Ying-Qing Yu
Jennifer Fournier
Martin Gilar
John C. Gebler

Waters Corporation, Milford, MA, USA

Original Paper

Phosphopeptide enrichment using microscale titanium dioxide solid phase extraction

Identification of phosphopeptides by MS is challenging due to their relatively low abundance in proteomic samples and their limited ionization efficiency. Various affinity enrichment methods have been used in the literature. Titanium dioxide SPE devices have been recently proposed as an alternative to immobilized metal affinity chromatography for phosphopeptide enrichment. This study evaluates the TiO_2 method using sorbent packed in a 96 well microscale extraction plate operated using a vacuum manifold. The phosphopeptide recovery and enrichment selectivity were investigated at various loading conditions. The effectiveness of organic additives such as dihydroxybenzoic acid derivatives and other nonaliphatic carboxylic acids on enrichment selectivity was examined. The performance of TiO_2 was compared to IMAC sorbent. The results suggest that various additives improve the enrichment selectivity by effectively interfering with the acidic peptides binding to TiO_2 sorbent. Interaction of phosphopeptides with sorbent is also affected, which leads to overall reduction in phosphopeptide recovery. The new SPE device was successfully utilized for the extraction of phosphopeptides from yeast lysate digest using 2,5-dihydroxybenzoic acid to minimize the interference from nonphosphorylated peptides.

Keywords: Displacers / Mass spectrometry / Phosphopeptide enrichment / Solid phase extraction / Titanium oxide

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1 Introduction

Protein phosphorylation plays a vital role in cellular events and is one of the most common post-translational modifications (PTM) [1, 2]. Phosphorylation analysis is a significant analytical challenge due to the low abundance of phosphoproteins and low phosphorylation stoichiometry [3, 4].

In shotgun proteomics, phosphopeptides of interest are typically masked by nonphosphorylated peptides present in greater abundance in a highly complex sample. Various methods have been used for a selective phosphopeptide enrichment, including strong cation-exchange chromatography (SCX) [5, 6], IEF [7, 8], immobilized metal affinity chromatography (IMAC) [9, 10], and metal oxide affinity chromatography (MOAC) [11 – 16].

Correspondence: Dr. Martin Gilar, Waters Corporation, 34 Maple St., Milford, MA 01757, USA
E-mail: martin_gilar@waters.com
Fax: +1-508-482-3085

Abbreviations: DDA, data dependent acquisition; **DHB,** 2,5-dihydroxybenzoic; **IMAC,** immobilized metal affinity chromatography; **OSA,** 1-octanesulfonic acid

IMAC methods are popular for offline phosphopeptide enrichment [17], but tend to retain acidic nonphosphorylated peptides, co-enriching them along with the target phosphopeptides [18]. Extraction at low pH [18] or blocking the acidic groups by methyl esterification [19, 20] was shown to improve the phosphopeptide extraction selectivity.

The ability of titanium dioxide (TiO_2) and zirconium dioxide (ZrO_2) to selectively retain organic phosphates is well documented [21 – 23]. More recently, MOAC was evaluated as an alternative to IMAC [24, 25] for the extraction of phosphopeptides. In addition, metal oxides were also used for the selective extraction of organic phosphates [26], phosphopeptides, phospholipids [27], and sialylated glycopeptides [28, 29].

Pinkse *et al.* [25] demonstrated an online extraction of phosphopeptides with a TiO_2 trapping column. Compared to IMAC, the MOAC method was easier to implement in a nano-LC MS format [12, 30]. Larsen *et al.* [24, 29] evaluated the selectivity of manually packed gel loader tips (0.4 mg of TiO_2) and compared their performance to IMAC. The authors utilized additives such as 2,5-dihydroxybenzoic (DHB) and others to improve the selectivity of enrichment. The additives compete for adsorption

with the acidic nonphosphorylated peptides, while the enrichment of phosphopeptides is virtually unaffected [24].

In addition to DHB, several modifiers (displacers) have been shown to enhance the extraction selectivity [11, 15, 16, 26, 31]. The search for alternative displacers was in part motivated by the limited DHB compatibility with online nano-LC MS (MS inlet contamination) [11, 14].

Larsen *et al*. [24] observed that DHB displacer performed comparably well to other hydroxycarboxylic aromatic acids, such as salicylic or phthalic acids, while benzoic or cyclohexane acids were less potent. In a later report, Jensen and Larsen [16] evaluated additional displacers and concluded that aromatic hydroxycarboxylic compounds (gallic, and phthalic acids), and aliphatic hydroxycarboxylic compound (lactic, oxalic, glycolic, and citric acids) can also improve the enrichment selectivity. Neither report estimated the impact of displacers on phosphopeptide recovery.

Sugiyama *et al*. [14] studied displacers such as DHB, glycolic, lactic, malic, tartaric, and 3-hydroxypropanoic acids. In contrast to Larsen *et al*. [24] and Jensen and Larsen [16] the authors obtained significantly better enrichment selectivity with lactic and 3-hydroxypropanoic acids than with DHB and glycolic acid.

Yu *et al*. [31] proposed the ammonium salt of glutamate as a displacer for TiO_2. According to the authors, the dicarboxylic compounds, such as glutamate, successfully reduce the adsorption of acidic nonphosphorylated peptides. Mazanek *et al*. [11] investigated 1-octanesulfonic acid (OSA) as another type of displacer in conjunction with acetic acid. The authors term OSA as an ion-pairing displacer, however, the mechanism of action was not further elucidated. The recovery of phosphopeptides was measured to be within a wide range of 33 – 100%. Surprisingly large amounts of nonphosphorylated peptides were co-extracted using both OSA and DHB protocols [11].

The extraction selectivity in many published reports was evaluated using mixtures of phosphopeptides and nonphosphorylated peptides at 1:1 molar ratio. Evaluating the extraction protocol with more realistic samples (low amounts of phosphopeptides spiked in a complex background) could reveal more conclusively the performance of the selected method [32]. A relevant comparison for simple samples was performed by Larsen *et al*. using 1:1, 1:10, and 1:50 molar ratios of phospho- to nonphosphorylated peptides. The study concluded that TiO_2 selectivity with DHB displacer meets or exceeds IMAC sorbents.

The extracts from highly complex proteomic samples are typically contaminated with significant numbers of nonphosphorylated peptides. Due to MS/MS experimental workflow it is often difficult to assess the phosphopeptide extraction selectivity and recovery. Ratios of phosphopeptides to nonphosphorylated peptides detected have been used as a crude estimate of extraction method selectivity [13, 14, 31, 33].

Some of the discrepancies in MOAC performance evaluation found in the literature could be explained by different source of sorbents (Sachtopore, or GL Sciences), different formats of SPE devices, and different detection techniques. Larsen *et al*. [24] used GELoader tip (3 mm long sorbent bed, ~0.4 mg of sorbent) with MALDI MS, while others implemented commercially available Glygen tips (4 or 10 mg of sorbent) [11], home-made tips (3 mg, 200 μL tip) [14] or packed trapping columns (0.5 mm × 50 mm, 5.5 mg of sorbent) [31], and analyzed extracts with nano-LC MS and MS/MS. The different aspect ratios of SPE devices and load/wash volumes may have affected the recovery and selectivity of enrichment methods. The impact of loading and eluting conditions is not fully understood [34, 35].

