Journal of Proteomics 234 (2021) 104102

Contents lists available at ScienceDirect

Journal of Proteomics

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

FAIMS-MS might contribute to phosphopeptides identification in plasma

ARTICLE INFO

Keywords
phosphopeptides
phosphoproteomics
FAIMS
compensation voltages

ABSTRACT

FAIMS interface is gaining popularity because of the impressive 100-fold signal to noise enhancement in addition to the recent coupling to the Orbitrap technology, the most important analyzer developed in the last 20 years. The selection of group of ions and effective removal of single-charged ones at particular compensation voltages increases around 50% the proteome coverage at expenses of lower peptides coverage. However, specific setting for phosphoproteome analysis is yet poorly described. Here we have found the maximum transmission for several tryptic phosphopeptides isolated from a single complex mixture and we have set an experimental method based on five compensation voltages partially different to the ones described previously, demonstrating the relevance of voltages higher than 47 V, with an increase of around 20% of unique phosphopeptides. Using this experimental setup two complex phosphoproteomes isolates (SH-SY5Y cell line and plasma) were analyzed and found increments of 50% on phosphopeptides identification with the proposed method with respect to a previous one, for the cell line extract. Meanwhile for plasma 109 of the detected phosphopeptides are found for first time in this body fluid, presumably due to the release of intracellular proteins. With this FAIMS setup, 60% of the proteins identified are classified as very low abundant proteins.

FAIMS is being under development for more than twenty years, as an analytical interface to separate ions at atmospheric pressure according to their mobilities in gas phase, prior to the mass spectrometer [1,2]. The differential mobility of ions is provoked by the asymmetric waveform induced by alternating low and high electric fields during different periods of time. A dc voltage (termed compensation voltage, CV) is used to alter the trajectory of the ions to be transmitted into the MS. The mobility, and thus the appropriate CV to use, cannot be easily predicted and must be empirically determined.

In 2018, Hebert et al. [3] proposed the use of three (−50, −65 and −85 V) or four (−45, −60, −75, and −90 V) CVs within a single LC-MS experiment, to allow the maximal number of protein identifications in single-shot proteomics experiments. Schweppe et al. [4] also concluded than at least three CVs should be used for good sensitivity and quantitative accuracy.

Recently, Bekker et al. [5] described the best parameters for the newest Thermo Exploris 480 mass spectrometer using FAIMS interface for DDA and DIA experiments. These authors scanned the CVs from -80 to -30 V (stepped 5 V) for unmodified peptides from HELA cell line and found the maximum peptide identification at -50 V and the higher number of protein identifications at -75 V.

Previously, some few works were done with FAIMS interface on detection of phosphopeptides and phosphoproteomics. Due to the negative charge introduced by the phosphate group a different CV range should be expected for phosphopeptides. In 2010, Cooper reported the separation of phosphopeptide isomers by FAIMS [6,7] by using stepping voltages from -45 down to -25 V. Cooper later studied the Fibroblast Growth Factor Signaling by using -50 to -22.5 V range with a 2.5 V step, identifying a total of 3741 phosphopeptides from SUM52 breast cancer carcinoma cells, after SCX/TiO_2 pre-fractionation [8]. In routine practice, the work with many CVs values in parallel is less convenient than defining a set of three to five values, as the duty cycle is increased with certainly a detrimental effect on the number of detected peptides. Sample fractionation could alleviate this effect, at expense of increasing substantially the execution time. The use of only five CVs (-24, -28, -32, -38, -42 V) for phosphopeptides analysis was introduced by Bridon et al. [9] reporting an enhancement on phosphopeptide detection isolated from *D. melanogaster*, with up to 2277 phosphopeptides detected by combining CAD and ETD, isolated after TiO_2 and 2D-LC-MS/MS.

Most of the studies define the working CVs based on the number of identified proteins while analyzing a complex mixture enriched in phosphopeptides. However, those complex mixtures usually contain as many non-phosphopeptides as phosphorylated counterparts (even after phosphopeptides enrichment) which could be misleading the determination of the optimal CVs for the identification of phosphorylated peptides. Recently, Muehlbauer et al. [10] also used a phosphopeptides extract for CV determination and proposed three or four CVs method with best outputs at voltages ranging between -35 to -75 volts.

