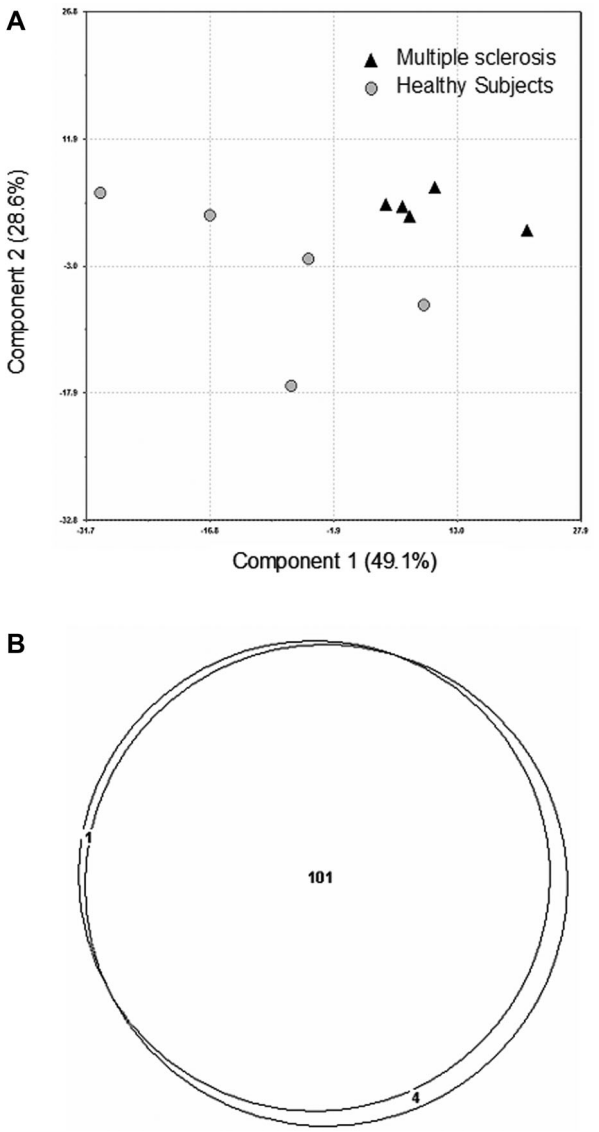

Figure 1. Discrimination experiment. (A) PCA plot of the samples based on PSM identities from CSF from multiple sclerosis patients versus controls. First component explains 49% of the variation, while the second component explains 29%. (B) Venn diagram based on number of identified proteins. Multiple sclerosis versus healthy subjects; 101 of the identified proteins are common for the two groups, four proteins are unique for the healthy group and one protein is unique for the multiple sclerosis group.