In this study, we describe the evaluation of a prototype 96 well SPE plate packed in-house with 2.5 mg of 20 μm TiO_2 particles *per* well (~2 mm bed length). The performance of this SPE plate for phosphopeptide isolation was evaluated from the point of reproducibility, selectivity, and sample recovery. Comparison of various additives used in the loading step was carried out in order to understand how to effectively reduce the nonspecific co-extraction of nonphosphorylated peptides. The performance of the TiO_2 SPE sorbent was compared to IMAC materials packed in the same plate format.

2 Experimental

2.1 Materials and reagents

Four synthetic phosphopeptides mixed with yeast enolase tryptic digest (commercially available as MassPREP™ enolase digest, Waters, Milford, MA, USA) at various molar ratios (1:1 – 1:50) were used as model samples. The synthetic phosphopeptides were purchased from SynPep (Dublin, CA, USA) and purified in house using HPLC. The amino acid sequence, phosphorylation site(s) and the mass for the singly and doubly charged ions are listed in Table 1. Phosphorylation sites include serine, threonine, and tyrosine; the amino acid primary sequence of these peptides is consistent with yeast enolase tryptic peptides T18, T19, and T43 residues (Table 1). T43_2P peptide contains two phosphoserine sites. Trypsin (Promega, Madison, WI, USA) was used as a protease to cleave proteins. High purity 2,5-DHB were purchased from two sources, Bruker BioSciences and Waters Corporation. Other materials were purchased from Sigma – Aldrich if not specified otherwise.

The format of the SPE was a 96 well micro-elution plate packed with 2.5 mg of TiO_2 particles in each well. TiO_2 sorbent (20 μm, 300 Å) was purchased from Sachtopore

Table 1. Four synthetic tryptic phosphopeptides derived from yeast enolase used in this study

Phosphopeptide description	Sequence	MW $(M + H)^+$	MW $(M + H)^{2+}$
T18_1P	NVPL*p*YK	813.39	407.20
T19_1P	HLADL*p*SK	863.40	432.21
T43_1P	VNQIG*p*TLSESIK	1368.68	684.84
T43_2P	VNQIGTL*p*SE*p*SIK	1448.64	724.83

The sequence, phosphorylation site(s), and mass to charge ratio for the singly and doubly charged ions are listed.

(Sachtleben, Germany). The sample reservoirs of the extraction plate accommodate ~0.7 mL of sample. Due to the conical shape of the bottom part, wells resemble pipette tips. The sorbent bed length is ~2 mm, sandwiched between two spherical ball frits.

Two IMAC sorbents used in this study were Poros MC (AB Biosystem) and Ni-NTA agarose gel (Qiagen). The IMAC sorbents were packed in a 96 well micro-elution plate; 2.5 mg Poros MC or 50 μL of the Ni-NTA agarose slurry was packed in the wells. The IMAC materials were washed with 50 mM EDTA and chelated with Fe (III).

2.2 TiO_2 SPE protocol

Extraction with 96 well micro-elution plate was performed using vacuum manifold. The extraction plate housing is identical to the commercially available μElution plate device manufactured by Waters for the extraction of small molecules and fits to standard vacuum manifold [36]. Each micro-elution plate well was equilibrated with 200 μL water, followed by 200 μL methanol before use. The micro-elution plate protocol is summarized in Table 2 for TiO_2 and IMAC sorbents. The additives concentration in loading step was 0.1% TFA with 250 mg/mL of 3-hydroxyl benzoic acid, salicylic acid, and 2,5-DHB. In case of glycolic acid, we used saturated 1 M solution (~80 mg/mL).

Sample load was performed at minimum vacuum setting (~2.5 inch of Hg), loading 200 μL of sample can take ~1 min. The overall sample processing time was less than 15 min. Multiple samples can be extracted at once if higher throughput is required. A significant volume of the sample can be easily processed and concentrated with multiple loading steps or using multiple wells. The recommended elution volume is 100 μL, but substantial elution yield can be accomplished in 25 μL [36].

2.3 Phosphopeptide enrichment from type 1 yeast tryptic digests

Type 1 yeast was purchased from Sigma (St. Louis, MO, USA). Proteins were extracted using Pierce Y-*per* Yeast protein extraction reagent as recommended by the manufacturer. Approximately 2.4 mg of yeast lysate was separated from the membrane *via* centrifugation at 50 000 rpm for 30 min. Benzonase, 25 units, was used to degrade the nucleic acids at 37°C for 1 h. A molecular weight cutoff spin column (>5000 Da) was used to retain proteins, while removing DNA degradation products and other low molecular weight contaminants. Final protein concentration was determined to be 56 μg/μL by Bradford Assay. Approximately 2 mg of the sample was reduced with 10 mM DTT and alkylated with 15 mM iodoacetamide, followed with trypsin digestion; about 40 μg of trypsin was used.

The entire digest was mixed in 500 μL of 50 mg/mL of DHB additive that was solubilized in 0.2% TFA, 50% MeCN before loading onto the TiO_2 SPE. The washing and elution steps were the same as stated in Table 2. The eluent was neutralized and lyophilized before LC/MS/MS analysis.

2.4 LC/MS and MALDI TOF MS analysis

An Alliance HPLC system (Waters) interfaced with a single quadrupole ZQ mass spectrometer (Waters) was used

Table 2. SPE protocols for TiO_2 and IMAC sorbent

Sorbent type	Loading	Washing	Elution
TiO_2	0.1% TFA in 50 – 80% MeCN (no additives), 200 μL 0.1% TFA in 50 – 80% MeCN (with additives[a]) 200 μL	(1) 0.1% TFA in 50 – 80% MeCN, 200 μL followed by (2) 0.1% TFA in water, 200 μL	0.3 M Ammonium hydroxide, 100 μL
IMAC	1% Acetic acid with 20% MeCN, 200 μL	1% Acetic acid with 20% MeCN, 200 μL, repeat once	

[a] The additives such as 2,5-DHB were used to improve the selectivity of phosphopeptide extraction. See Fig. 2 and Section 2.2 for details.

to perform quantitation of the enriched phosphopeptides. Quantitation was based on total ion current peak areas. The analytical column used was a reversed phase C_{18} column (2.1 mm × 100 mm Atlantis™ dC_{18}, 3 μm, Waters). Mobile phase (A) was 0.1% formic acid in aqueous and mobile phase (B), 0.1% formic acid in 100% MeCN. Samples were solubilized in water, and after each injection, a linear gradient of 0 – 42% B in 50 min was applied. The single quadrupole mass spectrometer was operated using a capillary voltage of 3.2 kV, and a desolvation temperature at 150°C.

Nano-LC/MS analysis (Nano-ACQUITY nano-LC system, Waters) was also performed on the isolated phosphopeptides on a nanoscale column 75 μm × 100 mm NanoEase™ Atlantis dC_{18} RP column (Waters). We did not observe the doubly phosphorylated peptide (T43_2P) when phosphopeptide standards were reconstituted in water and injected on the Nanoscale column. Such phenomenon was not observed when the 2.1 mm analytical column was used. To overcome this problem, we solubilized the extracted phosphopeptides in 50 mM $(NH_4)_2HPO_4$ with 25 mM EDTA prior to LC MS analysis. This solution minimized the chelation of phosphopeptides on the metal surfaces of the nano-LC system [37, 38]. The mobile phases were (A) 0.1% formic acid in water and (B) 0.1% formic acid in 100% MeCN. A C_{18} trap column (180 μm × 20 mm, Symmetry) was used to remove contaminants such as salts; the washing period was 3 min with mobile phase A. Peptides were eluted from the trap column and separated on the nanoscale analytical column with a gradient of 0 – 50% B at 1% *per* minute.