Unlike previous reports, in the present work we have determined maximal CVs for some phosphorylated peptides that were selectively isolated from a simple mixture of caseins, meaning without influence of non-modified species, and according to these values we have chosen five CVs for further analysis of complex mixtures to improve the identification of phosphopeptides. Fig. 1A shows the CV scan of the casein tryptic digest (without any enrichment) directly infused with a nano-emitter into the MS and representative mass spectra at particular CVs. Therefore, we focused on the detected phosphopeptides and scanned the CV at low and high FAIMS resolution to get their maximal transmission. In our opinion low resolution FAIMS would be more convenient for proteomic experiments commonly seen as comprehensive experiments meanwhile higher resolution FAIMS would be needed for selective isolation of particular species within less complex samples. Supplementary fig. 1

https://doi.org/10.1016/j.jprot.2021.104102
Received 26 December 2020; Accepted 3 January 2021
Available online 4 January 2021

shows the high resolution FAIMS compensation voltage scanning for specific phosphopeptides as well as the selected ion chromatograms (XIC) obtained while scanning CV at low resolution. Different peptide signals were detected at discrete CVs values (fig. 1B, C and D), therefore considering the previous voltages found for the casein phosphopeptides we defined CV values close to those experimental values but separated at least 4 volts therefore the experiments were carried out at -33, -37, -41, -48 and -52 V. Higher voltage did not increase the number of phosphopeptides detected for this sample. Certain phosphopeptides were only detected at voltages of -48 and -52 and were not detected at voltages below -48 V. Signal at m/z 1031.41 (2+) with a maximum CV at -42 V corresponds to a mono-phosphorylated peptide meanwhile its corresponding triple-charged ion (m/z 687.9469) is detected at -52 V. Supplementary table 1 shows all the phosphoforms detected for caseins by using different CV settings and LC-MS/MS runs. A relevant increment of the number of phosphoforms identified is observed when using five CV values instead of four values, and particularly the use of voltages of -48 and -52 allowed the detection of new phosphopeptides in presence of many other non-phosphopeptides. For the caseins sample more than 10 new phosphoforms were identified with the high resolution five CV method that were not found with the four CV method or even the low resolution five CV method which demonstrate the added value of those high voltages used. However, as our purpose is phosphoproteomics we used low resolution FAIMS on the next complex samples expecting to detect more instead of being more selective. Although our objective was not the characterization of caseins, and we do not know the exact composition of phosphopeptides in this sample, according to the annotations in UniprotKB, we have detected most of the phosphosites for these four constituent caseins but we realized that, two phosphopeptides (tetra and penta-phosphorylated) from alpha-S1- and alpha-S2-casein were not detected. The non-phosphorylated counterparts were also found for most of these peptides, except for these two regions, which is an aspect that suggest some difference with the sequence variants available in the database which could lead to a missed identification.

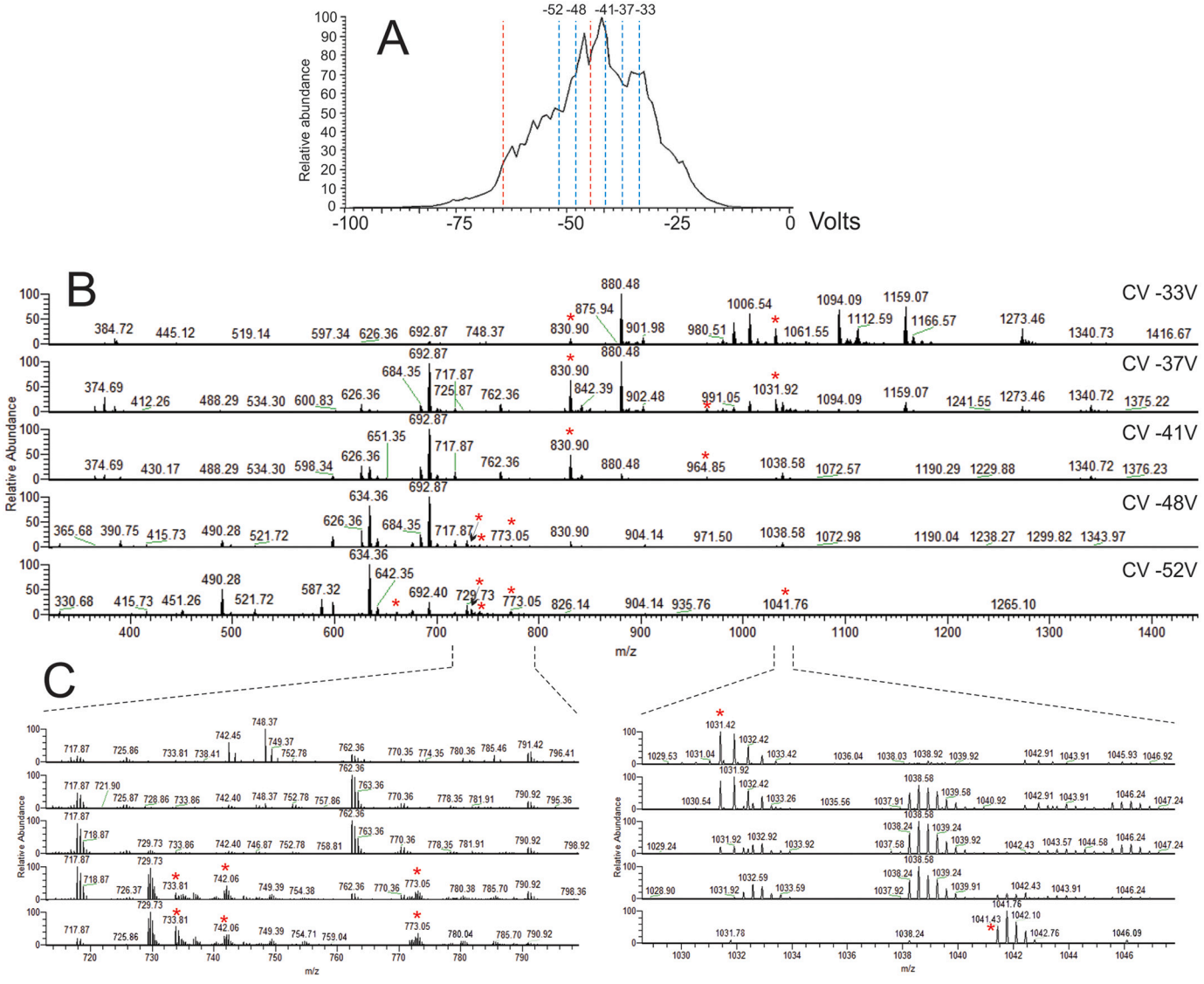

Fig. 1. Direct infusion of Casein trypsin digestion: A) TIC obtained while scanning the compensation voltage (CV), dotted red lines represent the voltages commonly used in our laboratory for positive (2+ charges or more) non-phosphopeptides, dotted blue lines are the voltages used in this work for phosphopeptides detection; B) ESI mass spectra obtained at the proposed compensation voltages (-33, -37, -41, -48 and -52 V), C) expanded m/z ranges 713.00-799.00 (left panel) and 1028.50-1047.80 (right panel) to highlight some of the different phosphopeptides that were uniquely detected at -52 V. Phosphopeptides highlighted are FQSEEQQQTEDELQDK, [1P] 3+ (m/z 687.9469); TVDMESTEVFTK, [1P] 2+ (m/z 733.8105); TVDMESTEVFTK, [1P, oxidized Met] 2+ (m/z 741.8068); TVDMESTEVFTK, [2P] 2+ (m/z 773.7869); VPQLEIVPNSAEER, [1P] 2+ (m/z 830.9033); DIGSESTEDQAMEDIK, [2P] 2+ (m/z 964.3499); FQSEEQQQTEDELQDK, [1P] 2+ (m/z 1031.4111); RELEELNVPGEIVESLSSSEESITR, [4P] 3+ (m/z 1041.4274). * phosphopeptides detected.

There are other possible reasons including the database searching parameters, nonetheless the use of phosphopeptide enrichment and the combined fragmentation HCD/ETD could also help to overcome this issue. Curiously we found two phosphosites for kappa-casein (supplementary table 1, peptides sPAQILQWQVLSNTVPAK and yIPIQYVLSR) different to the ones that have been described in UniprotKB as well as tyrosines 106 and 109 of alpha-s1-casein (see peptide YLGYLEQLLR) were also found phosphorylated. Considering the maximum transmission for each phosphopeptide and the results observed in fig. 1 we set the five CVs values (-33, -37, -41, -48 and -52 V) for further sample studies. For this relatively simple sample the use of FAIMS at high or low resolution is not so relevant, but it is expected than for complex samples low resolution could cover more diverse phosphopeptides structures in short duty cycles and chromatographic runs.

