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# Comparison of solid-phase extraction methods for efficient purification of phosphopeptides with low sample amounts

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ABSTRACT

Efficient phosphoproteomic analysis of small amounts of biological samples (e.g. tissue biopsies) requires carefully selected enrichment and purification steps prior to the nanoflow HPLC-MS/MS analysis. Solid-phase extraction (SPE) is one of the most commonly used approaches for sample preparation. Several stationary phases are available for peptide SPE purification, however, most of the published methods are not optimized to provide good recoveries of phosphorylated peptides. Our goal was to investigate the performance of 13 self-packed and 3 commercial centrifugal SPE cartridges/spin tips, thus enhancing the efficiency of the phosphoproteomic analysis of small amounts of complex protein mixtures. Eight reversed-phase (RP), five graphite, two ion-exchange, and one hydrophilic-lipophilic balance (HLB) stationary phase were evaluated. Two RP, one graphite, and the HLB self-packed centrifugal SPE tips provided excellent results for the purification of 1 µg tissue and cell line digests. Using these methods, the sample loss was significantly reduced compared to one of the commercial SPE methods, 22-58% more unique phosphopeptides were identified, and the recovery was higher by 132-155%.



## 1. Introduction

Reversible phosphorylation is one of the most common post-translational modifications (PTMs) of proteins, which plays a key role in many biological processes [1,2]. The most widespread technique for high-throughput analysis of complex biological samples is shotgun proteomics based on nanoflow HPLC-MS investigations and bioinformatics [3,4]. During this process, proteins are enzymatically cleaved into peptides (digestion) facilitating better separation and identification of the target compounds. This approach requires several sample preparation steps such as enrichment and purification of the phosphopeptide mixtures for reproducible and efficient analytical measurements [5,6]. Sample clean-up is a vital step in proteomics since the interfering contaminants (e.g. salts, detergents, buffers, and remaining enzymes) can highly influence the ionization efficiency and sensitivity of peptides and phosphopeptides (PPs). In particular, commonly used reagents during PP enrichment (e.g. hydroxy acids and glycerin) tend to stick to the metal parts of the instrument, resulting in clogging, peak tailing, and reduced stability of the spray. Thus, the purification after PP enrichment is inevitable with the additional benefit of prolonging the lifetime of the columns and HPLC-MS equipment.

The most common method for purifying protein digests is solid phase-extraction (SPE) with reversed-phase (RP) loading [7–10]. The primarily used stationary phase in the field of peptide cleaning is silica-based sorbents functionalized by $C_{18}$ chains. Hydrophilic-lipophilic balance (HLB) polymeric sorbent is also favorable in proteomic sample preparation due to its ability to retain a wide spectrum of polar and nonpolar compounds [11,12]. There are many comparative studies in the literature about different RP SPE methods for the analysis of various biological samples, like salivary proteome, porcine retinal protein markers, or human plasma [13–17]. Most of these studies focus on different aspects of performance like the number of identified proteins, reproducibility, binding capacity, desalting efficiency, or analysis time. Several parameters may be optimized to increase the efficiency of RP SPE approaches for the purification of the hydrophilic PPs. For example, cooling the spin tips extends the identification coverage of PPs and enhances the precision of the quantitative analysis [18].

Graphite-based stationary phases are commonly used in the chromatographic separation of polar components due to their excellent recovery and chromatographic efficiency [19]. Their proteomic application is currently on the rise, being mainly used in the investigation of polar post-translational modifications (e.g.

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glycosylation) and small hydrophilic peptides in both chromatographic and SPE setups [20–23]. Graphite-based SPE methods may enhance the detection of PPs and provide complementary selectivity since a significant number of PPs are not retained well on conventional RP sorbents [24].

Electrostatic repulsion hydrophilic interaction liquid chromatography (ERLIC), strong cation exchange (SCX), hydrophilic interaction liquid chromatography (HILIC), and high-pH RP methods are also applicable for phosphoproteomic sample preparation. ERLIC and SCX chromatography are feasible mainly for the isolation of the non-, mono-, and multi-phosphorylated peptides, while HILIC and high-pH RP chromatography are suitable for additional separation to RP chromatography during the HPLC-MS analysis [5,25-28].

Based on our previous experience, sample loss of 50-60% may occur during the purification of phosphoproteomic samples in the case of commonly used $C_{18}$ SPE methods. Despite a large number of stationary phases available on the market, detailed screening of phosphoproteomic-centered methods has still been lacking. In this study, we investigated the purification performance of 13 self-packed and 3 commercial centrifugal SPE cartridges/spin tips and outlined optimized methods for phosphoproteomic analysis of small amounts of complex protein mixtures.