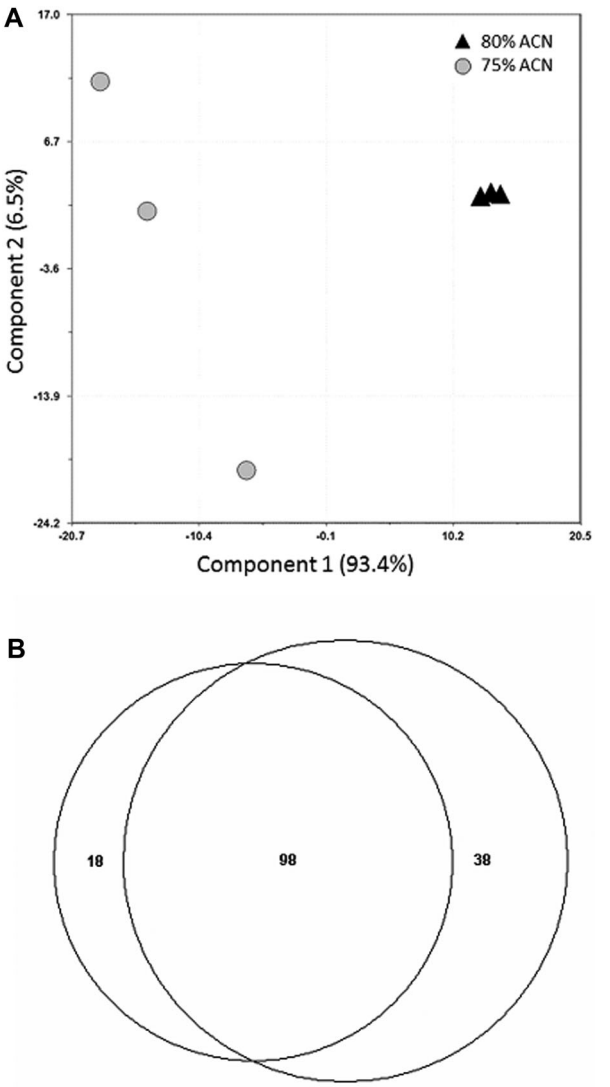

Figure 2. Reproducibility. (A) PCA plot of the samples based on PSM. The 75 and 80% groups are well separated. First component explains 93% of the variation, while the second component explains 7%. (B) Venn diagram based on number of identified proteins. Seventy percent ACN versus 80% ACN; 98 of the identified proteins are common for the two groups, 38 proteins are unique for the 75% group and 18 proteins are unique for the 80% group.

4 Discussion

We found that our sample preparation method can distinguish between healthy subjects and patients with multiple sclerosis. Although the present study examined few patients, the results are encouraging. Using ACN as depletion medium followed by direct addition of trypsin decreases the required preparation steps and is less time consuming compared to other methods, as discussed by Kay et al. [4] and Strader et al. [18]. ACN depletion therefore offers an alternative to immunoaffinity, a commonly used technique for removing HAPs from serum. Moreover, it has been demonstrated that this method was optimal for the detection of low-abundance proteins (i.e. insuline-like growth factor-I) at physiological level in serum [4, 18].

In MS-based proteomics, the statistical significance of a peptide-spectrum or PSM is an important indicator of the correctness of the peptide or protein identification [24]. We used PSMs for semiquantitative comparison of our sample groups. Based on PSM validation, our method rapidly and

© 2014 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

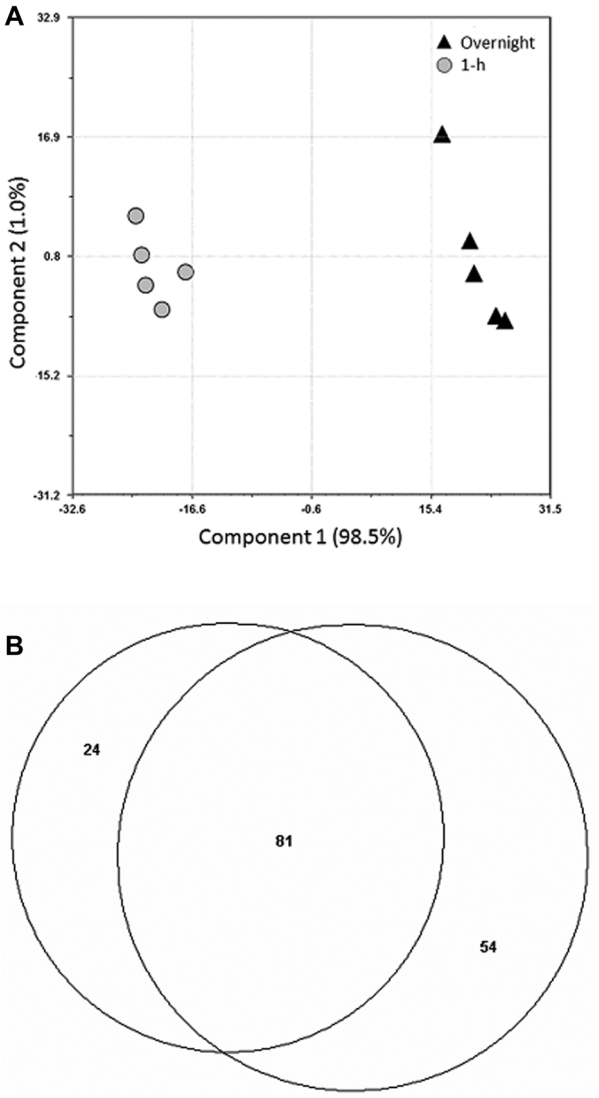

Figure 3. Trypsination reaction time experiment. (A) PCA plot of the samples based on PSM identities from CSF from healthy subjects, 1 h versus overnight trypsination. First component explains 99% of the variation, while the second component explains 1%. (B) Venn diagram based on number of identified proteins. One-hour trypsination versus overnight trypsination (16 h); 81 of the identified proteins are common for the two groups, 24 proteins are unique for the 1 h group, and 54 proteins are unique for the overnight group.

