Anal. Chem. **2010,** *82,* 848–858

Isobaric Tagging-Based Selection and Quantitation of Cerebrospinal Fluid Tryptic Peptides with Reporter Calibration Curves

Loïc Dayon,[†] Natacha Turck,[†] Stefan Kienle,[‡] Peter Schulz-Knappe,[‡] Denis F. Hochstrasser,[†,§] Alexander Scherl,[†] and Jean-Charles Sanchez*[,†]

Biomedical Proteomics Group, Department of Structural Biology and Bioinformatics, Faculty of Medicine, University of Geneva, Geneva, Switzerland, Proteome Sciences R&D GmbH & Co. KG, Altenhöferallee 3, 60438 Frankfurt am Main, Germany, and Clinical Proteomics Group, Genetics and Laboratory Medicine Department, Geneva University Hospital, Geneva, Switzerland

In the past few years, mass spectrometry (MS) has emerged as an efficient tool for the multiplexed peptide and protein concentration determination by isotope dilution. Despite the growing use of isobaric tagging to perform relative quantitation for the discovery of potential biomarkers in biological fluids, no real application has so far been presented for their absolute quantitation. Isobaric tandem mass tags (TMTs) were used herein for the selection and quantitation of tryptic peptides derived from brain damage related proteins in cerebrospinal fluid (CSF). Proteotypic tryptic peptide analogues were synthesized, prepared in four reference amounts, differentially labeled with four isobaric TMTs with reporter-ions at m/z = 128.1, 129.1, 130.1, and 131.1, and mixed with CSF sample previously labeled with TMT 126.1. Off-gel electrophoresis (OGE) was used as first-dimension separation of the pooled sample. The resulting fractions were analyzed with reversed-phase liquid chromatography (RP-LC) tandem mass spectrometry (MS/MS), using tandem time-of-flight (TOF/TOF) and hybrid linear ion trap−orbitrap (LTQ−OT) instruments. Under collision-induced dissociation (CID) or higher-energy C-trap dissociation (HCD), the release of the reporter fragments from the TMT-labeled peptide standards provided an internal calibration curve to assess the concentration of these peptides in the CSF. This tool also allowed identifying selectively these peptides in CSF as only the targeted peptides showed specific fragmentation pattern in the TMT reporter-ion zone of the tandem mass spectra. Assays for the concentration measurements of peptides from PARK7, GSTP1, NDKA, and S100B proteins in CSF were further characterized using this novel, efficient, and straightforward approach.

The proteomic toolbox offers a set of alternatives for the discovery of protein markers with mass spectrometry (MS) and tandem MS (MS/MS).[1] During the past few years, isobaric tagging, with the introduction of tandem mass tags (TMTs)[2,3] and isobaric tags for relative and absolute quantification (iTRAQ),[4] has played an increasing role for the comparison of biological samples using shotgun proteomic methods. Quantitative comparisons of biological fluids were carried out using these techniques to evidence increased or decreased protein levels in pathologies such as cancer[5,6] and brain damage.[2,7,8] Many biomedical projects pointed out lists of proteins, which might be used for the diagnosis and the prognosis of these pathologies. Nevertheless, subsequent verification and validation of the discovered potential biomarkers are rather long and laborious, principally relying on antibody-based assays.[9] The development of these assays requires the production of recombinant proteins and antibodies, which are sensitive and specific for the protein markers, and can encounter many obstacles such as high background, poor specific signal-to-noise ratio, and lack of reproducibility. Hence, the costly and time-consuming immunoassay development excludes the systematic verification of all potential markers highlighted in the discovery phase. To circumvent this limitation and help the rationalization of biomarker discovery, MS has recently emerged as a practical option for the absolute and multiplexed measurements of these markers in various biological fluids such as plasma, serum, and cerebrospinal fluid (CSF). Indeed, MS platforms are sufficiently versatile to

* To whom correspondence should be addressed. E-mail: Jean-Charles.Sanchez@unige.ch.
[†] University of Geneva.
[‡] Proteome Sciences R&D GmbH & Co. KG.
[§] Geneva University Hospital.

(1) Ong, S. E.; Mann, M. *Nat. Chem. Biol.* **2005**, *1*, 252–262.
(2) Dayon, L.; Hainard, A.; Licker, V.; Turck, N.; Kuhn, K.; Hochstrasser, D. F.; Burkhard, P. R.; Sanchez, J. C. *Anal. Chem.* **2008**, *80*, 2921–2931.
(3) Thompson, A.; Schafer, J.; Kuhn, K.; Kienle, S.; Schwarz, J.; Schmidt, G.; Neumann, T.; Hamon, C. *Anal. Chem.* **2003**, *75*, 1895–1904.
(4) Ross, P. L.; Huang, Y. L. N.; Marchese, J. N.; Williamson, B.; Parker, K.; Hattan, S.; Khainovski, N.; Pillai, S.; Dey, S.; Daniels, S.; Purkayastha, S.; Juhasz, P.; Martin, S.; Bartlet-Jones, M.; He, F.; Jacobson, A.; Pappin, D. J. *Mol. Cell. Proteomics* **2004**, *3*, 1154–1169.
(5) Chaerkady, R.; Harsha, H. C.; Nalli, A.; Gucek, M.; Vivekanandan, P.; Akhtar, J.; Cole, R. N.; Simmers, J.; Schulick, R. D.; Singh, S.; Torbenson, M.; Pandey, A.; Thuluvath, P. J. *J. Proteome Res.* **2008**, *7*, 4289–4298.
(6) DeSouza, L.; Diehl, G.; Rodrigues, M. J.; Guo, J. Z.; Romaschin, A. D.; Colgan, T. J.; Siu, K. W. M. *J. Proteome Res.* **2005**, *4*, 377–386.
(7) Abdi, F.; Quinn, J. F.; Jankovic, J.; McIntosh, M.; Leverenz, J. B.; Peskind, E.; Nixon, R.; Nutt, J.; Chung, K.; Zabetian, C.; Samii, A.; Lin, M.; Hattan, S.; Pan, C.; Wang, Y.; Jin, J.; Zhu, D.; Li, G. J.; Liu, Y.; Waichunas, D.; Montine, T. J.; Zhang, J. *J. Alzheimer's Dis.* **2006**, *9*, 293–348.
(8) Choe, L.; D'Ascenzo, M.; Relkin, N. R.; Pappin, D.; Ross, P.; Williamson, B.; Guertin, S.; Pribil, P.; Lee, K. H. *Proteomics* **2007**, *7*, 3651–3660.
(9) Rifai, N.; Gillette, M. A.; Carr, S. A. *Nat. Biotechnol.* **2006**, *24*, 971–983.

10.1021/ac901854k
Published on Web 01/08/2010

develop various MS-based assays and offer enough throughputs for the concomitant verification of several biomarkers in a relevant number of samples. MS-based assays are therefore expected to provide attractive solution to the protein marker selection bottleneck. They are further projected to be reliable for validation and routine clinical use.[10]

Peptide and protein absolute quantitation with MS relies mainly on isotope dilution (ID).[11] ID-MS has been widely employed for small molecules.[12] It consists of the spiking of a known amount of isotopically stable heavy analogues into the sample and comparing of their MS signals (in term of intensity) with those of the unknowns. Standards and analytes appear as doublets in the mass spectra, and because of their identical physicochemical properties such as retention time (t_R) and ionization efficiency, their peak intensities/areas or ideally their elution profiles are directly compared to provide quantitation of the unknowns. The gold standard for such measurements is carried out on triple quadrupole (QQQ) mass spectrometers and is termed selected reaction monitoring (SRM).[13] Basically, a precursor ion of interest is selected according to its m/z ratio in the first quadrupole, activated by collision-induced dissociation (CID) in the second quadrupole, and a specific m/z fragment is recorded in the third quadrupole. This so-called transition is monitored over time to quantify the molecule of interest. Several approaches have been reported in the proteomic field, namely, absolute quantification (AQUA),[14] quantification with concatemers (QconCAT),[15,16] and protein standard absolute quantification (PSAQ).[17,18] These approaches do not differ with regards to the MS measurement principle but according to the standard materials. AQUA uses heavy synthetic peptides which are spiked into the samples. QconCAT employs artificial stable isotope proteins that are concatemers of proteolytic peptides of the group of proteins under study. PSAQ is based on the use of isotope-labeled protein standards. These three approaches offer therefore different possibilities. Whereas the former two are suitable for shotgun proteomics, the PSAQ enables protein sample prefractionation and is not influenced by the proteolytic digestion extent. Chemical tagging approaches have also been proposed to induce a mass difference between the probed peptides and their synthetic analogues. The mTRAQ[19] (a nonisobaric variant of the iTRAQ available in two versions) technique was reported for quantitation of potential endometrial cancer markers.[20]

