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Characterisation and evaluation of metal-loaded iminodiacetic acid–silica of different porosity for the selective enrichment of phosphopeptides

L. Trojer, G. Stecher*, I. Feuerstein, S. Lubbad, G.K. Bonn

Institute of Analytical Chemistry and Radiochemistry, Leopold-Franzens University, Innrain 52a, 6020 Innsbruck, Austria

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Abstract

Silica particles of different porosity were functionalised with iminodiacetic acid (IDA) and loaded with Fe(III) to yield immobilised metal affinity chromatography stationary phases (Fe(III)–IDA–silica) for phosphopeptide enrichment. The elution step of bound phosphopeptides was optimised with a ^{32}P radioactive labelled peptide by a comprehensive study. Several elution systems, including phosphate buffers of different pH and concentration and ethylenediaminetetraacetic acid solutions were employed. Furthermore the effect of support porosity on elution behaviour was investigated. Under best conditions recoveries higher than 90% were achieved. A solid-phase extraction (SPE) protocol was developed for fractionation of phosphorylated and non-phosphorylated peptides and desalting of the fractions which is essential for subsequent mass spectrometric analysis by the combination of Fe(III)–IDA–silica and C_{18}–silica particles. The pH of the loading buffer was found to be a critical parameter for the efficiency of the SPE protocol. As tryptic digests of α-lactalbumin, lysozyme and ribonuclease A mixed with three synthetic phosphopeptides were fractionated, pH 2.5 provided minimal proportion of unspecific bound peptides when comparing the fractions after μ-LC–electrospray ionization MS separation. The effect of a sample derivatisation reaction (methylation) on the efficiency of phosphopeptide enrichment was further investigated. Blocking carboxylate groups by methyl ester formation totally prevented unspecific interaction with the immobilised Fe(III) ions, but generated partially methylated phosphopeptides that increased the complexity of the phosphorylated fraction.

Keywords: Silica particles; Phosphopeptides; Electrospray ionization

1. Introduction

Immobilised metal affinity chromatography (IMAC), employing complexed hard metal ions like Fe(III) or Ga(III), has been used for a number of years for the selective enrichment of phosphopeptides from complex mixtures. IMAC, which is based on different interaction of the analytes to metal cations bound to a support material by chelating ligands, was first introduced in 1975 by Porath et al. [1], who loaded metal ions into a carrier previously anchored by chelating iminodiacetic acid (IDA). Shortly after, it was reported that the 20 standard amino acids exhibit different retention on IMAC materials [2], which were loaded with middle hard metal ions, according to the HSAB-principle postulated by Pearson [3]. More studies demonstrated the ability of IMAC as stationary phases for protein separation [2,4]. An IMAC system, based on Ni(II) loaded nitrilotriacetic acid (NTA), was found to show high affinity toward two neighbouring histidin residues [5]. Nowadays six attached histidin residues (HisTag) are routinely proteins used to purify from complex mixtures in a single step reaching purities higher than 90%. In 1983 Porath et al. showed that immobilised hard metal ions, such as Fe(III), Ga(III) or Al(III), offer binding properties for different proteins and peptides beside those with good affinity towards the commonly used metal ions [Cu(II), Ni(II), Zn(II)] [6,7]. Hard ions exhibit high binding tendency of ligand atoms, like OH^-, O_2^- or F^-, as later on the potential of these new phases for isolating phosphorylated peptides was recovered. The commonly used supports for IMAC are porous [2,8]

* Corresponding author. Tel.: +43 512 507 5176; fax: +43 512 507 2767.
E-mail address: guenther.stecher@uibk.ac.at (G. Stecher).

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and non-porous [9] silica, beads of natural polymers such as agarose [10], sepharose [11] or cellulose [12], and synthetic polymer beads mainly crosslinked poly (styrene divinylbenzene) [13,14]. In addition surface derivatisation of matrix-assisted laser desorption ionisation (MALDI) targets was described for use in surface enhanced laser desorption ionisation (SELDI). These IMAC systems usually employ Cu(II) [15] and Ni(II) [16] aiming at histidin-rich analytes for biomarker discovery.

Phosphorylation is one of the most important post-translational modifications of proteins, where serine, threonine and tyrosine residues feature common phosphorylation sites [17–19]. Enrichment of phosphorylated proteins and peptides is generally required before analysis because their abundance is frequently low. Among various methods used in enrichment of phosphorylated species such as two-dimensional phosphopeptide mapping, radioactive labelling with ^{32}P-orthophosphate, mass spectrometry (LC/MS/MS; MALDI-TOF-TOF, where TOF = time-of-flight), immobilised antibodies or isotope-coded affinity tags (ICAT) [20,21], IMAC is considered a very efficient method [8–10,12,13,19–22,30].

The efficiency of an IMAC system in isolating phosphorylated species is influenced by many factors such as the choice of the metal ion immobilised, type, composition and pH of the loading and elution buffer. Optimisation of these parameters have to be performed with regard to the subsequent analysis by mass spectrometry (MS), since high salt concentration requires desalting steps, which are time consuming and always lead to sample loss. In addition the presence of unspecific (non-phosphorylated) bound peptides demonstrates another challenge, since these peptides severely limit the investigation of phosphorylation sites in complex biological samples. These retained unphosphorylated peptides were shown to be mostly peptides that are rich in acidic residues [13,22]. Ficarro et al. overcame this problem by blocking carboxylate groups through formation of methyl esters [13]. Mass spectrometric analysis of phosphopeptides is hindered by the presence of phosphate groups, which reduce ionisation efficiency [23]. The problem of coelution of unspecific compounds as well as low ionisation efficiency can be solved by derivatisation of phosphopeptides using partial chemical tagging [24].

In this study the influence of the support porosity and the composition of the elution solvent on the efficiency of the elution step of phosphopeptides, retained on Fe(III)–IDA–silica, was investigated. The results of these experiments were employed for establishing a SPE protocol that allows fractionation and desalting of peptide mixtures at the same time by combining a reversed phase material with Fe(III) loaded IDA–silica. The protocol was evaluated by the fractionation of protein digests mixed with synthetic phosphopeptides followed by μLC separation and detection with eletrospray ionisation (ESI) MS. Non-methylated and methylated samples were fractionated to evaluate the derivatisation reaction and to determine SPE conditions for minimal proportions of unspecific bound peptides.

