Published in final edited form as:

Anal Biochem. 2011 November 15; 418(2): 197–203. doi:10.1016/j.ab.2011.07.026.

Sequential Abundant Ion Fragmentation Analysis (SAIFA): An alternative approach for phosphopeptide identification using an ion trap mass spectrometer

Marla Chesnik^{1,2}, Brian Halligan², Michael Olivier^{2,3}, and Shama P. Mirza^{*,1,2}

¹Department of Biochemistry, Medical College of Wisconsin, Milwaukee WI 53226

²Biotechnology & Bioengineering Center, Medical College of Wisconsin, Milwaukee WI 53226

³Department of Physiology, Medical College of Wisconsin, Milwaukee WI 53226

Abstract

Phosphorylation has been the most studied of all the post-translational modifications of proteins. Mass spectrometry has emerged as a powerful tool for phosphomapping on proteins/peptides. Collision-induced dissociation (CID) of phosphopeptides leads to the loss of phosphoric or metaphosphoric acid as a neutral molecule, giving an intense neutral loss product ion in the mass spectrum. Dissociation of the neutral loss product ion identifies peptide sequence. This method of data-dependent constant neutral loss (DDNL) scanning analysis has been commonly used for mapping phosphopeptides. However, preferential losses of groups other than phosphate are frequently observed during CID of phosphopeptides. Ions that result from such losses are not identified during DDNL analysis due to predetermined scanning for phosphate loss. In this study, we describe an alternative approach for improved identification of phosphopeptides by sequential abundant ion fragmentation analysis (SAIFA). In this approach, there is no predetermined neutral loss molecule, thereby undergoing sequential fragmentation of abundant peak, irrespective of the moiety lost during CID. In addition to improved phosphomapping, the method increases the sequence coverage of the proteins identified, thereby increasing the confidence of protein identification. To the best of our knowledge, this is the first report to use SAIFA analysis for phosphopeptide identification.

Keywords

Phosphoproteomics; ion trap-mass spectrometry; sequential abundant ion fragmentation; data-dependent neutral loss scanning; posttranslational modification

Introduction

Phosphoproteins are the key regulators of cellular function. About one third of the proteins present in a typical mammalian cell are covalently bound to phosphate, and are

© 2011 Elsevier Inc. All rights reserved.

*Address for Correspondence: Shama P. Mirza, Ph.D., Assistant Professor, Department of Biochemistry, Biotechnology & Bioengineering Center, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee WI 53226 USA, Ph: 414-955-2231, Fax: 414-955-6568.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

phosphorylated at one time or the other. [1; 2; 3; 4] In addition to various cellular mechanisms like enzyme activity, protein interactions, or movement of proteins between subcellular compartments, many human diseases have also been recognized to be associated with abnormal phosphorylation of cellular proteins. [3; 5; 6]

In eukaryotes, the hydroxyl (-OH) groups of serine, threonine and tyrosine residues are the most common targets for phosphorylation. Due to phosphorylation, the molecular mass of the protein/peptide increases by 80 Da for each site of modification. This increase in molecular mass can be measured by mass spectrometry (MS), which has become the most common approach for phosphopeptide identification in the recent years[7]. However, confident identification of phosphopeptides by mass spectrometry has remained a challenge due to low stoichiometry, inefficient ionization, and signal suppression of phosphopeptides in presence of non-phosphopeptides. Recent advancements in phosphopeptide enrichment strategies have overcome these problems to some extent[8; 9; 10; 11; 12; 13].

However, even after enrichment, the difficulty in identifying phosphopeptides remains because of the formation of ambiguous spectra due to the loss of phosphate moiety during tandem mass spectrometry. Collision-induced dissociation (CID) of phosphopeptides usually removes the phosphate group as a neutral molecule and generates an intense peak from the resulting peptide ion, leaving other fragment ions at very low intensity in the mass spectrum.

Traditionally, the loss of a phosphate group as a neutral molecule (H_3PO_4 , 98 Da or HPO_3 , 80 Da) has been used as a signature for the identification of phosphopeptides. In a method commonly known as data-dependent constant neutral loss (DDNL) scanning, the abundant neutral loss peak is further fragmented for sequence identification[10; 14]. However, other non-phosphate modifications are also lost during CID, but these remain unidentified due to the requirement for loss of a phosphate group. Hence a method that is not biased towards identifying the neutral loss of the phosphate group enhances both the identification of proteins/peptides and the sites of phosphorylation or other protein modifications.

