Rev Anal Chem 31 (2012): 29–41 © 2012 by Walter de Gruyter • Berlin • Boston. DOI 10.1515/revac-2011-0025

# Enrichment strategies for phosphoproteomics: state-of-the-art

**Barbora Salovska[1,2,*], Ales Tichy[1,2], Martina Rezacova[1], Jirina Vavrova[2] and Eva Novotna[2]**

[1] Department of Medical Biochemistry, Faculty of Medicine in Hradec Kralove, Charles University in Prague, Hradec Králové, Czech Republic, e-mail: salob5aa@lfhk.cuni.cz

[2] Department of Radiobiology, Faculty of Military Health Sciences, University of Defence, Hradec Kralove, Czech Republic

* Corresponding author

## Abstract

Protein phosphorylation is a key regulator in many biological processes, such as homeostasis, cellular signaling and communication, transcriptional and translational regulation, and apoptosis. The defects in this tightly controlled reversible post-translational modification have been described to contribute to genesis and progression of various diseases, emphasizing the importance of a systematic research of this phenomenon. Although considerable effort has been devoted to improving the analysis of phosphorylation by mass spectrometry, which is currently the method of choice to study protein phosphorylation, the detection and identification of phosphorylation sites remains challenging because of the low abundance and low ionization efficacy of phosphoproteins in comparison with nonphosphorylated proteins. To overcome this obstacle, different enrichment strategies for phosphorylated peptides/proteins have been established and optimized for subsequent mass spectrometry analysis. In this review, we will give an overview of the methods currently available for the enrichment of phosphorylated proteins and peptides including immunoprecipitation, chemical derivatization and affinity enrichment techniques.

**Keywords:** metal oxide affinity chromatography; phosphopeptide enrichment; phosphoproteomics.

## Abbreviations

| | |
|---|---|
| BSA | bovine serum albumin |
| DHB | 2,5-dihydroxybenzoic acid |
| ERLIC | electrostatic repulsion hydrophilic interaction chromatography |
| HAP | hydroxyapatite chromatography |
| HILIC | hydrophilic interaction chromatography |
| IDA | imidoacetic acid |
| IMAC | immobilized metal affinity chromatography |
| LC | liquid chromatography |
| LPD | liquid phase deposition |
| MIP | molecularly imprinted polymer |
| MOAC | metal oxide affinity chromatography |
| MS | mass spectrometry |
| NTA | nitriloacetic acid |
| PTMs | post-translational modifications |
| pS | phosphoserine |
| pT | phosphothreonine |
| pY | phosphotyrosine |
| RPLC | reversed phase liquid chromatography |
| SAX | strong anion-exchange chromatography |
| SCX | strong cation-exchange chromatography |

## Introduction

The human genome involves approximately 30,000 protein-coding genes; the human proteome contains several million different protein effectors. This is due to alternative splicing of genes and post-translational modifications (PTMs). Several hundred PTMs are currently known, among them protein phosphorylation is the most studied and one of the most important in nature (Pinkse and Heck 2010).

Protein phosphorylation is a transient, reversible PTM, which is involved in many cellular processes including homeostasis, cellular signaling and communication, proliferation, differentiation, metabolism, transcriptional and translational regulation, degradation of proteins and cell survival (Cohen 2002). It is one of the most widespread regulatory mechanisms; it has been estimated that more than 50% of the proteins in mammalian cells are phosphorylated at some point during their life time (Reinders and Sickman 2005). Four types of phosphorylation are known: O-phosphorylation which occurs on serine, threonine and tyrosine residues, N- (Knezevic et al. 2000), S- (Weigt et al. 1995) and acyl- (Sanders et al. 1989) phosphorylation which are far less common and occur on histidine, lysine, cysteine, aspartic and glutamic residues. In eukaryotic cells phosphorylation on serine, threonine and tyrosine residues is considered to be predominant (Sickmann and Meyer 2001). Phosphorylation also occurs on histidine residues; a total of 6% of the total phosphorylation in eukaryotes consists of phosphohistidine residues (Matthews 1995). Nevertheless, phosphohistidine residues are not normally observed in proteins due to rapid hydrolysis of the phosphoryl group under acidic conditions (Hultquist 1968). The distribution proportions of phosphoserine (pS), phosphothreonine (pT) and phosphotyrosine (pY) sites were reported for the first time in 1980 by Hunter and Sefton who determined them in chicken cells as 92.19%, 7.77% and 0.03%, respectively (Hunter and Sefton 1980). Since then, several studies have been performed, e.g., a study on HeLa cells, which showed that the distribution proportions of pS, pT and phosphotyrosine pY sites were 86.4%, 11.8%

and 1.8% (Olsen et al. 2006) or a study performed on the same cell line reported by Chen et al. (2011b), with 84.98%, 14.26% and 0.76%, respectively. Additionally, in *Arabidopsis thaliana*, a popular model organism in plant biology and genetics, relative abundances of pS, pT and pY were estimated to be 85%, 10.7% and 4.3% (Sugiyama et al. 2008). This indicates that there are substantial differences in distribution proportions mainly due to various methodical approaches and dynamic range of particular phosphoproteome used in a given study. Cellular protein phosphorylation events are site-specific; they often occur at multiple sites within a protein and it has been established that more than 100,000 phosphorylation sites may exist in the human proteome (Zhang et al. 2002). Some of them are always quantitatively phosphorylated, whereas others are only transiently phosphorylated up to 0.5% (Reinders and Sickman 2005).

Phosphorylation is mediated by protein kinases, which compose one of the largest enzyme superfamilies in higher eukaryotes (Manning et al. 2002). It has been estimated that 2–3% of all eukaryotic genes are coding protein kinases (Manning et al. 2002). The reverse reaction, dephosphorylation, is mediated by protein phosphatases. Tight cooperation of protein kinases and protein phosphatases is essential for regulation of biological processes in a cell and dysregulation of these processes has been described to contribute to genesis and progression of cancer and other diseases (Blume-Jensen and Hunter 2001). The dysregulation is mostly induced by mutations in genes coding protein kinases, overexpression of kinases or defects in negative regulatory mechanisms. Technological advances in the recent past led to the development of phosphoproteomic approaches that allow researchers to identify aberrantly activated signaling pathways and determine the appropriate therapeutic targets that can be, for example, specifically targeted by small molecule kinase inhibitors (reviewed in Harsha and Pandey 2010).

Mass spectrometry (MS) is currently the method of choice to describe dynamic changes in protein phosphorylation. We covered in detail various MS approaches of phosphoproteomic analysis in our previous work (Tichy et al. 2011). Nevertheless, the MS analysis of phosphoproteins is still complicated because of the relatively low abundance of phosphorylated proteins in eukaryotic cells. Also, the ionization efficiency is often described as a reason for difficulties in phosphopeptide identification. It was suggested that ionization efficiencies and therefore signals of phosphopeptides in MS are lower compared with their nonphosphorylated analogs (Craig et al. 1994, Liao et al. 1994). Steen et al. (2006) tested the ionization/detection efficiencies of the synthetic peptide/phosphopeptide pairs by using online liquid chromatography (LC) electrospray ionization MS. In this study, it was shown that the statement about lower ionization efficiency is not valid in general and it is highly dependent on the MS instrumentation used in the particular study. The authors concluded that phosphopeptides are difficult to identify in phosphoprotein digests because of the substoichiometric nature of phosphorylation, not because of their low ionization efficiencies.

Phosphorylation is a very dynamic process, the phosphorylation sites on proteins might vary and thus any phosphoprotein can exist in several different phosphorylated forms. Additionally, most of the techniques used for the study of protein phosphorylation have a limited dynamic range. Hence, major phosphorylation sites might be identified easily, whereas minor sites might be difficult to detect. The complications caused by low ionization efficiency and low abundance of phosphorylated proteins and peptides can be reduced by phosphospecific enrichment prior to their characterization by MS. The separation of phosphorylated peptides from nonphosphorylated can be performed either on the protein level or on the peptide level.

Tyrosine phosphorylated proteins can be enriched with the immunoprecipitation with phosphotyrosine specific antibodies that have been employed successfully in some studies (Blagoev et al. 2004, Rush et al. 2005, Schumacher et al. 2007). Although antibodies against specific phosphorylated motifs in phosphothreonine and phosphoserine have been used in some studies (Grønborg et al. 2002, Zhang et al. 2002), immunoprecipitation is not capable for enriching of phosphoserine- and phosphothreonine-containing proteins. Phosphoproteins can also be precipitated using lanthanum ions (Pink et al. 2011, Verma et al. 2011). Protein kinases can be specifically captured with immobilized low-molecular weight inhibitors, e.g., bisindolylmaleimide compounds used by Brehmer and his colleagues (2004). Commercial kits for the enrichment of phosphoproteins, e.g., Phosphoprotein Purification Kit (QIAGEN, Hilden, Germany; Holland et al. 2011) or Thermo Scientific Pierce Phosphoprotein Enrichment Kit (Thermo Fischer Scientific, Rockford, IL, USA; Nilsson et al. 2010) are also available and used for phosphoprotein enrichment.

Many methods for the enrichment of phosphopeptides have been developed so far. However, any one of them is not able to yield comprehensive information about the phosphoproteome of complex biological samples. Therefore, the approaches described thereinafter are often combined to obtain complete information about the phosphopeptide pool in cells, biofluids, etc. Phosphopeptide enrichment techniques comprise chemical derivatizations, affinity enrichment methods and some alternative methods, e.g., barium (Ruse et al. 2008) and calcium (Zhang et al. 2007) precipitation. An interesting approach for identification of protein kinases substrates was presented in 2009 when phosphospecific antibody against a peptide library that represented the mitogen-activated protein kinases (MAPKs) consensus phosphorylated motif was developed. Immunoprecipitation with this antibody led to identification of 449 candidate substrates for MAPKs and 82 specific phosphorylation sites in 34 proteins including a novel phosphorylation site (Ser-447) discovered in δ-catenin (Edbauer et al. 2009).