2. Experimental

2.1. Chemicals and reagents

Irregular silica gel (63–200 μm) was obtained from Fluka (Buchs, Switzerland), all spherical 5 μm silica materials from Grom Analytik (Rottenburg-Hailfingen, Germany) and a 20 g sample of irregular particles with 35–70 μm was donated by Grace Vydac (Columbia, MD, USA). Iminodiacetic acid (IDA) (≥99%), acetone (HPLC grade), nitric acid (analytical-reagent grade), diethyl ether (for spectroscopy), sodium carbonate (analytical-reagent grade), acetic acid (analytical-reagent grade), magnesium acetate (analytical-reagent grade), iron(III) chloride (analytical-reagent grade), urea (analytical-reagent grade), ammonium bicarbonate and sodium acetate (for microbiology) were purchased from Merck (Darmstadt, Germany). Tris(hydroxymethyl)aminomethane (≥99%), disodium ethylenediamine tetraacetic acid (analytical-reagent grade), Triton X-100 (for molecular biology), iodoacetic acid (analytical-reagent grade), 2-mercapto-ethanol (for molecular biology) and trifluoroacetic acid (TFA) (for protein sequence analysis) were obtained from Fluka (Buchs, Switzerland). Myoglobin (from horse heart, ≥90%), α-lactalbumin (from bovine milk, ∼85%), lysozyme (from chicken egg white, ∼95%) and ribonuclease A (from bovine pancrease), further kemptide (≥97%), protein kinase A (catalytic subunit from porcine heart), DL-dithiothreitol (99%) and adenosine-5′-triphosphate (ATP) (99%) were from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade methanol and acetonitrile (ACN) were obtained from Riedel-de Haën, 3-glycidoxypropyltrimethoxysilane (GLYMO) (98%) and acetyl chloride (98%) from Aldrich (Milwaukee, WI, USA), γ-^{32}P-ATP (2 mCi/ml) from Amersham Biosciences (Uppsala, Sweden) and trypsin (sequencing grade modified ∼) from Promega (Madison, WI, USA). Three synthetic phosphopeptides were used, one of them, having a sequence of Ac-pY-EEIE (M_r: 803.73), was obtained from Bachem (Bubendorf, Switzerland), the other two peptides with the sequences TTPSFVGFTD-pT-ER (M_r: 1537.57) and SFDVPPIDASSPF-pS-QK (M_r: 1801.88) were from Biosynthan (Berlin, Germany). Water used was purified by a NANOpure Infinity unit (Barnstead, Boston, MA, USA). All chemicals were used without further purification.

2.2. Instrumentation

A μLC system coupled via an ESI interface to MS (LCQ, Thermo-Finnigan, San Jose, CA, USA) was used for reversed phase analysis of peptides. The LC device consisted of a micro pump (Rheos 2000, Flux Instruments, Basel, Switzerland), a degasser (Knauer, Berlin, Germany), a split function

to reduce the flow rate through the system, a 6-way injection valve (Vici E60 CE, Valco Instruments, Houston, TX, USA) and a monolithic PS/DVB column (50 x 0.2 mm) (LC-Packings, Amsterdam, The Netherlands), incorporated into a column oven. The ESI-interface was build up of a fused-silica spray capillary (I.D.: 20 μm, O.D.: 90 μm), located in a fitting, which was linked with a *x*,*y*,*z*-manipulator. This arrangement allowed precise, video controlled focussing of the spray capillary to the heated capillary. The ESI-interface was driven with the following settings: heated capillary temperature: 200 °C, spray voltage: 1.6 kV, flow rate of sheath gas: 100 arb, flow rate of auxiliary gas: 0 arb. The MS was operated in the full scan mode (500–2000 *m*/*z*), number of microscans: 2, maximum injection time: 400 ms. Tuning of the MS was performed with a cytochrome C solution (1 ng/μl) in 20% ACN, 0.05% TFA at regular intervals.

2.3. Synthesis of IDA–silica under absolute and aqueous conditions

Silica gel was activated and purified by several washing steps with 20% HNO_3, 0.5 M NaCl, H_2O, acetone and diethyl ether, followed by desiccation for 4 h at 150 °C under vacuum to remove physisorbed water. The surface was derivatised with GLYMO according to a procedure by Larsson at al. [25]. The epoxide groups were further reacted with IDA using following protocol: 1 g IDA was dissolved in 9 ml of an aqueous Na_2CO_3 solution and the pH adjusted to 9.6 by further addition of Na_2CO_3. The epoxide-silica was suspended in this alkaline solution and agitated at 65 °C for 4 h. The reaction mixture was then centrifuged and the IDA–silica purified by two-fold suspension in 5 ml H_2O, 10% HOAc, H_2O and MeOH. The product was dried at room temperature (RT) under vacuum. The synthesis of IDA–silica under aqueous conditions was done following a protocol that can be found in [26].

2.4. Evaluation of Fe(III) loaded IDA–silica regarding elution behaviour and recoveries

2.4.1. ^{32}P-Radiolabelling of kemptide

Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) was chosen as a target peptide for radiolabelling with ^{32}P. The reaction was performed employing γ-^{32}P-ATP and protein kinase A which is able to transfer the γ-phosphate group of ATP on a peptide substrate [27]. The procedure was carried out in a similar way as described in [28]. 267 μl of a 100 mM Tris-HCl buffer (pH 7.5) were combined with 400 μl of a 75 μM kemptide solution (50 mM Tris-HCl buffer (pH 7.5) with 0.005% Triton X-100). Then 200 μl of a 1.5 mM ATP/γ-^{32}P-ATP in a 37.5 mM $Mg(OAc)_2$ solution were added and the mixture was heated to 30 °C. The labelling reaction was initialised by the addition of 267 μl of a 20 mM Tris-HCl buffer (pH 7.5), which contained an appropriate amount of protein kinase A. The reaction was allowed to take place for 3 h at 30 °C. Excessive ATP and γ-^{32}P-ATP was removed by anion exchange [29], The purified, radioactive labelled peptide solution was lyophilised and the product was stored at −20 °C.

2.4.2. Elution experiments

One hundred milligrams of IDA–silica were put in a SPE-cartridge of 6 mm diameter (Separtis, Grenzach-Wyhlen, Germany) and sealed with a PE-frit (Separtis, Grenzach-Wyhlen, Germany). The IDA–silica in the cartridge was then loaded with 5 ml of a 0.1 M $FeCl_3$ solution, followed by a washing and equilibration step with 1% HOAc (pH 2.5), containing NaCl (50 mM) (equilibration buffer) to remove unspecific bound metal ions. The lyophilised radioactive labelled kemptide was dissolved in the equilibration buffer, 1.5 nmol assigned to the cartridge. Then the loaded material was washed with 1200 μl equilibration buffer before initiating the detachment of the bound phosphorylated peptide by flushing with 2200 μl of different elution buffers. Five fractions (flow-through and washing steps) were collected at the outlet of the cartridge to get information about the amount of non-retained peptide and 11 fractions (eluate) of equal volume to investigate the elution behaviour. All fractions were mixed with 10 ml of a scintillation fluid (Ultima Gold AB, Perkin-Elmer, Boston, MA, USA) and measured with a β-counter (bf betaszint 5000, Bertold, Bad Wildbach, Germany). The device was calibrated with standard solutions in the range of 30–600 pmol. The resulting standard-curve fitted a polynomic function of second order.