The analysis of a TiO_2 phosphopeptides isolate from the SH-SY5Y neuroblastoma cell line gaves a total of 2151 phosphopeptides, from 1289 phosphoproteins, which represent 54% of the total peptides in the isolate. Worth mentioning that the phosphoproteomic of this cell line was previously studied and gave as much as 2283 phosphopeptides and 1321 phosphoproteins, after extensive fractionation by IMAC-TiO_2 and further HILIC-HPLC [11]. When comparing the number of peptide-spectrum-match (PSM) and number of phosphopeptides identified the proposed setting (-33, -37, -41, -48 and -52 V) allowed 59% and 50% increase over the output obtained using the CVs -24, -28, -32, -38, -42 V proposed by Bridon[9] (fig. 2A and 2B). Supplementary table 2 contains the phosphopeptides identified and the PSMs found using both settings. Fig. 2C shows that 20% of the phosphopeptides from this isolate were uniquely detected with -48 V (5%) or -52 V (9%), or with both voltages (6%). Meanwhile 41% of the phosphopeptides were found using the CVs -33, -37 and -41V and 39% of them have possibility to be detected simultaneously at all of these five CVs.

A second study was performed with phosphopeptides isolated from plasma proteins obtained from one healthy donor. Less than 80 phosphoproteins have been described in plasma samples from healthy patients and 300-400 more phosphoproteins were found in a couple of disease-associated studies [12,13]. There is only a core of proteins that are common to most individuals, and some others that are presumably released into blood after cell death or due to inflammatory processes. Only 10 µL plasma sample (aprox. 600 µg total proteins and with no abundant proteins depletion) was digested and the

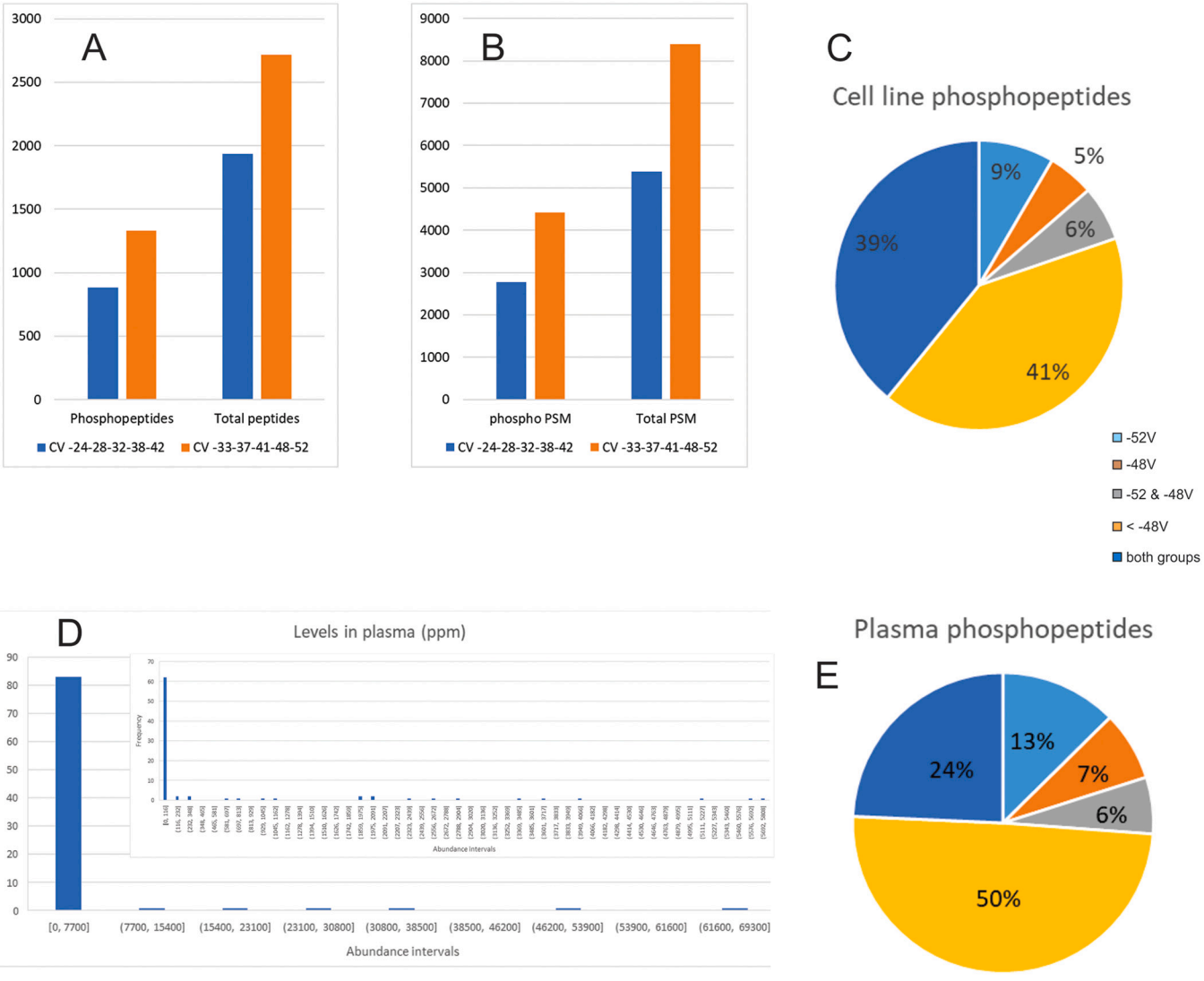

Fig. 2. Comparison of two methods with different CV settings for phosphopeptides analysis, (A) Number of phosphopeptides identified and (B) peptide-spectrum-matches for phosphopeptides isolated from a cell line. (C) Distribution of phosphopeptides detected with CVs of -48 and -52V in comparison to voltages of -33, -37 and -41 for the cell line TiO_2 isolate. (D) Histogram with the abundance in plasma (according to PAXdb (https://pax-db.org/) of the proteins that were detected phosphorylated. Inset covering the lowest abundance level showing that the phosphoproteins identified are presumably released intracellular proteins. (E) Distribution of phosphopeptides detected with CVs of -48 and -52V in comparison to voltages of -33, -37 and -41 for the plasma proteins TiO_2 isolate.

phosphopeptides enriched by using TiO_2 beads were analyzed by LC-MS/MS with a 15 cm length column and 90 min total gradient run. No bias toward the detection of more abundant proteins were found (fig. 2D) suggesting that neither the isolation procedure nor the CV setting favored this group of proteins that very often hamper the proteome coverage in proteomics experiments of human plasma. More than 80% of the identified proteins are classified as low abundant proteins in plasma. Fig. 2E shows that 26% of phosphopeptides were detected uniquely with voltages of -48 and -52 V, while 50% were detected with the voltages -33, -37 and -42. In total, 135 phosphosites from 95 phosphoproteins were identified. Phosphosite location was sometime ambiguous therefore it was not considered. Many isoforms mostly due to oxidation and deamidation generated during sample processing, were detected. As far as we know, 109 of the detected phosphopeptides are found for first time in plasma, which presumably are due to proteins released from cells. Counting the isoforms as only one peptide region, only 31 out of 140 phosphopeptides were previously reported as phosphoproteins found in plasma (see Supplementary table 3). Further work must be done with more plasma samples to recognize whether it is or not normal in healthy donors. In any case, this result again raises the interest to monitor phosphorylated proteins released to blood as biomarkers for certain diseases, where FAIMS could play an important role, due to its enhanced sensitivity. In general terms we have observed lower CVs are required for phosphopeptides than for the non-phospho counterpart, as well as lower CVs are required for oxidated and deamidated species, with respect to the phosphorylated or non-modified peptides.

In conclusions, we have arrived to an experimental method for FAIMS with CV values that could enhance the detection of phosphopeptides. We have found an important increase in the number of phosphopeptides detected when using the proposed method, which could be consequence of using voltages values derived from the analysis of selected phosphopeptides. However, this work also suggests that further studies with more diverse phosphopeptide structures as well as well-defined isomers would be beneficial to find even more appropriate CV settings suitable for wide proteome studies based on this novel interface of the Exploris 480. Considering that we have been able to identify phosphopeptides from very low abundant proteins, we foresee the improved detection of phosphoproteins as biomarkers from plasma or serum, for early detection of certain diseases.

1. Material and Methods

Casein was purchased from SIGMA. This is a single mixture containing four casein proteins (alpha-S1-casein, alpha-S2-casein, beta-casein and kappa-casein). Lysyl endopeptidase/trypsin 100:1 (w/w) at 37 ^{o}C overnight was used to digest 100 μg of total protein that later the peptides were desalted with stagetips before MS analysis. Phosphopeptide isolates were obtained from the neuroblastoma SH-SY5Y cell line and from human plasma (one healthy donor) by using TiO_2 beads (according to Tingholm, et al. [14]) in a batch format after reduction and carbamidomethylation of cysteines. For both complex samples phosphopeptides were obtained after overnight Lysyl endopeptidase/Trypsin digestion. A nano-emitter was used for direct infusion of casein through ESI into a Thermo Exploris 480 mass spectrometer. For LC-MS/MS runs the peptide mixtures corresponding to 1-2 μg proteins were injected via nanoLC Ultima 3000 coupled with a Pepmap column (75 μm x 150 mm) to the MS. Gradients of 80% acetonitrile in 0.1% formic acid was performed in 90 minutes at 300 nL/min flow rate. FAIMS experiments were performed at high (70^{o}C inner and 100^{o}C outer voltages) and low (100^{o}C inner and outer voltages) resolution and at compensation voltages as described along the text. Compensation voltages were scanned at 1-Volt steps. The mass spectrometer was operated in data dependent analysis (DDA) mode with dynamic exclusion of 30 s and full-scan MS spectra (m/z 350–1400) with resolution of 60,000 (m/z 200), followed by fragmentation of the most intense ions within 1 s cycle time with high energy collisional dissociation (HCD), normalized collision energy (NCE) of 30.0 and resolution of 15,000 (m/z 200) in MS/MS scans.

Peptide/protein identification was performed by Proteome Discoverer v.2.4.0.287 (Thermo Scientific, USA) using the Swissprot database (May/2018, 20348 sequences) and defining as fixed modification the cysteine carbamidomethylation and dynamic the deamidation (N/Q), methionine oxidation and phosphorylation (S/T/Y). A false discovery rate of 0.05 was considered for proper identification, but further inspection of every MS/MS assignment was performed keeping only those spectra with sufficient y" and b ion series that covered at least partial peptide sequences.

Data availability

Data will be made available on request.

Acknowledgements

The authors thank the collaboration of Jie Dai for the excellent technical assistance. This work was financed through the collaboration program between CIGB and CCBJIC.

Declaration of Competing Interest

None

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2021.104102.

References

[1] I.A. Buryakov, E.V. Krylov, E.G. Nazarov, U.K. Rasulev, A new method of separation of multi-atomic ions by mobility at atmospheric pressure using a high-frequency amplitude-asymmetric strong electric field, Int. J. Mass Spectrom. Ion Processes 128 (1993) 143–148.

[2] R.W. Purves, R. Guevremont, Electrospray ionization high-field asymmetric waveform ion mobility spectrometry-mass spectrometry, Anal Chem 71 (13) (1999) 2346–2357, https://doi.org/10.1021/ac981380y.

[3] Hebert AS, Prasad S, Belford MW, et al. Comprehensive Single-Shot Proteomics with FAIMS on a Hybrid Orbitrap Mass Spectrometer. Anal Chem. 2018;90(15): 9529-9537. doi:10.1021/acs.analchem.8b02233.

[4] D.K. Schweppe, S. Prasad, M.W. Belford, et al., Characterization and Optimization of Multiplexed Quantitative Analyses Using High-Field Asymmetric-Waveform Ion Mobility Mass Spectrometry [published correction appears in Anal Chem. 2020 Mar 17;92(6):4690], Anal Chem 91 (6) (2019) 4010–4016, https://doi.org/10.1021/acs.analchem.8b05399.

[5] D.B. Bekker-Jensen, A. Martínez-Val, S. Steigerwald, et al., A Compact Quadrupole-Orbitrap Mass Spectrometer with FAIMS Interface Improves Proteome Coverage in Short LC Gradients, Mol Cell Proteomics 19 (4) (2020) 716–729, https://doi.org/10.1074/mcp.TIR119.001906.

[6] A.A. Shvartsburg, A.J. Creese, R.D. Smith, H.J. Cooper, Separation of peptide isomers with variant modified sites by high-resolution differential ion mobility spectrometry, Anal Chem 82 (19) (2010) 8327–8334, https://doi.org/10.1021/ac101878a.

[7] A.J. Creese, J. Smart, H.J. Cooper, Large-scale analysis of peptide sequence variants: the case for high-field asymmetric waveform ion mobility spectrometry, Anal Chem 85 (10) (2013) 4836–4843, https://doi.org/10.1021/ac400646m.

[8] H. Zhao, D.L. Cunningham, A.J. Creese, J.K. Heath, H.J. Cooper, FAIMS and Phosphoproteomics of Fibroblast Growth Factor Signaling: Enhanced Identification of Multiply Phosphorylated Peptides, J Proteome Res 14 (12) (2015) 5077–5087, https://doi.org/10.1021/acs.jproteome.5b00713.

[9] G. Bridon, E. Bonneil, T. Muratore-Schroeder, O. Caron-Lizotte, P. Thibault, Improvement of phosphoproteome analyses using FAIMS and decision tree fragmentation. application to the insulin signaling pathway in Drosophila melanogaster S2 cells, J Proteome Res 11 (2) (2012) 927–940, https://doi.org/10.1021/pr200722s.

[10] L.K. Muehlbauer, A.S. Hebert, M.S. Westphall, E. Shishkova, J.J. Coon, Global Phosphoproteome Analysis Using High-Field Asymmetric Waveform Ion Mobility Spectrometry on a Hybrid Orbitrap Mass Spectrometer, Anal Chem 92 (24) (2020) 15959–15967, https://doi.org/10.1021/acs.analchem.0c03415. Dec 15.

[11] J.R. Murillo, L. Goto-Silva, A. Sánchez, Nogueira FCS, G.B. Domont, M. Junqueira, Quantitative proteomic analysis identifies proteins and pathways related to neuronal development in differentiated SH-SY5Y neuroblastoma cells, EuPA Open Proteom 16 (2017) 1–11, https://doi.org/10.1016/j.euprot.2017.06.001.

[12] S.D. Garbis, T.I. Roumeliotis, S.I. Tyritzis, K.M. Zorpas, K. Pavlakis, C. A. Constantinides, A novel multidimensional protein identification technology approach combining protein size exclusion prefractionation, peptide zwitterion-ion hydrophilic interaction chromatography, and nano-ultraperformance RP chromatography/nESI-MS2 for the in-depth analysis of the serum proteome and phosphoproteome: application to clinical sera derived from humans with benign prostate hyperplasia, Anal Chem 83 (3) (2011 Feb 1) 708–718, https://doi.org/10.1021/ac102075d.

[13] J.A. Jaros, D. Martins-de-Souza, H. Rahmoune, M. Rothermundt, F.M. Leweke, P. C. Guest, S. Bahn, Protein phosphorylation patterns in serum from schizophrenia patients and healthy controls, J Proteomics (2012), https://doi.org/10.1016/j.jprot.2012.05.027. Dec 5;76 Spec No.:43-55. Epub 2012 May 26. PMID: 22641159.

[14] T.E. Thingholm, M.R. Larsen, The Use of Titanium Dioxide for Selective Enrichment of Phosphorylated Peptides, Methods Mol Biol 1355 (2016) 135–146, https://doi.org/10.1007/978-1-4939-3049-4_9.

V. Besada[a,b,*], Y. Ramos[a], L.A. Espinosa[a], W. Fu[b], Y. Perera[a,b], L. J. González[a]

[a] *Mass Spectrometry Laboratory, Department of Proteomics, Center for Genetic Engineering and Biotechnology (CIGB), Havana CP: 10600, Cuba*
[b] *Proteomics Laboratory, China-Cuba Biotechnology Joint Innovation Center (CCBJIC), Yongzhou Zhong Gu Biotechnology Co., Ltd, Yangjiaqiao Street, Lengshuitan District, Yongzhou City, Hunan Province CP: 425000, China*

[*] Corresponding author at: Mass Spectrometry Laboratory, Department of Proteomics, Center for Genetic Engineering and Biotechnology (CIGB), Havana, Cuba. CP: 10600.
E-mail addresses: vladimir.besada@cigb.edu.cu (V. Besada), yassel.ramos@cigb.edu.cu (Y. Ramos), luis.espinosa@cigb.edu.cu (L.A. Espinosa), fu@ccbjic.com (W. Fu), yasser.perera@cigb.edu.cu (Y. Perera), luis.javier@cigb.edu.cu (L.J. González).