Advanced methods such as electron-transfer dissociation[15; 16; 17; 18] and electron-capture dissociation[19; 20; 21; 22; 23] have been developed for the gentle fragmentation of phosphopeptides to keep the phosphate moiety intact. However, these methods require additional instrumentation. In this study, we demonstrate that phosphopeptide identification can be significantly improved on an ion-trap instrument using a sequential abundant ion fragmentation analysis. As shown in our approach, the SAIFA method identified phosphopeptides that are missed by standard DDNL scanning approach as demonstrated from alpha-casein, a standard phosphoprotein and human liver carcinoma (HepG2) cells to represent a complex proteome.

Experimental Methods

Materials and Reagents

All chemicals and material were purchased from Sigma-Aldrich Corporation, St. Louis, MO, USA, unless specified otherwise. Dithiothreitol (DTT) and micro BCA assay kit were purchased from Pierce, Rockford, IL, USA. Mass spectrometry-grade trypsin (Trypsin Gold) used was obtained from Promega, Madison, WI, USA. Methanol, acetonitrile, and water were HPLC grade solvents from Burdick & Jackson, Muskegon, MI, USA.

Instrumentation

Liquid chromatography (LC) – mass spectrometry experiments were performed on an LTQ XL ion trap mass spectrometer (ThermoElectron, USA) coupled with a Surveyor Plus HPLC system (ThermoElectron, USA) equipped with an autosampler. The instrument was

interfaced with a capillary column (100 × 0.1 mm), in-house packed with 5 μm C₁₈ RP particles (Luna C₁₈, Phenomenex, USA). The fused silica capillaries (Polymicro Technologies, AZ, USA) for the columns were pulled by a micropipette puller P-2000 (Sutter Instrument Company, CA, USA) and packed with C₁₈ resin using a bomb-loader.

In-solution protein digestion

Protein samples were dissolved in 50 mM ammonium bicarbonate and 1M urea. Alpha-casein (1 pmol) was reduced and alkylated with DTT and iodoacetamide (IAA) at 10 mM and 55 mM concentration, respectively. Membrane proteins from HepG2 cells were extracted as described previously[24]. Protein (50 μg total protein as measured by micro BCA assay) was reduced and alkylated with DTT and IAA as mentioned above. The denatured protein was digested in-solution by the addition of trypsin (1:20 protease:protein) and incubating the reaction mixture overnight at 37°C. The protein digestion was stopped by the addition of 1% formic acid.

Phosphopeptide enrichment

The HepG2 tryptic peptides were reconstituted in 3% formic acid (pH 2) for phosphopeptide enrichment using Supel-Tips Ti and Supel-Tips Zr in tandem as per the manufacturer's instructions. Briefly, the Supel-Tips are equilibrated with 3% formic acid by aspirating and dispensing (A/D) for three times. The peptide sample is aspirated into the tip and the A/D cycle is repeated ten times, and washed with DI water three times. The phosphopeptides enriched on the Supel-Tips are eluted into 20 μl of 0.5% piperidine (pH 11.5) into a microcentrifuge tube by five or more A/D cycles. The sample is then dried in a speedvac and reconstituted in buffer A (5% acetonitrile in 0.1% formic acid) for mass spectral analysis.

Nano-HPLC-ESI mass spectrometry

The protein digests were analyzed using an ion trap LTQ XL mass spectrometer interfaced with a nanoHPLC system through an electrospray ionization (ESI) source. The samples were loaded through an autosampler onto a C₁₈ capillary column. The solvents A and B used for chromatographic separation of peptides were 5% acetonitrile in 0.1% formic acid and 95% acetonitrile in 0.1% formic acid, respectively. The peptides injected onto the microcapillary column were resolved at the rate of 200nl/min, by the following gradient conditions: 0-30min 0-5% B, 30-180 min 5-35% B, 180-240 min 35-65% B, 240-250 min 65-100% B. 100% B was held for 10 min, then switched to 100% A and held for another 40 min.

The ions eluted from the column were electrosprayed at a voltage of 1.8 kV. The capillary voltage was 45 V and the temperature was kept at 200°C. No auxiliary or sheath gas was used. Helium was used in the trap, which was also used as a collision gas for fragmentation of ions. Data were acquired in two different modes: 1) Sequential abundant ion fragmentation analysis, 2) Data-dependent constant neutral loss scanning for the loss of phosphate moiety.

Sequential abundant ion fragmentation analysis—In this approach, the instrument is cycled through acquisition of a full-scan mass spectrum (*m/z* 300-2000) followed by MS/MS of the most abundant ion from full-scan mass spectrum. Sequential fragmentation of the most abundant ion from the previous scan is carried out through the fourth stage (MS⁵) fragmentation (Figure 1). To retain spectral quality, the minimum signal threshold is set at 500 and fragmentation is allowed only if the signal is above the set signal intensity. Dynamic exclusion is enabled for 30 seconds. The chromatographic and mass spectral functions are controlled by the Xcalibur data system (ThermoFinnigan, Palo Alto, CA).

Data-dependent constant neutral loss scanning—The method is developed based on the loss of the phosphate group upon CID of phosphopeptides. The DDNL scanning involves a full scan mass spectrum (m/z 300–2000) followed by MS/MS by CID of the most abundant ion from MS¹. Whenever a loss of phosphate group as a neutral molecule is observed (98 for +1, 49 for +2 and 32.7 for +3 charged ions), third stage fragmentation (MS³) is triggered on the dephosphorylated peptide. Dynamic exclusion is enabled for 30 seconds. The chromatographic and mass spectral functions were controlled by the Xcalibur data system (ThermoFinnigan, Palo Alto, CA).

Data Analysis

In order to search the MSⁿ spectra using conventional search engines such as MASCOT[25], the individual MSⁿ sub-spectra were combined so as to mimic the situation in which all of the fragmentation had occurred in a single stage. To do this, the individual spectra were extracted from the raw data file as a Mascot Generic Format (.mgf) file using the `extract_ms` program provided by Thermo Fisher and a modified version of the .mgf creation script provided by Matrix Science. The .mgf file was then processed using the `combine_mgf` program, available from the MCW Proteomics Center (<http://proteomics.mcw.edu>). This program collects the spectra into groups of fragmentation spectra that arise from initial MS/MS spectra. If more than one spectrum is found in a group, ie. MSⁿ fragmentation was performed, the spectra are combined by binning the normalized intensity values from all spectra in the group into 0.01 Da mass bins. The data is filtered to remove low intensity peaks and smoothed to produce a composite spectrum for the group. The composite spectra, and optionally the individual subspectra, are output into a new .mgf file. The resulting .mgf file produced by `combine_mgf` was searched using MASCOT algorithm against the Uniprot database v49.1 (bovine for alpha-casein and human for membrane proteins). The search was limited to tryptic peptides, and allowed five missed cleavages. Methionine oxidation, carbamidomethylation of cysteine, and phosphorylation on serine, threonine and tyrosine were searched for as variable modifications. Mass tolerance was set to 2 Da for parent ion mass and 0.6 Da for fragment ion mass. Peptides identified with MASCOT score of 50 or above were considered potential positive identifications. All proteins were identified by two or more peptides, and those identified with a single peptide were included in the analysis summary only if identified in two or more scans. Finally, the peptides listed were manually verified for correct identification by comparing the experimental spectra with the theoretical *b* and *y* ion spectra.

Results

Sequential abundant ion fragmentation analysis (SAIFA) and data-dependent constant neutral loss (DDNL) scanning methods were used for identifying phosphosites from alpha-casein, and membrane proteins from human liver carcinoma (HepG2) cells. The various aspects that are advantageous of using SAIFA approach are detailed.

Improved phosphopeptide identification

Initial experiments were carried out using commercially available alpha-casein as it is a highly phosphorylated protein with mono, di and multiphosphorylated peptides. Using our SAIFA approach, we identified ten peptides in total, of which four were identified to be phosphopeptides (two monophospho, one diphospho and one tetraphosphopeptide). The DDNL scanning method identified seven peptides of which three were phosphopeptides. The multiply phosphorylated peptide (QMEAEpSIpSpSpSEEIVPNSVEQK) was identified only by the SAIFA approach. The peptides that are identified by the two methods are shown in Table 1. It can be seen that the SAIFA method identified all four phosphopeptides with eight phosphosites on serine residues in alpha-casein.

In addition to identifying multiply-phosphorylated peptides, mono-phosphopeptides were also more reliably identified using this approach. With the DDNL approach, peptides with a different neutral loss molecule that does not match the loss of phosphate moiety will not undergo second stage excitation (MS^3), and can therefore never be identified. In our approach, such peptides can be identified, due to the unbiased fragmentation of the most abundant peak in the spectrum. We have analyzed membrane proteins from HepG2 cells using both the SAIFA and DDNL approaches. In these MS runs, a peptide at m/z 993.6 was observed to lose m/z 53 giving an intense peak at m/z 940.4 in MS^2 . In DDNL analysis, this peptide was not identified due to limited fragment ions in the MS^2 spectrum, and it was also not selected for further fragmentation due to non-predetermined neutral loss molecule. However, in SAIFA run, that peak was further fragmented and was identified as a peptide with sequence $KELIT_{cam}CPTPGCDG_pSGHV_pTGDYASHR$ (Figure 2). This peptide was doubly phosphorylated with the modifications at the S14 and T18 residues. In addition, cysteine residue (C6) is carbamidomethylated in this peptide due to the reaction with iodoacetamide during sample preparation (methods section). In SAIFA analysis the first stage fragmentation (MS^2) led to the loss of m/z 53. This is further fragmented and the resulting MS^3 gave the first phosphate neutral loss (m/z 49). This is further continued to lose another phosphate group (m/z 49) in MS^4 . The resulting peak at m/z 842.3 in MS^4 is excited further to fragment the peptide backbone for sequence identification. The loss of m/z 53 is not characterized in this study, but it is found to be highly labile modification on the peptide sequence that resembles thiocresyl or thioanisyl moiety (106 Da).

Increased sequence coverage

In addition to the identification of phosphopeptides, the SAIFA method also improves the sequence coverage of the proteins identified regardless of the presence or absence of posttranslational modifications (PTMs). During the analysis of alpha-casein, identification of one of the non-phosphorylated peptides (HIQKEDVPSEK) was possible only by our approach. The peptide is identified from MS^3 spectrum during SAIFA analysis. In the full MS scan, the peak at m/z 690.7 is fragmented to give an abundant ion at m/z 669.1, a loss of 43 Da possibly from a carbamylated (CONH) peptide. It is a common modification on primary amines due to the reaction of cyanate, especially when urea is used to dissolve protein samples.[26; 27] This peptide could not be identified by other MS approaches, which can only reach up to MS^2 . The loss in 43 Da was not identified by DDNL when scanning for phosphorylation and hence this peptide could not be identified. However, in the SAIFA analysis, it was further fragmented to give a rich daughter ion spectrum with several fragment ions and is identified as HIQKEDVPSEK peptide (Figure 3). Similarly, the peptide EGIHAQQK from alpha-casein with m/z 456 is losing a water molecule (-18 Da) on collision-induced dissociation (CID) giving an intense ion at m/z 447 in MS^2 spectrum (data not shown). The peptide is identified by SAIFA scanning only (Table 1). The sequence coverage of alpha-casein using SAIFA approach is 61% and using DDNL scanning is 52%.

Similarly, figure 2 shows an abundant neutral loss peak (loss of m/z 53; +2 ion) that is not a phosphate loss, and is only identified by our approach. As these examples show, the SAIFA approach clearly identifies more peptides, thereby increasing the sequence coverage during protein identification.

Application of SAIFA analysis to complex biological samples

Analysis of membrane proteins from HepG2 cells—To test the suitability of the MS approach to the analysis of global phosphorylation in a biological sample, we analyzed proteins isolated from human carcinoma cell (HepG2) membranes. Whole cell lysate from HepG2 cells was fractionated by sucrose gradient centrifugation to obtain the membrane fraction. The insoluble fraction was chloroform-extracted as described in our previous

work[24]. The tryptic digest of the membrane proteome was enriched for phosphopeptides using TiO_2 and ZrO_2 tips to capture as many phosphopeptides as possible. This overcomes the limitation of enriching mono or multiple modifications using each of these enrichment strategies in tandem. The enriched peptides were analyzed by both SAIFA and DDNL approaches to compare the methods.

In the SAIFA analysis, 211 proteins were identified, whereas DDNL scanning identified 276 proteins from the same amount of sample injected into the mass spectrometer. However, the number of phosphopeptides identified using our approach was higher than from DDNL scanning. The SAIFA approach identified 77 phosphopeptides (43 were mono-, 20 di- and 14 multi-phosphopeptides). Using DDNL approach, 67 phosphopeptides were identified, of which 42 were mono-, 17 di- and 8 multi-phosphopeptides (Table 2).

Comparing the efficiencies of the two methods, the average number of peptides identified per protein was higher using the SAIFA approach. However, the average number of scans per protein was lower compared to DDNL scanning. An average of three peptides and six scans were identified per protein using SAIFA, compared to two peptides and eight scans per protein identified using DDNL scanning (Figure 4).

Discussion

The data dependent constant neutral loss (DDNL) scanning has been an established method for phosphomapping using mass spectrometry. However, the method is limited to identifying peptides with a neutral loss of phosphoric acid during initial fragmentation. Though several improvements have been made such as multi-stage activation during neutral loss scanning,[28] the method is limited to peptides that show neutral loss of phosphate moiety during excitation in MS^2 . During fragmentation, peptides often lose different ions that are either attached due to modifications, or represent the loss of a residue from the backbone sequence itself.[29; 30] Most of the sample preparation techniques for protein identification in bottom-up approaches involve the use of iodoacetamide for alkylating the proteins before enzymatic digestion. Iodoacetamide usually alkylates the thiol groups that are opened by reducing the disulfide bonds of cysteines. During this process, methionine also can get alkylated to form S-carbamidomethylmethionine (camMet) as a side reaction. [31] Upon collision-induced dissociation, carbamidomethylated peptides lose 2-(methylthio)acetamide ($\text{C}_3\text{H}_7\text{NOS} = 105.025 \text{ Da}$) as a neutral molecule. This can be observed as loss of 105, 52.5 or 35 mass units from +1, +2 or +3 charged ions. In addition to carbamylation of proteins *in vivo*[26; 32; 33], the use of urea for dissolving protein during sample preparation may lead to carbamylation of primary amines that adds 43 mass units (CONH) to the peptide[34]. This modification is usually observed when old stocks of urea are used, or if urea is exposed to elevated temperatures during sample preparation.

Under electrospray ionization conditions, peptides form sodium and/or potassium adducts. [35; 36] When these adducts are selected as precursor ions for fragmentation, the alkali metal ion is easily lost and the side-chain fragmentation is diminished. Thus, a loss of 22 or 38 Da is often observed from sodium or potassium adducted peptides. Even without any chemical modifications or adductions, peptides lose water or amine as neutral molecules from acidic and basic amino acids, respectively.[37; 38] These are some of the common neutral loss molecules that are observed during collision-induced dissociation of peptide samples. However, including all these ions in the neutral loss mass list in the data-dependent scanning is not possible due to the limitation in the number of ions that can be listed. Usually, for phosphopeptide identification, only the losses of 98, 49 and 32.7 (+1, +2 and +3 charged ions) are screened for during analysis. Hence, peptides that exhibit preferential loss of the above-mentioned moieties before the phosphate group is lost are not identified by

DDNL method. In contrast, SAIFA fragments the most abundant ion in the spectrum independent of a specific neutral loss, and is able to identify these peptides.

Furthermore, the SAIFA method was found to be especially useful for the identification of multiply phosphorylated peptides, especially when the phosphate groups are lost one after the other during excitation. During the DDNL scanning of multiply phosphorylated peptides, the richness (number and intensity) of daughter-ion peaks does not improve even after the initial phosphate group loss. This is due to the loss of other phosphate groups in the next stage fragmentation, rather than the production of *b* and *y* ions, making the peptide identification difficult. Hence, sequential fragmentation of the most abundant peak leads to the formation of informative *b* and *y* ions helping to identify these peptides. Using the exceptional capability of exciting an ion to 12th stage in ion traps, we developed the SAIFA method by exciting the ion up to fourth stage to get better sequence information. However, since the ion current decreases at each stage of fragmentation, we set a threshold value of 500 ion counts for daughter ion spectra. If the set threshold is reached, it does not proceed for further fragmentation and returns to the next cycle of analysis, thereby retaining the data quality.

Though SAIFA analysis certainly is better than DDNL approach in identifying proteins and their PTMs, there are certain limitations that need to be considered. The duty cycle of SAIFA method (the time over which the experiment takes place) is relatively longer than that of DDNL scanning, especially for the peptides that undergo fragmentation up to third or fourth stage. Hence, the time spent on each peptide is longer, thereby missing the peptides that are eluted within the fragmentation process of the SAIFA cycle. This results in loss of protein identification data, especially during the analysis of complex biological samples. Nevertheless, this limitation can be overcome by using longer LC gradients, repeated analysis or prior fractionation of protein/peptide samples. Peptides can be better resolved with an increase in LC gradient time; thereby more peptides can be analyzed by MS and thus data loss can be minimized. Another approach is to do repeat analysis, possibly by excluding the precursor ions that resulted in positive peptide identification in the previous MS run. However, it should be noted that the difference in protein identification is not very significant comparing the two approaches and SAIFA definitely identified more number of modified residues, which is the focus of the methodology.

Several studies have shown the use of sequential product ion MS experiments for structural elucidation of small molecules[39; 40]. However, to our knowledge, this is the first report to use sequential abundant ion fragmentation analysis in proteomics studies, specifically for phosphopeptide identification. Overall, the SAIFA approach appears to be better than data-dependent neutral loss scanning in its ability to identify phosphopeptides. As the method is not biased towards any neutral loss molecule, and there is no predetermined mass scanning, the method can be applied not only to the identification of phosphorylation, but also other protein PTMs. The real power of the method underlies in dealing with unknown posttranslational modifications on proteins. Even without prior knowledge of the modification on a protein, our SAIFA approach has the potential to identify it. Several different modifications can be identified from a single mass spectral run, thus handling lesser amounts of sample, which is vital for precious samples like clinical tissue biopsies, cerebrospinal fluid etc. Further investigation of identifying various modifications from a single mass spectral analysis using SAIFA approach is underway.

Acknowledgments

This work was funded by National Heart, Lung and Blood Institute of the National Institutes of Health (N01-HV-28182) and Wisconsin Center of Excellence in Genomics Science (NIH/NHGRI - P50 HG004952). The LTQ-XL mass spectrometer used was partially funded by Advanced Healthier Wisconsin (AHW-5520058).

References

1. Macek B, Mann M, Olsen JV. Global and Site-Specific Quantitative Phosphoproteomics: Principles and Applications. *Annu Rev Pharmacol Toxicol*. 2008
2. Lorts A, Burroughs T, Shanley T. Elucidating the role of reversible protein phosphorylation in sepsis-induced myocardial dysfunction. *Shock*. 2008
3. Zhong J, Molina H, Pandey A. Phosphoproteomics. *Curr Protoc Protein Sci Chapter*. 2007; Chapter 24(Unit 24 4)
4. Hubbard MJ, Cohen P. On target with a new mechanism for the regulation of protein phosphorylation. *Trends Biochem Sci*. 1993; 18:172–7. [PubMed: 8392229]
5. Goedert M, Jakes R, Crowther RA, Six J, Lubke U, Vandermeeren M, Cras P, Trojanowski JQ, Lee VM. The abnormal phosphorylation of tau protein at Ser-202 in Alzheimer disease recapitulates phosphorylation during development. *Proc Natl Acad Sci U S A*. 1993; 90:5066–70. [PubMed: 8506352]
6. Puig B, Vinals F, Ferrer I. Active stress kinase p38 enhances and perpetuates abnormal tau phosphorylation and deposition in Pick's disease. *Acta Neuropathol*. 2004; 107:185–9. [PubMed: 14689204]
7. Salih E. Phosphoproteomics by mass spectrometry and classical protein chemistry approaches. *Mass Spectrom Rev*. 2005; 24:828–46. [PubMed: 15538747]
8. Reinders J, Sickmann A. State-of-the-art in phosphoproteomics. *Proteomics*. 2005; 5:4052–61. [PubMed: 16196093]
9. Barnouin KN, Hart SR, Thompson AJ, Okuyama M, Waterfield M, Cramer R. Enhanced phosphopeptide isolation by Fe(III)-IMAC using 1,1,1,3,3,3-hexafluoroisopropanol. *Proteomics*. 2005; 5:4376–88. [PubMed: 16294313]
10. Yu LR, Zhu Z, Chan KC, Issaq HJ, Dimitrov DS, Veenstra TD. Improved titanium dioxide enrichment of phosphopeptides from HeLa cells and high confident phosphopeptide identification by cross-validation of MS/MS and MS/MS/MS spectra. *J Proteome Res*. 2007; 6:4150–62. [PubMed: 17924679]
11. Mirza SP, Olivier M. Methods and approaches for the comprehensive characterization and quantification of cellular proteomes using mass spectrometry. *Physiol Genomics*. 2008; 33:3–11. [PubMed: 18162499]
12. Mazanek M, Mituloviae G, Herzog F, Stingl C, Hutchins JR, Peters JM, Mechtler K. Titanium dioxide as a chemo-affinity solid phase in offline phosphopeptide chromatography prior to HPLC-MS/MS analysis. *Nat Protoc*. 2007; 2:1059–69. [PubMed: 17545998]
13. Mazanek M, Roitinger E, Hudecz O, Hutchins JR, Hegemann B, Mitulovic G, Taus T, Stingl C, Peters JM, Mechtler K. A new acid mix enhances phosphopeptide enrichment on titanium- and zirconium dioxide for mapping of phosphorylation sites on protein complexes. *J Chromatogr B Analyt Technol Biomed Life Sci*. 878:515–24.
14. Beausoleil SA, Jedrychowski M, Schwartz D, Elias JE, Villen J, Li J, Cohn MA, Cantley LC, Gygi SP. Large-scale characterization of HeLa cell nuclear phosphoproteins. *Proc Natl Acad Sci U S A*. 2004; 101:12130–5. [PubMed: 15302935]
15. Coon JJ, Shabanowitz J, Hunt DF, Syka JE. Electron transfer dissociation of peptide anions. *J Am Soc Mass Spectrom*. 2005; 16:880–2. [PubMed: 15907703]
16. Gunawardena HP, Emory JF, McLuckey SA. Phosphopeptide anion characterization via sequential charge inversion and electron-transfer dissociation. *Anal Chem*. 2006; 78:3788–93. [PubMed: 16737238]
17. Syka JE, Coon JJ, Schroeder MJ, Shabanowitz J, Hunt DF. Peptide and protein sequence analysis by electron transfer dissociation mass spectrometry. *Proc Natl Acad Sci U S A*. 2004; 101:9528–33. [PubMed: 15210983]
18. Chi A, Huttenhower C, Geer LY, Coon JJ, Syka JE, Bai DL, Shabanowitz J, Burke DJ, Troyanskaya OG, Hunt DF. Analysis of phosphorylation sites on proteins from *Saccharomyces cerevisiae* by electron transfer dissociation (ETD) mass spectrometry. *Proc Natl Acad Sci U S A*. 2007; 104:2193–8. [PubMed: 17287358]

19. Creese AJ, Cooper HJ. Liquid chromatography electron capture dissociation tandem mass spectrometry (LC-ECD-MS/MS) versus liquid chromatography collision-induced dissociation tandem mass spectrometry (LC-CID-MS/MS) for the identification of proteins. *J Am Soc Mass Spectrom.* 2007; 18:891–7. [PubMed: 17350280]
20. Shi SD, Hemling ME, Carr SA, Horn DM, Lindh I, McLafferty FW. Phosphopeptide/phosphoprotein mapping by electron capture dissociation mass spectrometry. *Anal Chem.* 2001; 73:19–22. [PubMed: 11195502]
21. Stensballe A, Jensen ON, Olsen JV, Haselmann KF, Zubarev RA. Electron capture dissociation of singly and multiply phosphorylated peptides. *Rapid Commun Mass Spectrom.* 2000; 14:1793–800. [PubMed: 11006587]
22. Sweet SM, Cooper HJ. Electron capture dissociation in the analysis of protein phosphorylation. *Expert Rev Proteomics.* 2007; 4:149–59. [PubMed: 17425452]
23. Zubarev RA. Electron-capture dissociation tandem mass spectrometry. *Curr Opin Biotechnol.* 2004; 15:12–6. [PubMed: 15102460]
24. Mirza SP, Halligan BD, Greene AS, Olivier M. Improved method for the analysis of membrane proteins by mass spectrometry. *Physiol Genomics.* 2007; 30:89–94. [PubMed: 17341690]
25. Perkins DN, Pappin DJ, Creasy DM, Cottrell JS. Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis.* 1999; 20:3551–67. [PubMed: 10612281]
26. Lapko VN, Smith DL, Smith JB. In vivo carbamylation and acetylation of water-soluble human lens alphaB-crystallin lysine 92. *Protein Sci.* 2001; 10:1130–6. [PubMed: 11369851]
27. Lapko VN, Smith DL, Smith JB. Methylation and carbamylation of human gamma-crystallins. *Protein Sci.* 2003; 12:1762–74. [PubMed: 12876325]
28. Schroeder MJ, Shabanowitz J, Schwartz JC, Hunt DF, Coon JJ. A neutral loss activation method for improved phosphopeptide sequence analysis by quadrupole ion trap mass spectrometry. *Anal Chem.* 2004; 76:3590–8. [PubMed: 15228329]
29. Martin DB, Eng JK, Nesvizhskii AI, Gemmill A, Aebersold R. Investigation of neutral loss during collision-induced dissociation of peptide ions. *Anal Chem.* 2005; 77:4870–82. [PubMed: 16053300]
30. Talaty ER, Chueachavalit C, Osburn S, Van Stipdonk MJ. Investigation of the neutral loss of a full amino acid mass during collision-induced dissociation of the b(3)+ ion derived from a model peptide containing a 4-aminobutyric acid residue. *Rapid Commun Mass Spectrom.* 2007; 21:2529–37. [PubMed: 17610213]
31. Kruger R, Hung CW, Edelson-Averbukh M, Lehmann WD. Iodoacetamide-alkylated methionine can mimic neutral loss of phosphoric acid from phosphopeptides as exemplified by nano-electrospray ionization quadrupole time-of-flight parent ion scanning. *Rapid Commun Mass Spectrom.* 2005; 19:1709–16. [PubMed: 15912474]
32. Wang Z, Nicholls SJ, Rodriguez ER, Kummu O, Horkko S, Barnard J, Reynolds WF, Topol EJ, DiDonato JA, Hazen SL. Protein carbamylation links inflammation, smoking, uremia and atherogenesis. *Nat Med.* 2007; 13:1176–84. [PubMed: 17828273]
33. Mun KC, Golper TA. Impaired biological activity of erythropoietin by cyanate carbamylation. *Blood Purif.* 2000; 18:13–7. [PubMed: 10686438]
34. Lippincott J, Apostol I. Carbamylation of cysteine: a potential artifact in peptide mapping of hemoglobins in the presence of urea. *Anal Biochem.* 1999; 267:57–64. [PubMed: 9918655]
35. Medzihradszky KF, Guan S, Maltby DA, Burlingame AL. Sulfopeptide fragmentation in electron-capture and electron-transfer dissociation. *J Am Soc Mass Spectrom.* 2007; 18:1617–24. [PubMed: 17629708]
36. Shirran S, Garnaup P, Daff S, McMillan D, Barran P. The formation of a complex between calmodulin and neuronal nitric oxide synthase is determined by ESI-MS. *J R Soc Interface.* 2005; 2:465–76. [PubMed: 16849206]
37. Paizs B, Suhai S. Towards understanding the tandem mass spectra of protonated oligopeptides. 1: mechanism of amide bond cleavage. *J Am Soc Mass Spectrom.* 2004; 15:103–13. [PubMed: 14698560]

38. Sun S, Yu C, Qiao Y, Lin Y, Dong G, Liu C, Zhang J, Zhang Z, Cai J, Zhang H, Bu D. Deriving the probabilities of water loss and ammonia loss for amino acids from tandem mass spectra. *J Proteome Res.* 2008; 7:202–8. [PubMed: 18092745]
39. Liu S, Cui M, Liu Z, Song F, Mo W. Structural analysis of saponins from medicinal herbs using electrospray ionization tandem mass spectrometry. *J Am Soc Mass Spectrom.* 2004; 15:133–41. [PubMed: 14766280]
40. Minamisawa T, Suzuki K, Hirabayashi J. Multistage mass spectrometric sequencing of keratan sulfate-related oligosaccharides. *Anal Chem.* 2006; 78:891–900. [PubMed: 16448065]

Figure 1. Sequential Abundant Ion Fragmentation Analysis: The method is unbiased towards neutral loss molecule and fragments the most abundant ion in the spectrum. Excitation is continued up to four stages, unless the signal intensity reaches the defined threshold.

Figure 3.

Identification of alpha-casein peptide HIQKEDVPSEER using SAIFA method. a) Full scan MS depicting m/z 690.7 that is fragmented in next scan, b) CID of m/z 690.7 giving an abundant ion at m/z 669.1 due to neutral loss of 43 mass units, c) CID of m/z 669.1 is used for the sequence identification.

Figure 4.

Plot representing the average number of peptides and average number of scans each protein is identified from using the SAIFA method and DDNL approach. The two methods are compared by the analysis of membrane proteins from HepG2 cells to validate the method for the analysis of complex protein mixtures.

Table 1

Identification of alpha-casein phosphopeptides using sequential abundant ion fragmentation analysis and data-dependent constant neutral loss scanning methods: A '✓' mark against the peptide represents identification of the peptide by the respective methods. A tetraphosphopeptide QMEAEpSIpSpSpSEEIVPNSVEQK is identified by the SAIFA approach, but not by DDNL scanning. A non-phosphopeptide HIQKEDVPSEK also is identified by SAIFA approach only. The sequence coverage of alpha-casein using SAIFA approach is 61% compared to 52% using DDNL scanning.

Peptide Sequence	MSF	DDNL
QMEAEpSIpSpSpSEEIVPNSVEQ K	✓	-
EPMIGVNQELAYFYPELFR	✓	✓
DIGpSEpSTEDQAMEDIK	✓	✓
HQGLPQEVLENENLLR	✓	✓
VPQLEIVNpSAEER	✓	✓
FFVAPFPEVFGK	✓	✓
YLGYLEQLLR	✓	✓
EGIHAQQK	✓	-
HIQKEDVPSEK	✓	-
VNELpSK	✓	✓

Table 2

Summary of the results obtained from sequential abundant ion fragmentation analysis and data-dependent constant neutral loss scanning methods for the analysis of complex protein mixtures obtained from HepG2 cell membranes. Fewer proteins are identified using the SAIFA approach, but the number of phosphopeptides identified is higher than in DDNL scanning method.

SAIFA analysis		DDNL scanning	
Proteins id:	211	Proteins id:	276
Peptides id:	678	Peptides id:	749
Phosphopeptides:		Phosphopeptides:	
mono	43	mono	42
di-	20	di	17
multi-	14	multi	08