Data dependent acquisition (DDA) was used to confirm the yeast phosphopeptide sequences and to locate the phosphorylation site(s) using a QTof MS system in positive detection mode (Waters). The amount of sample injected corresponds to 80 μg of initial yeast lysate tryptic digest. Ions with +2, +3, and +4 charges were subjected to CID at elevated energies from 15 to 40 V. The total DDA duty cycle was about 10 s, interrogating three precursors by MS/MS in parallel fashion. The mass measurement accuracy was corrected by measuring the mass of the $(M + 2H)^{2+}$ ions of a standard peptide (GluFib) that was delivered continually through an auxiliary pump to the ion source. MASCOT Distiller (Version 2.0) was used to process the spectra and identify the peptides *via* the database search.

MALDI TOF MS were used to evaluate the microscale SPE device for the phosphopeptide extraction. The MALDI TOF instrument (MALDI micro-MX™, Waters) was equipped with a pulsed N_2 laser (337 nm) and a 2.3 m flight. This instrument was operated in positive reflectron mode with delayed extraction (500 ns). Re-crystallized α-cyano-4-hydroxy cinnamic acid (CHCA) matrix was used for the ionization of phosphopeptides; the CHCA was prepared as 10 mg/mL in 70% MeCN with 0.1% TFA.

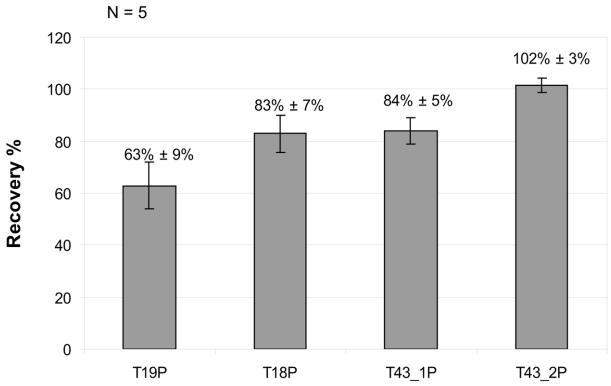

Figure 1. The extraction recovery and repeatability of the TiO_2 micro-elution plate. The four synthetic phosphopeptides were extracted using the TiO_2 with protocol listed in Table 2 (loading and washing with 0.1% TFA in 80% MeCN, washing with 0.1% TFA in 80% MeCN and 0.1% TFA in water, elute with 0.3 M ammonium hydroxide). Parallel analyses $N = 5$ were conducted using five individual wells. The recovery and the RSD for each phosphopeptide were calculated using the extracted ion chromatograms.

3 Results and discussion

3.1 TiO_2 micro-elution plate performance and extraction repeatability

Out of several protocols for phosphopeptide enrichment using TiO_2 published recently only few have evaluated the extraction recovery [11, 14]. Extraction repeatability was addressed by a single report [14]. The majority of the reports recommend using displacers to reduce the adsorption of nonphosphorylated peptides on TiO_2 to improve extraction selectivity.

Judging from the reports, we suspect that displacers may affect the overall enrichment experiment recovery. While Mazanek *et al.* [11] observed overall good recovery of phosphopeptides with TopTips SPE devices, Sugiyama *et al.* [14] reported significantly lower yield for selected phosphopeptides (10% or less using 3 mg, 200 μL tip). The mass balance of the SPE experiment was not performed; the sources of the sample loss remain unclear. Neither report [11, 14] investigated the phosphopeptide recovery in a baseline experiment without displacers.

Using the protocols outlined in Table 2 we measured the extraction recovery and repeatability for phosphopeptides listed in Table 1. Five wells were processed in parallel using ~600 pmole *per* peptide (~2 μg total mass load).

The data for experiments performed without displacer (only 0.1% TFA in the loading solution) are summarized in Fig. 1. The recovered fractions and extracts were analyzed 2.1 mm × 100 mm HPLC with MS detection (see Section 2) and compared to the original sample composition

prior to the extraction. All peptides except T19_1P show recovery greater than 80% with less than ±7% RSD. The recovery of the T19_1P peptide was 63 ± 9%. The performance of the 96-well micro-elution plate is adequate for routine phosphopeptide enrichment experiments.

In order to investigate the sources of the recovery loss, we analyzed load and wash fractions. Small breakthrough was observed, in particular for T19_1P (data not shown). Higher recovery of T43_2P compared to the other three peptides suggests that TiO_2 has greater affinity toward multiply phosphorylated species.

The loading capacity based on cumulative mass load of four phosphopeptides (Table 1) is about 100 μg; above this limit a significant breakthrough of all phosphopeptides was observed by LC/MS. The 100 μg mass load limit should be treated with caution, since it is difficult to determine the loading capacity for complex proteomic samples.

3.2 Extraction selectivity with different displacers

Infrared spectroscopic and chromatographic studies confirm that phosphates, dicarboxylic, and hydroxycaroboxilic compounds exhibit distinct affinity toward TiO_2 surface [39 – 41]. Presumably, phosphates are adsorbed stronger than glutamate and aspartate residues. The rationale for using displacers is that they reduce the binding of nonphosphorylated peptides, while the phosphopeptides remain strongly adsorbed.

The protocol shown in Table 2 utilizes additives to improve the extraction selectivity. Larsen *et al*. [24] first proposed that 2,5-DHB additive in the load step effectively interferes with the adsorption of acidic nonphosphorylated peptides. Some other displacers were utilized with various degrees of success [11, 14, 16, 24, 26, 29, 31]. In this study, we use DHB and several structurally similar additives in an attempt to further clarify the impact of displacers in the phosphopeptide enrichment selectivity and recovery.

Besides displacer(s) the loading solution is comprised of other components, such as acids and organic solvent. Some protocols included high concentrations of acid [11, 13]. In such cases, the acid can possibly act as displacers themselves (competing with acidic peptides for adsorption sites). Surprisingly little attention was paid to the role of organic solvent in the extraction [34]. Loading solutions typically contain 50 – 80% MeCN [16, 24, 29]. Our experiments suggest that at least 50% MeCN concentration is necessary to achieve good enrichment selectivity with TiO_2 sorbents. Loading samples at lower organic concentration results in co-extraction of nonphosphorylated peptides (data not shown). This effect in part explains why significant amounts of nonphosphorylated peptides were recovered in a protocol published by Mazanek *et al*. (30% MeCN in load), despite using displacers [11].

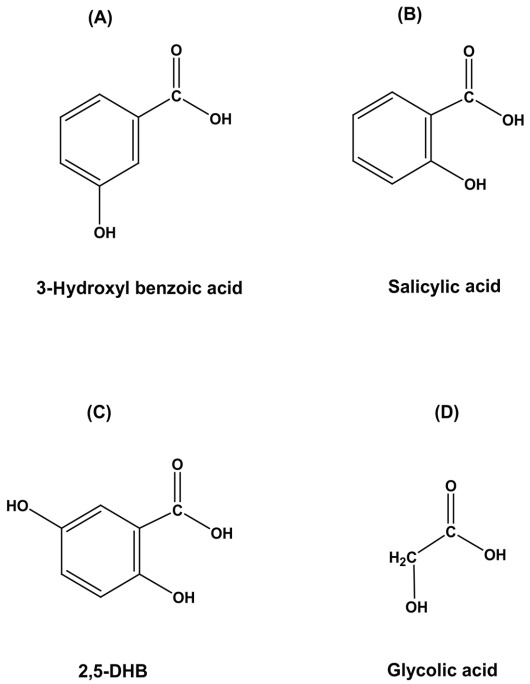

Figure 2. The structures of the displacing agents used for phosphopeptide extraction. (A) 3-hydroxyl benzoic acid, (B) salicylic acid, (C) 2,5-DHB, and (D) glycolic acid.

From the literature reports [16, 24, 29], we deduce that compounds with carboxylic moiety alone are not efficient displacers. Two functional groups such as in 2,5-DHB may be required for the displacer to work effectively – a carboxylic acid group and a hydroxyl group. This hypothesis is supported by Larsen *et al*. [24], who found that 2,5-DHB, salicyclic acid, and phthalic acid (all having carboxylic and hydroxyl groups) are the most effective additives for phosphopeptide enrichment. We hypothesize that potent displacers have two functional groups in close proximity, preferably in a spatially locked position on the aromatic ring.

The structures of compounds used in this study are show in Fig. 2. Except for glycolic acid (Fig. 2D), the displacers are aromatic hydroxyl-carboxylic acids. 2,5-DHB (Fig. 2C) is an aromatic carboxylic acid with two hydroxyl groups in *ortho* and *meta* position. Salicylic acid (Fig. 2B) has a single OH group in *ortho* position, while 3-hydroxylbenzoic acid (Fig. 2A) has a single OH group in *meta* position.

The sample used in the enrichment experiment was enolase tryptic digest spiked with four phosphopeptide standards (Table 1). The peptide mixture was dissolved in 0.1% TFA with 250 mg/mL of 3-hydroxyl benzoic acid, salicylic acid, 2,5-DHB, or saturated glycolic acid (1 M solution, ~80 mg/mL) in 80% MeCN prior to the TiO_2 SPE extraction. Both MALDI-TOF MS and LC/MS were used to compare the effectiveness of these displacing reagents in

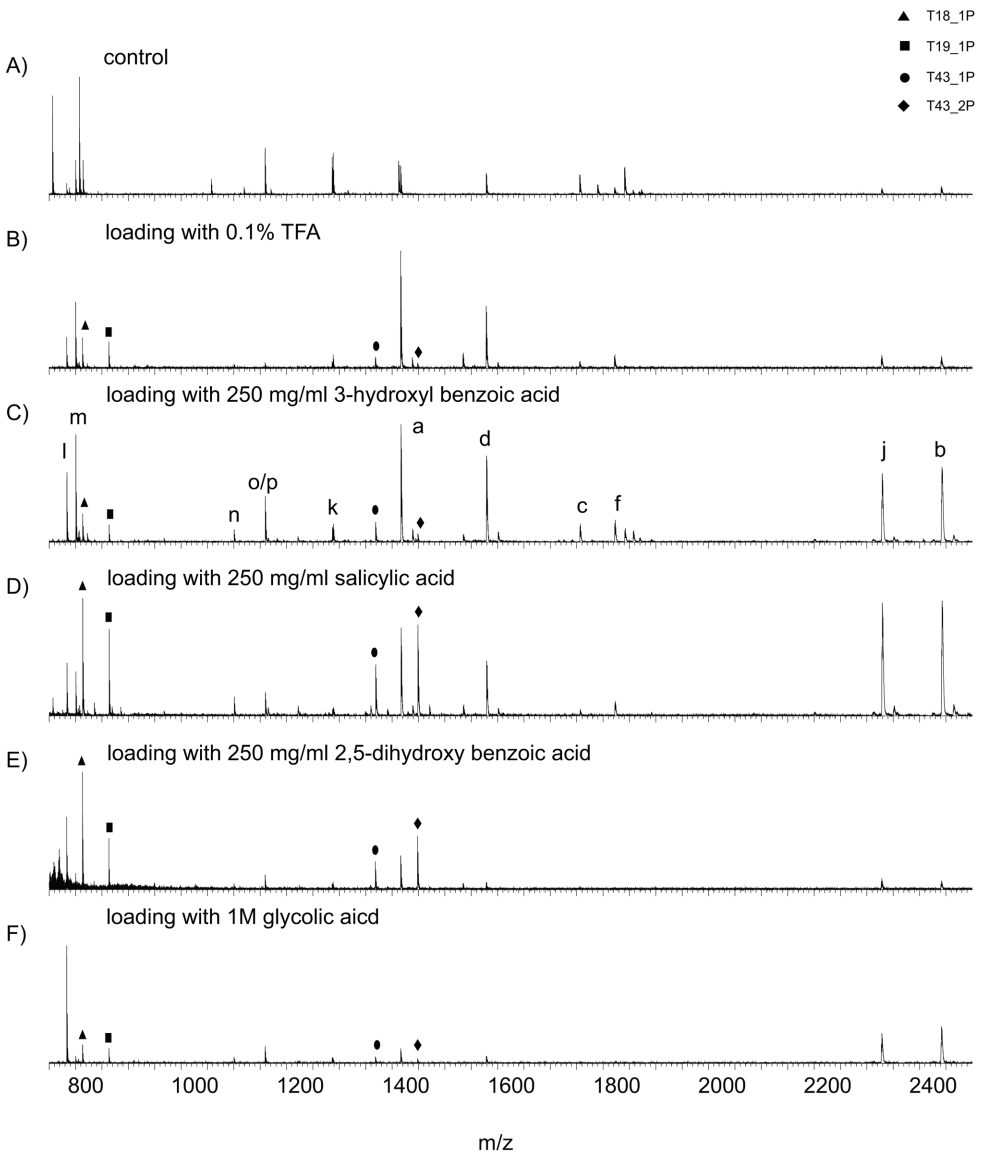

Figure 3. A mixture of yeast enolase tryptic peptides with four phosphopeptide standards in 50:1 molar ratio (250:5 pmol) was used to compare different loading conditions by changing the additives. The MALDI-TOF spectra from (A) a control sample (enolase peptide with four phosphopeptides), (B) TiO_2 loading with 0.1% TFA, (C) loading with 250 mg/mL of 3-hydroxyl benzoic acid, (D) loading with 250 mg/mL of salicylic acid, (E) loading with 250 mg/mL of 2,5-DHB, and (F) loading with saturated glycolic acid (1 M). Sequences of labeled peptides are shown in Tables 1 and 4.

terms of reducing the content of nonphosphorylated peptides in the TiO_2 extract.

We decided to challenge our SPE protocol with the sample containing (50:1) excess of the enolase tryptic digest to phosphopeptide standards. This sample mimics more realistically the complex biological sample and allows for more meaningful evaluation of enrichment selectivity. The enolase tryptic digest was chosen because it contains multiple acidic peptides.

Figure 3A shows the MALDI MS analysis of the original sample prior to the extraction. Because of the molar excess of nonphosphorylated enolase peptides the phosphopeptide signals are not evident in this control sample. After TiO_2 affinity extraction at five different loading conditions, all four phosphopeptides were enriched to certain degrees (Figs. 3B – F). DHB and salicylic acid loadings gave the best results in terms of eliminating the signals of nonphosphorylated peptides. Glycolic acid, 3-hydroxyl benzoic acid, and TFA experiments showed decreasing trend of extraction selectivity. Since the MALDI experiment is essentially nonquantitative, the phosphopeptide extraction recovery cannot be ascertained from the experiment shown in Fig. 3.

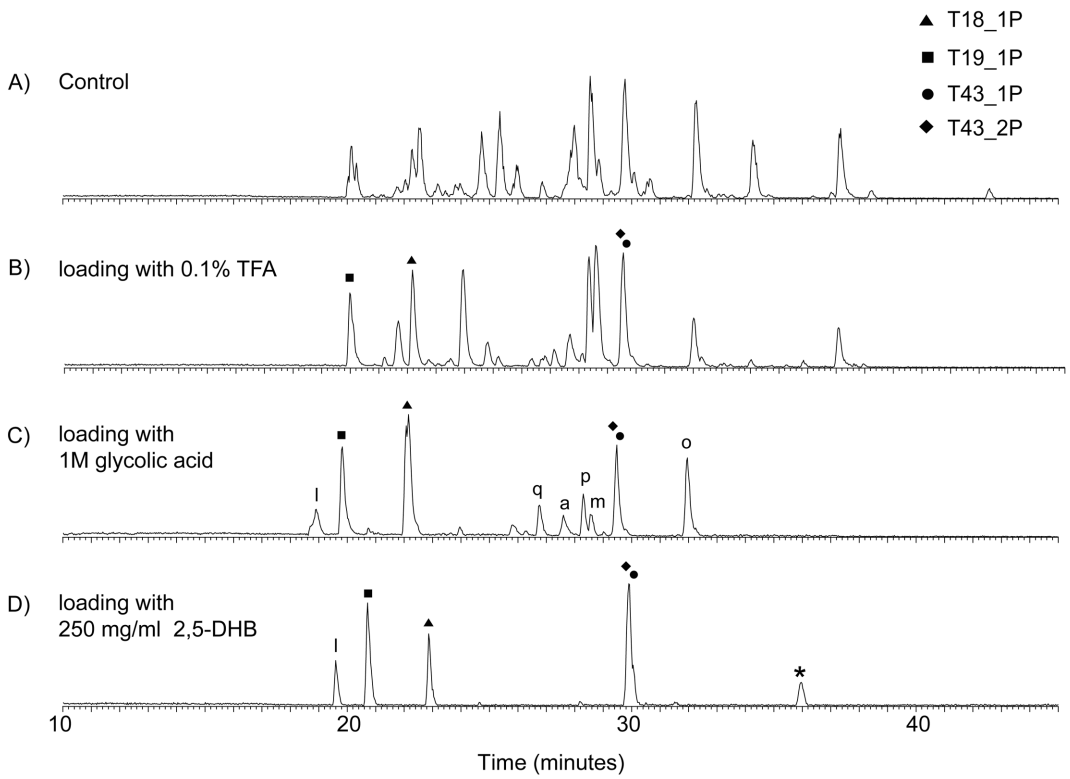

Figure 4. A mixture of yeast enolase tryptic peptides mixed with four phosphopeptide standards in 50:1 molar ratio (250:5 pmol) was used to compare different loading conditions by changing the additives. The LC/MS total ion current results from (A) a control sample (enolase tryptic digest spiked with four phosphopeptides), (B) loading in 0.1% TFA, (C) loading in saturated glycolic acid, and (D) loading in 250 mg/mL of 2,5-DHB solutions. The asterisk indicates an unidentified impurity originating from the raw DHB material. Sequences of labeled peptides are shown in Tables 1 and 4. The data for 3-hydroxyl benzoic and salicylic acid acids are similar to glycolic acid, chromatograms are not shown.

Both TFA and 3-hydroxyl benzoic acid experiments gave rather similar MALDI-TOF spectra which suggests a less selective extraction of phosphopeptides. More supporting data were obtained in the LC/MS experiment shown in Fig. 4, which compares the TiO_2 extraction selectivity using TFA, glycolic acid, and 2,5-DHB additives in the loading step. Again, 2,5-DHB provides the best selectivity of phosphopeptide extraction followed by glycolic acid; TFA is shown to give the least desirable results. A small unknown impurity peak was marked in Fig. 3D. We observed this impurity peak consistently when using the 2,5-DHB obtained from Bruker. This impurity peak was not observed when using an alternative source of DHB (Waters, MassPREP MALDI matrix).

Further analysis of the results in Figs. 3 and 4 sheds some light on the importance of a hydroxyl group on a benzene ring. The comparison of 2,5-DHB, salicylic acid, 3-hydroxyl benzoic acid, and nonaromatic carboxylic acid (glycolic acid) suggests that the OH group in *ortho* position (DHB and salicylic acid) is potentially more important for reducing the nonspecific peptide interaction than OH group in *meta* position.

The 2,5-DHB was found to be the most potent additive, fully in agreement with Larsen *et al*. [24] and other reports [16, 29]. The selectivity improvements correlated directly with the displacer nature and concentration as discussed in the next section.

3.3 Recovery of phosphopeptide enrichment

The phosphopeptide extraction selectivity and yield were evaluated for both IMAC and TiO_2 SPE methods. The comparison of phosphopeptide yield is shown for four synthetic phosphopeptides (Table 1) spiked into the enolase tryptic digest in 1:1 molar ratio.

As discussed earlier, the co-extraction of nonphosphorylated peptides can be minimized by including the 2,5-DHB in the loading step (for TiO_2 SPE, Figs. 3 and 4). According to Larsen *et al*. [24], the 2,5-DHB preferentially affects the binding of the acidic peptides to TiO_2 sorbent. However, the results in Table 3 suggest that the use of additive also reduces the phosphopeptide extraction yield compared to loading in 0.1% TFA. Presumably, a

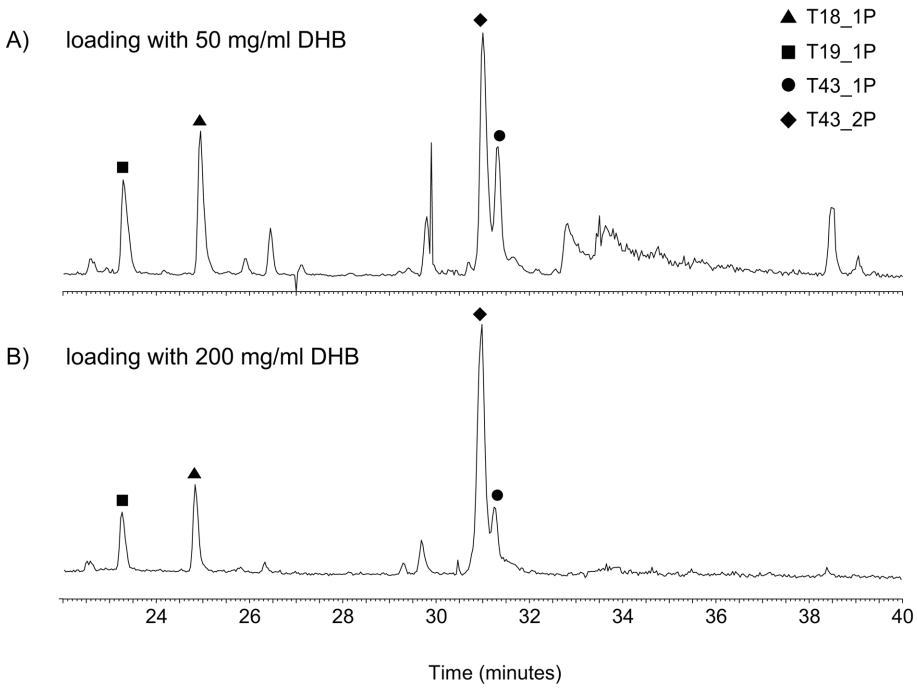

Figure 5. A mixture of yeast enolase tryptic peptides mixed with four phosphopeptide standards in 50:1 molar ratio (250:5 pmol) was processed using the TiO_2 micro-elution SPE plate. An estimated 400 fmol of extract (phosphopeptides) was injected into nano-LC/MS. The two normalized LC/MS chromatograms show the difference in phosphopeptide recovery using two different 2,5-DHB loading amounts. (A) The peptide mixture was solubilized in 50 mg/mL of 2,5-DHB in 0.1% TFA and 80% MeCN. The total SPE loading volume was 200 μL. (B) 200 mg/mL of 2,5-DHB in 0.1% TFA and 80% MeCN was used in the SPE loading step (200 μL).

Table 3. The phosphopeptide recovery using both TiO_2 and IMAC extraction methods

Phosphopeptide recovery	T18_P (%)	T19_P (%)	T43_1P (%)	T43_2P (%)
TiO_2/TFA	83	63	84	102
TiO_2/glycolic acid	74	59	64	69
TiO_2/DHB	40	22	21	61
IMAC/Poros (Fe III)	55	76	18	94

Average recovery is given for TFA experiment ($N = 5$), $N = 3$ for the remaining TiO_2 results and *$N = 2$ for IMAC experiments.*

portion of phosphopeptides is lost as sample breakthrough.

Reduction in recovery correlates with the 2,5-DHB concentration used in loading step. Figure 5 shows the nano-LC/MS analysis of two different 2,5-DHB loading concentrations; 50 and 200 mg/mL. Nonphosphorylated peptides were more effectively removed with higher DHB concentration (Fig. 5; the y-axis range in Figs. 5A and B is the same), while the greater phosphopeptide recovery was obtained with lower concentration. The loss of recovery is most apparent for peak T43_1P. The unmarked peaks in Fig. 5A are co-extracted acidic enolase peptides.

The experiment confirms that 2,5-DHB can effectively minimize the nonspecific binding of nonphosphorylated peptides when used in sufficiently high concentration. However, the singly phosphorylated peptides exhibiting lower affinity toward TiO_2 are likely to be also partially displaced by 2,5-DHB.

Some recovery loss was also observed when using glycolic acid (1 M) as the loading additive, but to a lesser extent than for 2,5-DHB (250 mg/mL, 1.6 M). The recovery difference between TiO_2 and IMAC (Poros MC) shown in Table 3 is based on an average of two measurements. The overall IMAC recovery using the chosen phosphopeptides is lower than with TiO_2 using TFA and glycolic acid loading conditions, but better than using 2,5-DHB as the displacing agent. We also noticed that IMAC provides for relatively more efficient extraction of doubly phosphorylated T43_2P, while failing to recover T43_1P with phosphothreonine motif. It appears that not only the number of phosphate groups but also the type of phosphorylation (phosphothreonine *vs.* phosphoserine *vs.* phosphotyrosine) and potentially the sequence motif affect the adsorption affinity toward both TiO_2 and IMAC sorbents. Not surprisingly, we also observed a different performance and selectivity for IMAC sorbents based on different chelating ligands [32], as discussed in the next section.

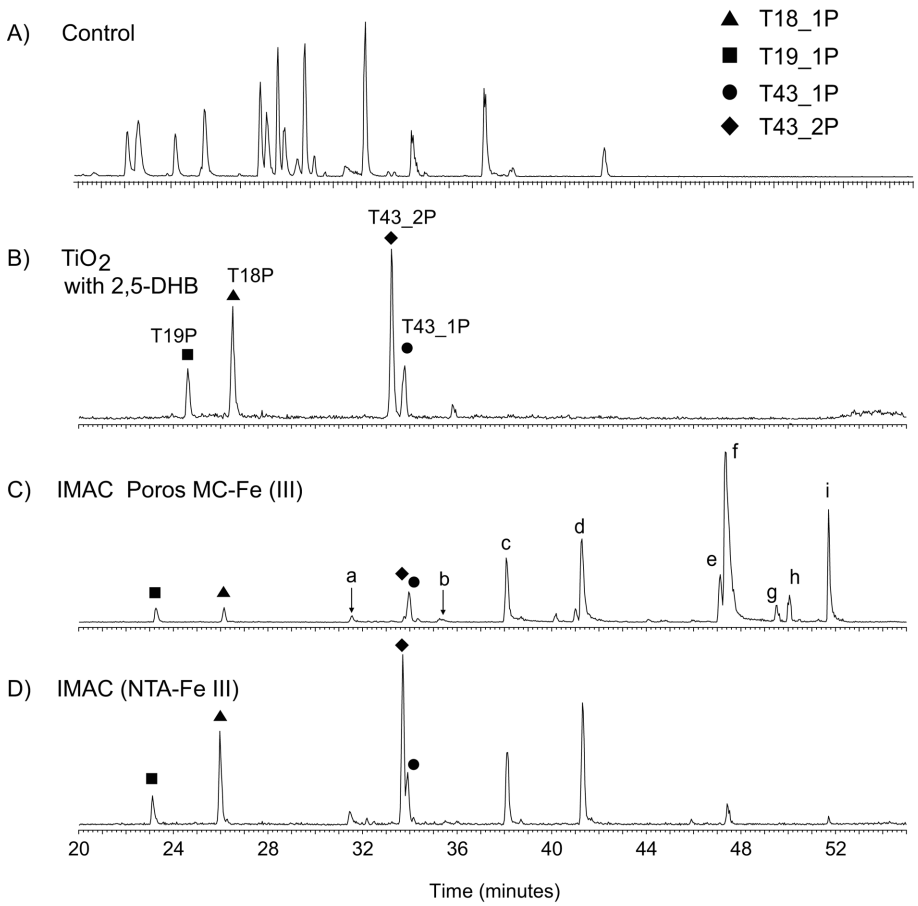

Figure 6. A mixture of enolase tryptic peptides with four synthetic phosphopeptides in a 50:1 molar ratio (250:5 pmol) was used to compare the phosphopeptide selectivity between TiO_2 and IMAC. LC/MS was used to analyze the extracts. (A) LC/MS of the control sample, (B) TiO_2 method with 250 mg/mL of 2,5-DHB in the loading step, (C) IMAC Poros MC enrichment, and (D) Qiagen NTA enrichment. Both IMAC devices were chelated with Fe (III). Approximately 400 fmol of sample was injected onto the nano-LC column.

3.4 TiO_2 and IMAC SPE methods comparison

Figure 6 shows the selectivity difference between TiO_2 (with 2,5-DHB loading) and IMAC in more detail. The SPE extract was analyzed with nano-LC/Q-Tof MS. The control sample is 250 pmol of enolase peptide mixed with 5 pmol of the standard phosphopeptides (50:1), ~0.4 pmol of phosphopeptides was injected on nano-LC column for analysis. The phosphopeptides are present only as minor signals in the control sample (Fig. 6A). Figure 6B once again confirms that extraction with TiO_2 and 2,5-DHB (250 mg/mL) in the loading step provides for good selectivity toward phosphopeptides. The nonphosphopeptides were nearly completely eliminated from the extract. In contrast, the IMAC sorbents retained both phosphopeptides and acidic enolase peptides (Figs. 6C and D).

The list of peptides with their p*I* values is provided in Table 4. Figure 6 clearly indicates that some of the acidic peptides were co-extracted along with the target phosphopeptides, and their relative abundance was enriched compared to the control sample shown in Fig. 6A. Some of the acidic peptides were also observed in TiO_2 extracts in Figs. 3 and 4, along with other peptides with p*I* values as high as 7.3. The TiO_2 sorbent exhibits affinity toward glutamic and aspartic amino acids [39] (perhaps in certain motifs), rather than toward acidic peptides in general (see also Table S5 of Supporting Information).

NTA-(Fe III) agarose sorbent retained acidic peptides to a lesser extent compared to Poros MC (Fe III) (Figs. 6C and D). Trace amounts of DHB were retained on the TiO_2 in the loading step and were eluted with the phosphopeptides as a significant peak detectable by UV. We believe that the residual amount of DHB has an impact on the retention selectivity of the peptides in an RP LC separation. This is why the phosphopeptide peaks in the TiO_2 and IMAC experiments did not align between LC/MS chromatograms as expected. The retention and selectiv-

Table 4. Nonphosphorylated peptides co-enriched along with phosphopeptides

		Peptide fragment number and sequences	p*I*
a	T4	GNPTVEVELTTEK	4.3
b	T51-52	IEEELGDNAVFAGENFHHGDKL	4.3
c	T38	TAGIQIVADDLTVTNPK	4.1
d	T14	AVDDFLISLDGTANK	3.9
e	T44-45	AAQDSFAAGWGVMVSHRSGETEDTFIADLVVGLR	4.4
f	T45	SGETEDTFIADLVVGLR	3.9
g	T37	YPIVSIEDPFAEDDWEAWSHFFK	4.0
h	T2-4	VYARSVYDSRGNPTVEVELTTEK	4.9
i	T27	YGASAGNVGDEGGVAPNIQTAEEALDLIVDAIK	3.7
j	T51	IEEELGDNAVFAGENFHHGDK	4.3
k	T30	IGLDCASSEFFK	4.3
l	T19	HLADLSK	7.3
m	T32	YDLDFK	4.1
n	T23	IGSEVYHNLK	7.3
o	T11	NVNDVIAPAFVK	6.2
p	T43	VNQIGTLSESIK	6.4
q	T31-32	DGYDLDFK	4.3

Peptides a – i are shown in Fig. 6 (IMAC protocol). Peptides j – q were observed in Figs. 3 and 4, co-extracted in TiO_2 experiment.

ity shift for DHB experiments were consistent and reproducible (data not shown).

The experiment depicted in Fig. 6 illustrates that TiO_2 SPE in combination with 2,5-DHB additive provides for highly selective enrichment of phosphopeptides from complex peptide mixtures. Interestingly, the recent proteomic study suggests that IMAC method outperforms TiO_2 enrichment in both the number of identified phosphopeptides and selectivity and that in those terms the phthalic acid is superior to DHB displacer [33]. Naturally, the format of SPE device and specific protocol conditions will affect the results and may account for the discrepancy. As far as we know, only one systematic study was published to date comparing the performance of IMAC and MOAC methods in proteomic experiment [33].

3.5 Phosphopeptide extraction from yeast

The 96-well micro-SPE TiO_2 plate was tested for phosphopeptide extraction from a complex biological sample. The extraction method is simple and could be accomplished in 15 min without the need for derivatization, or other sample pretreatment. Type 1 yeast lysate was processed using the TiO_2 SPE, loading with DHB additive (50 mg/mL) using protocol shown in Table 2.

The selectivity of enrichment can be estimated from the ratio of identified phosphopeptides *versus* co-extracted nonphosphorylated peptides [14, 31]. A total of 57 unique yeast phosphopeptides along with 40 nonphosphorylated peptides were identified by LC/MS/MS (DDA) experiment using MASCOT Distiller. The average MASCOT ion score was 60. Among the 57 phosphopeptides, 21 of them were multiply phosphorylated. A detailed list of peptides including 40 nonphosphorylated ones is shown in Table S5 of Supporting Information. More extensive list of phosphopeptides (198 unique peptides, 151 proteins) identified using combination of mixed-mode chromatography and MOAC methods was published elsewhere [28].

The phosphopeptide identification limits can be roughly estimated for a given nano-LC MS/MS system. Approximately 5 – 50 fmole peptides should be introduced on column to achieve MS/MS spectra quality suitable for peptide identification. Assuming ~50% recovery and the fact that only ~10% of sample processed by SPE is injected on column, the minimal amount of starting phosphopeptide (phosphoprotein) material should be 0.1 – 1 pmole.

4 Conclusions

A new TiO_2 micro-elution SPE device in a 96-well format achieved good performance in terms of ease of use, phosphopeptide extraction recovery, and selectivity. Similarly the selectivity of TiO_2 micro-SPE phosphopeptide enrichment depends strongly on the extraction conditions. When loading the sample with 0.1% TFA and 80% MeCN, the extraction selectivity was comparable to IMAC sorbents or worse.

The nonphosphorylated peptides containing aspartic and/or glutamic acid can be effectively displaced from the TiO_2 binding sites when loading the samples on SPE in DHB or salicylic acid solutions. The extraction selectivity decreases for displacers as follows: DHB – salicylic acid > 3-hydroxyl benzoic acid – glycolic acid > 0.1% TFA (no displacer). The selectivity depends on the nature and

concentrations of displacer. High concentration of displacer improves the selectivity, but compromises the phosphopeptide recovery. Quantitative data suggest that phosphopeptide recovery is between ~60 and 100% when using 0.1% TFA/80% MeCN in the loading solutions. The yield is reduced to ~20 – 60% when 250 mg/mL DHB was included in the load.

Data from literature and our results lead us to speculate that certain displacers are more effective because of the conformation and spatial orientation of the hydroxyl and carboxyl functional moieties (which mimic the spatial geometry of aspartate and glutamate residues). Our results are in general agreement with Larsen *et al*. [24], however, similarly to Sugiyama *et al*. [14] we found that glycolic acid performance is less favorable than observed by Jensen and Larsen [16].

There is considerable interest in a selective and sensitive phosphopeptide enrichment using online nano-LC methods using trapping columns. In online setup, the choice of displacers becomes crucial. Our data suggest that sample loading in 0.1% TFA is not ideal from the point of extraction selectivity. DHB and gallic acid are strongly adsorbed on the TiO_2 (and other metal oxide sorbents) as demonstrated by the permanent sorbent discoloration [16]. It may be difficult to regenerate the trapping columns in order to perform repeatable experiments with these displacers. Besides glutamate salt [31], 3-hydroxypropanoic acid, lactic acid [14], and other aliphatic hydroxycarboxylic acid [16], there potentially are other displacers more suitable for online nano-LC MS analyses.

The authors declared no conflict of interest.

5 References

[1] Hubbard, M. J., Cohen, P., *Trends Biochem. Sci.* 1993, *18*, 172 – 177.

[2] Figeys, D., *Anal. Chem.* 2003, *75*, 2891 – 2905.

[3] Reinders, J., Lewandrowski, U., Moebius, J., Wagner, Y., Sickmann, A., *Proteomics* 2004, *4*, 3686 – 3703.

[4] Reinders, J., Sickmann, A., *Proteomics* 2005, *5*, 4052 – 4061.

[5] Ballif, B. A., Villen, J., Beausoleil, S. A., Schwartz, D., Gygi, S. P., *Mol. Cell. Proteomics* 2004, *3*, 1093 – 1101.

[6] Lim, K. B., Kassel, D. B., *Anal. Biochem.* 2006, *354*, 213 – 219.

[7] Hung, C. W., Kubler, D., Lehmann, W. D., *Electrophoresis* 2007, *28*, 2044 – 2052.

[8] Xu, C. F., Wang, H., Li, D., Kong, X. P., Neubert, T. A., *Anal. Chem.* 2007, *79*, 2007 – 2014.

[9] Brill, L. M., Salomon, A. R., Ficarro, S. B., Mukherji, M., Stettler-Gill, M., Peters, E. C., *Anal. Chem.* 2004, *76*, 2763 – 2772.

[10] Hart, S. R., Waterfield, M. D., Burlingame, A. L., Cramer, R., *J. Am. Soc. Mass Spectrom.* 2002, *13*, 1042 – 1051.

[11] Mazanek, M., Mituloviae, G., Herzog, F., Stingl, C., Hutchins, J. R., Peters, J. M., Mechtler, K., *Nat. Protoc.* 2007, *2*, 1059 – 1069.

[12] Pinkse, M. W., Mohammed, S., Gouw, J. W., van Breukelen, B., Vos, H. R., Heck, A. J., *J. Proteome Res.* 2008, *7*, 687 – 697.

[13] Schlosser, A., Vanselow, J. T., Kramer, A., *Anal. Chem.* 2005, *77*, 5243 – 5250.

[14] Sugiyama, N., Masuda, T., Shinoda, K., Nakamura, A., Tomita, M., Ishihama, Y., *Mol. Cell. Proteomics* 2007, *6*, 1103 – 1109.

[15] Thingholm, T. E., Jorgensen, T. J., Jensen, O. N., Larsen, M. R., *Nat. Protoc.* 2006, *1*, 1929 – 1935.

[16] Jensen, S. S., Larsen, M. R., *Rapid Commun. Mass Spectrom.* 2007, *21*, 3635 – 3645.

[17] Nuhse, T., Yu, K., Salomon, A., *Curr. Protoc. Mol. Biol.* 2007, Chapter 18, Unit 18.13.

[18] Posewitz, M. C., Tempst, P., *Anal. Chem.* 1999, *71*, 2883 – 2892.

[19] He, T., Alving, K., Feild, B., Norton, J., Joseloff, E. G., Patterson, S. D., Domon, B., *J. Am. Soc. Mass Spectrom.* 2004, *15*, 363 – 373.

[20] Molloy, M. P., Andrews, P. C., *Anal. Chem.* 2001, *73*, 5387 – 5394.

[21] Ikeguchi, Y., Nakamura, H., *Anal. Sci.* 1997, *13*, 479 – 483.

[22] Kawahara, M., Nakamura, H., Nakajima, T., *Anal. Sci.* 1989, *5*, 763 – 764.

[23] Matsuda, H., Nakamura, H., Nakajima, T., *Anal. Sci.* 1991, *6*, 911 – 912.

[24] Larsen, M. R., Thingholm, T. E., Jensen, O. N., Roepstorff, P., Jorgensen, T. J., *Mol. Cell. Proteomics.* 2005, *4*, 873 – 886.

[25] Pinkse, M. W., Uitto, P. M., Hilhorst, M. J., Ooms, B., Heck, A. J., *Anal. Chem.* 2004, *76*, 3935 – 3943.

[26] Sano, A., Nakamura, H., *Anal. Sci.* 2007, *23*, 1285 – 1289.

[27] Ikeguchi, Y., Nakamura, H., *Anal. Sci.* 2000, *16*, 541 – 543.

[28] Gilar, M., Yu, Y. Q., Ahn, J., Fournier, J., Gebler, J. C., *J. Chromatogr. A* 2008, *1191*, 162 – 170.

[29] Larsen, M. R., Jensen, S. S., Jakobsen, L. A., Heegaard, N. H., *Mol. Cell. Proteomics* 2007, *6*, 1778 – 1787.

[30] Ficarro, S. B., Salomon, A. R., Brill, L. M., Mason, D. E., Stettler-Gill, M., Brock, A., Peters, E. C., *Rapid Commun. Mass Spectrom.* 2005, *19*, 57 – 71.

[31] Yu, L. R., Zhu, Z., Chan, K. C., Issaq, H. J., Dimitrov, D. S., Veenstra, T. D., *J. Proteome Res.* 2007, *6*, 4150 – 4162.

[32] Zhou, H., Ye, M., Dong, J., Han, G., Jiang, X., Wu, R., Zou, H., *J. Proteome Res.* 2008, *7*, 3957 – 3967.

[33] Bodenmiller, B., Mueller, L. N., Mueller, M., Domon, B., Aebersold, R., *Nat. Methods* 2007, *4*, 231 – 237.

[34] Cantin, G. T., Shock, T. R., Park, S. K., Madhani, H. D., Yates, J. R., III, *Anal. Chem.* 2007, *79*, 4666 – 4673.

[35] Simon, E. S., Young, M., Chan, A., Bao, Z. Q., Andrews, P. C., *Anal. Biochem.* 2008, *377*, 234 – 242.

[36] Mallet, C. R., Lu, Z., Fisk, R., Mazzeo, J. R., Neue, U. D., *Rapid Commun. Mass Spectrom.* 2003, *17*, 163 – 170.

[37] Liu, S., Zhang, C., Campbell, J. L., Zhang, H., Yeung, K. K., Han, V. K., Lajoie, G. A., *Rapid Commun. Mass Spectrom.* 2005, *19*, 2747 – 2756.

[38] Tuytten, R., Lemiere, F., Witters, E., Van Dongen, W., Slegers, H., Newton, R. P., Van Onckelen, H., Esmans, E. L., *J. Chromatogr. A* 2006, *1104*, 209 – 221.

[39] Roddick-Lanzilotta, A. D., McQuillan, A. J., *J. Colloid Interface Sci.* 2000, *227*, 48 – 54.

[40] Tani, K., Ozawa, M., *J. Liq. Chromatogr. Relat. Technol.* 1999, *22*, 843 – 856.

[41] Connor, P. A., McQuillan, A. J., *Langmuir* 1999, *15*, 2916 – 2921.