## 2. Materials and methods

### 2.1. Reagents

Acetonitrile (ACN), LC-MS grade water ($H_2O$), methanol (MeOH), and LC-MS grade formic acid (FA) were purchased from VWR International (Debrecen, Hungary). Citric acid (CA), trifluoroacetic acid (TFA), and heptafluorobutyric acid (HFBA) were purchased from Sigma-Aldrich (Budapest, Hungary).

### 2.2. Samples

A mixture of 1 µg of rat smooth muscle digest enriched for PPs and 250 fmol Enolase MassPrep Phosphopeptide mix (Waters Hungary, Budapest, Hungary) was used for testing the purification performance of the 16 different SPE cartridges/spin tips. Male Wistar rats (170–250 g, Charles River Laboratories-Semmelweis University, Budapest) were kept on a standard semisynthetic diet. Our research conforms to the Guide for the Care and Use of Laboratory Animals (NIH, 8th edition, 2011) as well as national legal and institutional guidelines for animal care. They were approved by the Animal Care Committee of the Semmelweis University, Budapest and by Hungarian authorities (No. 001/2139-4/2012).

The second set of experiments was performed on SPE cartridges/spin tips considered to be the most effective for PP purification. 1 µg Pierce HeLa tryptic digest (Unicam Plc., Budapest, Hungary) enriched for PPs mixed with 250 fmol Enolase MassPrep Phosphopeptide mix was used for these experiments.

### 2.3. Tryptic digestion of rat smooth muscle cells

Rat smooth muscle cells were isolated as previously described [29], and lysed using the cOmplete Protease Inhibitor (Roche Applied Science, Basel, Switzerland), the cells were incubated at 60°C for 30 min, sonicated for 45 sec, and then centrifuged at 4°C for 10 min with 18000 g. The pellet was removed, and the buffer of the supernatant was exchanged to 50 mM ammonium bicarbonate. Then the proteins were unfolded by 0.5% Rapigest and reduced with 200 mM dithiothreitol in 5% MeOH + 50 mM ammonium bicarbonate solution, incubated at 60°C for 30 minutes. Then proteins were alkylated with 200 mM iodoacetamide in 200 mM ammonium hydrogen carbonate solution and incubated for 30 minutes at room temperature in dark. Then proteins were digested with LysC-Trypsin mixture for 1 hour (1:100 protein:enzyme ratio, 37°C), and with trypsin for 2 hours (1:25 protein:enzyme ratio, 37°C). The digestion was stopped with FA and the solvents were evaporated. Cleaning of the peptide mixture was performed using Isolute $C_{18}$ (EC) SPE 100 mg/1 mL columns (Biotage, Uppsala, Sweden) as follows. The column was activated with 1.5 mL 100% ACN, with 1.5 mL 50 mM citric acid in ACN/$H_2O$, 50:50 (v/v) and with 1.5 mL 0.1% TFA in ACN/$H_2O$, 50:50 (v/v), then equilibrated with 1.5 mL 0.5% TFA in ACN/$H_2O$, 5:95 (v/v), and with 1.5 mL loading solvent (0.1% TFA in MeOH/$H_2O$, 5:95 (v/v)). The samples were loaded onto the column in 60 µL loading solvent and washed with 1.5 mL of loading solvent. Elution was performed with 1.5 mL 0.1% TFA in ACN/$H_2O$, 70:30 (v/v). Then the samples were lyophilized and stored at -20°C until usage.

### 2.4. Phosphopeptide enrichment

Pierce™ $TiO_2$ Spin Tips (Unicam Plc., Budapest, Hungary) were used for the enrichment of PPs of both rat smooth muscle digest and HeLa digest as previously described [30]. Briefly, the column was activated with 2 × 50 µL wash buffer (0.1% TFA in ACN/$H_2O$, 40:60 (v/v)) and conditioned with 2 × 50 µL loading buffer (50 mM citric acid, 1.5% TFA in ACN/$H_2O$, 80:20 (v/v)). The sample was loaded and re-loaded in 150 µL loading buffer and washed with 2 × 50 µL loading buffer and with 2 × 50 µL wash buffer. The PPs were eluted with 1 × 50 µL $NH_3$ (25 *m/m%* in $H_2O$)/ACN/$H_2O$, 16:80:4 (v/v)) and with 2 × 50 µL 4 *m/m%* $NH_3$ (in $H_2O$). After every step, tips were centrifuged at 2000 g for 2 minutes, except for sample loading (1000 g for 10 minutes) and elution (1000 g for 5 minutes). The enriched samples were lyophilized and stored at –20°C until further use.