efficiently decreased HAP levels, thereby separating the CSF proteome into subproteomes and allowed us to study low-abundance proteins, previously described as a necessary practice [3, 4, 15]. We further confirmed the previous report by Kay et al. [4], finding that the use of ACN was an effective precipitation strategy for HAP depletion.

Due to its specificity (cutting after arginine and lysine residues), trypsin is the most commonly used enzyme for proteolytic cleavage [25]. Here, we compared two trypsination approaches, finding that the 1 h digestion was both more efficient and more reproducible than the overnight one. It has been previously demonstrated that the addition of an organic solvent, such as 80% ACN, leads to the most complete digestion, producing more peptide identifications than other solvents [5, 18]. ACN assist in digestion by unfolding and solubilizing the proteins [26]. Although overnight digestion resulted in a higher total amount of detected peptides, the reproducibility was better with the presence of an organic solvent. Moreover, short trypsination time is important for reduction of semitryptic peptides [18].

In neuroscience, there is a constant need for novel biomarkers that can be used to improve the diagnosis, predict the disease progression, improve understanding of the pathology, or serve as therapeutic targets for neurodegenerative and autoimmune diseases.

Overall, here we have further developed a method for HAP removal and rapid trypsination based on previous approaches [4, 14, 15]. Our results indicate that this method is well-suited for use in other CSF proteome studies. Compared to currently used methods, the newly described method is less time consuming and is relatively inexpensive.

E.L. was supported by a PhD grant from The Western Norway Regional Health Authority (WNRHA, "Helse Vest"), grant number 911613.

The authors have declared no conflict of interest.

5 References

[1] Stangel, M., Fredrikson, S., Meinl, E., Petzold, A. et al., The utility of cerebrospinal fluid analysis in patients with multiple sclerosis. *Nat. Rev. Neurol.* 2013, *9*, 267–276.

[2] Romeo, M. J., Espina, V., Lowenthal, M., Espina, B. H. et al., CSF proteome: a protein repository for potential biomarker identification. *Expert Rev. Proteomics* 2005, *2*, 57–70.

[3] Kroksveen, A. C., Guldbrandsen, A., Vedeler, C., Myhr, K. M. et al., Cerebrospinal fluid proteome comparison between multiple sclerosis patients and controls. *Acta Neurol. Scand. Suppl.* 2012, 90–96.

[4] Kay, R., Barton, C., Ratcliffe, L., Matharoo-Ball, B. et al., Enrichment of low molecular weight serum proteins using acetonitrile precipitation for mass spectrometry based proteomic analysis. *Rapid Commun. Mass Spectrom.* 2008, *22*, 3255–3260.

[5] Hervey, W. J., Strader, M. B., Hurst, G. B., Comparison of digestion protocols for microgram quantities of enriched protein samples. *J. Proteome Res.* 2007, *6*, 3054–3061.

[6] Kroksveen, A. C., Opsahl, J. A., Aye, T. T., Ulvik, R. J., Berven, F. S., Proteomics of human cerebrospinal fluid: discovery and verification of biomarker candidates in neurodegenerative diseases using quantitative proteomics. *J. Proteomics* 2011, *74*, 371–388.

[7] Schmidt, O., Schulenborg, T., Meyer, H. E., Marcus, K., Hamacher, M., How proteomics reveals potential biomarkers in brain diseases. *Expert Rev. Proteomics* 2005, *2*, 901–913.

© 2014 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

[8] Stephane, M., Kuskowski, M., McClannahan, K., Surerus, C., Nelson, K., Evaluation of speech misattribution bias in schizophrenia. *Psychol. Med.* 2010, *40*, 741–748.

[9] Azadi, G., Gustafson, E., Wessel, G. M., Tripathi, A., Rapid detection and quantification of specific proteins by immunodepletion and microfluidic separation. *Biotechnol. J.* 2012, *7*, 1008–1013.