2.5. Sample preparation

Five hundred microlitres of denaturation solution (8 M urea, 0.5 M NH_4HCO_3) were added to 1 mg of protein, degassed with argon and shaken for 30 min at 37 °C. After addition of 50 μl DL-dithiothreitol (30 mM), the mixture was incubated for 4 h at 37 °C. Afterwards, the mixture was cooled down and spiked with 25 μl iodoacetic acid (100 mM). The reaction was allowed to proceed for 15 min at darkness and RT. Excess of iodoacetic acid was destroyed by addition of 50 μl of a 100 mM 2-mercaptoethanol. Finally, the carboxymethylated protein was dialysed (cut off 2000 Da) for at least 3 h. The reduced and derivatised proteins were digested with 20 μg trypsin. The digest was allowed to proceed overnight at 37 °C and then terminated by the addition of 25 μl of a 2.5% TFA. The acid digest solution was stored at −20 °C.

Digests of myoglobin, lysozyme, α-lactalbumin and ribonuclease A thus produced were – single or combined – mixed with three synthetic phosphopeptides in ratios of 1:2, 1:5 and 1:10 (phosphopeptide to protein digest). After lyophilisation the samples were stored at −20 °C. Some were further derivatised by methylation of the carboxylic acid functions [13]. This was performed by dissolving the samples in 100 μl of a 2 M methanolic HCl. The reaction was terminated by lyophilisation after 2 h.

Table 1
Manufacturer's specifications of the silica particles derivatised to IDA–silica

Name	Particle diameter (μm)	Pore diameter (Å)	Surface area (m^2/g)	Pore volume (ml)
Silicagel 60	63–200	60	500	No specification
GromSIL 60Si	5	60	580	0.95
GromSIL 120Si	5	120	300	1.00
GromSIL 300Si	5	300	100	1.05
GromSIL 1000Si	5	1000	30	1.10
Davisil XWP	35–70	1000	30–55	1.05

2.6. SPE experiments

One hundred milligrams of C_{18}-silica filled in SPE-cartridges (Waters, Milford, MA, USA) were overlaid by 100 mg IDA–silica and sealed with a PE-frit. The IMAC-silica material was loaded with a 0.1 M $FeCl_3$ solution and equilibrated with a solution of 50 mM NaCl in a 170 mM acetate buffer. The samples were dissolved in 100 μl equilibration buffer and loaded on the cartridge. All non-phosphorylated peptides were flushed out of the IMAC-particles by 4 ml equilibration buffer, followed by a desalting step with 3 ml 0.1% TFA/H_2O. Then they were detached from the reversed phase by elution with 2 ml 0.1% TFA/70% ACN. To activate the C_{18}-phase for the rebinding of analytes, the cartridge was rinsed with 3 ml 0.1% TFA/H_2O. Finally phosphorylated constituents were eluted from Fe(III)–IDA–silica with 4 ml of a 100 mM phosphate buffer (pH 8), the cartridge was desalted with 3 ml 0.1% TFA/H_2O and the phosphorylated fraction removed from the reversed phase with 2 ml 0.1% TFA/70% ACN. Both the unphosphorylated and phosphorylated fractions were collected on the outlet, lyophilised and analysed directly or stored at −20 °C.

3. Results and discussion

3.1. Synthesis of IDA–silica under absolute and aqueous conditions

One silica material was synthesised via both reaction channels described in Section 2.3 to work out differences of the IDA–silica ascribed to synthesis. The material chosen was irregular Silicagel 60 (see Table 1 for further specification). Fig. 1 shows the determined elution curves for both Fe(III) loaded materials (absolute and aqueous synthesis). Elution of the bound radioactive labelled kemptide was achieved by a 50 mM phosphate buffer (pH 8). It was demonstrated, that the synthesis had low influence on the quality of the IDA–silica concerning elution properties. Both curves have their maximum elution at the same volume. Moreover the calculated amount of unretained phosphopeptide and their recoveries for the two materials synthesised under absolute and aqueous conditions were almost similar with values of 1.8, 75.6% and 2.3, 73.6%, respectively. All other silica particles described in this study were then synthesised under absolute conditions to assure comparability and uniformity.

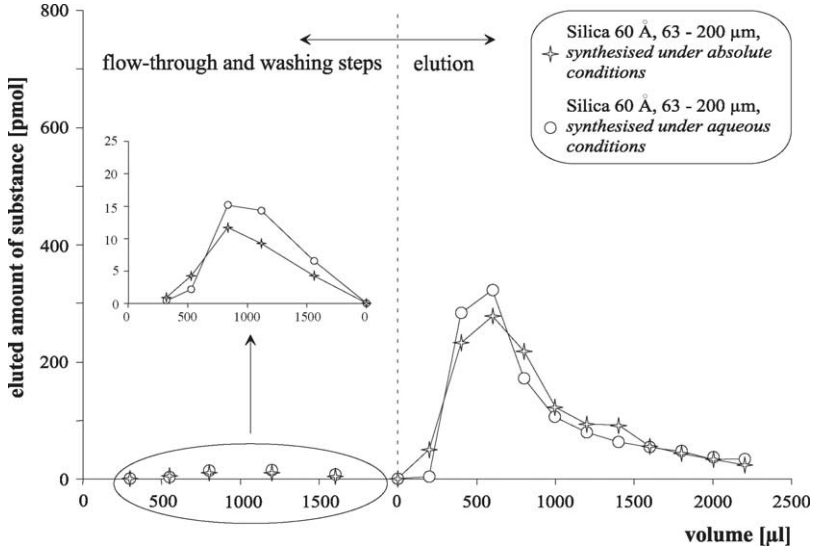

Fig. 1. Elution behaviour of radioactive labelled kemptide bound to Fe(III) loaded IDA–silica particles synthesised via the two different reaction channels in Section 2.3. Elution was performed by a 50 mM phosphate buffer pH 8.