The affinity enrichment methods are a large group of chromatographic techniques exploiting the typical properties of phosphopeptides, which allows them to interact with the chromatographic resin in a different way from nonphosphopeptides. These methods include IMAC (immobilized metal affinity chromatography), MOAC (metal oxide affinity chromatography), HAP (hydroxyapatite chromatography), SCX (strong cation-exchange chromatography) and SAX (strong anion-exchange chromatography), HILIC (hydrophilic interaction chromatography) and ERLIC (electrostatic repulsion hydrophilic interaction chromatography). This review gives an overview of the methods currently available for the enrichment of phosphorylated proteins and peptides (Table 1).

**Table 1** An overview of the main methods used in phosphoproteomics.

| Method | Principle | | References |
|---|---|---|---|
| SCX | Interaction between positively charged peptides and negatively charged column resin (pH<3) | + Effective prefractionation tool prior to subsequent phosphopeptide enrichment | Beausoleil et al. (2004), Gruhler et al. (2005), Benschop et al. (2007) |
| SAX | Interaction between negatively charged peptides and positively charged column resin | + Effective prefractionation tool prior to subsequent phosphopeptide enrichment<br>+ Suitable for more acidic peptides and more effective for phosphopeptide fractionation than SCX | Nühse et al. (2003), Wu et al. (2009) |
| IEF | Differences in p*I* values of more acidic phosphopeptides and less acidic nonphosphorylated peptides | + Prefractionation tool prior to subsequent phosphopeptide enrichment | Maccarrone et al. (2006), Xu et al. (2007), Chen et al. (2010) |
| HILIC | Partitioning between a water-enriched layer of stagnant eluent which hydrates the polar stationary phase and a relatively hydrophobic bulk eluent; retention time of an analyte increases with the increasing polarity of the peptide | + Effective prefractionation tool prior to subsequent phosphopeptide enrichment<br>+ High degree of orthogonality to RP-LC | Gilar et al. (2005), McNulty and Annan (2008), Albuquerque et al. (2008) |
| ERLIC | Combination of the electrostatic attraction with hydrophilic interactions | + Effective prefractionation tool prior to subsequent phosphopeptide enrichment | Chen et al. (2011b), Chien et al. (2011) |
| IMAC | Affinity of positively charged metal cations ($Fe^{3+}$, $Al^{3+}$, $Ga^{3+}$, $Zr^{4+}$ or $Co^{2+}$) to negatively charged phosphopeptides at acidic pH | + Effective phosphopeptide enrichment from complex samples in combination with some prefractionation method<br>- High level of nonspecific binding; biased towards multiphosphorylated peptides; affected by various buffers, detergents and other reagents | Bodenmiller et al. (2007), Liang et al. (2007) |
| MOAC | Affinity of metal oxides ($TiO_2$, $ZrO_2$) for phosphate ions at acidic pH | + Robust method of phosphopeptide enrichment from complex samples; high recovery and selectivity<br>+ Metal oxides are more stable than metal ions, tolerant towards many reagents | Wu et al. (2007), Sugiyama et al. (2008), Li et al. (2009), Montoya et al. (2011) |
| Chemical modification | - β-Elimination and Michael addition reaction | - Nonspecific labeling of cysteines and O-glycosylated peptides<br>- Inability of p-Tyr residues to undergo the β-elimination<br>- Sample loss, side products | Goshe et al. (2001), Oda et al. (2001), Arrigoni et al. (2006) |
| | - Phosphoramidate chemistry (PAC) | + Available also for p-Tyr<br>- Sample loss, side products | Zhou et al. (2001), Lansdell and Tepe (2004), Tao et al. (2005) |

## Chemical modifications

In chemical derivatization techniques, the phosphorylated residues are chemically altered with an affinity tag that is selectively captured. Most of the methods exploit the lability of phosphate groups on serine and threonine residues under alkaline conditions. In the presence of strong bases, e.g., NaOH or $Ba(OH)_2$, the phosphoserine and phosphothreonine residues undergo the β-elimination reaction to form dehydroalanine or dehydrobutyric acid, respectively, which serve as Michael acceptors. This reaction is followed by Michael addition reaction with different nucleophiles such as ethanedithiol (Goshe et al. 2001, 2002, Oda et al. 2001, Poot et al. 2006), dimethylaminoethanethiol (Steen and Mann 2002) or mercaptoethylpyridine (Arrigoni et al. 2006) that can be linked to an affinity tag (e.g., biotin; Oda et al. 2001) or immobilizing agent (e.g., dithiopyridine resin; Thaler et al. 2003). The major drawbacks of methods relying on a β-elimination reaction are associated with nonspecific labeling of cysteines and O-glycosylated peptides and inability of p-Tyr residues to undergo the β-elimination. The nonspecific labeling could, however, be reduced by blocking the sulfhydryl group of cysteins by alkylation or oxidation or by performing enzymatic deglycosylation in the case of O-glycosylated peptides.

Another covalent enrichment technique is the carbodiimide catalyzed condensation reaction with excess amine to form phosphoramidate (i.e., phosphate that has an $NR_2$ instead of an OH group). In comparison with the β-elimination chemistry, this approach is also capable for the enrichment of tyrosine-phosphorylated peptides. Zhou et al. (2001) presented this method in a multistep approach (six steps), where cystamines attached to phosphate groups by a condensation reaction were further reduced to form free sulfhydryl groups that provided the attachment of phosphopeptides to a solid phase by reacting with iodoacetyl groups immobilized on glass beads. Although the final yield is only approximately 20% due to considerable sample loss, this approach is very selective providing contaminant-free phosphopeptides. Tao et al. (2005) applied this chemistry in a simpler procedure including the methylation of the carboxyl groups, the condensation reaction with a dendrimer (synthetic polyamine) in the presence of carbodiimide and imidazole, and finally the acid hydrolysis of the phosphoramidate bonds among phosphopeptides and the dendrimer. This method provides higher recovery of phosphopeptides than previous approaches; however, wider utilization of it is hampered by extremely slow conversion during the carbodiimide catalyzed condensation step. Another approach suitable for phosphorylated serine, threonine and tyrosine peptides that included chemical derivatization was presented in 2004. Carboxylic groups were protected by methylation and the peptide mixture was subjected to the reaction with α-diazo functionalized resin, which resulted in the formation of a covalent phosphopeptide-resin bond that was later cleaved with acid hydrolysis (Lansdell and Tepe 2004).

## SCX, SAX, IEF, HILIC and ERLIC

In strong cation exchange (SCX) chromatography, peptides are retained on a column on the basis of the interaction between positively charged peptide groups and negatively charged column resin. Under acidic conditions (pH<3) tryptic peptides become positively charged by protonation at the N termini and side chains of arginine, lysine and histidine, whereas carboxyl groups and the C termini become neutrally charged. Phosphoryl groups stay negatively charged at low pH and thus peptides containing phosphoryl groups have lower affinity for the negatively charged resin. Hence, phosphopeptides are eluted in the earlier fractions during SCX fractionation (Grimsrud et al. 2010). SCX was originally known as a component of multidimensional protein identification technology for shotgun proteomics (MudPIT; Washburn et al. 2001, Wolters et al. 2001). In 2004, SCX was initially described for phosphopeptide enrichment of HeLa cell nuclear phosphoproteins (Beausoleil et al. 2004). Since then, SCX has been exploited as a prefractionation method before IMAC (Villén et al. 2007, Gruhler et al. 2005, Swaney et al. 2009, Stone et al. 2011, Zhou et al. 2011) and titanium dioxide ($TiO_2$) enrichment (Benschop et al. 2007, Wu et al. 2007, Lemeer et al. 2008a,b, Lee et al. 2009) and also as a single method for phosphopeptide enrichment after combined cleavage with trypsin and Lys-N (Gauci et al. 2009, Taouatas et al. 2009).

Strong anion exchange (SAX) chromatography is a method of peptides separation that is based on the level of negative charges and thus can retain more acidic peptides than SCX; the use of SAX for phosphopeptide enrichment should not result in serious loss of phosphopeptides. Another significant advantage of using SAX for phosphoproteome analysis is that SAX has the ability to fractionate phosphopeptides. In 2003, Nühse et al. successfully used SAX chromatography with salt gradient elution as a prefractionation step before IMAC for the identification of plasma membrane phosphoproteins (Nühse et al. 2003). More recently, SCX, SAX and reversed phase liquid chromatography (RP-LC) with pH gradient elution were combined in the Yin-yang multidimensional liquid chromatography tandem mass spectrometry method (MDLC-MS/MS; Dai et al. 2007). The Yin-yang MDLC-MS/MS approach in combination with SILAC was further used for the quantification of phosphoproteome changes during adipocyte differentiation (Wu et al. 2009).

SAX was shown to be complementary with the $Fe^{3+}$-IMAC method; however, in comparing these two techniques, more peptides, especially monophosphorylated, were identified by the SAX approach than by the $Fe^{3+}$-IMAC method. Enrichment and fractionation of phosphopeptides by SAX was then applied to phosphoproteomics analysis of human liver tissue (Han et al. 2008). A similar conclusion was made when SAX chromatography was compared with the method combining SCX and $TiO_2$ enrichment (Dai et al. 2009). AFET (anion exchange followed by flow-through enrichment with titanium dioxide) is a phosphopeptide identification strategy where SAX is used as the first step for the separation and the enrichment of phosphopeptides that is online coupled with

LC-MS/MS. In this approach, the flow-through fraction from SAX chromatography is further enriched with $TiO_2$ chromatography to obtain more comprehensive phosphoproteome of the studied cell line (Nie et al. 2010).

Isoelectric focusing (IEF) was also presented as a method of phosphopeptide enrichment that exploits the difference in p*I* values of more acidic phosphopeptides and less acidic nonphosphorylated peptides (Maccarrone et al. 2006, Xu et al. 2007). A recent study introduced new methodology that includes the separation of proteins by IEF, phosphopeptides enrichment with IMAC and analysis by LC-MS/MS for mapping of the phosphoproteome in the human prostate cell line LNCaP (Chen et al. 2010).

HILIC has been more commonly used for small polar solutes (e.g., pharmaceuticals, saponins, urea, aminoglycoside antibiotics, glucosinolates, sugars and glycans, folic acid and its metabolites, nicotine and its metabolites, glycoalkaloids) than for peptides fractionation. The retention of an analyte is believed to be caused by partitioning between a water-enriched layer of stagnant eluent which hydrates the polar stationary phase and a relatively hydrophobic bulk eluent, with mobile phase usually being 10–40% water in acetonitrile. Naturally, the retention time of an analyte increases with the increasing polarity of the peptide (Alpert 1990). The mechanism was discussed in detail by Hemström and Irgum (2006). Gilar et al. (2005) showed that HILIC has the highest degree of orthogonality to RP-LC of all commonly used peptides fractionation techniques, which probably reflects their different mechanisms of actions. More recently, McNulty and Annan (2008) used HILIC as a first dimension separation for 2D LC proteomics for investigation of HeLa cells phosphopeptides. Applying this approach, they identified more than 700 novel phosphorylation sites in HeLa cells phosphoproteome. Additionally, they revealed that HILIC performed before IMAC enrichment (HILIC-IMAC) dramatically improved the selectivity of IMAC, whereas HILIC performed after IMAC enrichment (IMAC-HILIC) was found to be less beneficial for the selectivity of IMAC. The authors of that study stated that the fractions provided by hydrophilicity-based separation contained peptides with the same polarity ensuring very effective competition between peptides and phosphopeptides within these fractions for IMAC binding sites (McNulty and Annan 2008). Contrary to this statement, Albuquerque et al. (2008) performed IMAC enrichment before HILIC prefractionation followed by RP-LC-MS/MS with very satisfactory results; thousands of phosphopeptides (n=8764) from yeast phosphoproteome were identified.

The newest chromatographic approach developed especially for the enrichment and fractionation of phosphopeptides is ERLIC which combines the electrostatic attraction with hydrophilic interactions to selectively capture phosphopeptides. At low pH (pH 2) phosphate groups still retain their negative charge, whereas carboxyl groups become neutrally charged and basic amino groups positively charged. Phosphate groups are electrostatically bound to the column; nevertheless, their affinity is not sufficient to overcome electrostatic repulsion from the positively charged amino groups. Thus, hydrophilic interactions of the phosphate group should be enhanced by using high concentrations of an organic solvent (e.g., 70% acetonitrile; Alpert 2008). In a current study performed on HeLa cells, SCX, HILIC and ERLIC based fractionation methods were combined for phosphopeptides separation prior to sequencing by RP-LC-MS/MS. A total of 9069 unique phosphopeptides were identified with only 1697 unique phosphopeptides (18.7%) found in all fractionation methods, which indicated that these three techniques are complementary and can be combined for more comprehensive phosphoproteome analysis (Chen et al. 2011b). Chien et al. (2011) introduced an IMAC step following ERLIC separation to reduce the abundance of the nonphosphorylated (mostly highly acidic) peptides in eluted fraction and subsequent RP-LC-MS/MS for phosphoproteomic characterization of Marek's disease virus-infected chicken embryonic fibroblast (CEF) cells.

## Immobilized metal affinity chromatography (IMAC)

IMAC was originally used for the fractionation of proteins that was based on the affinity of histidine and cysteine residues to the IMAC resin (Porath et al. 1975); however, in 1986 Andersson and Porath, for the first time, demonstrated the binding of phosphoproteins and phosphoamino acids to ferric ions immobilized on an iminodiacetate-agarose gel (Andersson and Porath 1986). Since then, the IMAC technique has been further extended and widely used for enrichment of phosphopeptides prior to MS analysis.

IMAC exploits the high affinity of positively charged metal cations ($Fe^{3+}$, $Al^{3+}$, $Ga^{3+}$, $Zr^{4+}$ or $Co^{2+}$) to negatively charged phosphopeptides. Metal ions are chelated to nitriloacetic acid or imidoacetic acid coated surfaces to form the IMAC materials. So far, several types of matrices, including polymer beads (Qi et al. 2010), the inner wall of capillaries (Xue et al. 2009) and monoliths (Dong et al. 2007, Zhang et al. 2011) have been employed for metal ion immobilization. The enrichment of phosphopeptides appears to be dependent on the type of metal ions, pH value, ionic strength and organic phase (reviewed in Thingholm et al. 2009).

The major drawback of this method is a high level of nonspecific binding of nonphosphorylated proteins containing multiple acidic amino acid residues that co-elute with the phosphopeptides during the enrichment of highly complex peptide samples. This problem may be solved by O-methylesterification of the free carboxylic groups on acidic amino acids residues in peptide under acidic conditions (Ficarro et al. 2002) or by decreasing the pH resulting in protonation of the carboxyl groups on the highly acidic amino acid residues which reduces nonspecific binding (Posewitz and Tempst 1999). Another approach to increase the efficiency of IMAC is the prefractionation of the peptide samples by various methods (described thereinbefore). In addition, the IMAC method has some other disadvantages. It was reported that IMAC is more selective for multiphosphorylated peptides than monophosphorylated peptides, as multiply phosphorylated peptides are more strongly bound to the IMAC

resin (Ficarro et al. 2002). The technique is also affected by various buffers, detergents and other reagents that are used in biochemical and cell biological procedures. Therefore, prepurification steps are necessary prior to IMAC enrichment (Bai and Wang 2009).

Bodenmiller et al. (2007) compared three phosphopeptide isolation methods: phosphoramidate chemistry, IMAC and $TiO_2$ enrichment. Among these methods, IMAC was the most specific and led to the identification of the most phosphopeptides (n=555, n=8 nonphosphorylated peptides). $TiO_2$ enrichment was the least specific with 366 phosphopeptides identified and 96 nonphosphopeptides nonspecifically bound. In addition, 2,5-dihydroxybenzoic acid (DHB) further decreased the efficiency of $TiO_2$ enrichment with only 156 phosphopeptides identified. Nevertheless, it is important to mention that the comparison was done with a specific setup and by a specific laboratory. More results are needed to confirm or to confute these conclusions suggesting that IMAC is more specific than $TiO_2$ enrichment. Liang et al. (2007) applied iTRAQ labeling of phosphopeptide standard mixtures to quantitatively evaluate IMAC and MOAC resins. The results indicated that $Fe^{3+}$ coated magnetic beads reached the same efficiency for phosphopeptide enrichment as $TiO_2$ coated magnetic beads and chromatographic $TiO_2$ spheres (Titansphere, GL Sciences, Torrance, CA, USA).

SIMAC (sequential elution from IMAC) strategy was designed to separate monophosphorylated and multiple phosphorylated peptides from complex biological samples. Monophosphorylated peptides were eluted from the IMAC resin using an acidic solution (1% TFA, 20% acetonitrile), while multiphosphorylated peptides were subsequently eluted under basic conditions (ammonia water, pH 11.3). The acidic eluent was further enriched using $TiO_2$ chromatography to sequester the pool of monophosphorylated peptides from acidic nonphosphorylated peptides (Thingholm et al. 2008). A similar procedure that involved $TiO_2$ enrichment of non-retained fractions from IMAC was used for the characterization of human T lymphocytes phosphoproteome (Carrascal et al. 2008). Another study used the combination of IMAC and $TiO_2$ enrichment to produce a large data set with only a small degree of overlap between these two methods suggesting the complementary nature of these methods (Wilson-Grady et al. 2008). Some of recent phosphoproteomic studies applying IMAC were mentioned thereinbefore in context with the prefractionation chromatographic methods. Other studies used IMAC enrichment without any prefractionation followed by LC-MS/MS. For instance, Chen et al. (2011a) studied the phosphoproteome of human prostate cancer specimens obtained from tissue depository to evaluate the phosphoproteomic analysis for the investigation of cancer-relevant phosphoproteins from archived tumor specimens to search for a prostate cancer biomarker.

## Metal oxide/hydroxide affinity chromatography (MOAC)

MOAC is one of the most powerful and promising approaches that have appeared in recent years. Metal oxides have been shown to have affinity for phosphate ions at acidic pH; the mechanism of phosphate-metal oxide interaction is based on ion-exchange where metal oxide acts as a Lewis acid, whereas the phosphopeptide group acts as a Lewis base (Matsuda et al. 1990, Ikeguchi and Nakamura 1997, Tani and Suzuki 1997).

The nature of this interaction implicates that phosphopeptides are most effectively enriched under low pH conditions. In experiments, a pH range of 2–3 is used to prevent nonspecific binding of nonphosphorylated proteins, where carboxyl groups are protonated and cannot bind to metal oxide. It has been established that especially nonphosphorylated peptides containing greater proportions of aspartic and glutamic acid bind nonspecifically (Klemm et al. 2006). Therefore, various additives have been used to increase binding specificity. Namely, trifluoroacetic acid or formic acid for pH control, acetonitrile (50–70%) to prevent hydrophobic interactions with the sorbent, and different monocarboxylic or dicarboxylic acids that are supposed to compete for binding sites with nonphosphorylated peptides; however, they do not decrease the binding affinity of phosphopeptides as they are stronger Lewis bases. The first of these presented was DHB (Larsen et al. 2005); nevertheless, lactic acid (Sugiyama et al. 2007), glutamic acid (Wu et al. 2007) or ammonium glutamate (Yu et al. 2007) have also been reported to be advantageous. The concentration of these additives might have a strong influence on the nature of phosphopeptides that are observed. Using high concentration during the binding step may improve specificity, but more weakly bound phosphopeptides could be lost, especially monophosphorylated peptides. Another approach how to minimize the level of nonspecifically bound peptides is chemical derivatization before the enrichment, e.g., methylesterification of peptides (Ficarro et al. 2002, Simon et al. 2008). A variety of pH conditions during the binding or the elution step have been employed to optimize the protocol for enrichment on MOAC resins (Simon et al. 2008, Park and Maudsley 2011).

Two different strategies have been described to enrich phosphopeptides using MOAC. The most widely followed experimental approach is the offline strategy. Metal oxide particles may be embedded in pipette tips and the enrichment proceeds during multiple passing of the peptide solution through the sorbent or mixed and incubated with proteins in a microtube, which may be more favorable because of the longer contact time of the sample with the particles. The second is the online strategy, which does not allow so much flexibility in the protocol in comparison with the offline strategy but has other undeniable advantages, e.g., higher sensitivity and automation. With the online strategy the metal oxides are integrated into the LC-MS system in the form of precolumns (e.g., Pinkse et al. 2004, Cantin et al. 2007).

A variety of metal oxides have been used for phosphopeptide enrichment to date, including $TiO_2$, zirconium dioxide ($ZrO_2$) (Kweon and Håkansson 2006), aluminum hydroxide (Wolschin and Weckwerth 2005), gallium oxide (Li et al. 2008), iron oxide (Lee et al. 2008), niobium pentoxide (Ficarro et al. 2008), tin dioxide (Leitner et al. 2009, 2010), hafnium oxide (Rivera et al. 2009) and tantalum oxide (Qi

et al. 2009); however, most of the phosphoproteomic studies have been based on $TiO_2$ enrichment.

## Titanium dioxide ($TiO_2$) enrichment

$TiO_2$ enrichment is the most popular metal oxide resin used for phosphopeptide enrichment because of its outstanding enrichment behavior and commercial availability (e.g., Titansphere, GL Sciences). $TiO_2$ is highly selective to preferentially bind phosphopeptides in suitable conditions during sample loading, rinsing and peptide elution. The great advantage of $TiO_2$ enrichment is that $TiO_2$ is more robust and tolerant towards many reagents normally utilized in biochemical and cell biological procedures in comparison with conventional IMAC which is severely affected by various buffers, detergents and other low molecular molecules. In addition, various detergents were shown to enhance the efficiency of $TiO_2$ enrichment (Jensen and Larsen 2007).

This method was introduced in 2004 as a novel promising strategy for the selective phosphopeptide enrichment prior to 2D-nano-LC-ESI-MS/MS alternative to IMAC (Pinkse et al. 2004). In that setup, $TiO_2$ was used in the form of an online $TiO_2$ precolumn coupled to a reversed phase capillary column, loading solution containing 0.1 M acetic acid and the elution was performed with ammonium bicarbonate (pH 9.0). Phosphorylated peptides were successfully enriched; nevertheless, nonphosphorylated peptides were also retained on the $TiO_2$ precolumn. As it was estimated that the retention of nonphosphorylated peptides was caused by their acidic nature, the use of various "nonphosphopeptide excluders" in loading and washing solutions for $TiO_2$ has been examined.

In 2005, Larsen and his coworkers investigated the effect of different aromatic carboxylic acids and aliphatic carboxylic acids in loading buffer. DHB and other substituted aromatic carboxylic acids (salicylic acid, phthalic acid) followed by monofunctional carboxylic aromatic and aliphatic acids (benzoic acid, cyclohexanecarboxylic acid, phosphoric acid, trifluoroacetic and acetic acid) showed the best efficacy in inhibition of adsorption of nonphosphorylated peptides (Larsen et al. 2005). DHB was also found to be the most potent additive in a study by Yu et al. (2009). These results suggest the importance of a hydroxyl group in *ortho* position on a benzene ring that is probably more relevant for reducing the nonspecific peptide binding than a hydroxyl group in *meta* position, probably because the interactions between substituted aromatic carboxylic acids and the surface of $TiO_2$ are based on a coordination bond that forms a chelating bidentate (Dobson and McQuillan 2000).

By contrast, Sugiyama et al. (2007) reported that DHB added to loading buffer decreased the number of phosphopeptides identified in the LC-ESI-MS system, probably because DHB was eluted at the same time range as phosphopeptides and the suppression of phosphopeptide ionization occurred. In addition, DHB caused various problems within this system, e.g., column clogging, precipitation around the orifice and loss of sensitivity over time. They also compared the effect of hydroxy acids (DHB, glycolic acid, lactic acid, malic acid, tartaric acid, hydroxypropanoic acid) in the loading buffer on phosphopeptide enrichment with various MOAC tips. Among the hydroxy acids used, lactic acid and hydroxypropanoic acid were the most effective for $TiO_2$ and $ZrO_2$ enrichment. Furthermore, aliphatic hydroxy acids are hydrophilic enough to be removed during the desalting step and thus they did not appear to affect the LC-MS system, as described in the case of DHB.

Jensen and Larsen (2007) further examined the effect of hydroxy acids, namely phthalic, glycolic, oxalic, lactic, gallic and citric acids. Among these compounds, 1 M glycolic acid was shown to be the most advantageous for the minimization of nonspecific absorption of acidic nonphosphorylated peptides without effect on the binding of phosphopeptides to $TiO_2$. Contrary to their results, Aryal and Ross (2010) found that the addition of glycolic acid to the loading solution reduced the specificity towards phosphopeptides. A possible explanation of this discordance is variability in properties of titania that was used for the particular study. It was shown that structure and retention properties of titania are strongly dependent on the calcination temperature of the beads (Tani et al. 2002) and thus can differ from one study to another.

The optimized protocol for $TiO_2$ enrichment presented by Wu et al. (2007) included the addition of glutamic acid in the sample loading buffer as an effective nonphosphopeptide excluding agent. Other investigations showed that loading buffer consisting of a combination of high concentrations of 1-octanesulfonic acid and low concentrations of DHB also improved the selectivity for phosphopeptides without affecting the LC-MS system, as also observed when DHB was used as a nonphosphopeptide excluder alone (Mazanek et al. 2007). This protocol was further optimized with the addition of heptafluorobutyric acid (Mazanek et al. 2010).

Peptide-to-$TiO_2$ ratio was also investigated as a substantial factor for the selectivity of phosphopeptide enrichment. The optimum ratio for HeLa cell lysate was from 1:2 to 1:8 (mass/mass); less or more $TiO_2$ beads decreased the selectivity. Interestingly, multiple phosphorylated peptides were identified by deficient $TiO_2$ beads, whereas with the increasing beads dosage, the monophosphorylated peptides became dominant (Li et al. 2009). Similar findings were obtained for acute myeloid leukemia (AML) cell line P31 (Montoya et al. 2011).

Recently, the $TiO_2$ enrichment method has been further improved by developing and using various nanoparticles, nanocomposites and microspheres. For example, nano-titanium dioxide composites were synthesized from $TiO_2$ nanoparticles via photopolymerization in the presence of a diacrylate crosslinker. The enrichment efficacy of these nanocomposites was determined to be two to five times larger compared to 5 μm $TiO_2$ particles. Moreover, crosslinking of the $TiO_2$ nanoparticles helps to prevent loss of the particles from the packed cartridges during washing procedures (Liang et al. 2006). Lin and coworkers deposited a thin $TiO_2$ layer onto the inner surface of a capillary column by the LPD (liquid phase deposition) technique. This $TiO_2$ nanoparticle-deposited capillary column was then applied offline with MALDI-TOF MS or online with ESI-QTOF MS and nano-LC-ESI-

MS/MS and a good capability for enriching of phosphopeptides was demonstrated, i.e., phosphopeptides from α-casein were detected in a mixture of tryptic peptides from α-casein and bovine serum albumin (BSA) at the femtomole level (Lin et al. 2008). Mesoporous nanostructured $TiO_2$ clusters were also exploited for selective separation of phosphopeptides. $TiO_2$ nanocrystals were first self-assembled and then further modified to form submicrometer clusters with relatively uniform mesoscale pores and a hydrophilic and negatively charged surface that enhanced the water dispersibility of the clusters. The incorporation of various components that further facilitate the separation of phosphopeptides, e.g., superparamagnetic nanocrystals, was demonstrated to be feasible because of the self-assembly process (Lu et al. 2010). The affinity material particles could also be immobilized on MALDI plates. The on-plate enrichment for subsequent MALDI-MS analysis has been shown to have some advantages in comparison with the conventional resin-based techniques such as minimal sample handling and thus lower sample loss (e.g., Qiao et al. 2007).

## Zirconium dioxide ($ZrO_2$) enrichment

The utilization of $ZrO_2$ enrichment for phosphopeptide isolation prior to MS analysis was first demonstrated by Kweon and Håkansson (2006). The selectivities for phosphopeptides of microtips filled with $ZrO_2$ and $TiO_2$ microtips were compared, and both $ZrO_2$ and $TiO_2$ were highly specific for phosphopeptides. Nevertheless, $ZrO_2$ provided more selective enrichment for monophosphorylated peptides, whereas $TiO_2$ was more selective for multiphosphorylated peptides (Kweon and Håkansson 2006). The observed difference in binding selectivity of $ZrO_2$ and $TiO_2$ is probably due to the fact that $ZrO_2$ is a stronger Lewis acid than $TiO_2$ under acidic conditions, together with different coordination numbers of zirconia and titania in crystalline forms (7 and 6, respectively; Kweon and Håkansson 2006). Contrary to that report, no obvious affinity difference towards singly and multiple phosphorylated peptides was observed when $ZrO_2$ nanoparticles were used. Compared with microparticles, nanoparticles have a higher surface area and thus provide higher sample capacity (Zhou et al. 2007). As in the case of $TiO_2$, the $ZrO_2$ enrichment method has been further improved by using various loading, washing and elution conditions and by preparing different $ZrO_2$ containing microspheres, nanoparticles and nanocomposites. Sugiyama and coworkers (2007) examined the effect of various hydroxy acids (β-hydroxypropanoic, DHB, glycolic, lactic, malic and tartaric acids) in the loading solution, and among them the β-hydroxypropanoic acid was shown to be the most effective nonphosphopeptide excluder. Lo et al. (2007) presented iron oxide nanocomposites of magnetic particles coated with zirconia with high surface-to-volume ratio improving the trapping capacity and reducing the time required for enrichment; phosphopeptides could be enriched by pipetting the sample with the particles for only 30 s. In addition, magnetic property enabled easy isolation by application of an external magnetic field. Li et al. (2007) prepared $Fe_3O_4/ZrO_2$ core shell microspheres with well-defined core shell structure and higher selectivity than $ZrO_2$ coated magnetic particles (no core shell structure) described previously. Furthermore, $Fe_3O_4/ZrO_2$ core shell microspheres were more selective in comparison with commercially available IMAC material (PHOS-select iron affinity beads, Sigma, St. Louis, MO, USA). In 2009, mesoporous $ZrO_2$ nanomaterial with very large surface areas and many surface sites which provide higher loading capacity for binding of phosphate groups than microparticles and nanoparticles was first applied for phosphopeptide enrichment (Nelson et al. 2009). However, mesoporous $ZrO_2$ has some disadvantages including its thermal instability and hence mesoporous silica microspheres coated with zirconia layer were synthesized to improve its thermal stability. Owing to the interactions of metal oxide and silica support, the physical-chemistry properties of metal oxide differ greatly to the bulk crystalline metal oxides and enable high phosphopeptide recovery, especially for multiphosphorylated peptides, which was shown to be even higher than that of the widely used commercial $TiO_2$ microparticles (Wan et al. 2010).

## Hydroxyapatite enrichment

Ceramic hydroxyapatite based enrichment is a new method presented in 2010 (Mamone et al. 2010), which was shown to be employable for both phosphoproteins and phosphopeptides enrichment and also for on-particle tryptic digestion (Pinto et al. 2010). Hydroxyapatite is a crystalline form of calcium phosphate with a chemical formula $Ca_{10}(PO_4)_6(OH)_2$, which was originally used for the separation of proteins (Tiselius et al. 1956) and nucleic acids (Bernardi 1969). Binding between hydroxyapatite crystal surface that contains positively charged $Ca^{2+}$ ions and negatively charged $PO_4^{3-}$ groups occurs by the complexing of protein carboxyls or phosphates with $Ca^{2+}$ ions. The specificity of hydroxyapatite enrichment for phosphopeptides is based on the fact that phosphogroups of the protein bind more strongly with $Ca^{2+}$ than carboxyl groups (Gorbunoff 1984). The HAP method was compared with the $TiO_2$ technique and it was shown that HAP enrichment increased specificity towards multiphosphorylated peptides by reducing the amount of monophosphorylated peptides and nonphosphorylated peptides (Mamone et al. 2010).

## Molecularly imprinted polymers (MIPs)

MIPs can also be exploited to target phosphorylated peptides. A phosphate-selective molecularly imprinted polymer has been prepared using 1-allyl-2-urea as a functional monomer and a diphenylphosphate template. The imprinted polymer exhibited high binding ability and selectivity for phosphate, with 70% phosphate recovery (Kugimiya and Takei 2008). Recently, phosphotyrosine MIP-based receptors have been developed using two urea-based monomers and an N,O-protected p-Tyr template. These receptors have been demonstrated to have high selectivity, sensitivity and affinity for peptides containing the imprinted amino acid (pTyr) and hence could be used to

capture pTyr-containing peptides at the femtomole level in the presence of four orders of magnitude higher concentrations of other nonphosphorylated or pSer/Thr-phosphorylated peptide fragments (Helling et al. 2011).

## Conclusion

In recent years, the advances made in MS technologies have spurred the progress of phosphoproteomics. The development of new methods for phosphoproteomics research is motivated by the importance and omnipresence of protein phosphorylation in nature. The characterization of cellular networks and signal transduction via phosphorylation and dephosphorylation is essential to understand both physiological and pathological cellular processes. Although the potential for phosphopeptide enrichment and rapid analysis has improved dramatically over the past few years, a comprehensive description of phosphoproteome is still a great challenge for many researchers. The combinations of enrichment techniques or the utilization of a variety of prefractionation techniques prior to phosphopeptide enrichment are employed to obtain more complete information about the phosphopeptide pool in real samples. Furthermore, the application of proteases other than the most used trypsin such as Lys-N could be promising. Nevertheless, further improvements in enrichment techniques are necessary to achieve the truly comprehensive and thus reproducible experiments, to improve sensitivity to allow researchers to work with only small amounts of starting material, and also to simplify and speed up phosphoproteomic analysis that is often very time-consuming.

## Acknowledgments

This work was supported by the Grant Agency of the Czech Republic (project P206/12/P338) and by the grant of the "Internal competition of the Faculty of medicine in Hradec Králové". The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

## References

Albuquerque, C. P.; Smolka, M. B.; Payne, S. H.; Bafna, V.; Eng, J.; Zhou, H. A multidimensional chromatography technology for in-depth phosphoproteome analysis. *Mol. Cell. Proteomics* **2008**, *7*, 1389–1396.

Alpert, A. J. Hydrophilic-interaction chromatography for the separation of peptides, nucleic acids and other polar compounds. *J. Chromatogr.* **1990**, *19*, 177–196.

Alpert, A. J. Electrostatic repulsion hydrophilic interaction chromatography for isocratic separation of charged solutes and selective isolation of phosphopeptides. *Anal. Chem.* **2008**, *80*, 62–76.

Andersson, I.; Porath, J. Isolation of phosphoproteins by immobilized metal ($Fe^{3+}$) affinity chromatography. *Anal. Biochem.* **1986**, *154*, 250–254.

Arrigoni, G.; Resjö, S.; Levander, F.; Nilsson, R.; Degerman, E.; Quadroni, M.; Pinna, L. A.; James, P. Chemical derivatization of phosphoserine and phosphothreonine containing peptides to increase sensitivity for MALDI-based analysis and for selectivity of MS/MS analysis. *Proteomics* **2006**, *6*, 757–766.

Aryal, U. K.; Ross, A. R. Enrichment and analysis of phosphopeptides under different experimental conditions using titanium dioxide affinity chromatography and mass spectrometry. *Rapid Commun. Mass. Spectrom.* **2010**, *24*, 219–231.

Bai, Z. F.; Wang, H. X. Advances in separation and enrichment approach of phosphoproteome reasearches. *Chin. J. Anal. Chem.* **2009**, *37*, 1382–1389.

Beausoleil, S. A.; Jedrychowski, M.; Schwartz, D.; Elias, J. E.; Villén, J.; Li, J.; Cohn, M. A.; Cantley, L. C.; Gygi, S. P. Large-scale characterization of HeLa cell nuclear phosphoproteins. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 12130–12135.

Benschop, J. J.; Mohammed, S.; O'Flaherty, M.; Heck, A. J.; Slijper, M.; Menke F. L. Quantitative phosphoproteomics of early elicitor signaling in Arabidopsis. *Mol. Cell. Proteomics* **2007**, *6*, 1198–1214.

Bernardi, G. Chromatography of nucleic acids on hydroxyapatite. I. Chromatography of native DNA. *Biochim. Biophys. Acta* **1969**, *174,* 423–434.

Blagoev, B.; Ong, S. E.; Kratchmarova, I.; Mann, M. Temporal analysis of phosphotyrosine-dependent signaling networks by quantitative proteomics. *Nat. Biotechnol.* **2004**, *22*, 1139–1145.

Blume-Jensen, P.; Hunter T. Oncogenic kinase signalling. *Nature* **2001**, *411*, 355–365.

Bodenmiller, B.; Mueller, L. N.; Mueller, M.; Domon, B.; Aebersold, R. Reproducible isolation of distinct, overlapping segments of the phosphoproteome. *Nat. Methods* **2007**, *4*, 231–237.

Brehmer, D.; Godl, K.; Zech. B.; Wissing, J.; Daub, H. Proteome-wide identification of cellular targets affected by bisindolylmaleimide-type protein kinase C inhibitors. *Mol. Cell. Proteomics* **2004**, *3*, 490–500.

Cantin, G. T.; Shock, T. R.; Park, S. K.; Madhani, H. D.; Yates, J. R. Optimizing $TiO_2$-based phosphopeptide enrichment for automated multidimensional liquid chromatography coupled to tandem mass spectrometry. *Anal. Chem.* **2007**, *79*, 4666–4673.

Carrascal, M.; Ovelleiro, D.; Casas, V.; Gay, M.; Abian, J. Phosphorylation analysis of primary human T lymphocytes using sequential IMAC and titanium oxide enrichment. *J. Proteome Res.* **2008**, *7*, 5167–5176.

Chen, L.; Giorgianni, F.; Beranova-Giorgianni, S. Characterization of the phosphoproteome in LNCaP prostate cancer cells by in-gel isoelectric focusing and tandem mass spectrometry. *J. Proteome Res.* **2010**, *9*, 174–178.

Chen, L.; Fang, B.; Giorgianni, F.; Gingrich, J. R.; Beranova-Giorgianni, S. Investigation of phosphoprotein signatures of archived prostate cancer tissue specimens via proteomic analysis. *Electrophoresis* **2011a**, *32*, 1984–1991.

Chen, X.; Wu, D.; Zhao, Y.; Wong, B. H.; Guo, L. Increasing phosphoproteome coverage and identification of phosphorylation motifs through combination of different HPLC fractionation methods. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **2011b**, *879*, 25–34.

Chien, K. Y.; Liu, H. C.; Goshe, M. B. Development and application of a phosphoproteomic method using electrostatic repulsion-hydrophilic interaction chromatography (ERLIC), IMAC, and LC-MS/MS analysis to study Marek's disease virus infection. *J. Proteome Res.* **2011**, *10*, 4041–4053.

Cohen, P. The origins of protein phosphorylation. *Nat. Cell. Biol.* **2002**, *4*, 127–130.

Craig, A. G.; Hoeger, C. A.; Miller, C. L.; Goedken, T.; Rivier, J. E.; Fischer, W. H. Monitoring protein kinase and phosphatase

reactions with matrix-assisted laser desorption/ionization mass spectrometry and capillary zone electrophoresis: comparison of the detection efficiency of peptide-phosphopeptide mixtures. *Biol. Mass Spectrom.* **1994**, *23*, 519–528.

Dai, J.; Jin, W. H.; Sheng, Q. H.; Shieh, C. H.; Wu, J. R.; Zeng, R. Protein phosphorylation and expression profiling by Yin-yang multidimensional liquid chromatography (Yin-yang MDLC) mass spectrometry. *J. Proteome Res.* **2007**, *6*, 250–262.

Dai, J.; Wang, L. S.; Wu, Y. B.; Sheng, Q. H.; Wu, J. R.; Shieh, C. H.; Zeng, R. Fully automatic separation and identification of phosphopeptides by continuous pH-gradient anion exchange online coupled with reversed-phase liquid chromatography mass spectrometry. *J. Proteome Res.* **2009**, *8*, 133–141.

Dobson, K. D.; McQuillan, A. J. In situ infrared spectroscopic analysis of the absorption of aromatic carboxylic acids to $TiO_2$, $ZrO_2$, $Al_2O_3$, and $Ta_2O_5$ from aqueous solutions. *Spectrochim. Acta A Mol. Biomol. Spectrosc.* **2000**, *56*, 557–565.

Dong, J.; Zhou, H.; Wu, R.; Ye, M.; Zou, H. Specific capture of phosphopeptides by Zr4+-modified monolithic capillary column. *J. Sep. Sci.* **2007**, *30*, 2917–2923.

Edbauer, D.; Cheng, D.; Batterton, M. N.; Wang, C. F.; Duong, D. M.; Yaffe, M. B.; Peng, J.; Sheng, M. Identification and characterization of neuronal mitogen-activated protein kinase substrates using a specific phosphomotif antibody. *Mol. Cell. Proteomics* **2009**, *8*, 681–695.

Ficarro, S. B.; McCleland, M. L.; Stukenberg, P. T.; Burke, D. J.; Ross, M. M.; Shabanowitz, J.; Hunt, D. F.; White, F. M. Phosphoproteome analysis by mass spectrometry and its application to *Saccharomyces cerevisiae*. *Nat. Biotechnol.* **2002**, *20*, 301–305.

Ficarro, S. B.; Parikh, J. R.; Blank, N. C.; Marto, J. A. Niobium(V) oxide($Nb_2O_5$): application to phosphoproteomics. *Anal. Chem.* **2008**, *80*, 4606–4613.

Gauci, S.; Helbig, A. O.; Slijper, M.; Krijgsveld, J.; Heck, A. J.; Mohammed, S. Lys-N and trypsin cover complementary parts of the phosphoproteome in a refined SCX-based approach. *Anal. Chem.* **2009**, *81*, 4493–4501.

Gilar, M.; Olivova, P.; Daly, A. E.; Gebler, J. C. Orthogonality of separation in two-dimensional liquid chromatography. *Anal. Chem.* **2005**, *77*, 6426–6434.

Gorbunoff, M. J. The interaction of proteins with hydroxyapatite. *Anal. Biochem.* **1984**, *136*, 425–445.

Goshe, M. B.; Conrads, T. P.; Panisko, E. A.; Angell, N. H.; Veenstra, T. D.; Smith, R. D. Phosphoprotein isotope-coded affinity tag approach for isolating and quantitating phosphopeptides in proteome-wide analyses. *Anal. Chem.* **2001**, *73*, 2578–2586.

Goshe, M. B.; Veenstra, T. D.; Panisko, E. A.; Conrads, T. P.; Angell, N. H.; Smith, R. D. Phosphoprotein isotope-coded affinity tags: application to the enrichment and identification of low-abundance phosphoproteins. *Anal. Chem.* **2002**, *74*, 607–616.

Grimsrud, P. A.; Swaney, D. L.; Wenger, C. D.; Beauchene, N. A.; Coon, J. J. Phosphoproteomics for the masses. *ACS Chem. Biol.* **2010**, *5*, 105–119.

Grønborg, M.; Kristiansen, T. Z.; Stensballe, A.; Andersen, J. S.; Ohara, O.; Mann, M.; Jensen, O. N.; Pandey, A. A mass spectrometry-based proteomic approach for identification of serine/threonine-phosphorylated proteins by enrichment with phospho-specific antibodies: identification of a novel protein, Frigg, as a protein kinase A substrate. *Mol. Cell. Proteomics* **2002**, *1*, 517–527.

Gruhler, A.; Olsen, J. V.; Mohammed, S.; Mortensen, P.; Faergeman, N. J.; Mann, M.; Jensen, O. N. Quantitative phosphoproteomics applied to the yeast pheromone signaling pathway. *Mol. Cell Proteomics* **2005**, *4*, 310–327.

Han, G.; Ye, M.; Zhou, H.; Jiang, X.; Feng, S.; Jiang, X.; Tian, R.; Wan, D.; Zou, H.; Gu, J. Large-scale phosphoproteome analysis of human liver tissue by enrichment and fractionation of phosphopeptides with strong anion exchange chromatography. *Proteomics* **2008**, *8*, 1346–1361.

Harsha, H. C.; Pandey, A. Phosphoproteomic in cancer. *Mol. Oncol.* **2010**, *4*, 482–495.

Helling, S.; Shinde, S.; Brosseron, F.; Schnabel, A.; Müller, T.; Meyer, H. E.; Marcus, K.; Sellergren, B. Ultratrace enrichment of tyrosine phosphorylated peptides on an imprinted polymer. *Anal. Chem.* **2011**, *83*, 1862–1865.

Hemström, P.; Irgum, K. Hydrophilic interaction chromatography. *J. Sep. Sci.* **2006**, *29*, 1784–1821.

Holland, C.; Schmid, M.; Zimny-Arndt, U.; Rohloff, J.; Stein, R.; Jungblut, P. R.; Meyer T. F. Quantitative phosphoproteomics reveals link between *Helicobacter pylori* infection and RNA splicing modulation in host cells. *Proteomics* **2011**, *11*, 2798–2811.

Hultquist, D. E. The preparation and characterization of phosphorylated derivatives of histidine. *Biochim. Biophys. Acta* **1968**, *153*, 329–340.

Hunter, T.; Sefton, B. M. Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. *Proc. Natl. Acad. Sci. USA* **1980**, *77*, 1311–1315.

Ikeguchi, Y.; Nakamura, H. Determination of organic phosphates by column-switching high performance anion-exchange chromatography using on-line preconcentration on titania. *Anal. Sci.* **1997**, *13*, 479–483.

Jensen, S. S.; Larsen, M. R. Evaluation of the impact of some experimental procedures on different phosphopeptide enrichment techniques. *Rapid Commun. Mass. Spectrom.* **2007**, *21*, 3635–3645.

Klemm, C.; Otto, S.; Wolf, C.; Haseloff, R. F.; Beyermann, M.; Krause, E. Evaluation of the titanium dioxide approach for MS analysis of phosphopeptides. *J. Mass. Spectrom.* **2006**, *41*, 1623–1632.

Knezevic, I.; Bachem, S.; Sickmann, A.; Meyer, H. E.; Stülke, J.; Hengstenberg, W. Regulation of the glucose-specific phosphotransferase system (PTS) of *Staphylococcus carnosus* by the antiterminator protein GlcT. *Microbiology* **2000**, *146*, 2333–2342.

Kugimiya, A.; Takei, H. Selectivity and recovery performance of phosphate-selective molecularly imprinted polymer. *Anal. Chim. Acta* **2008**, *606*, 252–256.

Kweon, H. K.; Håkansson, K. Selective zirconium dioxide-based enrichment of phosphorylated peptides for mass spectrometric analysis. *Anal. Chem.* **2006**, *78*, 1743–1749.

Lansdell, T. A.; Tepe, J. J. Isolation of phosphopeptides using solid phase enrichment. *Tetrahedron Lett.* **2004**, *45*, 91–93.

Larsen, M. R.; Thingholm, T. E.; Jensen, O. N.; Roepstorff, P.; Jørgensen, T. J. Highly selective enrichment of phosphorylated peptides from peptide mixtures using titanium dioxide microcolumns. *Mol. Cell Proteomics* **2005**, *4*, 873–886.

Lee, A.; Yang, H. J.; Lim, E. S.; Kim, J.; Kim, Y. Enrichment of phosphopeptides using bare magnetic particles. *Rapid Commun. Mass. Spectrom.* **2008**, *22*, 2561–2564.

Lee, H. J.; Na, K.; Kwon, M. S.; Kim, H.; Kim, K. S.; Paik, Y. K. Quantitative analysis of phosphopeptides in search of the disease biomarker from the hepatocellular carcinoma specimen. *Proteomics* **2009**, *9*, 3395–3408.

Leitner, A.; Sturm, M.; Smått, J. H.; Järn, M.; Lindén, M.; Mechtler, K.; Lindner, W. Optimizing the performance of tin dioxide microspheres for phosphopeptide enrichment. *Anal. Chim. Acta* **2009**, *638*, 51–57.

Leitner, A.; Sturm, M.; Hudecz, O.; Mazanek, M.; Smått, J. H.; Lindén, M.; Lindner, W.; Mechtler, K. Probing the phosphoproteome of HeLa cells using nanocast metal oxide microspheres for phosphopeptide enrichment. *Anal. Chem.* **2010**, *82*, 2726–2733.

Lemeer, S.; Pinkse, M. W.; Mohammed, S.; van Breukelen, B.; den Hertog, J.; Slijper, M.; Heck, A. J. Online automated in vivo zebrafish phosphoproteomics: from large-scale analysis down to a single embryo. *J. Proteome Res.* **2008a**, *7*, 1555–1564.

Lemeer, S.; Jopling, C.; Gouw, J.; Mohammed, S.; Heck, A. J.; Slijper, M.; den Hertog, J. Comparative phosphoproteomics of zebrafish Fyn/Yes morpholino knockdown embryos. *Mol. Cell. Proteomics* **2008b**, *7*, 2176–2187.

Li, Y.; Leng, T.; Lin, H.; Deng, C.; Xu, X.; Yao, N.; Yang, P.; Zhang, X. Preparation of Fe3O4@ZrO2 core-shell microspheres as affinity probes for selective enrichment and direct determination of phosphopeptides using matrix-assisted laser desorption ionization mass spectrometry. *J. Proteome Res.* **2007**, *6*, 4498–4510.

Li, Y.; Lin, H.; Deng, C.; Yang, P.; Zhang, X. Highly selective and rapid enrichment of phosphorylated peptides using gallium oxide-coated magnetic microspheres for MALDI-TOF-MS and nano-LC-ESI-MS/MS/MS analysis. *Proteomics* **2008**, *8*, 238–249.

Li, Q. R.; Ning, Z. B.; Tang, J. S.; Nie, S.; Zeng, R. Effect of peptide-to-$TiO_2$ beads ratio on phosphopeptide enrichment selectivity. *J. Proteome Res.* **2009**, *8*, 5375–5381.

Liang, S. S.; Makamba, H.; Huang, S. Y.; Chen, S. H. Nano-titanium dioxide composites for the enrichment of phosphopeptides. *J. Chromatogr. A* **2006**, *1116*, 38–45.

Liang, X.; Fonnum, G.; Hajivandi, M.; Stene, T.; Kjus, N. H.; Ragnhildstveit, E.; Amshey, J. W.; Predki, P.; Pope, R. M. Quantitative comparison of IMAC and TiO2 surfaces used in the study of regulated, dynamic protein phosphorylation. *J. Am. Soc. Mass Spectrom.* **2007**, *18*, 1932–1944.

Liao, P. C.; Leykam, J.; Andrews, P. C.; Gage, D. A.; Allison, J. An approach to locate phosphorylation sites in a phosphoprotein: mass mapping by combining specific enzymatic degradation with matrix-assisted laser desorption/ionization mass spectrometry. *Anal. Biochem.* **1994**, *219*, 9–20.

Lin, B.; Li, T.; Zhao, Y.; Huang, F. K.; Guo, L.; Feng, Y. Q. Preparation of a TiO2 nanoparticle-deposited capillary column by liquid phase deposition and its application in phosphopeptide analysis. *J. Chromatogr. A* **2008**, *1192*, 95–102.

Lo, C. Y.; Chen, W. Y.; Chen, C. T.; Chen, Y. C. Rapid enrichment of phosphopeptides from tryptic digests of proteins using iron oxide nanocomposites of magnetic particles coated with zirconia as the concentrating probes. *J. Proteome Res.* **2007**, *6*, 887–893.

Lu, Z.; Duan, J.; He, L.; Hu, Y.; Yin, Y. Mesoporous TiO(2) nanocrystal clusters for selective enrichment of phosphopeptides. *Anal. Chem.* **2010**, *82*, 7249–7258.

Maccarrone, G.; Kolb, N.; Teplytska, L.; Birg, I.; Zollinger, R.; Holsboer. F.; Turck, C. W. Phospho-peptide enrichment by IEF. *Electrophoresis* **2006**, *27*, 4585–4595.

Mamone, G.; Picariello, G.; Ferranti, P.; Addeo, F. Hydroxyapatite affinity chromatography for the highly selective enrichment of mono- and multi-phosphorylated peptides in phosphoproteome analysis. *Proteomics* **2010**, *10*, 380–393.

Manning, G.; Whyte, D. B.; Martinez, R.; Hunter, T.; Sudarsanam, S. The protein kinase complement of human genome. *Science* **2002**, *298*, 1912–1934.

Matsuda, H.; Nakamura, H.; Nakajima, T. New ceramic titania: selective adsorbent for organic phosphates. *Anal. Sci.* **1990**, *6*, 911–912.

Matthews, H. R. Protein kinases and phosphatases that act on histidine, lysine, or arginine residues in eukaryotic proteins: a possible regulator of the mitogen-activated protein kinase cascade. *Pharm. Ther*. **1995**, *67*, 323–350.

Mazanek, M.; Mituloviae, G.; Herzog, F.; Stingl, C.; Hutchins, J. R.; Peters, J. M.; Mechtler, K. Titanium dioxide as a chemo-affinity solid phase in offline phosphopeptide chromatography prior to HPLC-MS/MS analysis. *Nat. Protoc.* **2007**, *2*, 1059–1069.

Mazanek, M.; Roitinger, E.; Hudecz, O.; Hutchins, J. R.; Hegemann, B.; Mitulović, G.; Taus, T.; Stingl, C.; Peters, J. M.; 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.* **2010**, *878*, 515–524.

McNulty, D. E.; Annan, R. S. Hydrophilic interaction chromatography reduces the complexity of the phosphoproteome and improves global phosphopeptide isolation and detection. *Mol. Cell. Proteomics* **2008**, *7*, 971–980.

Montoya, A.; Beltran, L.; Casado, P.; Rodríguez-Prados, J. C.; Cutillas, P. R. Characterization of a $TiO_2$ enrichment method for label-free quantitative phosphoproteomics. *Methods*. **2011**, *54*, 370–378.

Nelson, C. A.; Szczech, J. R.; Xu, Q.; Lawrence, M. J.; Jin, S.; Ge, Y. Mesoporous zirconium oxide nanomaterials effectively enrich phosphopeptides for mass spectrometry-based phosphoproteomics. *Chem. Commun.* (*Camb.*) **2009**, *43*, 6607–6609.

Nie, S.; Dai, J.; Ning, Z. B.; Cao, X. J.; Sheng, Q. H.; Zeng, R. Comprehensive profiling of phosphopeptides based on anion exchange followed by flow-through enrichment with titanium dioxide (AFET). *J. Proteome Res.* **2010**, *9*, 4585–4594.

Nilsson, C. L.; Dillon, R.; Devakumar, A.; Shi, S. D.; Greig, M.; Rogers, J. C.; Krastins, B.; Rosenblatt, M.; Kilmer, G.; Major, M.; Kaboord, B. J.; Sarracino, D.; Rezai, T.; Prakash, A.; Lopez, M.; Ji, Y.; Priebe, W.; Lang, F. F.; Colman, H.; Conrad, C. A. Quantitative phosphoproteomic analysis of the STAT3/IL-6/HIF1alpha signaling network: an initial study in GSC11 glioblastoma stem cells. *J. Proteome Res.* **2010**, *9*, 430–443.

Nühse, T. S.; Stensballe, A.; Jensen, O. N.; Peck, S. C. Large-scale analysis of in vivo phosphorylated membrane proteins by immobilized metal ion affinity chromatography and mass spectrometry. *Mol. Cell. Proteomics* **2003**, *2*, 1234–1243.

Oda, Y.; Nagasu, T.; Chait, B. T. Enrichment analysis of phosphorylated proteins as a tool for probing the phosphoproteome. *Nat. Biotechnol.* **2001**, *19*, 379–382.

Olsen, J. V.; Blagoev, B.; Gnad, F.; Macek, B.; Kumar, C.; Mortensen, P.; Mann, M. Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. *Cell* **2006**, *127*, 635–648.

Park, S. S.; Maudsley, S. Discontinuous pH gradient-mediated separation of TiO2-enriched phosphopeptides. *Anal. Biochem.* **2011**, *409*, 81–88.

Pink, M.; Verma, N.; Polato, F.; Bonn, G. K.; Baba, H. A.; Rettenmeier, A. W.; Schmitz-Spanke, S. Precipitation by lanthanum ions: a straightforward approach to isolating phosphoproteins. *J. Proteomics* **2011**, *75*, 375–383.

Pinkse, M. W. H.; Uitto, P. M.; Hilhorst, M. J.; Ooms, B.; Heck, A. J. R. Selective isolation at the femtomole level of phosphopeptides from proteolytic digests using 2D-nanoLC-ESI-MS/MS and titanium oxide precolumns. *Anal. Chem.* **2004**, *76*, 3935–3943.

Pinkse, M. W. H.; Heck, A. J. R. Essential enrichment strategies in phosphoproteomics. *Drug Discov. Today Technol.* **2010**, *3*, 331–337.

Pinto, G.; Caira, S.; Cuollo, M.; Lilla, S.; Fierro, O.; Addeo, F. Hydroxyapatite affinity chromatography for the highly selective enrichment of mono- and multi-phosphorylated peptides in phosphoproteome analysis. *Proteomics* **2010**, *10*, 380–393.

Poot, A. J.; Ruijter, E.; Nuijens, T.; Dirksen, E. H.; Heck, A. J.; Slijper, M.; Rijkers, D. T.; Liskamp, R. M. Selective enrichment of Ser-/Thr-phosphorylated peptides in the presence of Ser-/Thr-glycosylated peptides. *Proteomics* **2006**, *6*, 6394–6399.

Porath, J.; Carlsson, J.; Olsson, I.; Belfrage, G. Metal chelate affinity-chromatography, a new approach to protein fractionation. *Nature* **1975**, *258*, 598–599.

Posewitz, M. C.; Tempst, P. Immobilized gallium(III) affinity chromatography of phosphopeptides. *Anal. Chem.* **1999**, *71*, 2883–2892.

Qi, D.; Lu, J.; Deng, C.; Zhang, X. Development of core-shell structure Fe3O4@Ta2O5 microspheres for selective enrichment of phosphopeptides for mass spectrometry analysis. *J. Chromatogr. A* **2009**, *1216*, 5533–5539.

Qi, D.; Mao, Y.; Zhang, X. Phosphate-functionalized magnetic microspheres for immobilization of $Zr^{4+}$ ions for selective enrichment of the phosphopeptides. *J. Chromatogr. A* **2010**, *1217*, 2606–2617.

Qiao, L.; Roussel, C.; Wan, J.; Yang, P.; Girault, H. H.; Liu, B. Specific on-plate enrichment of phosphorylated peptides for direct MALDI-TOF MS analysis. *J. Proteome Res.* **2007**, *6*, 4763–4769.

Reinders, J.; Sickman, A. State-of-the-art in phosphoproteomics. *Proteomics* **2005**, *5*, 4052–4061.

Rivera, J. G.; Choi, Y. S.; Vujcic, S.; Wood, T. D.; Colón, L. A. Enrichment/isolation of phosphorylated peptides on hafnium oxide prior to mass spectrometric analysis. *Analyst.* **2009**, *134*, 31–33.

Ruse, C. I.; McClatchy, D. B.; Lu, B.; Cociorva, D.; Motoyama, A.; Park, S. K.; Yates, J. R., 3rd. Motif-specific sampling of phosphoproteomes. *J. Proteome Res.* **2008**, *7*, 2140–2150.

Rush, J.; Moritz, A.; Lee, K. A.; Guo, A.; Goss, V. L.; Spek, E. J.; Zhang, H.; Zha, X. M.; Polakiewicz, R. D.; Comb, M. J. Immunoaffinity profiling of tyrosine phosphorylation in cancer cells. *Nat. Biotechnol.* **2005**, *23*, 94–101.

Sanders, D. A.; Gillece-Castro, B. L.; Stock, A. M.; Burlingame, A. L.; Koshland, D. E. Identification of the site of phosphorylation of the chemotaxis response regulator protein, CheY. *J. Biol. Chem.* **1989**, *264*, 21770–21778.

Schumacher, J. A.; Crockett, D. K.; Elenitoba-Johnson, K. S.; Lim, M. S. Evaluation of enrichment techniques for mass spectrometry: identification of tyrosine phosphoproteins in cancer cells. *J. Mol. Diagn.* **2007**, *9*, 169–177.

Sickmann, A.; Meyer, H. E. Phosphoamino acid analysis. *Proteomics* **2001**, *1*, 200–206.

Simon, E. S.; Young, M.; Chan, A.; Bao, Z. Q.; Andrews, P. C. Improved enrichment strategies for phosphorylated peptides on titanium dioxide using methylesterification and pH gradient elution. *Anal. Biochem.* **2008**, *377*, 234–242.

Steen, H.; Mann, M. A new derivatization strategy for the analysis of phosphopeptides by precursor ion scanning in positive ion mode. *J. Am. Soc. Mass. Spectrom.* **2002**, *13*, 996–1003.

Steen, H.; Jebanathirajah, J. A.; Rush, J.; Morrice, N.; Kirschner, M. W. Phosphorylation analysis by mass spectrometry: myths, facts, and the consequences for qualitative and quantitative measurements. *Mol. Cell Proteomics* **2006**, *5*, 172–181.

Stone, M. D.; Chen, X.; McGowan, T.; Bandhakavi, S.; Cheng, B.; Rhodus, N. L.; Griffin, T. J. Large-scale phosphoproteomics analysis of whole saliva reveals a distinct phosphorylation pattern. *J. Proteome Res.* **2011**, *10*, 1728–1736.

Sugiyama, N.; Masuda, T.; Shinoda, K.; Nakamura, A.; Tomita, M.; Ishihama, Y. Phosphopeptide enrichment by aliphatic hydroxy acid-modified metal oxid chromatography. *Moll. Cell. Proteomics* **2007**, *6*, 1103–1109.

Sugiyama, N.; Nakagami, H.; Mochida, K.; Daudi, A.; Tomita, M.; Shirasu, K.; Ishihama, Y. Large-scale phosphorylation mapping reveals the extent of tyrosine phosphorylation in Arabidopsis. *Mol. Syst. Biol.* **2008**, *4*, 193.

Swaney, D. L.; Wenger, C. D.; Thomson, J. A.; Coon, J. J. Human embryonic stem cell phosphoproteome revealed by electron transfer dissociation tandem mass spectrometry. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 995–1000.

Tao, W. A.; Wollscheid, B.; O'Brien, R.; Eng, J. K.; Li, X. J.; Bodenmiller, B.; Watts, J. D.; Hood, L.; Aebersold, R. Quantitative phosphoproteome analysis using a dendrimer conjugation chemistry and tandem mass spectrometry. *Nat. Methods* **2005**, *2*, 591–598.

Taouatas, N.; Altelaar, A. F.; Drugan, M. M.; Helbig, A. O.; Mohammed, S.; Heck, A. J. Strong cation exchange-based fractionation of Lys-N-generated peptides facilitates the targeted analysis of post-translational modifications. *Mol. Cell. Proteomics* **2009**, *8*, 190–200.

Tani, K.; Suzuki, Y. Investigation of the ion-exchange behaviour of titania: application as a packing material for ion chromatography. *Chromatographia* **1997**, *46*, 623–627.

Tani, K.; Sumizawa, T.; Watanabe, M.; Tachibana, M.; Koizumi, H.; Kiba, T. Evaluation of titania as an ion-exchanger and as a ligand-exchanger in HPLC. *Chromatographia* **2002**, *55*, 33–37.

Thaler, F.; Valsasina, B.; Baldi, R.; Xie, J.; Stewart, A.; Isacchi, A.; Kalisz, H. M.; Rusconi, L. A new approach to phosphoserine and phosphothreonine analysis in peptides and proteins: chemical modification, enrichment via solid-phase reversible binding, and analysis by mass spectrometry. *Anal. Bioanal. Chem.* **2003**, *376*, 366–373.

Thingholm, T. E.; Jensen, O. N.; Robinson, P. J.; Larsen, M. R. SIMAC (sequential elution from IMAC), a phosphoproteomics strategy for the rapid separation of monophosphorylated from multiply phosphorylated peptides. *Mol. Cell. Proteomics* **2008**, *7*, 661–671.

Thingholm, T. E.; Jensen, O. N.; Larsen, M. R. Analytical strategies for phosphoproteomics. *Proteomics* **2009**, *9*, 1451–1469.

Tichy, A.; Salovska, B.; Rehulka, P.; Klimentova, J.; Vavrova, J.; Stulik, J.; Hernychova, L. Phosphoproteomics: searching for a needle in a haystack. *J. Proteomics* **2011**, *74*, 2786–2797.

Tiselius, A.; Hjerten, S.; Levin, O. Protein chromatography on calcium phosphate columns. *Arch. Biochem. Biophys.* **1956**, *65*, 132–155.

Verma, N.; Bäuerlein, C.; Pink, M.; Rettenmeier, A. W.; Schmitz-Spanke, S. Proteome and phosphoproteome of primary cultured pig urothelial cells. *Electrophoresis* **2011**, *32*, 3600–3611.

Villén, J.; Beausoleil, S. A.; Gerber, S. A.; Gygi, S. P. Large-scale phosphorylation analysis of mouse liver. *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 1488–1493.

Wan, H.; Yan, J.; Yu, L.; Zhang, X.; Xue, X.; Li, X.; Liang, X. Zirconia layer coated mesoporous silica microspheres used for highly specific phosphopeptide enrichment. *Talanta* **2010**, *82*, 1701–1707.

Washburn, M. P.; Wolters, D.; Yates, J. R., 3rd. Large-scale analysis of the yeast proteome by multidimensional protein identification technology. *Nat. Biotechnol.* **2001**, *19*, 242–247.

Weigt, C.; Korte, H.; Pogge von Strandmann, R.; Hengstenberg, W.; Meyer, H. E. Identification of phosphocysteine by electrospray mass spectrometry combined with Edman degradation. *J. Chromatogr. A* **1995**, *712*, 141–147.

Wilson-Grady, J. T.; Villén, J.; Gygi, S. P. Phosphoproteome analysis of fission yeast. *J. Proteome Res.* **2008**, *7*, 1088–1097.

Wolschin, F.; Weckwerth, W. Combining metal oxide affinity chromatography (MOAC) and selective mass spectrometry for robust identification of in vivo protein phosphorylation sites. *Plant Methods.* **2005**, *1*, 9.

Wolters, D. A.; Washburn, M. P.; Yates, J. R., 3rd. An automated multidimensional protein identification technology for shotgun proteomics. *Anal. Chem.* **2001**, *73*, 5683–5690.

Wu, J.; Shakey, Q.; Liu, W.; Schuller, A.; Follettie, M. T. Global profiling of phosphopeptides by titania affinity enrichment. *J. Proteome Res*. **2007**, *6*, 4684–4689.

Wu, Y. B.; Dai, J.; Yang, X. L.; Li, S. J.; Zhao, S. L.; Sheng, Q. H.; Tang, J. S.; Zheng, G. Y.; Li, Y. X.; Wu, J. R.; Zeng, R. Concurrent quantification of proteome and phosphoproteome to reveal system-wide association of protein phosphorylation and gene expression. *Mol. Cell. Proteomics* **2009**, *8*, 2809–2826.

Xu, Y.; Sprung, R.; Kwon, S. W.; Kim, S. C.; Zhao, Y. Isolation of phosphopeptides by pI-difference-based electrophoresis. *J. Proteome Res.* **2007**, *6*, 1153–1157.

Xue, Y.; Wei, J.; Han, H.; Zhao, L.; Cao, D.; Wang, J.; Yang, X.; Zhang, Y.; Qian, X. Application of open tubular capillary columns coated with zirconium phosphonate for enrichment of phosphopeptides. *J. Chromatogr. B* **2009**, *877*, 757–764.

Yu, L. R.; Zhu, Z.; Chan, K. C.; Issaq, H. J.; Dimitrov, D. S.; Veenstra, T. D. Improved titanium dioxide enrichment of phoshopeptides from HeLa cells and high confident phosphopeptide identification by cross-validation of MS/MS and MS/MS/MS spectra. *J. Proteome Res.* **2007**, *6*, 4150–4162.

Yu, Y. Q.; Fournier, J., Gilar, M.; Gebler, J. C. Phosphopeptide enrichment using microscale titanium dioxide solid phase extraction. *J. Sep. Sci.* **2009**, *32*, 1189–1199.

Zhang, H.; Zha, X.; Tan, Y.; Hornbeck, P. V. Phosphoprotein analysis using antibodies broadly reactive against phosphorylated motifs. *J. Biol. Chem*. **2002**, *277*, 39379–39387.

Zhang, X.; Ye, J.; Jensen, O. N.; Roepstorff, P. Highly efficient phosphopeptide enrichment by calcium phosphate precipitation combined with subsequent IMAC enrichment. *Mol. Cell. Proteomics* **2007**, *6*, 2032–2042.

Zhang, L.; Wang, H.; Liang, Z.; Yang, K.; Zhang, L.; Zhang, Y. Facile preparation of monolithic immobilized metal affinity chromatography capillary columns for selective enrichment of phosphopeptides. *J. Sep. Sci.* **2011**, *34*, 2122–2130.

Zhou, H.; Watts, J. D.; Aebersold, R. A systematic approach to the analysis of protein phosphorylation. *Nat. Biotechnol.* **2001**, *19*, 375–378.

Zhou, H.; Tian, R.; Ye, M.; Xu, S.; Feng, S.; Pan, C.; Jiang, X.; Li, X.; Zou, H. Highly specific enrichment of phosphopeptides by zirconium dioxide nanoparticles for phosphoproteome analysis. *Electrophoresis* **2007**, *28*, 2201–2215.

Zhou, H.; Low, T. Y.; Hennrich, M. L.; van den Toorn, H.; Schwend, T.; Zou, H.; Mohammed, S.; Heck, A. J. Enhancing the identification of phosphopeptides from putative basophilic kinase substrates using Ti (IV) based IMAC enrichment. *Mol. Cell. Proteomics.* **2011,** *10*, M110.006452.

Received October 7, 2011; accepted January 13, 2012;
previously published online February 25, 2012