[10] Shi, Y., Xiang, R., Horvath, C., Wilkins, J. A., The role of liquid chromatography in proteomics. *J. Chromatogr.* 2004, *1053*, 27–36.

[11] Kamihira, M., Iijima, S., Kobayashi, T., Stabilities of antigen and antibody under elution conditions in immunoaffinity chromatography using monoclonal antibody. *Bioseparation* 1992, *3*, 185–188.

[12] Barton, C., Kay, R. G., Gentzer, W., Vitzthum, F., Pleasance, S., Development of high-throughput chemical extraction techniques and quantitative HPLC-MS/MS (SRM) assays for clinically relevant plasma proteins. *J. Proteome Res.* 2010, *9*, 333–340.

[13] Chertov, O., Simpson, J. T., Biragyn, A., Conrads, T. P. et al., Enrichment of low-molecular-weight proteins from biofluids for biomarker discovery. *Expert Rev. Proteomics* 2005, *2*, 139–145.

[14] Fernandez, C., Santos, H. M., Ruiz-Romero, C., Blanco, F. J., Capelo-Martinez, J. L., A comparison of depletion versus equalization for reducing high-abundance proteins in human serum. *Electrophoresis* 2011, *32*, 2966–2974.

[15] Warder, S. E., Tucker, L. A., Strelitzer, T. J., McKeegan, E. M. et al., Reducing agent-mediated precipitation of high-abundance plasma proteins. *Anal. Biochem.* 2009, *387*, 184–193.

[16] Abdi, F., Quinn, J. F., Jankovic, J., McIntosh, M. et al., Detection of biomarkers with a multiplex quantitative proteomic platform in cerebrospinal fluid of patients with neurodegenerative disorders. *J. Alzheimers Dis.* 2006, *9*, 293–348.

[17] Zhang, J., Goodlett, D. R., Peskind, E. R., Quinn, J. F. et al., Quantitative proteomic analysis of age-related changes in human cerebrospinal fluid. *Neurobiol. Aging* 2005, *26*, 207–227.

[18] Strader, M. B., Tabb, D. L., Hervey, W. J., Pan, C., Hurst, G. B., Efficient and specific trypsin digestion of microgram to nanogram quantities of proteins in organic-aqueous solvent systems. *Anal. Chem.* 2006, *78*, 125–134.

[19] Polman, C. H., Reingold, S. C., Banwell, B., Clanet, M. et al., Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. *Ann. Neurol.* 2011, *69*, 292–302.

[20] Bjartmar, C., Trapp, B. D., Axonal and neuronal degeneration in multiple sclerosis: mechanisms and functional consequences. *Curr. Opin. Neurol.* 2001, *14*, 271–278.

[21] Berven, F. S., Kroksveen, A. C., Berle, M., Rajalahti, T. et al., Pre-analytical influence on the low molecular weight cerebrospinal fluid proteome. *Proteomics Clin. Appl.* 2007, *1*, 699–711.

[22] Borg, J., Campos, A., Diema, C., Omenaca, N. et al., Spectral counting assessment of protein dynamic range in cerebrospinal fluid following depletion with plasma-designed immunoaffinity columns. *Clin. Proteomics* 2011, *8*, 6. Available at http://www.ncbi.nlm.nih.gov/pubmed/21906361

[23] Rajalahti, T., Arneberg, R., Kroksveen, A. C., Berle, M. et al., Discriminating variable test and selectivity ratio plot: quantitative tools for interpretation and variable (biomarker) selection in complex spectral or chromatographic profiles. *Anal. Chem.* 2009, *81*, 2581–2590.

[24] Liu, X., Segar, M. W., Li, S. C., Kim, S., Spectral probabilities of top-down tandem mass spectra. *BMC Genomics* 2014, *15*(Suppl. 1), S9.

[25] Schutzer, S. E., Liu, T., Natelson, B. H., Angel, T. E. et al., Establishing the proteome of normal human cerebrospinal fluid. *PloS One* 2010, *5*, e10980.

[26] Griebenow, K., Klibanov, A. M., Lyophilization-induced reversible changes in the secondary structure of proteins. *Proc. Natl. Acad. Sci. U S A* 1995, *92*, 10969–10976.

© 2014 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim