Review Article

Strong Cation Exchange Chromatography in Analysis of Posttranslational Modifications: Innovations and Perspectives

Mariola J. Edelmann

Institute for Genomics, Bioinformatics & Biotechnology; Mississippi Agricultural and Forestry Experiment Station, Mississippi State University, 650 Stone Boulevard, MS 39762, USA

Correspondence should be addressed to Mariola J. Edelmann, mje100@mafes.msstate.edu

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Strong cation exchange (SCX) chromatography has been utilized as an excellent separation technique that can be combined with reversed-phase (RP) chromatography, which is frequently used in peptide mass spectrometry. Although SCX is valuable as the second component of such two-dimensional separation methods, its application goes far beyond efficient fractionation of complex peptide mixtures. Here I describe how SCX facilitates mapping of the protein posttranslational modifications (PTMs), specifically phosphorylation and N-terminal acetylation. The SCX chromatography has been mainly used for enrichment of these two PTMs, but it might also be beneficial for high-throughput analysis of other modifications that alter the net charge of a peptide.

1. Introduction

Posttranslational modifications (PTMs) regulate protein function, subcellular localization, and degradation. Although detection of the modified residues is crucial for understanding of the physiological roles of proteins as well as their mechanisms and pathways, it still represents a challenge. It is especially true in case of global proteome studies, in which hundreds or thousands of modified sites are monitored at the same time, and such analyses are becoming now more required due to an increasing popularity of the systems biology approaches. There is therefore a great demand for further development of efficient tools that could be used to characterize the entire complement of modified proteins in cells. Mass-spectrometry-based proteomics offers highly sensitive tools for modification mapping (reviewed in [1, 2]), where thousands of proteins can be concurrently analyzed to construct and validate comprehensive physiological models. Nevertheless, PTM analysis usually requires preceding enrichment methods due to the high complexity of the samples and the low abundance of modified peptides. In this paper I will discuss strong cation exchange (SCX) chromatography as an effective method for enrichment of PTMs, including phosphorylation and N-terminal acetylation (summarized in Table 1).

2. Principles of SCX Separation for Proteomic Applications

The SCX stationary phase usually contains aliphatic sulfonic acid groups that are negatively charged in aqueous solution, therefore tightly binding any strongly basic analytes. To recover the analyte, the resin is then washed with a solvent neutralizing this ionic interaction. Most tryptic peptides in acidic pH are characterized by a net charge of +2 and above, and they can be therefore separated by SCX from peptides possessing a net charge of +1, such as trypsin-generated phosphopeptides, C-terminal peptides, or peptides with blocked N-termini (i.e., peptides with blocked N-terminal free amine group, for instance, by N-acetylation), as well as from peptides containing higher charges, including ones containing missed cleavages and therefore more arginine and lysine residues [3]. Trypsin is not the only enzyme used to generate peptides for proteomic analyses, and other
Table 1: Overview of the SCX chromatography methods used for PTM analysis described in this paper.

| Sample type                          | Protein amount/cell number | A number of fractions collected | Mass spectrometer used | Experimental workflow | Other details | PTM analysis                  | References |
|--------------------------------------|---------------------------|---------------------------------|------------------------|-----------------------|---------------|--------------------------------|------------|
| HeLa cells                           | 8 mg protein              | 4 early fractions from each gel band | LCQ DECA XP ion trap   | SDS-PAGE (10 gel bands) → SCX → LC-MS/MS |               | 2002 phosphorylation sites identified from 967 proteins | [6]        |
| HeLa cells                           | 0.5 mg protein            | 10 fractions                    | LTQ-Orbitrap           | SCX → TiO₂ → LC-MS/MS |               | 722 nonredundant phosphorylation sites | [7]        |
| Human Chang liver cells              | 1.5 mg protein            | 40 fractions                    | Q-TOF and LCQ          | SCX → TiO₂ → LC-MS/MS | Lys-C digestion followed by trypsin digestion | 1035 phosphorylation sites from 607 phosphoproteins | [8]        |
| *Escherichia coli* cells             | 20 mg protein             | 10 fractions                    | LTQ-Orbitrap           | SCX → TiO₂ → LC-MS/MS |               | 81 phosphorylation sites from 79 proteins | [9]        |
| *Bacillus subtilis* cells            | 10 mg protein             | 15 fractions                    | LTQ-Orbitrap or LTQ-FT | SCX → TiO₂ → LC-MS/MS | Lys-C digestion followed by trypsin digestion | 78 phosphorylation sites from 78 proteins | [10]       |
| HeLa cells                           | 40 mg protein             | 25 fractions                    | QSTAR ELITE            | IMAC → SCX → LC-MS/MS |               | ~4512 phosphorylated sites        | [11]       |
| HEK293 cells                         | 1 mg protein              | 40 fractions                    | LTQ-Orbitrap           | SCX → LC-MS/MS        | Lys-N, Lys-C, and trypsin digestion | 5036 nonredundant phosphorylation sites | [4]        |
| Human embryonic stem cells           | 10 mg protein             | 12 fractions                    | LTQ-Orbitrap           | SCX → IMAC → LC-MS/MS |               | 10844 nonredundant phosphorylation sites | [12]       |
| Membrane fraction of human teratocarcinoma Nt2/d1 cells | 5 × 10⁶ cells | ~30 fractions                   | LTQ-Orbitrap           | SCX → LC-MS/MS        | Lys-C digestion followed by trypsin digestion | 116 N-acetylated sites | [13]       |
| HEK293 cells                         | 12.3 mg protein/6 × 10⁷ cells | 49 fractions                  | LTQ XL Linear ion trap | SCX → LC-MS/MS        |               | N-acetylated peptides                | [14]       |
| Cytoplasmic, nuclear, and membrane fractions from Kc 167 cells (*Drosophila melanogaster*) | n/a                        | n/a                             | HCT ion trap, LTQ linear ion trap, and XCT-Ultra ion trap | SCX → COFRADIC → LC-MS/MS |               | 861 N-acetylated sites                | [15]       |
| HEK293 cells                         | 1 mg protein              | ~40 fractions                   | LTQ-Orbitrap           | SCX → LC-MS/MS        | Lys-N, Lys-C, and trypsin digestion | 1391 N-acetylated peptides                | [16]       |

SCX: strong cation exchange; PTMs: post-translational modifications; IMAC: immobilized metal affinity chromatography; ETD: electron transfer dissociation; CID: collision-induced dissociation; COFRADIC: combined fractional diagonal chromatography; LC-MS/MS: liquid chromatography-tandem mass spectrometry; n/a: data not available.
proteolytic enzymes are also utilized, for instance, Lys-N, Lys-C, Glu-C, or elastase. Some of these enzymes can be used to obtain peptides characterized by specific, distinguishable net charges, which provide additional advantages for SCX separation of differently modified peptides [4, 5].

3. SCX in Phosphoproteome Studies

The complex system of signal transduction enables effective and targeted cell responses to external or internal stimuli, and PTMs play here a major role. Phosphorylation is one of the best characterized among these PTMs. This modification has been shown to affect approximately 30% of all the human proteins [17], although recent studies suggest that this percentage is even higher [18]. Protein phosphorylation has been associated with a wide range of cellular processes, such as proliferation, migration, differentiation, cell cycle progression, and apoptosis [19, 20]. The mass spectrometric analysis of phosphopeptides can be very challenging for several reasons. Firstly, these negatively charged peptides are not easily analyzed in a positive mode, and this method of ionization is typically used in the electrospray ionization- (ESI- ) based peptide mass spectrometry. Secondly, the hydrophilic property of phosphopeptides lowers their binding to the C18-based trapping columns used for purification of the peptide sample prior to its separation by HPLC. Thirdly, due to the ion suppression, detection of phosphopeptides by a mass spectrometer is problematic, especially in the complex samples containing unmodified or other more easily detectable peptides. Some of the above problems are still questionable, as discussed in [21], but the sole complexity of the peptide samples usually calls for prior enrichment in order to specifically select for phosphopeptides/phosphoproteins. These techniques include purification by phosphospecific antibodies [22, 23] and use of immobilized metal affinity chromatography (IMAC), where negatively charged phosphopeptides bind to positively charged metal ions, such as Fe$^{3+}$, Ga$^{3+}$, or Zr$^{4+}$ [2, 24–26], as well as separation by titanium dioxide (TiO$_2$) columns [27, 28]. Second chromatography dimension is also frequently utilized, and this might include hydrophilic interaction chromatography (HILIC) [29–32], electrostatic repulsion hydrophilic interaction chromatography (ERLIC) [11], and here discussed SCX.

The SCX chromatography is the most widely used HPLC fractionation method, and it has also found applications in phosphoproteomics (Table 1). The peptides are eluted from an SCX column using a low-pH buffer, and in these conditions phosphopeptides have a net charge of 0 or +1, as opposed to a majority of other tryptic peptides characterized by +2 or higher net charges. Therefore, most of the phosphopeptides elute in the first few SCX fractions, although this depends on the type of gradient used and on a number of fractions collected [3, 33–35].

Because of these features, the SCX chromatography can be employed as a stand-alone method for phosphopeptide mapping, as demonstrated by Beausoleil et al. [6], who used it without additional IMAC or TiO$_2$ phosphopeptide enrichment. In this experiment 8 mg of the nuclear protein extract derived from HeLa cells was first separated by SDS-PAGE, then ten gel bands were digested by trypsin and the peptides were resolved by SCX, where only the first four fractions were collected for subsequent LC-MS/MS analysis by LCQ DECA XP ion trap. This simple workflow allowed for identification of 2002 phosphorylation sites from 967 proteins. Adding another dimension to this separation, such as an off-gel fractionation, which is based on the isoelectric point of peptides/proteins, might result in even larger number of identified phosphopeptides without enrichment by IMAC, TiO$_2$, or phosphospecific antibodies.

Due to the complexity of the protein samples, SCX-based phosphopeptide separation alone is often ineffective, and the secondary phosphopeptide enrichment is necessary for identification of a larger number of phosphorylated residues [33, 36, 37]. For instance, an automated SCX-TiO$_2$-RP-LC-MS/MS method was used to separate HeLa protein digest, where 722 nonredundant phosphorylation sites were identified, including several doubly and triply phosphorylated peptides [7]. Briefly, 500 μg of HeLa protein extract was digested, and these peptide mixtures were separated either by SCX or strong anion exchange (SAX) columns. In case of SCX, each of ten SCX fractions was additionally subjected to the phosphopeptide enrichment by TiO$_2$. The samples were analyzed by the LTQ-Orbitrap mass spectrometer combined with C18-based HPLC. This SCX method was complementary to the on-line SAX separation, which however did not use subsequent TiO$_2$ enrichment. In comparison with SCX, the latter method was able to separate a higher number of multiply phosphorylated peptides [7], but this could be due to the TiO$_2$ enrichment used after the SCX chromatography which is known to be less ideal for multiply phosphorylated peptide analysis. Furthermore, Sui et al. used similar SCX separation followed by TiO$_2$ enrichment and LC-MS/MS analysis to map the phosphoproteome in human Chang liver cell line [8]. In this experiment, 1.5 mg protein was digested with trypsin and subjected to separation by SCX chromatography into forty fractions, followed by enrichment by TiO$_2$ and LC-MS/MS analysis by Q-TOF and LCQ mass spectrometers. This study identified 1035 phosphorylation sites on 607 phosphoproteins, including a large fraction of previously unknown phosphoproteins, and the functional analysis demonstrated that majority of these proteins were involved in liver metabolism. Finally, similar procedure has been used for the phosphoproteome analysis of a Gram-negative bacterium Escherichia coli, where 81 phosphorylation sites were identified on 79 proteins [9]. In this study, 20 mg protein was digested with Lys-C followed by tryptic digestion. The resulted peptides were split into two technical replicates and separated on SCX column into ten fractions, followed by TiO$_2$ phosphopeptide purification and analysis by HPLC coupled to LTQ-Orbitrap mass spectrometer. A comparison of this dataset to a phosphoproteomic study of bacterium Bacillus subtilis [10] indicated that both phosphoproteomes demonstrate high conservation levels, and the authors suggested that some phosphorylation sites are conserved from Archaea to humans [9]. The Bacillus subtilis study has been done using a very similar technique to the one described for E. coli [9], but 10 mg protein was
used for analysis and fifteen SCX fractions were collected, which were then subjected to the TiO₂ chromatography and analysis by HPLC coupled to LTQ-Orbitrap or LTQ-FT mass spectrometry.

Although SCX is a relatively old separation method, it still performs quite well if compared with newer HPLC techniques. Chen et al. [11] evaluated four different chromatography methods in terms of their capabilities to separate phosphopeptides of HeLa cells, and these included SCX, HILIC, ERLIC chromatography with nonvolatile solvent system (ERLIC-nV), and ERLIC with volatile solvent system (ERLIC-V). Each chromatography method was performed on peptides obtained from 40 mg protein. Phosphopeptides were first enriched on IMAC, followed by SCX, HILIC, ERLIC-nV, or ERLIC-V-based separation into multiple fractions (25 fractions in case of SCX) and finally analyzed by LC-MS/MS using QSTAR ELITE mass spectrometer. As a result, each technique yielded between 4000 and 5000 phosphopeptides, including a large number of nonoverlapping phosphopeptides for each HPLC method. Together, the data from all four chromatography analyses generated 9069 unique phosphopeptides, demonstrating a complementary nature of these different chromatography methods. Interestingly, the phosphopeptide resolving power was the greatest for the SCX method (60.52% nonrepetitive phosphopeptides), followed by ERLIC-nV (50.79% nonrepetitive phosphopeptides), while the lowest performance was assigned to HILIC (50.83% nonrepetitive phosphopeptides), which also had the worst orthogonality relative to reversed-phase chromatography.

Apart from combining SCX separation with additional phosphopeptide enrichment methods, another innovation is to utilize other proteolytic enzymes for peptide generation in order to complement the data obtained from trypsin-digested samples. An example is Lys-N [4, 5] that generates phosphopeptides characterized by distinct net charges, which can be thus efficiently separated by SCX and distinguished from the N-acetylated peptide populations [4]. This feature of Lys-N has been taken advantage of in a study where a parallel use of Lys-N, trypsin, and Lys-C digestion followed by SCX and LC-MS/MS analysis provided a total of 5036 nonredundant phosphopeptides [4]. In this study only 1 mg protein was subjected to digestion with one of these proteolytic enzymes. The peptides were then desalted on C18 columns and subjected to the SCX-based fractionation. Forty thus obtained SCX fractions were analyzed by HPLC combined with LTQ-Orbitrap mass spectrometry. Lys-N increased the phosphopeptide coverage by 72% if compared with the trypsin-digested sample, whereas trypsin accounted for only 25% of the increase. Clearly, future exploration of other proteolytic enzymes can additionally benefit the global phosphoproteome studies.

In addition, the mass spectrometry methods can also be modified to fully exploit the phosphopeptide separation capabilities provided by SCX. As an example, electron transfer dissociation (ETD) has emerged as an alternative to collision-induced dissociation (CID) fragmentation method used in mass spectrometry. ETD is especially suitable for analysis of highly charged peptides and for PTMs that are labile upon CID fragmentation methods [38]. As an example, this approach has been used to characterize phosphoproteome of human embryonic stem cells [12]. In this study, peptides obtained from 10 mg protein lysate were subjected to the SCX separation into twelve fractions, followed by IMAC phosphopeptide purification and LC-MS/MS analysis by the LTQ-Orbitrap mass spectrometer. The peptides were analyzed using ETD and CID fragmentation methods, which resulted in the identification of 10,844 nonredundant phosphorylation sites. Importantly, the ETD method yielded a much larger number of unique phosphopeptides than CID (8,087 in comparison with 3,868). In this study, ETD fragmentation analysis has been also shown to more frequently localize the phosphorylation site to a specific residue, which is often problematic when using CID fragmentation [12].

Finally, my preliminary data suggests that SCX chromatography resins can be further optimized in terms of their phosphopeptide enrichment properties. I used TiO₂ resin as well as cartridges containing three different SCX resins to separate phosphorylated peptides of several forms of casein, followed by LS-MS/MS analysis (please, refer to the Supplementary methods in Appendix). The SCX resins included sulfonic acid (SCX-1), propyl sulfonic acid (SCX-2), and ethylbenzene sulfonic acid (SCX-3), and their performance varied in terms of their phosphopeptide enrichment potential. In this experiment, SCX-3 has been shown to be the most efficient column among the tested resins (Figure 1). Due to the presence of an aromatic ring, SCX-1 and SCX-3 resins have nonpolar character, therefore enhancing nonpolar secondary interactions with the analyte and increasing its binding to the column. However, SCX-3 might have also other characteristics that enable for a slightly better separation of phosphopeptides. This preliminary study should be repeated using a more complex sample, but it demonstrates that there might be a bigger potential for the SCX-based separation methods if these resins are further optimized specifically for phosphorylation analysis.

4. Utilizing SCX Chromatography in Analysis of N-Terminal Acetylation

The N-terminal acetylation is a common protein modification with a relatively unclear physiological role. It affects a variety of protein functions, such as enzymatic activity, DNA-binding, and protein-protein interactions [39]. It might also represent a specific degron site, thus targeting proteins for ubiquitination and subsequent degradation [40]. The SCX-based separation of N-terminal acetylated peptides is based on the fact that these peptides lack the N-terminal free amine, which contributes to their earlier elution in the SCX chromatography [13] and enables their separation from slightly later migrating phosphopeptides (Table 1; [4, 14]).

One of the first attempts to use SCX for analysis of the N-acetylated and C-terminal peptides showed that this approach, if adequately optimized, can provide 95% specificity for this peptide class [13]. The membrane-enriched fraction from 5 × 10⁶ human teratocarcinoma NT2/d1 cells was digested with Lys-C, followed by tryptic digestion. The peptides were desalted with a C18 column
and separated by SCX into approximately thirty fractions, which were then analyzed by the LC-ITQ-Orbitrap mass spectrometry. This workflow proved to be successful, and it allowed for identification of 116 N-terminal acetylated peptides, although a relatively small amount of protein material was used.

Moreover, similarly as in case of phosphoproteome analysis, the SCX-based separation of N-terminal acetylated peptides has been combined with the ETD fragmentation method [14], yielding results complementary to the ones obtained by the CID fragmentation. Digestion with proteolytic enzymes such as Lys-N and Lys-C has also been shown to be advantageous, for instance, due to already mentioned separation of the N-terminally acetylated peptides from phosphopeptides [4, 14, 16].

Furthermore, there are additional methods suitable for enrichment of the N-terminal acetylated peptides that can be used in conjunction with SCX, and these include combined fractional diagonal chromatography (COFRADIC; summarized in [41]). For instance, SCX combined with COFRADIC and LC-MS/MS analysis by an ion trap mass spectrometer has been used to identify over 1,200 mature protein N-termini, including 861 N-terminal acetylation sites in cytoplasmic, nuclear, and membrane fractions obtained from Drosophila melanogaster cells [15].

In summary, the SCX methods used for mapping of the N-terminal acetylation sites are expected to shed more light on the abundance and function of this prevalent but not well-understood modification.

5. Conclusions

Although current efforts are focused on optimizing the SCX chromatography for analysis of phosphorylation as a key signaling modification, this HPLC method has a potential to be used in analysis of other PTMs that alter a net charge of the peptides. For example, SCX could be possibly used in glycomics, based on the assumption that many extracellular N-glycans contain sialic acid residues, thereby reducing the net charge of glycopeptides in comparison with the unmodified peptides [42]. Moreover, modifications of lysine and arginine residues, such as methylation or acetylation, also account for a shift in SCX elution time in comparison with their unmodified counterparts [43]. When compared to the whole complement of peptides, these modifications might not cause a sufficient change in elution properties in order to monitor these modifications on a global scale, but they can still be optimized to study modifications of specific proteins, such as histones that are characterized by multiple lysine and arginine PTMs. Moreover, differential elution of peptides containing modified arginine or lysine residues in comparison with peptides containing these amino acids in an unmodified form can be used as an additional validation of the proteomic results. All this further underlines the importance of the SCX chromatography in proteomic applications focused on investigation of protein modifications.

Appendix

Supplementary Methods: Phosphopeptide Enrichment and Mass Spectrometry Analysis

50 μg of casein (Sigma-Aldrich, UK) was digested with trypsin, as we previously described [44–46]. SCX (here referred to as SCX-1, SCX-2, and SCX-3 cartridges (ISOLUTE SCX 200 mg/3 mL; Kinesis, UK) were washed with 5 mL wash solvent containing 0.1% formic acid and 5% acetonitrile. The digested protein samples were loaded onto SCX-1, SCX-2, or SCX-3 columns and washed twice with 5 mL wash solvent. The peptides were eluted sequentially with buffers A, B, C, and D, containing 5% acetonitrile as well as 50, 100, 200, or 500 nM ammonium acetate pH 3, respectively. All the elution procedures were performed on a vacuum manifold (Kinesis, UK) connected to a vacuum pump.

For the phosphopeptide enrichment with TiO2 pipette tip (Gl Sciences, Japan), TiO2 tips were preconditioned with 100% acetonitrile, conditioned with 0.2 M phosphate buffer pH7, and equilibrated with solvent containing 50% acetonitrile and 0.1% formic acid. The peptide sample was then loaded onto the TiO2 tips, washed six times with buffer containing 50%, 1% formic acid, and 0.1 M KCl, and eluted with 0.2 M phosphate buffer pH 7 and 0.5% aqueous ammonia.
The volume of all eluates was reduced in a vacuum centrifuge (Thermo, UK) and adjusted to appropriate volume with solvent containing 2% acetonitrile and 0.1% formic acid. The triplicates of samples (∼1 μg protein) were analyzed on HPLC (Ultimate 3000, Dionex, UK) attached to a 3D high-capacity ion trap mass spectrometer (Esquire HCTplus, Bruker Daltonics, UK), exactly as we previously described [44–46]. The obtained peak lists were submitted to the Mascot search engine, where phosphorylation on serine, threonine, and tyrosine was added as a variable modification in the search parameters. The peptide and fragment mass tolerances were 2.5 and 0.8, respectively. Individual MS/MS spectra for peptides with a Mascot Mowse score lower than 40 were inspected manually and only included in the statistics if a series of at least four continuous y or b ions were observed. Standard variations for triplicate samples were calculated using Excel (Microsoft).

**Abbreviations**

- **SCX**: Strong cation exchange
- **RP**: Reversed phase
- **PTM**: Posttranslational modification
- **ESI**: Electrospray ionization
- **IMAC**: Immobilized metal affinity chromatography
- **ETD**: Electron transfer dissociation
- **CID**: Collision-induced dissociation
- **HILIC**: Hydrophilic interaction chromatography
- **ERLIC**: Electrostatic repulsion hydrophilic interaction chromatography
- **COFRADIC**: Combined fractional diagonal chromatography
- **LC-MS/MS**: Liquid chromatography-tandem mass spectrometry

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**References**

[1] M. Mann, S. E. Ong, M. Gronborg, H. Steen, O. N. Jensen, and A. Pandey, “Analysis of protein phosphorylation using mass spectrometry: deciphering the phosphoproteome,” *Trends in Biotechnology*, vol. 20, no. 6, pp. 261–268, 2002.

[2] H. Y. Wu and P. C. Liao, “Analysis of protein phosphorylation using mass spectrometry,” *Chang Gung Medical Journal*, vol. 31, no. 3, pp. 217–227, 2008.

[3] S. Mohammed and A. J. Heck, “Strong cation exchange (SCX) based analytical methods for the targeted analysis of protein post-translational modifications,” *Current Opinion in Biotechnology*, 2010.

[4] S. Gauci, A. O. Helbig, M. Slijper, J. Krijgsveld, A. J. R. Heck, and S. Mohammed, “Lys-N and trypsin cover complementary parts of the phosphoproteome in a refined SCX-based approach,” *Analytical Chemistry*, vol. 81, no. 11, pp. 4493–4501, 2009.

[5] N. Tautouas, M. M. Drugan, A. J. R. Heck, and S. Mohammed, “Straightforward ladder sequencing of peptides using a Lys-N metalloendopeptidase,” *Nature Methods*, vol. 5, no. 5, pp. 405–407, 2008.

[6] S. A. Beausoleil, M. Jedyrchowski, D. Schwartz et al., “Large-scale characterization of HeLa cell nuclear phosphoproteins,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 33, pp. 12130–12135, 2004.

[7] J. Dai, L. S. Wang, Y. B. Wu et al., “Fully automatic separation and identification of phosphopeptides by continuous pH-gradient anion exchange online coupled with reversed-phase liquid chromatography mass spectrometry,” *Journal of Proteome Research*, vol. 8, no. 1, pp. 133–141, 2009.

[8] S. Sui, J. Wang, B. Yang et al., “Phosphoproteome analysis of the human Chang liver cells using SCX and a complementary mass spectrometric strategy,” *Proteomics*, vol. 8, no. 10, pp. 2024–2034, 2008.

[9] B. Macek, F. Gnädig, B. Soufi et al., “Phosphoproteome analysis of E. coli reveals evolutionary conservation of bacterial Ser/Thr/Tyr phosphorylation,” *Molecular and Cellular Proteomics*, vol. 7, no. 2, pp. 299–307, 2008.

[10] B. Macek, I. Mijakovic, J. V. Olsen et al., “The serine/threonine/tyrosine phosphoproteome of the model bacterium *B. subtilis*,” *Molecular and Cellular Proteomics*, vol. 6, no. 4, pp. 697–707, 2007.

[11] X. Chen, D. Wu, Y. Zhao, B. H. Wong, and L. Guo, “Increasing phosphoproteome coverage and identification of phosphorylation motifs through combination of different HPLC fractionation methods,” *Journal of Chromatography B*, vol. 879, no. 1, pp. 25–34, 2011.

[12] D. L. Swaney, C. D. Wenger, J. A. Thomson, and J. J. Coon, “Human embryonic stem cell phosphoproteome revealed by electron transfer dissociation tandem mass spectrometry,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 4, pp. 995–1000, 2009.

[13] W. Dormeyer, S. Mohammed, B. Van Breukelen, J. Krijgsven, and A. J. R. Heck, “Targeted analysis of protein termini,” *Journal of Proteome Research*, vol. 6, no. 12, pp. 4634–4645, 2007.

[14] N. Tautouas, A. F. M. Altelaar, M. M. Drugan, A. O. Helbig, S. Mohammed, and A. J. R. Heck, “Strong cation exchange-based fractionation of Lys-N-generated peptides facilitates the targeted analysis of post-translational modifications,” *Molecular and Cellular Proteomics*, vol. 8, no. 1, pp. 190–200, 2009.

[15] S. Goeztze, E. Qeli, C. Mosimann et al., “Identification and functional characterization of N-terminally acetylated proteins in *Drosophila melanogaster*,” *PLoS Biology*, vol. 7, no. 11, Article ID e1000236, 2009.

[16] A. O. Helbig, S. Gauci, R. Rajimakers et al., “Profiling of N-acetylated protein termini provides in-depth insights into the N-terminal nature of the proteome,” *Molecular and Cellular Proteomics*, vol. 9, no. 5, pp. 928–939, 2010.

[17] P. Cohen, “The role of protein phosphorylation in human health and disease. The Sir Hans Krebs Medal Lecture,” *European Journal of Biochemistry*, vol. 268, pp. 5001–5010, 2001.

[18] J. V. Olsen, B. Blagoev, F. Gnädig et al., “Global, in vivo, and site-specific phosphorylation dynamics in signaling networks,” *Cell*, vol. 127, no. 3, pp. 635–648, 2006.