Isobaric tagging is not commonly used for absolute concentration determination of peptides or proteins because of the lack of specificity of the reporter-ion signals. However, with sufficient fractionation to avoid cofragmentation of peptides with those of interest, absolute quantitation can be obtained.[21] Advantageously, concomitant spiking of synthetic peptides labeled with a different version of the tag provides internal calibration curves as shown herein. Moreover, the technique is feasible on different MS platforms used for biomarker discovery. Six-plex isobaric TMTs were used in this study for the selection and quantitation of tryptic peptides representative of eight CSF proteins. These proteins (i.e., protein S100-B (S100B),[22−26] glial fibrillary acidic protein (GFAP),[27,28] fatty acid-binding protein, heart (FAPBH),[29,30] prostaglandin-H2 D-isomerase (PTGDS),[31,32] protein DJ-1 (PARK7),[33,34] nucleoside diphosphate kinase A (NDKA),[33,35] ubiquitin fusion degradation protein 1 (UFD1),[36] and glutathione S-transferase P (GSTP1)[37]) were previously reported to be related to brain damage disorders. After tryptic digestion, postmortem CSF sample and four samples of synthetic peptides (in four known quantities) were differentially modified with TMTs. After sample mixing, off-gel electrophoresis (OGE)[38−40] and reversed-phase liquid chromatography (RP-LC) were used as orthogonal separation methods prior to MS analysis. MS/MS was performed using matrix-assisted laser desorption ionization (MALDI) tandem time-of-flight (TOF/TOF) and electrospray ionization (ESI) hybrid linear ion trap−orbitrap (LTQ−OT) instruments. As the synthetic peptide standards were

(10) McIntosh, M.; Fitzgibbon, M. *Nat. Biotechnol.* **2009**, *27*, 622–623.
(11) Kusmierz, J. J.; Sumrada, R.; Desiderio, D. M. *Anal. Chem.* **1990**, *62*, 2395–2400.
(12) Deleenheer, A. P.; Thienpont, L. M. *Mass Spectrom. Rev.* **1992**, *11*, 249–307.
(13) Lange, V.; Picotti, P.; Domon, B.; Aebersold, R. *Mol. Syst. Biol.* **2008**, *4*, 222.
(14) Gerber, S. A.; Rush, J.; Stemman, O.; Kirschner, M. W.; Gygi, S. P. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 6940–6945.
(15) Beynon, R. J.; Doherty, M. K.; Pratt, J. M.; Gaskell, S. J. *Nat. Methods* **2005**, *2*, 587–589.
(16) Pratt, J. M.; Simpson, D. M.; Doherty, M. K.; Rivers, J.; Gaskell, S. J.; Beynon, R. J. *Nat. Protoc.* **2006**, *1*, 1029–1043.
(17) Brun, V.; Dupuis, A.; Adrait, A.; Marcellin, M.; Thomas, D.; Court, M.; Vandenesch, F.; Garin, J. *Mol. Cell. Proteomics* **2007**, *6*, 2139–2149.
(18) Dupuis, A.; Hennekinne, J. A.; Garin, J.; Brun, V. *Proteomics* **2008**, *8*, 4633–4636.
(19) DeSouza, L. V.; Taylor, A. M.; Li, W.; Minkoff, M. S.; Romaschin, A. D.; Colgan, T. J.; Siu, K. W. M. *J. Proteome Res.* **2008**, *7*, 3525–3534.
(20) DeSouza, L. V.; Romaschin, A. D.; Colgan, T. J.; Siu, K. W. M. *Anal. Chem.* **2009**, *81*, 3462–3470.
(21) Quaglia, M.; Pritchard, C.; Hall, Z.; O'Connor, G. *Anal. Biochem.* **2008**, *379*, 164–169.
(22) Buttner, T.; Weyers, S.; Postert, T.; Sprengelmeyer, R.; Kuhn, W. *Stroke* **1997**, *28*, 1961–1965.
(23) Missler, U.; Wiesmann, M.; Friedrich, C.; Kaps, M. *Stroke* **1997**, *28*, 1956–1960.
(24) Romner, B.; Ingebrigtsen, T.; Kongstad, F.; Borgesen, S. E. *J. Neurotrauma* **2000**, *17*, 641–647.
(25) Rothermundt, M.; Peters, M.; Prehn, J. H. M.; Arolt, V. *Microsc. Res. Tech.* **2003**, *60*, 614–632.
(26) Wiesmann, M.; Missler, U.; Hagenstrom, H.; Gottmann, D. *Acta Neurochir. (Wien)* **1997**, *139*, 1155–1160.
(27) Herrmann, M.; Vos, P.; Wunderlich, M. T.; de Bruijn, C.; Lamers, K. J. B. *Stroke* **2000**, *31*, 2670–2677.
(28) Rosengren, L. E.; Wikkelso, C.; Hagberg, L. *J. Neurosci. Methods* **1994**, *51*, 197–204.
(29) Wunderlich, M. T.; Hanhoff, T.; Goertler, M.; Spener, F.; Glatz, J. F. C.; Wallesch, C. W.; Pelsers, M. *J. Neurol.* **2005**, *252*, 718–724.
(30) Zimmermann-Ivol, C. G.; Burkhard, P. R.; Le Floch-Rohr, J.; Allard, L.; Hochstrasser, D. F.; Sanchez, J. C. *Mol. Cell. Proteomics* **2004**, *3*, 66–72.
(31) Harrington, M. G.; Fonteh, A. N.; Biringer, R. G.; Huhmer, A. F. R. *Dis. Markers* **2006**, *22*, 73–81.
(32) Lescuyer, P.; Gandini, A.; Burkhard, P. R.; Hochstrasser, D. F.; Sanchez, J. C. *Electrophoresis* **2005**, *26*, 4563–4570.
(33) Allard, L.; Burkhard, P. R.; Lescuyer, P.; Burgess, J. A.; Walter, N.; Hochstrasser, D. F.; Sanchez, J. C. *Clin. Chem.* **2005**, *51*, 2043–2051.
(34) Waragai, M.; Wei, J. S.; Fujita, M.; Nakai, M.; Ho, G. J.; Masliah, E.; Akatsu, H.; Yamada, T.; Hashimoto, M. *Biochem. Biophys. Res. Commun.* **2006**, *345*, 967–972.
(35) Kim, S. H.; Fountoulakis, M.; Cairns, N. J.; Lubec, G. *Biochem. Biophys. Res. Commun.* **2002**, *296*, 970–975.
(36) Allard, L.; Turck, N.; Burkhard, P. R.; Walter, N.; Rosell, A.; Gex-Fabry, M.; Hochstrasser, D. F.; Montaner, J.; Sanchez, J. C. *Biomarker Insights* **2007**, *7*, 155–163.
(37) Turck, N.; Burkhard, P. R.; Robin, X.; Rosell, A.; Dayon, L.; Walter, N.; Fouda, C.; Wagner, G.; Gex-Fabry, M.; Hochstrasser, D. F.; Mueller, M.; Montaner, J.; Sanchez, J. C., **2010**, submitted for publication.
(38) Horth, P.; Miller, C. A.; Preckel, T.; Wenz, C. *Mol. Cell. Proteomics* **2006**, *5*, 1968–1974.
(39) Michel, P. E.; Reymond, F.; Arnaud, I. L.; Josserand, J.; Girault, H. H.; Rossier, J. S. *Electrophoresis* **2003**, *24*, 3–11.
(40) Ros, A.; Faupel, M.; Mees, H.; van Oostrum, J.; Ferrigno, R.; Reymond, F.; Michel, P.; Rossier, J. S.; Girault, H. H. *Proteomics* **2002**, *2*, 151–156.

initially prepared at four known concentrations, their characteristic TMT reporter-ions allowed building a four-point internal calibration curve to retrieve the concentrations of the unknowns in CSF. This work demonstrates the applicability of this new method for efficient and straightforward early assessment of peptide markers, as illustrated by the proof-of-principle assays for the absolute quantitation of peptides of PARK7, GSTP1, NDKA, and S100B in CSF with the standard addition method.

EXPERIMENTAL SECTION

Materials. β-Lactoglobulin (LACB) from bovine milk (~90%), iodoacetamide (IAA, ≥ 99%), tris(2-carboxyethyl) phosphine hydrochloride (TCEP) 0.5 M, and α-cyano-4-hydroxycinnamic acid were purchased from Sigma (St. Louis, MO). Triethylammonium hydrogen carbonate buffer (TEAB) 1 M pH = 8.5, sodium dodecyl sulfate (SDS, ≥ 98%), and trifluoroacetic acid (TFA, ≥ 99.5%) were from Fluka (Büchs, Switzerland). Formic acid (FA, 99%) was from Biosolve (Valkenswaard, The Netherlands). Hydroxylamine solution 50 wt % in H_2O (99.999%) was from Aldrich (Milwaukee, WI). Hydrochloric acid (25%) and ammonium dihydrogen phosphate ($(NH_4)H_2PO_4$) were from Merck (Darmstadt, Germany). Water for chromatography LiChrosolv and acetonitrile Chromasolv for HPLC (≥99.9%) were, respectively, from Merck and Sigma-Aldrich (Büchs, Switzerland). The six-plex tandem mass tags (TMT) were provided by Proteome Sciences (Frankfurt am Main, Germany) and can be purchased from Thermo Scientific (Rockford, IL). Sequencing grade modified trypsin was from Promega (Madison, WI). Synthetic peptides were synthesized on solid phase using the Fmoc/*t*-Bu strategy and purified by preparative RP-HPLC.[41] Oasis HLB 1 cc (30 mg) extraction cartridges were from Waters (Milford, MA). Immobiline DryStryp pH 3−10, 24 cm and IPG buffer pH 3−10 were from GE Healthcare (Uppsala, Sweden). Glycerol 50% and mineral oil were from Agilent Technologies (Wilmington, DE).

CSF Samples. Postmortem CSF samples were collected by ventricular puncture at autopsy, 6 h after death on average. Clinical data of deceased patients were previously reported.[42] Each patient or patient's relatives gave informed consent prior to enrolment. The local institutional ethical committee board approved the clinical protocol. Two pooled samples ($n = 5$) were prepared and filtered with 0.20 μm filter (Vivascience, Hannover, Germany). The first pool was used for the prospective experiments (two aliquots named sample 1 and sample 2 were made), and the second one for the standard addition experiments. An amount of 155 μL of CSF (corresponding to an average of 50 μg of proteins) was used for each TMT analysis. Pooled postmortem CSF protein concentration after filtration was determined with Bradford assay[43] (Bio-Rad, Hercules, CA). Each sample was spiked with 1 μg of LACB and dried under vacuum.

Standard Peptide Preparation. Forty-one synthetic peptides representative of eight CSF proteins were used. They were diluted in water and stored at −20 °C. Mixtures of the 41 peptides were prepared in four standard amounts to give four reference ratios at 0.25, 0.5, 2, and 4 (equivalent concentrations C_1, C_2, C_3, and C_4) with respect to the expected protein amounts in postmortem CSF. The expected protein concentrations in postmortem CSF were taken from the literature[2,44] and preliminary experimental tests using the workflow reported herein (data not shown). These concentrations were 3.14×10^{-9} M^{-1} (~0.034 μg·mL^{-1}) for S100B, 3.44×10^{-9} M^{-1} (~0.17 μg·mL^{-1}) for GFAP, 7.83×10^{-9} M^{-1} (~0.12 μg·mL^{-1}) for FABPH, 7.17×10^{-7} M^{-1} (~15 μg·mL^{-1}) for PTGDS, 5.84×10^{-8} M^{-1} (~1.2 μg·mL^{-1}) for PARK7, 4.66×10^{-10} M^{-1} (~0.008 μg·mL^{-1}) for NDKA, 5.34×10^{-9} M^{-1} (~0.18 μg·mL^{-1}) for UFD1, and 8.59×10^{-8} M^{-1} (~2 μg·mL^{-1}) for GSTP1. The quantities of peptide standards to prepare were calculated with respect to 155 μL of CSF. Each peptide mixture was spiked with 1 μg of LACB and dried under vacuum.

Reduction, Alkylation, Digestion, and Labeling with TMT. The samples (the pooled postmortem CSF sample, and the four peptide standard mixtures) were dissolved in 100 μL of TEAB 100 mM adjusted to pH = 8 with diluted HCl. An amount of 1 μL of SDS 1% and 2 μL TCEP 50 mM was added to each tube. The reduction was carried out at 60 °C for 1 h. Alkylation was performed by adding 1 μL of IAA 400 mM during 30 min in the dark. An amount of 10 μL of trypsin 0.2 μg·μL^{-1} freshly prepared in the TEAB solution was added, and the digestion was proceeded overnight at 37 °C.

TMT labeling was achieved for 1 h, after addition of 40.3 μL of six-plex TMT reagent in CH_3CN (1.66 mg, 4.84×10^{-6} mol). CSF was labeled with TMT with reporter-ion at $m/z = 126.1$ and standard peptide mixtures at expected ratios 0.25, 0.5, 2, and 4 with respect to CSF with TMT with reporter-ions at $m/z = 128.1$, 129.1, 130.1, and 131.1, respectively. An amount of 8 μL of hydroxylamine 5% was added for 15 min reaction. The differentially TMT-labeled samples were pooled in a new tube and dried.

OGE. The sample was desalted with Oasis HLB 1 cc (30 mg) extraction cartridges. After drying, the sample was dissolved in 3232.8 μL H_2O with 345.6 μL glycerol 50% and 21.6 μL of carrier ampholytes IPG buffer pH 3−10. The IPG strips (pH 3−10, 24 cm) were assembled on the OGE trays and rehydrated for 30 min with a solution of 89.8% H_2O, 9.6% glycerol 50%, and 0.6% of carrier ampholytes. The samples were loaded on the 24 off-gel wells. The separation was carried out overnight using the 3100 OFFGEL fractionator (Agilent Technologies) with a limiting current of 50 μA and a limit of 50 kV·h before holding the voltage to 500 V. The fractions were collected, and their pH was measured (744 pH Meter and Biotrode from Metrohm (Herisau, Switzerland)). The fractions were dried, cleaned with C18 ultramicrospin column (Harvard Apparatus, Holliston, MA), and dried again. One-third of each fraction was used per LC MS/MS analysis.

LC MS/MS. MALDI TOF/TOF MS was performed on a 4800 proteomics analyzer from Applied Biosystems (Foster City, CA). The off-gel fractions were first separated with RP-LC using an Alliance system from Waters equipped with a flow splitter. A home-packed 5 μm 200 Å Magic C18 AQ (Michrom, Auburn, CA) 0.1 mm × 100 mm column was used. The separation was run for 60 min using a gradient of H_2O/CH_3CN/TFA 97%/2.9%/0.1%

(41) Fields, G. B.; Noble, R. L. *Int. J. Pept. Protein Res.* **1990**, *35*, 161–214.
(42) Lescuyer, P.; Allard, L.; Zimmermann-Ivol, C. G.; Burgess, J. A.; Hughes-Frutiger, S.; Burkhard, P. R.; Sanchez, J. C.; Hochstrasser, D. F. *Proteomics* **2004**, *4*, 2234–2241.
(43) Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248–254.
(44) Burgess, J. A.; Lescuyer, P.; Hainard, A.; Burkhard, P. R.; Turck, N.; Michel, P.; Rossier, J. S.; Reymond, F.; Hochstrasser, D. F.; Sanchez, J. C. *J. Proteome Res.* **2006**, *5*, 1674–1681.

(solvent A) and H_2O/CH_3CN/TFA 5%/94.9%/0.1% (solvent B). The gradient was run as follows: 0−10 min 98% A and 2% B, then to 90% A and 10% B at 12 min, 50% A and 50% B at 55 min, and 2% A and 98% B at 60 min at a flow rate estimated to 400 $nL \cdot min^{-1}$. One minute fractions were deposited onto MALDI plates using a homemade LC-robot. The matrix (α-cyano-4-hydroxycinnamic acid in H_2O/CH_3CN/TFA 50%/50%/0.1% with 10 mM $NH_4H_2PO_4$) was then spotted onto the plates. All mass spectra were acquired in positive ionization mode with an m/z scan range of 800−4000 (1000 shoots with laser intensity of 4000 au). After selection of 20 precursors at the maximum, MS/MS experiments (1500 shoots with laser intensity of 4500 au) were done from the less to the most intense precursors at medium collision energy.

ESI LTQ−OT MS was performed on a LTQ Orbitrap XL from Thermo Electron (San Jose, CA) equipped with a NanoAcquity system from Waters. Peptides were trapped on a homemade 5 μm 200 Å Magic C18 AQ (Michrom) 0.1 mm × 20 mm precolumn and separated on a homemade 5 μm 100 Å Magic C18 AQ (Michrom) 0.75 mm × 150 mm column with a gravity-pulled emitter. The analytical separation was run for 65 min using a gradient of H_2O/FA 99.9%/0.1% (solvent C) and CH_3CN/FA 99.9%/0.1% (solvent D). The gradient was run as follows: 0−1 min 95% C and 5% D, then to 65% C and 35% D at 55 min, and 20% C and 80% D at 65 min at a flow rate of 220 $nL \cdot min^{-1}$. For MS survey scans, the OT resolution was set to 60 000 and the ion population was 5×10^5 with an m/z window from 400 to 2000. A maximum of three precursors was selected for both CID in the LTQ and higher-energy C-trap dissociation (HCD) with analysis in the OT. For MS/MS in the LTQ, the ion population was 1×10^4 (isolation width of 2 m/z), whereas for MS/MS detection in the OT, it was 2×10^5 (isolation width of 4 m/z), with resolution of 7500, first mass at $m/z = 100$, and maximum injection time of 750 ms. The normalized collision energies were 35% for CID and 50% for HCD.[45] Dynamic time exclusion was 60 and 4 s, respectively, for data-dependent and inclusion list experiments.

Protein Identification. Peak lists were generated from TOF/TOF and LTQ−OT raw data using, respectively, the 4000 series Explorer software from Applied Biosystems and an in-house-written Perl script. CID and HCD data from LTQ−OT were merged using a custom-made program.[45] The mgf files, combined from the off-gel fractions, were searched against UniProt-Swiss-Prot database (56.6 of Dec 16, 2008) using Phenyx (GeneBio, Geneva, Switzerland). *Homo sapiens* taxonomy was specified for database search. Variable amino acid modifications were oxidized methionine. TMT-labeled peptide amino terminus and TMT-labeled lysine (+229.1629 Da) were set as fixed modifications as well as carbamidomethylation of cysteines (to evaluate the TMT labeling efficiency, TMT-labeled peptide amino terminus was set as variable in an independent search job; sample 1 and sample 2 in the prospective experiments exhibited 97% of completely TMT-labeled peptides). Trypsin was selected as the enzyme, with one potential missed cleavage, and the normal cleavage mode was used. Only one search round was used with selection of "turbo" scoring. The peptide p value was 1×10^{-6} for searching TOF/TOF data and 1×10^{-3} for LTQ−OT data. For TOF/TOF data, the protein and peptide scores were 7.5 and 7.0, respectively, for sample 1 and sample 2. The false peptide discovery rates were, respectively, 0.42% and 0.63%. The parent ion tolerance was 1.1 Da. The protein and peptide scores were 5.1 and 4.9 for sample 1 and sample 2 analyzed with LTQ−OT. The false peptide discovery rate was 0.63% and 0.79%, respectively. The parent ion tolerance was 5 ppm. For all analyses, only proteins matching two different peptide sequences were selected.

Protein Quantification. TOF/TOF areas of the TMT reporter-ions were extracted from the tandem mass spectra using the analysis tool of the 4000 series Explorer software. Reporter-ion intensities were directly extracted from the LTQ−OT peak lists using the dedicated Phenyx export. The reporter-ion areas or intensities were corrected according to the isotopic purities of the reporter-ions provided by the manufacturer, as already described.[2] Negative values after the correction were put to zero. No minimal ion count threshold was applied. No normalization was performed according to LACB as the specificity of its peptides in CSF had to be assessed first (see the Results and Discussion section). For each peptide, the relative abundance of each reporter-ion was calculated as the reporter-ion value over the sum of the reporter-ion values corresponding to the spiked standard peptides (i.e., at m/z = 128.1, 129.1, 130.1, and 131.1). No quantitation was performed for proteins, but only peptide relative abundances were calculated.

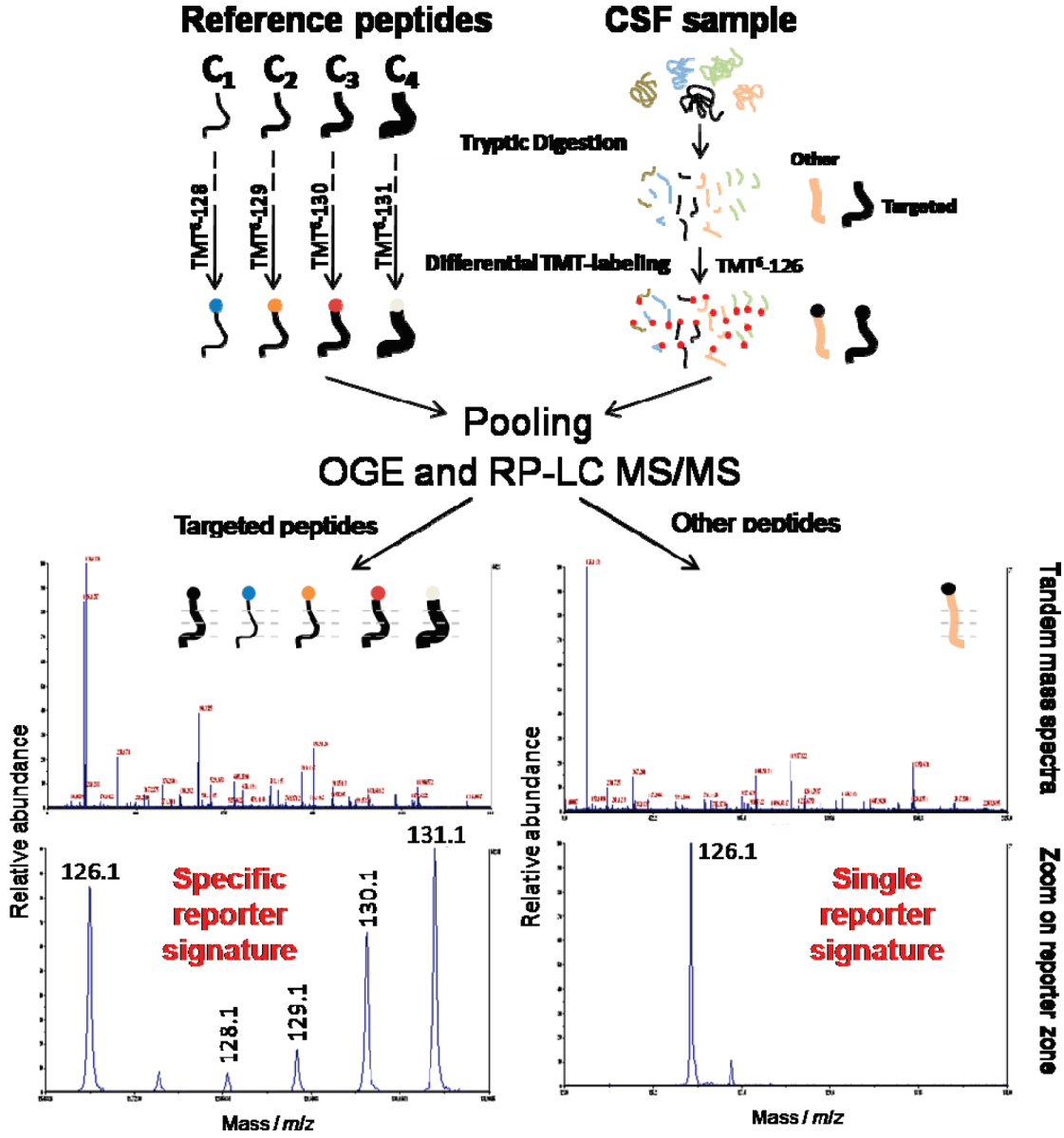
Further processing was carried out for the peptides related to the brain-damage markers. For peptides whose sequence was matched several times per LC MS/MS run (i.e., per off-gel fraction), only the peptide hits with the two most abundant reporter-ion signals were kept to be as more as possible at the chromatogram peak apex and avoid memory signals along the gradient. When multiple hits ($n \geq 3$) were found for a peptide (i.e., same peptide sequence matched several times), outliers were assessed on the relative abundances with Grubbs, Dixon, IQR, and/or Gauss tests using a testing outlier program.[46] Measurement outliers detected by one of these tests were removed. Peptide hits with missing reporter-ion signals were removed. An additional filter on t_R and isoelectric point (pI) was applied in case of inconstancy of some results (e.g., when a peptide was at an unusual t_R or pI, with respect to its other identified analogues). The relative standard deviation (RSD) of the quantitative measurements was calculated for each individual peptide. Finally, reporter-ion values were summed for each individual TMT channel in order to give a preponderant weight to the peptide hits with the most intense reporter-ion signals. The final relative abundance of each targeted peptide was calculated by division of the summed reporter-ion values over the sum of the summed reporter-ion values corresponding to the spiked standard peptides. The unknown peptide concentrations were determined from the calibration curves provided by the four standard points.

Standard Addition Experiments. The second pool of CSF samples ($n = 5$) was used for standard addition experiments and was divided in eight aliquots of 155 μL. Known quantities of the standard peptides were added into CSF sample aliquots (corre-

(45) Dayon, L.; Pasquarello, C.; Hoogland, C.; Sanchez, J. C.; Scherl, A. *J. Proteomics* **2009**, *1010*, DOI: 10.1016/j.jprot.2009.10.015.

(46) http://www.sediment.uni-goettingen.de/staff/dunkl/software/out_lier.html, accessed July 1, 2009.

Scheme 1. Schematic Representation of the Use of Isobaric Tag Reporter Calibration Curves for the Selection and the Quantitation of Brain-Damage Peptide Markers in CSF[a]



[a] Proteotypic peptides representative of CSF proteins are prepared at known concentrations and labeled with different versions of the isobaric tag. CSF is labeled with another version. After pooling of the samples and orthogonal two-dimensional separation, MS and MS/MS analyses are performed. Targeted peptides exhibit a characteristic distribution of the reporter-ions for absolute quantitation.

sponding to three incremental additions of 1-, 2-, and 3-fold with respect to the expected protein contents). The proteomic protocol described above was applied to determine the peptide concentrations C_0, $C_0 + \Delta C_0$, $C_0 + 2\Delta C_0$, and $C_0 + 3\Delta C_0$ in the samples. The experiments were duplicated. Only peptides with most consistent quantitation results in the prospective phase were assessed. A mass inclusion list of the targeted peptides was made for MS. Phenyx identification searches were restricted to the protein markers. The protein and peptide scores were 4.0 for all searches. Peptides not identified after automatic database search were manually recovered from the raw MS data according to their m/z, t_R, and the presence of the characteristic calibration curve. Their tandem mass spectra were checked by comparison to their matched analogues. Quantitation was performed as described above.

RESULTS AND DISCUSSION

The general procedure to select and quantify specific tryptic peptides in CSF is depicted in Scheme 1. Representative peptides of S100B, GFAP, FAPBH, PTGDS, PARK7, NDKA, UFD1, and GSTP1 were chosen because they were proteotypic in human and were for most of them previously detected on several MS platforms.[2,44] Two identical pooled CSF samples (sample 1 and sample 2) were processed to assess interassay performances in a prospective study. In a second time, standard addition experiments were carried out to further characterize the quantitative method.

Isobaric Labeling Hyphenation with OGE and RP-LC. Because peptide cofragmentation is an issue with isobaric tagging, efficient and complementary fractionation and separation methods are required. Isobaric labeling with iTRAQ was shown to be compatible with immobilized pH gradient (IPG) isoelectric focus-

Table 1. Workflow and OGE Fractionation Performances Evaluated with RP-LC MALDI TOF/TOF and RP-LC ESI LTQ−OT Analyses of Identical Sample 1 and Sample 2

fraction number	1	2	3	4	5	6	7	8	9	10	11	12
average pH	4.02	4.21	4.38	4.60	4.80	5.03	5.28	5.53	5.75	6.00	6.20	6.40
pH RSD/%	0.9	0.2	0.6	0.3	0.1	0.4	0.4	0.4	0.1	0.4	0.6	0.4
					TOF/TOF							
unique peptides (sample 1)	31	134	135	97	43	9	10	92	88	110	98	108
unique peptides (sample 2)	86	150	99	108	48	11	9	84	99	87	89	118
common peptides/%	27	59	43	55	57	54	33	49	60	54	51	50
					LTQ−OT							
unique peptides (sample 1)	210	376	373	330	213	65	80	316	336	350	363	359
unique peptides (sample 2)	206	344	256	333	185	55	57	274	307	312	297	334
common peptides/%	40	57	44	52	52	56	54	55	53	52	52	55

fraction number	13	14	15	16	17	18	19	20	21	22	23	24
average pH	6.61	6.80	7.04	7.27	7.52	7.75	7.96	8.13	8.42	8.71	9.11	9.33
pH RSD/%	0.0	0.2	0.3	0.5	0.1	0.4	0.3	0.6	0.6	0.4	0.8	1.6
					TOF/TOF							
unique peptides (sample 1)	52	46	26	43	68	25	2	16	48	26	55	27
unique peptides (sample 2)	97	81	35	64	40	35	3	42	59	77	88	65
common peptides/%	26	27	25	35	35	30	67	21	34	27	42	26
					LTQ−OT							
unique peptides (sample 1)	300	342	226	277	337	119	22	164	312	247	307	264
unique peptides (sample 2)	301	288	248	247	274	79	19	139	246	270	238	201
common peptides/%	55	56	48	55	53	46	41	54	38	45	49	50

ing (IEF),[47,48] and specially OGE.[49] We report here the use of TMT instead of iTRAQ and describe a robust protocol for the analysis of CSF proteins.[50] The analyses of the two identical CSF samples (so-called sample 1 and sample 2), separated into 24 off-gel fractions and analyzed with RP-LC MALDI TOF/TOF and RP-LC ESI LTQ−OT, proved excellent resolution and good reproducibility of the IEF separation. Indeed, 80% of the peptides were found in a unique fraction, and 49% were identified in the same off-gel fraction between sample 1 and sample 2 using a MS data-dependent acquisition mode (Table 1). When considering the 24 fractions together, 54% of the peptides were commonly identified in sample 1 and sample 2. This result was very close to the percentage obtained for individual fractions, supporting the reproducibility of the IEF. The pH measured in the 24 off-gel fractions after IEF increased linearly from 4.0 to 9.4. The RSD of pH values varied from 0.0% to 1.6% with an average value of 0.4%. In addition, OGE and RP-LC exhibited very good orthogonal properties (see Supporting Information SI1) for the separation of tryptic peptides.[51,52] The workflow enabled the identification of, respectively, 185 and 209 proteins in sample 1 and sample 2 with MALDI TOF/TOF, with 66% of commonly identified proteins between both samples (see Supporting Information SI2 and SI3). The ESI LTQ−OT analysis of both samples provided 588 and 615 proteins, with 66% of commonly identified proteins (see Supporting Information SI4 and SI5). Among the 41 spiked synthetic peptides, 38 were identified with Phenyx (29 with TOF/TOF, 35 with LTQ−OT, and 27 with both apparatus) (see Supporting Information SI6). From these peptides, the eight protein markers were recovered, but S100B and NDKA were identified only with LTQ−OT MS. In postmortem CSF, S100B and NDKA are indeed slightly lower concentrated (nanograms per milliliter). Finally, this workflow offers an efficient pipeline for TMT-based quantitation.

Selective MS/MS Detection with Spiked Standard Peptides and Their Reporter-Ions. Most of the targeted peptides were identified with Phenyx. Their characteristic TMT reporter signature could also be used to select them (Scheme 1). When reporting the reporter-ion abundances along the successive RP-LC MS/MS analyses of the off-gel fractions, a very different pattern was obtained for the reporter-ion at $m/z = 126.1$, specific to the CSF sample, with respect to the other reporter-ions, specific to the spiked peptide standards (Figure 1). Furthermore, the peak intensities increased from Figure 1b−e in accordance to the increasing amounts of the standards. Some peaks exhibited almost the same intensities and most likely corresponded to tryptic peptides of LACB, spiked in equal amount in all samples (see the Experimental Section).

The typical reporter signature was further exploited. The relative abundance of reporter-ion at $m/z = 126.1$ (i.e., normalized by the sum of the standard reporter-ion values at $m/z = 128.1$, 129.1, 130.1, and 131.1) allowed discriminating targeted peptides from other peptides in CSF with 95% specificity and 93% sensitivity at a ratio cutoff of 3.06 (see Supporting Information SI7a, computed with Prism 4 (GraphPad Software, La Jolla, CA)). The area under the receiver operator characteristic (ROC) curve reached 0.97 ($p < 0.0001$). These results were, however, highly dependent on the spiked amounts of peptide standards, which had to be chosen

(47) Lengqvist, J.; Uhlen, K.; Lehtio, J. *Proteomics* **2007**, *7*, 1746–1752.
(48) Scherl, A.; Francois, P.; Charbonnier, Y.; Deshusses, J. M.; Koessler, T.; Huyghe, A.; Bento, M.; Stahl-Zeng, J.; Fischer, A.; Masselot, A.; Vaezzadeh, A.; Galle, F.; Renzoni, A.; Vaudaux, P.; Lew, D.; Zimmermann-Ivol, C. G.; Binz, P. A.; Sanchez, J. C.; Hochstrasser, D. F.; Schrenzel, J. *BMC Genomics* **2006**, *7*, 296.
(49) Chenau, J.; Michelland, S.; Sidibe, J.; Seve, M. *Proteome Sci.* **2008**, *6*, 9.
(50) Waller, L. N.; Shores, K.; Knapp, D. R. *J. Proteome Res.* **2008**, *7*, 4577–4584.
(51) Busnel, J. M.; Lion, N.; Girault, H. H. *Anal. Chem.* **2007**, *79*, 5949–5955.
(52) Cargile, B. J.; Bundy, J. L.; Freeman, T. W.; Stephenson, J. L. *J. Proteome Res.* **2004**, *3*, 112–119.

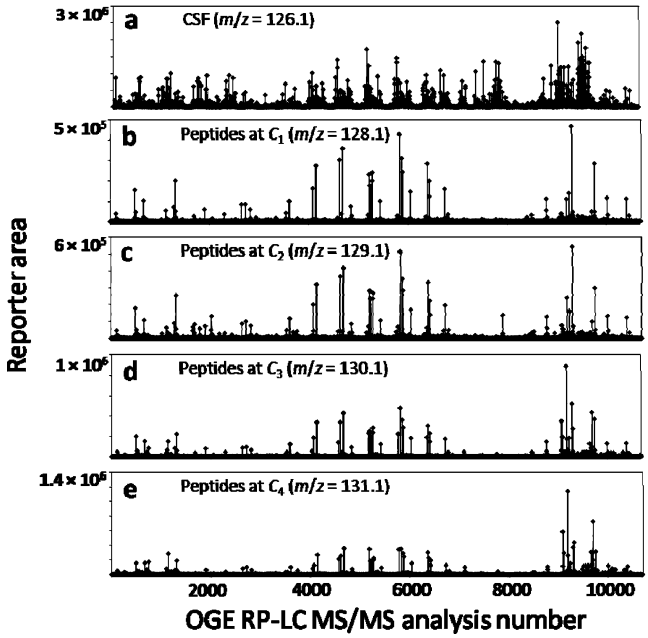


Figure 1. Raw reporter-ion area values obtained with MALDI TOF/TOF (sample 1) along off-gel fractions (24 fractions with measured pH from 4.0 to 9.4) separated with RP-LC and deposited onto MALDI plates. The CSF and peptide standards at four concentrations (C_1-C_4) were labeled, respectively, with TMT with reporter-ions at $m/z = 126.1$ (a), 128.1 (b), 129.1 (c), 130.1 (d), and 131.1 (e). Every sample was previously spiked with same amount of LACB.

adequately in the range of the targeted peptides. At the contrary, the slope of the linear regression for the relative abundance of reporter-ions at $m/z = 128.1$, 129.1, 130.1, and 131.1 with respect to the spiked quantities is theoretically 1 for the targeted peptides, and was assessed for selection (see Supporting Information SI7b). Indeed, 92% of the targeted peptides presented slope values between 0.9 and 1.1, whereas only 5% of the nontargeted peptides were in this range. With additional information such as pI, t_R, and mass, these findings suggest that the spiking of synthetic TMT-labeled peptides can be exploited to pick and characterize their natural analogues in a complex tryptic mixture. Another advantage is that product ion monitoring is applicable (see Supporting Information SI8). To focus the analysis on the targeted peptides, product ion scanning experiments were implemented. Data-dependent acquisition with pulsed Q dissociation (PQD) fragmentation and analysis in the ion trap was performed with the LTQ−OT mass spectrometer. When ions at $m/z = 131.1$ were detected, further HCD fragmentation was carried out to obtain precise quantitation.[45]

Nevertheless, the technique might lack of specificity since any peptides of CSF coeluting with a similar precursor ion m/z ratio contribute to the reporter-ion signal at $m/z = 126.1$. This situation was clearly illustrated by the quantitative data obtained for the spiked LACB. Considering that all LACB tryptic peptides provided the same quantitative response, the raw relative abundances were, respectively, measured at 0.30 (RSD of 116% on individual peptide values), 0.23 (RSD of 12%), 0.26 (RSD of 21%), 0.27 (RSD of 19%), and 0.24 (RSD of 17%) for reporter-ions at $m/z = 126.1$, 128.1, 129.1, 130.1, and 131.1 by analysis of sample 1 with TOF/TOF MS. These results highlighted contamination from peptides of CSF. The corrected relative abundances became 0.37 (RSD of 62%), 0.24 (RSD of 6%), 0.27 (RSD of 10%), 0.26 (RSD of 9%), and 0.23 (RSD of 7%) when only the proteotypic peptides in the human database were considered and the outliers filtered. The inspection of survey mass spectra confirmed that the 62% RSD for reporter-ion at $m/z = 126.1$ was due to accidental CID events, low peak intensities, and subsequent low reporter-ion signals in the tandem mass spectra (Table 2). In the following, peptide quantitation was therefore performed on the summed reporter-ion values for each peptide to give a preponderant weight to the peptide hits with the most intense signals (see the Experimental Section). The results obtained with LTQ−OT MS were better with respective corrected relative abundances of 0.30 (RSD of 18%), 0.25 (RSD of 7%), 0.28 (RSD of 10%), 0.27 (RSD of 8%), and 0.20 (RSD of 12%). Less cofragmentation might have occurred with this setup because the peptides were analyzed on the flight. As a matter of fact, careful evaluation of each peptide, even proteotypic, is mandatory to obtain sufficient specificity and perform quantitation.

Quantitation with Internal Reporter Calibration Curves. The benefit to use six-plex TMT labeling and spiked standard synthetic peptides at four concentrations (C_1-C_4) comes from the internal calibration curve (Scheme 1). Multiple-point internal calibration is achieved from isobaric standards, whose reporter-ions are only distinguishable with MS/MS. Because labeled standards and labeled analytes have the same mass, the global detection limit with MS is increased. Under MS/MS, several calibration points are available to improve quantitation accuracy.

Examples of such quantitation with both MS instruments are given in Figure 2. The calibration curves were obtained from the reporter-ions released by the spiked peptide standards with a low interassay RSD between sample 1 and sample 2. From all data (obtained with both mass spectrometers), 66% and 93% of the linear regression coefficients were, respectively, superior to 0.99 and 0.95, which attested for the quality and linearity of the calibration curves. The internal calibration curves enabled the accurate determination of the absolute concentrations of the tryptic peptides in the digested CSF. Each peptide was evaluated in terms of interassay RSD between sample 1 and sample 2 with both TOF/TOF and LTQ−OT MS. RSD values were also calculated between TOF/TOF and LTQ−OT analyses of the same sample (see Supporting Information SI6b). All together, the RSD values provided a rapid assessment of the quantitative performance of each peptide. As stated before for LACB, the obtained quantitative values were noticed to occasionally vary from a peptide to another, even though coming from the same protein. Quantitative differences and high RSD values might have resulted from different factors such as post-translational modifications, cofragmentation of several peptides, tryptic digestion artifacts, peptide recovery, and stability. As a first approximation, it was assumed that if several peptides provided the same quantitative response, then the probability of misleading quantitation decreased. Few peptides with good quantitative behaviors (see Supporting Information SI6, parts a and b) in the prospective experiments were assessed with the standard addition method to validate several proof-of-principle assays.

Assay Characterization with Standard Addition Experiments. A second pool of postmortem CSF ($n = 5$) was analyzed with the workflow described previously to further evaluate the quantitative capabilities of this new approach. Standard addition

Table 2. Identified and Quantified Proteotypic Peptides of LACB[a]

peptide sequence	fraction number	peak intensity in mass spectrum	satellite peak intensity/ %[b]	area of reporter-ion at $m/z = 126.1$	measured relative abundance
GLDIQK	16	3219	8	18762	0.37
	15	717	24	3634	0.99
	11	629	65	5364	0.86
	13	488	19	3890	0.55
	14	239	20	2482	0.64
IDALNENK	3	29000	7	57552	0.26
	2	1717	20	29777	1.37
IPAVFK	22	500	27	7771	1.50
	23	295	10	3635	0.74
LSFNPTQLEEQCHI	4	73000	5	67532	0.27
	5	2611	8	7967	0.85
TPEVDDEALEK	1	5444	37	27200	0.57
VLVLDTDYK	3	71000	28	113800	0.40
VYVEELKPTPEGDLEILLQK	3	1196	*c*	1131	0.13
WENGECAQK	2	29000	9	59009	0.31

[a] The results are from sample 1 analyzed with MALDI TOF/TOF MS. [b] The presence of another peak within a 4 m/z window in the survey mass spectrum was evaluated in terms of intensity with respect to the peak of interest. [c] Because of the high mass of the peptide and its natural isotopic distribution, it was difficult to evaluate the presence of another peak.

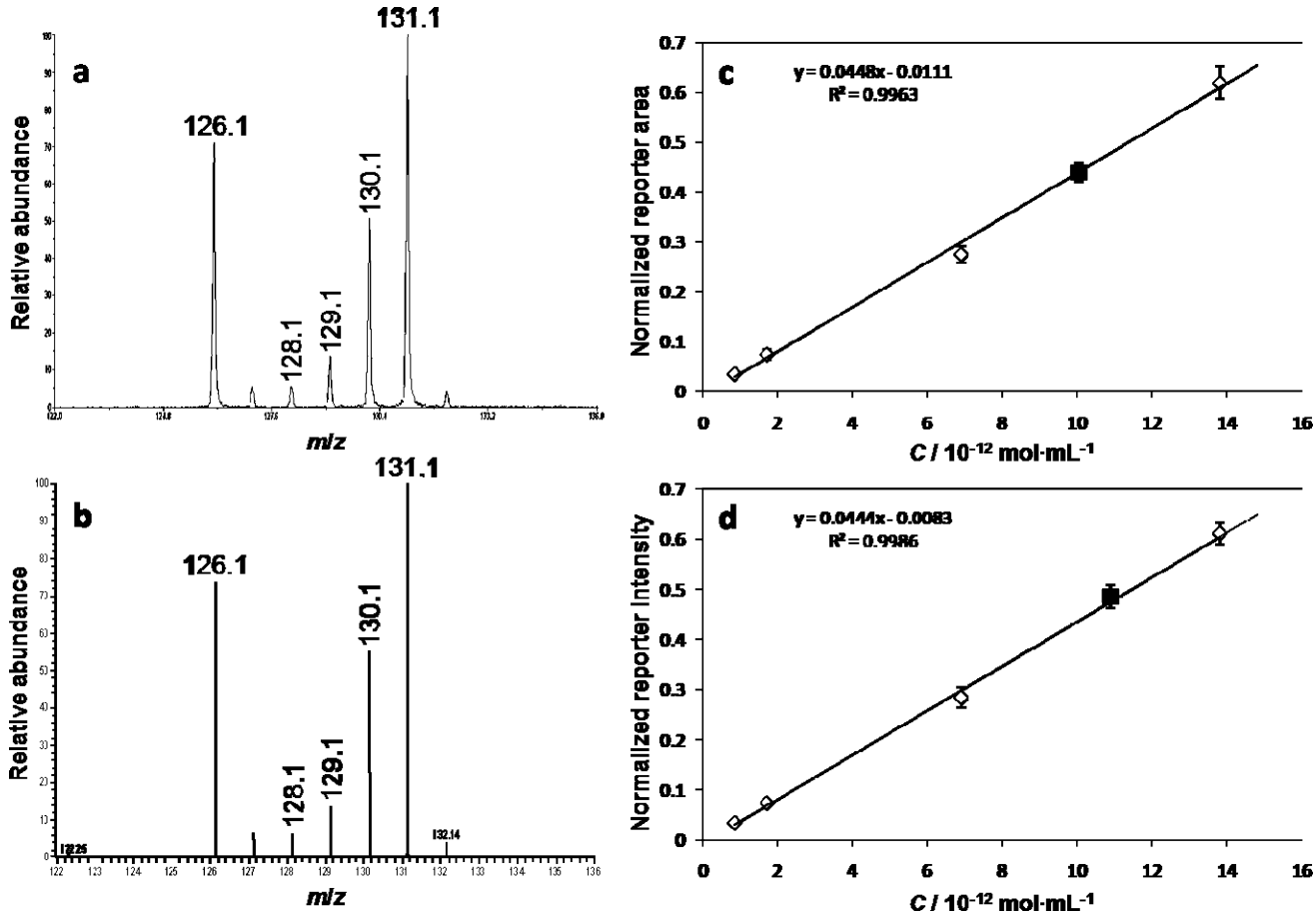


Figure 2. Zoomed tandem mass spectra of the TMT reporter-ion region obtained, respectively, with MALDI TOT/TOF (a) and ESI LTQ−OT (b). The tandem mass spectra were matched with HLQEYQDLLNVK of GFAP. Calibration fitting curves obtained from the peptidic standards releasing reporter-ions at $m/z = 128.1$, 129.1, 130.1, and 131.1 (unfilled diamonds) and determined concentrations of HLQEYQDLLNVK in CSF from reporter-ion at $m/z = 126.1$ (dark squares) (c and d). The two graphs correspond to the TOT/TOF (c) and LTQ−OT (d) measurements in both sample 1 and sample 2. Error bars correspond to interassay measurements.

experiments were carried out by spiking three increasing quantities of peptide standards inside CSF samples. Although the standard addition method requires that no other components than the one of interest interfere the measurement,[53] a similar behavior of several peptides over the studied dynamic range should evidence consistent quantitation (Table 3).

Figure 3 displays the quantitation results with TOF/TOF MS for two peptides of PARK7. The concentration responses were

Table 3. Results of the Standard Addition Experiments on CSF Sample for Peptides of PARK7, GSTP1, NDKA, and S100B[a]

protein name	peptide sequence	$C/10^{-12}$ mol·mL^{-1} (RSD)			
		C_0	$C_0 + \Delta C_0$	$C_0 + 2\Delta C_0$	$C_0 + 3\Delta C_0$
		TOF/TOF			
PARK7	VTVAGLAGK	30.4 (7%)	69.3 (18%)	115 (7%)	198 (14%)
	ALVILAK	32.1 (22%)	73.7 (16%)	101 (11%)	191 (13%)
		LTQ−OT			
PARK7	VTVAGLAGK	22.1 (12%)	64.0 (17%)	104 (4%)	196 (15%)
	ALVILAK	28.1 (40%)	69.3 (21%)	96.0 (25%)	207 (17%)
GSTP1	ALPGQLKPFETLLSQNQGGK	107 (23%)	174 (−)	251 (3%)	359 (−)
	ASCLYGQLPK	34.1 (−)	200 (−)	339 (5%)	419 (−)
NDKA	DRPFFAGLVK	5.94 (10%)	9.56 (7%)	14.8 (8%)	16.8 (3%)
	FMQASEDLLK	3.67 (0%)	8.88 (2%)	14.5 (11%)	18.2 (6%)
S100B	ELINNELSHFLEEIK	19.9 (6%)	46.9 (1%)	84.5 (1%)	123 (3%)

[a] RSDs correspond to interassay measurements.

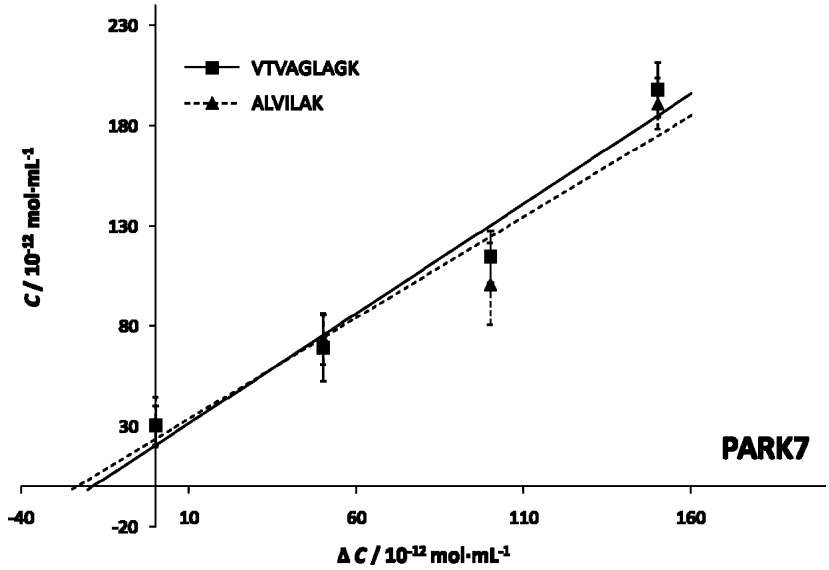


Figure 3. Standard addition experiments on a pooled CSF sample for two peptides of PARK7. The two peptides were detected in off-gel fraction 22 with MALDI TOF/TOF. The fitting line equations are $y = 1.0958x + 20.837$ ($R^2 = 0.97$) and $y = 1.0077x + 23.846$ ($R^2 = 0.94$), respectively, for VTVAGLAGK (squares) and ALVILAK (triangles). Error bars correspond to interassay measurements.

linear with a slope close to 1, reflecting the absence of matrix effect. This was reasonable as the measurement was obtained through internal calibration curve of the isotopically labeled reporter-ions. The two peptides responded identically with a mean RSD between them of 8%. The duplicate experiments showed mean interassay RSDs of 12% and 16% for VTVAGLAGK and ALVILAK, respectively (Table 3). The determined average concentration in the initial pooled postmortem CSF (C_0) was 31.3×10^{-12} mol·mL^{-1} (i.e., 622.6 μg·L^{-1}), whereas previously reported measurements of PARK7 with enzyme-linked immunosorbent assay (ELISA) of such samples gave 41.0×10^{-12} mol·mL^{-1} (i.e., 816.2 μg·L^{-1}).[2]

Several peptides were measured with LTQ−OT MS. Tandem mass spectra zoomed on the TMT reporter-ion region are provided in Supporting Information SI9 and show the increasing addition of standards in the CSF. The results for the two peptides of PARK7 previously assessed with TOF/TOF MS are presented in Figure 4a. The results were very similar since 7% of mean variation on the average values was observed between both RP-LC MS techniques.

Other examples are given in parts b and c of Figure 4, respectively, for the quantitation of peptides of GSTP1 and NDKA. For GSTP1, a few values were missing or exhibited very low reporter-ion signals, especially for ASCLYGQLPK (absence of error bars for few data points). The nonlinear response for this peptide was linked to limits of detection and quantitation, as shown by the number of missing reporter-ion signals. ALPGQLKPFETLLSQNQGGK peptide responded better for linear fitting with a slope of 1.0438 ($R^2 = 0.99$). The mean variation between both peptides was 29%. RSDs of 73%, 10%, 21%, and 11% were obtained when the concentration increased (Table 3). The dynamic behaviors of both peptides for the assay were therefore slightly different. The measured peptide concentration in postmortem CSF was in the range of previously determined protein concentrations,[44] which were measured up to 85.6×10^{-12} mol·mL^{-1} (i.e., 2000 μg·L^{-1}) for GSTP1 with ELISA (unpublished work).

As regard to NDKA (Figure 4c), FMQASEDLLK fitted with a line $y = 0.9824x + 3.959$ ($R^2 = 0.99$). Variations with DRPFFAGLK

(53) Saxberg, B. E. H.; Kowalski, B. R. *Anal. Chem.* **1979**, *51*, 1031–1038.

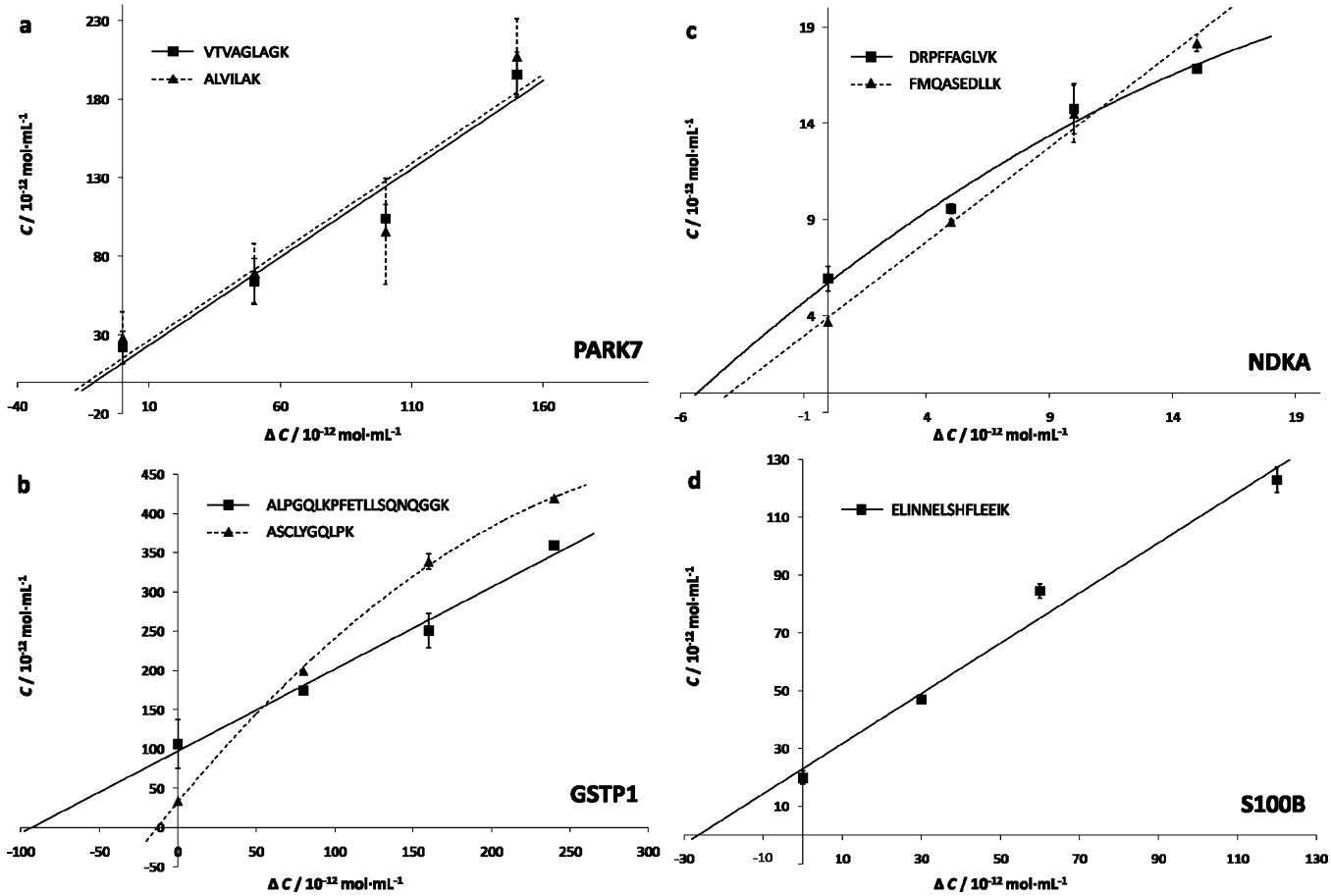


Figure 4. Standard addition experiments on a pooled CSF sample for peptides of PARK7 (a), GSTP1 (b), NDKA (c), and S100B (d) analyzed with LTQ−OT. Several peptides were evaluated, whose sequences are indicated in the graphs and in Table 3. Error bars correspond to interassay measurements.

were 33%, 5%, 1%, and 5% (Table 3). The less concentrated samples were measured with less precision, because they were retrieved in the low-intensity region of the reporter calibration curve (see Figure 2, and Supporting Information SI9). For DRPFFAGLVK, slight saturations of the reporter-ion signals were noticed in the tandem mass spectra for the most concentrated samples and could explain the nonlinear fitting. Optimized collision energy should be used in this case. The concentration of FMQASEDLLK in the initial pooled postmortem CSF was 3.67×10^{-12} mol·mL^{-1} (i.e., 62.9 ng·mL^{-1}), showing that promising limit of detection and quantitation are achievable with the technique. Indeed, all differentially labeled peptides contribute to the same signal in MS mode, and TMT reporter-ions are strongly released under HCD. The mixtures are not complicated by the spiking of heavy molecules in the samples. Furthermore, OGE fractionation with good resolution and orthogonal properties with RP-LC was performed.

At last, S100B was probed only with ELINNELSHFLEEIK, but charges +2 and +3 were assessed together. Mean interassay RSD on the quantitative values was below 6% (Table 3). The measured peptide concentration (19.9×10^{-12} mol·mL^{-1}; i.e., 213.2 μg·L^{-1}) was 50 times more elevated than a previously reported measurement in postmortem CSF for S100B.[2] But, some studies reported S100B values in CSF from 0.26 μg·L^{-1} in controls to, respectively, 55.7 and 630 μg·L^{-1} in patients with subarachnoid hemorrhage and severe traumatic brain injury.[54,55]

CONCLUSIONS

The TMT-based quantitation hyphenated with orthogonal IEF OGE and RP-LC separations was demonstrated to be an efficient platform for quantitative proteomics. Absolute quantitation of peptides through the use of reporter calibration curves obtained from labeled synthetic standards was shown. As a proof-of-principle, PARK7 peptide concentrations were determined in CSF with both MALDI TOF/TOF and ESI LTQ−OT MS in the range of ~0.6 μg·mL^{-1}. The technique is therefore applicable on MS platforms commonly used for biomarker discovery. Moreover, it is easily implementable (for instance, no collision energy optimization is required in most cases) and does not require the costly synthesis of heavy peptides. As the specificity of the assay is a crucial issue (proteotypic characteristics should be supported by uniqueness of mass as well as t_R and pI), the technique should benefit from the enrichment of the targeted peptides as described in the stable isotope standards and

(54) Hayakata, T.; Shiozaki, T.; Tasaki, O.; Ikegawa, H.; Inoue, Y.; Toshiyuki, F.; Hosotubo, H.; Kieko, F.; Yamashita, T.; Tanaka, H.; Shimazu, T.; Sugimoto, H. *Shock* **2004**, *22*, 102–107.
(55) Kay, A.; Petzold, A.; Kerr, M.; Keir, G.; Thompson, E.; Nicoll, J. *Stroke* **2003**, *34*, E240–E243.

capture by antipeptide antibodies (SISCAPA) approach,[56,57] as well as a narrow precursor selection window for MS/MS.

The standard addition experiments clearly showed that the method is accurate and allows measuring different ranges of analyte concentration. Besides, the variation of the technique (for instance, respectively, 16% and 6% for the assays relative to PARK7 and NKDA) is below the expected biological variation.[10] The tool can therefore be very useful to verify peptides of biomarker candidates, and make decision on which proteins are worth being validated.

As perspective, the feasibility to perform quantitation with TMT reporter calibration with recombinant protein standards should be evaluated. Other types of molecules that easily undergo modification may be probed using this technique.

(56) Anderson, L.; Hunter, C. L. *Mol. Cell. Proteomics* **2006**, *5*, 573–588.
(57) Anderson, N. L.; Anderson, N. G.; Haines, L. R.; Hardie, D. B.; Olafson, R. W.; Pearson, T. W. *J. Proteome Res.* **2004**, *3*, 235–244.

ACKNOWLEDGMENT

Dr. Carla Pasquarello and Dr. Ian Pike are thanked for very fruitful discussions. The authors thank Proteome Sciences R&D GmbH & Co. KG for financial support.

SUPPORTING INFORMATION AVAILABLE

Orthogonality between OGE and RP-LC, tables of identification and quantification for sample 1 and sample 2 with TOF/TOF MS/MS, tables of identification and quantification for sample 1 and sample 2 with LTQ−OT MS/MS, summary tables of identification and quantification of the targeted peptides for sample 1 and sample 2, ROC curve and histogram to distinguish the targeted from other peptides in CSF, product ion scanning chromatograms, tandem mass spectra zoomed on the reporter-ion region in the standard addition experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review August 17, 2009. Accepted December 16, 2009.

AC901854K