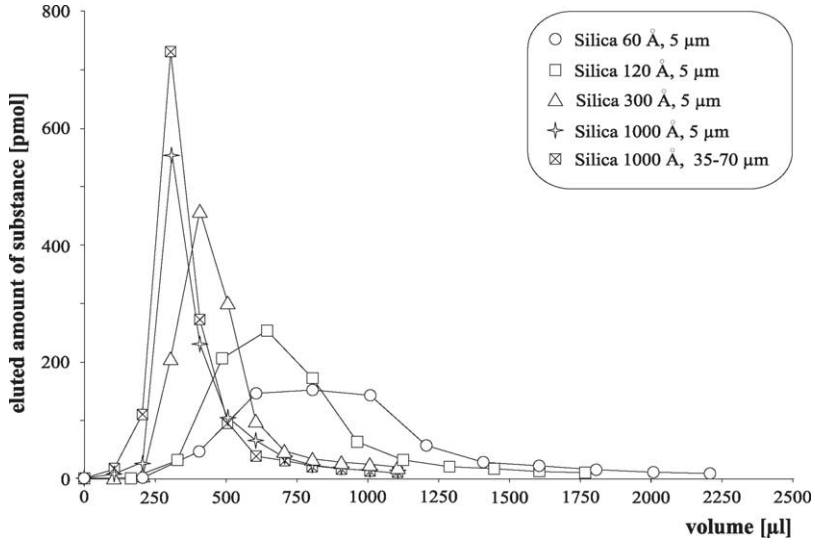

Fig. 2. Elution curves of radioactive labelled kemptide as a function of support porosity. Elution was performed with a 50 mM phosphate buffer pH 8.

3.2. Optimisation of the elution conditions of retained phosphopeptides bound to Fe(III)–IDA–silica

The denotation and some important physical properties of all synthesised silica gels are listed in Table 1. In order to determine the optimal elution conditions of bound phosphorylated species, the Fe(III)–IDA–silica materials were loaded with a radioactive labelled phosphopeptide (phosphorylated kemptide) which was then detached using different buffer systems.

3.2.1. Effect of support porosity on elution

The influence of the porosity of the carrier material on the elution behaviour of phosphorylated species was studied using a 50 mM phosphate buffer pH 8. It was noticed that the recovery improved and the elution maxima required lower amount of solvent with increasing the pore diameter (Fig. 2, Table 2). This might be due to the exponential decrease in the surface area (Table 1) which makes the wide-pore materials more preferred concerning elution properties. Fe(III)–IDA–silica (mean pore diameter = 1000 Å) showed 90% recovery of the loaded phosphopeptide which is about 20% higher than that of commercially available Fe(III)–IDA-PS/DVB (Porous 20 MC, PerSeptive Biosystems) [30] applying similar elution conditions. Recovery of a radioactive labelled phosphopeptide bound on derivatised cellulose gave similar results compared to wide-pore Fe(III)–IDA–silica [12]. It was further shown (Table 2), that even the capacity of wide-pore materials is sufficient to bind 1.5 nmol phosphopeptide, which is pointed out by the quantity of non-retained phosphopeptide, detected in the flow-through and the washing steps. This amount never exceeded 3%.

The elution of phosphorylated substance by a phosphate buffer is based on a competitive ligand that replaces the analytes at the complexed metal centre, while the immobilised metal ions themselves keep bound to the chelator. Another strategy is the elution of phosphopeptides by elution of the metal ions themselves, using a chelator with a higher affinity toward the metal ions. The potential of this strategy was examined by elution with a 50 mM Na_2EDTA solution. Surprisingly EDTA led to recoveries of 10–15% lower than those obtained with phosphate based elution (Table 2). All further

Table 2
Calculated recoveries and percentage rates of non-retained radioactive labelled kemptide as a function of support porosity

Material	Pore diameter (Å)	Surface area (m^2/g)	Flow-through[a] (%)	Recovery (%)
Elution with a 50 mM phosphate buffer, pH 8				
GromSIL 60Si	60	580	0.3	75.8
GromSIL 120Si	120	300	0.7	80.8
GromSIL 300Si	300	100	1.3	81.8
GromSIL 1000Si	1000	30	1.2	85.0
Davisil XWP	1000	30–55	0.5	89.7
Elution with a 50 mM Na_2EDTA solution				
GromSIL 60Si	60	580	0.8	66.0
GromSIL 120Si	120	300	1.2	68.1
GromSIL 300Si	300	100	1.9	72.9
GromSIL 1000Si	1000	30	2.9	71.1

[a] Sum of all detectable substance in the flow-through and in the washing steps.

Table 3
Calculated recoveries and percentage rates of non-retained radioactive labelled kemptide as a function of pH-value and ionic strength (elution with phosphate buffers)

Material[a]	Pore diameter (Å)	Concentration[b] (mmol/l)	pH value	Flow-through[c] (%)	Recovery (%)
Optimisation of the pH-value					
Silicagel 60	60	50	4.0	1.3	61.4
Silicagel 60	60	50	6.0	2.6	65.0
Silicagel 60	60	50	8.0	1.9	75.6
Silicagel 60	60	50	10.0	2.0	84.9
Optimisation of the ion strength					
Silicagel 60	60	20	8.0	3.9	55.9
Silicagel 60	60	50	8.0	3.9	74.2
Silicagel 60	60	100	8.0	4.1	82.9
Silicagel 60	60	200	8.0	5.6	80.1

[a] Synthesised under absolute conditions.
[b] Concentration of the phosphate buffer.
[c] Sum of all detectable substance in the flow-through and in the washing steps.

elution experiments were therefore performed using phosphate buffers of different concentration and pH.

3.2.2. Effect of pH on elution

To study the pH effect on elution, four phosphate buffer solutions (50 mM, pH = 4, 6, 8 and 10) were applied. Raising the pH from 4 to 10 improved the recovery from 60 to 85% (Table 3), but showed no change in the location of the elution maxima. These results could be explained by deprotonation of phosphate, when increasing the buffer pH, which makes them stronger competitive ligands. Another possibility is the replacement of phosphopeptides by OH^- ions that are more abundant at higher pH (basic elution) [22]. Silica undergoes hydrolysis under alkaline conditions (>pH 8), which limits the use of phosphate buffer of pH 10. The hydrolysis effect could be negligible for single use protocols (like SPE) but is significant in continuous flow through analysis (online phosphopeptide enrichment by two-dimensional fractionation with μLC). With regard to this, all further SPE experiments were performed at pH 8.

3.2.3. Effect of ionic strength on elution

Changing the concentration of the phosphate buffer solution (20, 50, 100 mM, pH 8) led to recovery improvement as 56, 74, 83%, respectively (Table 3). In contrast to the effect of pH variation, the elution maxima were shifted at lower elution volumes and the elution curves got narrower. However, increasing the buffer concentration to 200 mM showed no significant change from that of 100 mM. This is an important observation when MS is employed for subsequent detection of the phosphorylated fraction. The higher the concentration of the buffer, the more tedious the analysis is as high volume of water is needed for desalting which leads to sample loss.

3.3. Fe(III)–IDA–silica as SPE material for the selective enrichment of phosphopeptides

All SPE-experiments were performed using Davisil XWP as support material (see Table 1 for further specification). This carrier was selected due to its very favourable elution behaviour (Fig. 2 and Table 2). The elution step of retained phosphopeptides was executed using a 100 mM phosphate buffer pH 8, out of reasons explained above. Apart from phosphate and phosphoric acid mono-esters, it is known that carboxylate groups of glutamate and aspartate have affinity to hard metal ions [13,22]. The influence of carboxylate groups on the complexity of the phosphorylated fraction was studied by comparison of both non-methylated and methylated samples after SPE separation.

3.3.1. Analysis of methylated samples

Atryptic digest of myoglobin mixed with three synthetic phosphopeptides in a ratio of 2:1 (Fig. 3A–C) and 10:1 (Fig. 3D–F) was used. Both samples were methylated and 500 pmol (with respect to the myoglobin digest) introduced into the cartridge at a pH of 2.5. To get information about the amount of non-bound phosphopeptides - represented through the detectable amount of substance in the flow-through - a suitable mass range of each phosphopeptide of chromatograms 3B and 3C was extracted for comparison [858–861 Da for single charged P1 (Ac-pY-EEIE), 789–792 Da for double charged P2 (TTPSFVGFTD-pT-ER) and 921–924 Da for double charged P3 (SFDVPPIDASSPF-pS-QK)] (Fig. 4). Only traces of P2 (<2%) could be detected in the flow-through, P1 and P3 were completely retained on Fe(III)–IDA–silica which confirms the very good performing binding affinity of wide-pore Fe(III)–IDA–silica. However the location of phosphorylation (serine, threonine or tyrosine) does not seem to influence the binding properties towards Fe(III)–IDA–silica. Chromatogram B (Fig. 3) of the three phosphopeptides (P1, P2 and P3) shows no interference of unspecific bound peptides of myoglobin digest. Moreover the SPE approach was suited to detect even little amounts of phosphorylated species out of a complex mixture with high purities (Chromatogram E). Nevertheless some peaks (labelled with 1, 2, 3 and 4) can be found, that are not ascribed to totally methylated P1, P2 or P3. When decreasing the concentration of the phosphopeptides in the original sample,

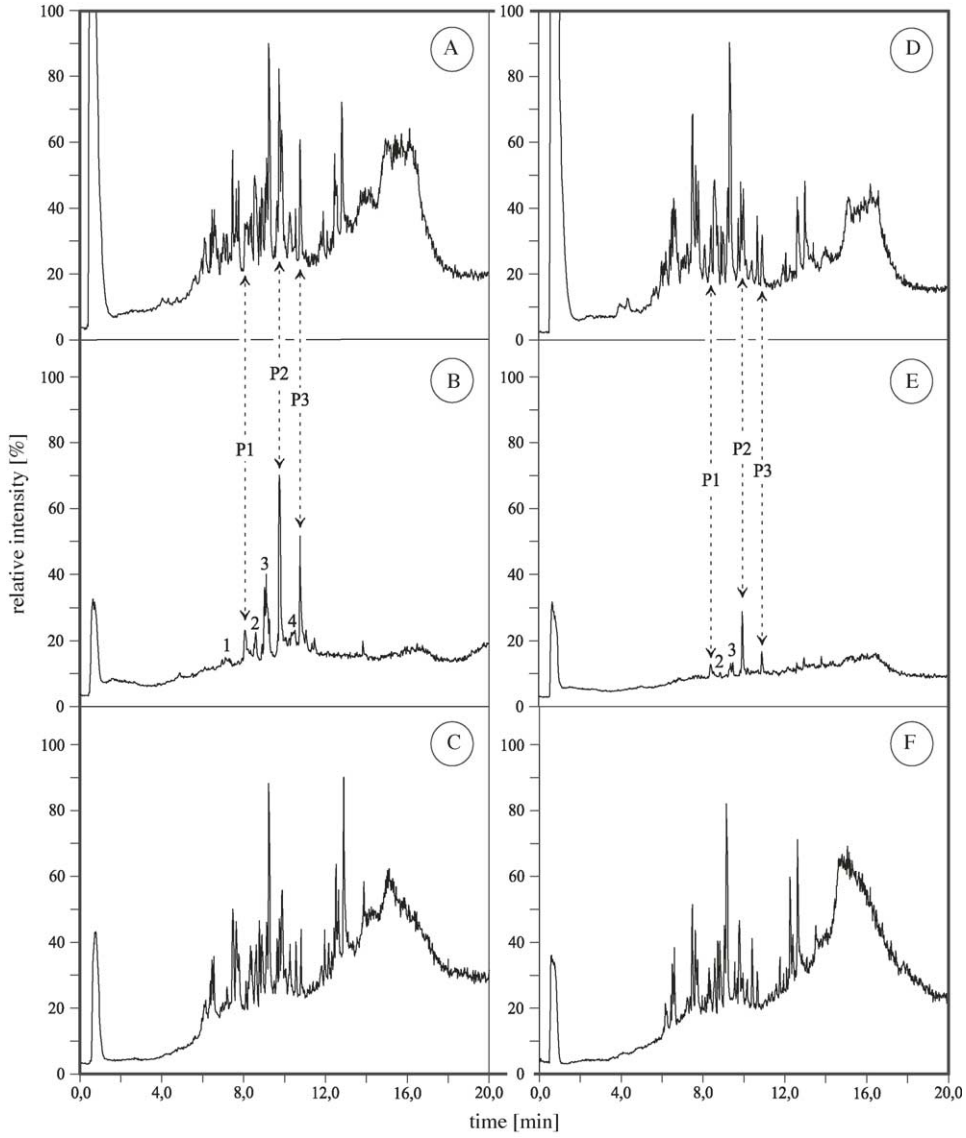

Fig. 3. Separation of a methylated sample after SPE fractionation. Solvent (A): 0.05% TFA/H_2O, solvent (B): 0.05% TFA/ACN, linear gradient: 0–70% (B) in 20 min, 60 °C, 2 μl/min, injection: 500 nl, separation capillary: PS/DVB-monolith (50 mm × 0.2 mm), detection: ESI-MS (TIC-mode), sample: 5 pmol/μl tryptic myoglobin digest mixed with three synthetic phosphopeptides (2.5 pmol/μl in case of (A) and 500 fmol/μl in case of D). (A) and (D): unfractionated samples, (B) and (E): phosphorylated fraction of the SPE, (C) and (F): non-phosphorylated fraction of the SPE (flow-through), P1, P2 und P3: totally methylated phosphopeptides, 1, 2, 3 und 4: partially methylated phosphopeptides.

the intensity of those peaks went down. This indicates that these signals do not represent unspecific (over carboxylate groups) bound myoglobin fragments, but are coupled with the phosphorylated ingredients of the sample. The peaks represent partially methylated phosphopeptides as 1 is three-fold methylated P1, 2 single, 3 two-fold methylated P2 and 4 is two-fold methylated P3. This was supported by mass match of the obtained and the theoretical values (Table 4). Apart from these partial methylated phosphopeptides, no other unspecific bound peptides could be detected.

3.3.2. Analysis of non-methylated samples

The separation of a non-methylated sample before and after SPE fractionation at different loading conditions is shown in Fig. 5. A mixture of three protein digests (α-lactalbumin, lysozyme, ribonuclease A) and three phosphopeptides in a ratio of 2:1 was employed, simulating a very complex mixture. 500 pmol (with respect to one protein digest) were loaded into the cartridge at different pH-values to optimise the conditions for minimal proportion of unspecific bound peptides. Fig. 5 shows that equilibration at pH 1 leads to a phosphorylated fraction of low intensity. The better part of the phosphopeptides was detected in the flow-through. Raising the pH to 2.5 demonstrated P2 and P3 to be highly retained while a number of unspecific bound peptides were present in the chromatogram. Further increase of pH up to 4.8 and finally 5.8 did not provide better results. Both the number and intensity of

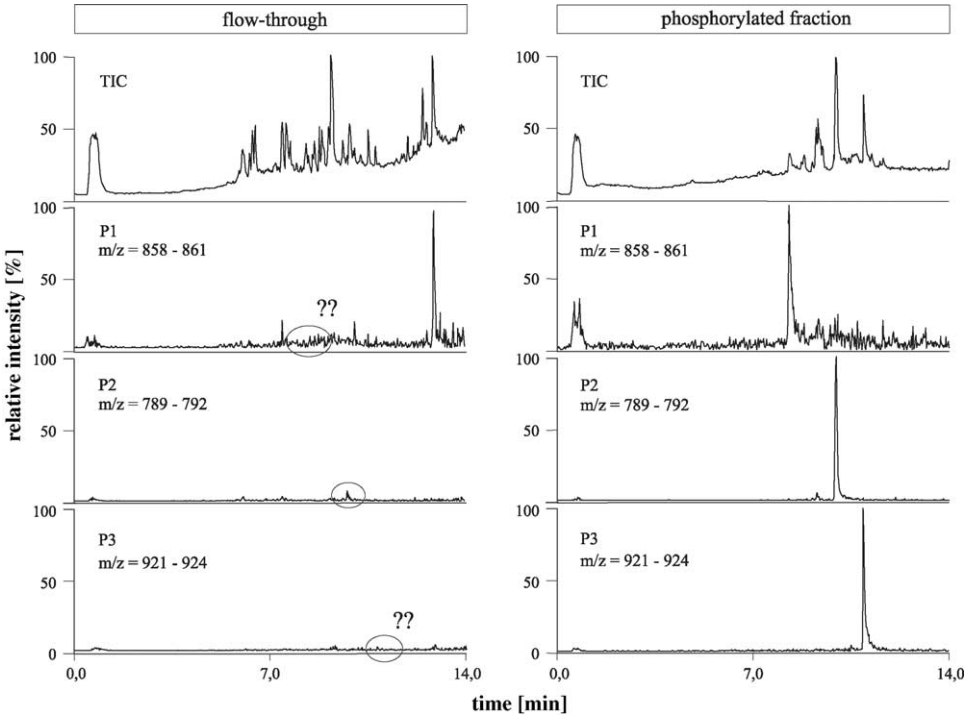

Fig. 4. Extraction of a suitable mass range of each phosphopeptide in chromatogram B and C (Fig. 3) to analyse the amounts of non-retained phosphopeptides in the flow-through. Separation conditions: see Fig. 3.

unspecific bound analytes strongly increased. These results are in agreement with earlier observations made with immobilised Ga(III), as peptide mixtures containing phosphorylated species were incubated on IMAC-particles at different pH-values and analysed with MALDI [30]. We suppose that low pH-values have a positive influence on specificity because of a favourable charge situation. At pH 2.5 nearly all carboxylate groups exist in the protonated form, whereas phosphate groups are not totally protonated because of their lower pKa-value. The protonation of the carboxylate groups reduce their ability to interact with Fe(III). The weak signal intensities at pH 1 can be explained by the protonation of the chelator (IDA) itself, which leads to metal ion bleeding. Strong evidence gives the fact that the yellow-orange coloured Fe(III)–IDA–silica loses its tinct when equilibrated at pH 1. The peaks obtained in the phosphorylated fraction at pH 1 can only be attributed to some immobilised residual cations.

Table 4
List of all grades of methylation for the three examined phosphopeptides P1, P2 and P3

Grade of methylation	Sequence[a]	Theoretical mass (Da)	Obtained mass (Da)
Non-methylated	Ac-*p* Y-EEIE	803.73	n.d.[b]
Non-methylated	TTPSFVGFTD-*p* T-ER	1537.57	n.d.
Non-methylated	SFDVPPIDASSPF-*p* S-QK	1801.88	n.d.
Single methylated	Ac-*p* Y-E^{m}EIE, Ac-*p* Y-EE^{m}IE, two possibilities of: Ac-*p* Y-EEIE^{m}	817.76	n.d.
Single methylated	TTPSFVGFTD^{m}-*p* T-ER, TTPSFVGFTD-*p* T-E^{m}R, TTPSFVGFTD-*p* T-ER^{m}	1551.60	1551.4
Single methylated	SFD^{m}VPPIDASSPF-*p* S-QK, SFDVPPID^{m}ASSPF-*p* S-QK, SFDVPPIDASSPF-*p* S-QK^{m}	1815.91	n.d.
Two-fold methylated	Ac-*p* Y-E^{m}E^{m}IE, Ac-*p* Y-E^{m}EIE^{m}, Ac-*p* Y-EE^{m}IE^{m}, Ac-*p* Y-EEIE^{2m}	831.79	n.d.
Two-fold methylated	TTPSFVGFTD^{m}-*p* T-E^{m}R, TTPSFVGFTD^{m}-*p* T-ER^{m}, TTPSFVGFTD-*p* T-E^{m}R^{m}	1565.63	1565.4
Two-fold methylated	SFD^{m}VPPID^{m}ASSPF-*p* S-QK, SFD^{m}VPPIDASSPF-*p* S-QK^{m}, SFDVPPID^{m}ASSPF-*p* S-QK^{m}	1829.94	1830.6
Three-fold methylated	Ac-*p* Y-E^{m}E^{m}IE^{m}, Ac-*p* Y-E^{m}EIE^{2m}, Ac-*p* Y-EE^{m}IE^{2m}	845.82	845.4
Totally methylated	TTPSFVGFTD^{m}-*p* T-E^{m}R^{m}	1579.66	1580.2
Totally methylated	SFD^{m}VPPID^{m}ASSPF-*p* S-QK^{m}	1843.97	1844.2
Totally methylated	Ac-*p* Y-E^{m}E^{m}IE^{2m}	859.85	859.5

[a] A prefixed *p* notes the location of the phosphorylation, a superscript m marks a methylation.
[b] Not detectable.

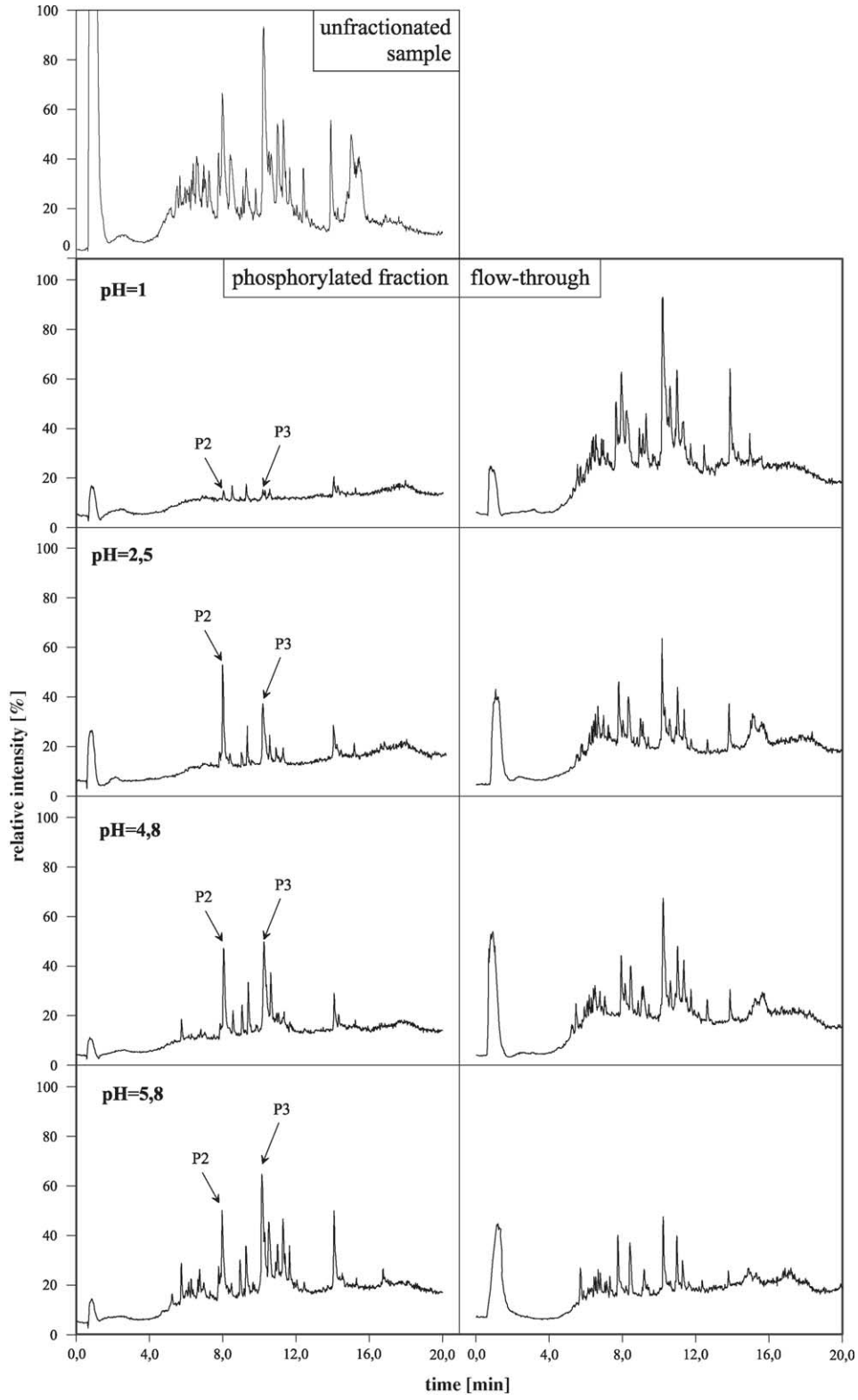

Fig. 5. Separation of a non-methylated sample before and after SPE fractionation for pH optimisation of the equilibration buffer. Solvent (A): 0.05% TFA/H_2O, solvent (B): 0.05% TFA/ACN, linear gradient: 0–70% (B) in 20 min, 60 °C, 2 μl/min, injection: 500 nl, separation capillary: PS/DVB-monolith, (50 mm × 0.2 mm), detection: ESI-MS (TIC-mode), sample: tryptic digests of α-lactalbumin, lysozyme and ribonuclease A (5 pmol/μl per protein) mixed with three synthetic phosphopeptides in a ratio of 2:1.

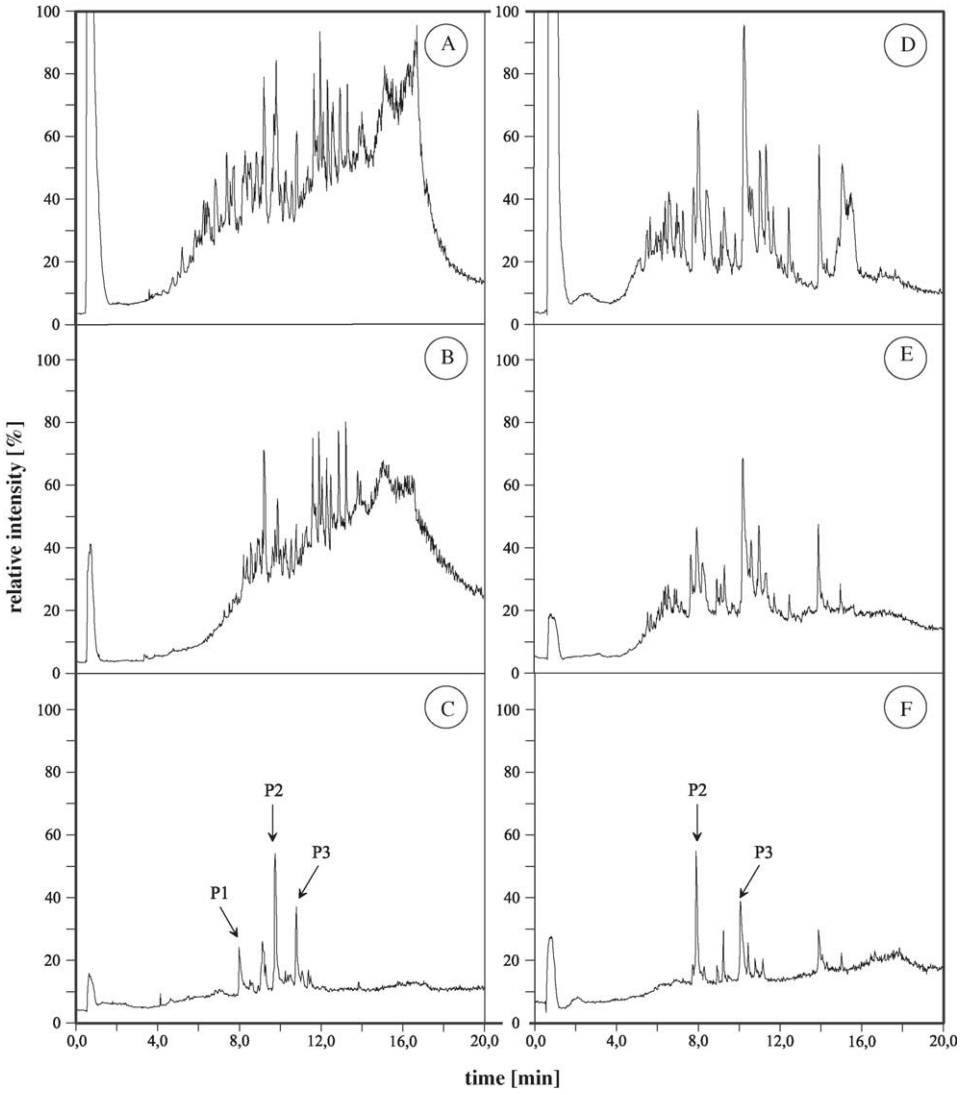

Fig. 6. Separation of SPE fractions for the comparison of methylated and non-methylated samples. Solvent (A): 0.05% TFA/H_2O, solvent (B): 0.05% TFA/ACN, linear gradient: 0–70% (B) in 20 min, 60 °C, 2 μl/min, injection: 500 nl, separation capillary: PS/DVB-monolith (50 mm × 0.2 mm), detection: ESI-MS (TIC-mode), sample: tryptic digests of myoglobin, α-lactalbumin, lysozyme and ribonuclease A (5 pmol/μl per protein) mixed with three synthetic phosphopeptides in a ratio of 2:1 used as methylated sample (A, B and C) and tryptic digests of α-lactalbumin, lysozyme and ribonuclease A (5 pmol/μl per protein) mixed with 3 synthetic phosphopeptides in a ration of 2:1 used as non-methylated sample (D, E and F). (A) and (D): original samples, (B) and (E): unphosphorylated fraction, (C) and (F): phosphorylated fraction.

3.3.3. Methylated versus unmethylated samples – a direct comparison

The methylation of peptide mixtures improves the analysis of phosphopeptides. This can be seen from Fig. 6 which represents a direct comparison of two SPE experiments deriving from methylated and non-methylated peptides applying the same conditions. A methylated sample (tryptic digest of myoglobin, lysozyme, α-lactalbumin, ribonuclease A mixed with three synthetic phosphopeptides in a ratio 2:1) and a non-methylated sample (tryptic digest of lysozyme, α-lactalbumin, ribonuclease A mixed with three synthetic phosphopeptides in a ratio 2:1) were analysed (Chromatograms A–C and D–F, respectively). 500 pmol (with respect to one protein digest) were loaded on the cartridge at pH 2.5. All three phosphopeptides (P1, P2 and P3) were detected for the methylated sample, whereas only P2 and P3 were seen after fractionation of the non-methylated sample (Fig. 6C and F). This can be justified by the increase of hydrophobicity with increasing grade of peptide methylation. This change in hydrophobicity is supported by higher retention times of P2 and P3 in chromatogram C compared to F. In non-methylated sample, P1 (Ac-pY-EEIE) composed of a very short polar sequence was not retained on the C_{18}-phase of SPE cartridge as it was not detected by LC/MS, whereas in the methylated sample it showed a retention time of 8 min.

A direct comparison of the fractionation of methylated and unmethylated samples can be summarised as following: Unmethylated samples show unspecific bound peptides and are

of higher polarity than methylated ones, what leads to a loss of hydrophilic components. In contrast, methylated samples exhibit no unspecific bound peptides. However phosphopeptides identification could be confusing after derivatisation due to the partial methylation of peptides as a result of incomplete derivatisation reaction.

4. Conclusions

The SPE approach presented here is a powerful tool to isolate phosphopeptides from complex mixtures. Under optimised loading and elution conditions, Fe(III)–IDA–silica exhibited high specificity towards phosphopeptides. Porosity of the IMAC support had high influence on recovery rates. Supports with mean pore diameter of 1000 Å showed the best recoveries (≥90%), while their capacity still allowed the adsorption of 1.5 pmol/mg. The usage of silica as support material for IMAC features advantages over other frequently used materials. Beside its commercial availability in almost every size, shape and porosity, silica particles are characterised by high mechanical stability. This is important for the establishment of a two-dimensional μLC system for online fractionation and separation of mixtures containing phosphopeptides as well as unphosphorylated species, where the IMAC is packed into stainless steel columns or capillaries and used as a first dimension.

The presence of carboxylate groups gave unspecific bound peptides on Fe(III)–IDA–silica. Both their number and amounts were strongly influenced by the pH-value of the loading and washing buffer where the optimum pH was found to be 2.5. Methylated samples showed no unspecific bound peptides in the phosphorylated fraction, due to the blocking of the oxygen-ligand by ester formation. Nevertheless the derivatisation reaction used in this study gave peptide mixtures of different methylation grades, which complicated the analysis of the phosphorylated fraction.

The SPE protocol can be suited for detection of low abundant phosphopeptides in biological samples, by miniaturising the system (for example two-dimensional μLC coupled to MS). The basics for the establishment of the 2 dimensional system however have been worked out in this study.

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