### 2.5. Preparation of the self-packed centrifugal SPE tips

Stationary phases of analytical columns and SPE cartridges (indicated in Table 1 with SP sign) were used for the preparation of the self-packed centrifugal SPE tips. 2 × 100 µL 50 mg/mL methanol suspension (10 mg resin in total) was pipetted into the empty Glygen fritless SPE pipette tip (SunChrom GmbH, Friedrichsdorf Germany) and then centrifuged at 5000 g for 2 minutes.

### 2.6. SPE sample purification

The SPE purifications were performed with 3 commercial and 13 self-packed centrifugal SPE tips. Altogether, 16 different stationary phases were investigated (Table 1), eight reversed phase (RP), five graphite (G), one strong cation exchanger (SCX), one weak anion exchanger (WAX), and one hydrophilic-lipophilic balance copolymer (HLB). Detailed protocols for each purification method are shown in Table S1. After elution, solvents were evaporated using a heated vacuum centrifuge and stored at –20°C until analysis. The resulting samples were reconstituted in 8 µL injection solvent (0.1% FA in ACN/$H_2O$, 2:98 (v/v)), of which 6 µL was injected.

1 µg rat smooth muscle digest and 1 µg HeLa digest enriched for PPs were used for testing the purification performance of each method. Both samples contained an additional 250 fmol Enolase MassPrep Phosphopeptide mix. Four parallel experiments were performed for the rat sample, and six for the HeLa sample. No unique control samples were prepared for each method, as it would have doubled the experimental work and instrument time. Rather we chose to use a universal control; 1 µg phosphopeptide enriched but unpurified mixture of rat/HeLa digest and 250 fmol Enolase MassPrep Phosphopeptide mix were used. This provided information about the hydrophobic and acidic nature of the sample and gave an estimation on the amount of phosphopeptides lost during purification.

**Table 1**
The applied stationary phases and their attributes. PGC: Porous Graphitic Carbon; SP: self-packed; C: commercial.

| ID | SORBENT | PARTICLE SIZE (µm) | MANUFACTURER/TYPE | SELF-PACKED/ COMMERCIAL | AMOUNT OF SORBENT USED (mg) |
|---|---|---|---|---|---|
| RP-1 | $C_{18}$ | 5 | Kromasil-100-5-$C_{18}$ | SP | 10 |
| RP-2 | RP | 5 | Phenomenex Ultracarb ODS(30) | SP | 10 |
| RP-3 | $C_{18}$ | 5 | Sigma-Aldrich Discovery HS $C_{18}$ | SP | 10 |
| RP-4 | $C_{18}$ | 5 | Waters XSelect HSS $C_{18}$ SB | SP | 10 |
| RP-5 | $C_8$ | 5 | Waters Sunfire $C_8$ | SP | 10 |
| RP-6 | RP | 5 | Phenomenex Ultracarb ODS(20) | SP | 10 |
| RP-7 | RP | 5 | Thermo Hypersil Gold | SP | 10 |
| RP-8 | $C_{18}$ | N.A. | Thermo Pierce $C_{18}$ | C | 9 |
| G-1 | PGC | 5 | Thermo Hypercarb | SP | 10 |
| G-2 | PGC | 3 | Thermo Hypercarb | SP | 10 |
| G-3 | Graphite | 37-125 | Supelco Envi-Carb | SP | 10 |
| G-4 | Graphite+$C_{18}$ | N.A. | Glygen TopTip | C | 10 |
| G-5 | Graphite | N.A. | Thermo Pierce Graphite | C | 10 |
| HLB | HLB | 30 | Waters Oasis HLB | SP | 10 |
| SCX | SCX | 5 | Phenomenex Luna SCX | SP | 10 |
| WAX | WAX | 60 | Waters Oasis WAX | SP | 10 |

### 2.7. Mass spectrometry and chromatography analysis

For nanoLC-MS/MS analysis, a Dionex Ultimate 3000 RSLC nanoLC (Dionex, Sunnyvale, CA, USA) coupled to a Bruker Maxis II Q-TOF (Bruker Daltonik GmbH, Bremen, Germany) via CaptiveSpray nanoBooster ionization source was used. Trapping was performed on an Acclaim PepMap100 $C_{18}$ trap column (5 µm, 100 µm × 20 mm, Thermo Fisher Scientific, Waltham, MA, USA) with 0.01% HFBA and 0.1% TFA ($H_2O$) transport liquid. Then peptides were separated on a Waters Acquity M-Class BEH130 $C_{18}$ analytical column at 48°C (1.7 µm, 75 µm × 250 mm) using gradient elution: isocratic hold at 4% Solvent B for 11 minutes, then elevating Solvent B to 20% in 75 minutes, and to 40% in 15 minutes. Solvent A was 0.1% FA in $H_2O$, Solvent B was 0.1% FA in ACN, and the flow rate was 300 nL $min^{-1}$.

For MS analysis, data-dependent acquisition measurements were performed. Spectra were collected with 2.5 sec cycle time and with a dynamic MS/MS exclusion of the same precursor for 2 min, or if its intensity was at least 3 times larger than before. Preferred charge states were set between +2 and +5. MS spectra were acquired at 3 Hz in the 150-2200 *m/z* range, collision-induced dissociation was performed on multiply charged precursors at 16 Hz (intensity > 40000) and 4 Hz (intensity < 40000) for abundant and low-abundance ones, respectively. Collision energies used were optimized previously to maximize peptide identification [31]. Internal calibration was performed by infusing sodium formate and data were automatically recalibrated using the Compass Data Analysis (v4.3; Bruker Daltonik GmbH, Bremen, Germany) software.

### 2.8. Data analysis

Byonic (v3.6.0, Protein Metrics Inc, San Carlos, CA, USA) was used for the database search as follows. Uniprot rat database (containing 29942 sequences, downloaded on 10/2020) was used for the rat smooth muscle sample. Uniprot human database (containing 75069 sequences, downloaded on 10/2020) was used for HeLa cell line sample. For the rat sample, a focused database was prepared with loose criteria (2% false discovery rate (FDR), other parameters same as the strict search), then the searches were performed against this focused database (containing 175 sequences) to maximize PTM identification performance. The parameters for the strict search and for the HeLa cell line sample were the following: precursor mass tolerance of 15 ppm, fragment mass tolerance of 20 ppm, cleavage at lysine and arginine C terminal, maximum 2 missed cleavages, and 1% FDR limit. The set PTMs were the following: Carbamidomethyl/+57.021464 @ C | fixed; Oxidation/+15.994915 @ M | common2; Gln->pyro-Glu/-17.026549 @ NTerm Q | rare1; Glu->pyro-Glu/-18.010565 @ NTerm E | rare1; Ammonia-loss/-17.026549 @ NTerm C | rare1; Acetyl/+42.010565 @ Protein NTerm | rare1; Phospho/+79.966331 @ S, T, Y | common3; Deamidated/+0.984016 @ N, Q | rare1; Methyl/+14.015650 @ NTerm, H, K, N, R | rare1. The common modifications were maximized in 3 instances, and the rare modifications were limited to 2 in the case of the rat sample, and it was 1 in the case of the HeLa sample. From the hits, only peptides with less than a 5% probability of false identification (AbsLogProb ≥ 1.3) were considered reliable hits.

Compass Data Analysis v4.3 was used for the integration of extracted ion chromatogram (EIC) peak areas (AUC). Recovery was calculated using the four synthetically phosphorylated Enolase peptides by dividing the given AUC with AUC values measured in the respective control samples. The isoelectric points were calculated using the IPC – Isoelectric Point Calculator by Kozlowsky [32], and GRAVY (Grand Average of Hydropathy) scores [33] were calculated by an in-house developed function.

### 2.9. Data visualization and availability

Data visualization was done using Microsoft Excel and VIB-BEG Venn-diagram maker [34]. The graphical abstract was created with BioRender.com. The mass spectrometry proteomics data have been deposited to the MassIVE data repository with the dataset identifier MSV000090215.

## 3. Results and discussion

We compared 16 different stationary phases to investigate the efficiency of the purification of complex phosphopeptide mixtures. The purification performance was primarily characterized based on the number of identified PPs and the recovery. A detailed comparison of the selectivity of the methods based on the hydrophobicity and isoelectric point distributions of the identified PPs was performed. The best-performing SPE methods were further investigated by the purification of phospho-enriched HeLa cell line digest.

During the experimental planning, our aim was to use the same protocols for the SPE methods with the same types of sorbents. Furthermore, in most of the cases, the manufacturer protocols of commercial SPE cartridges were used. For RP 1-8 SPE methods, an improved version of the manufacturer protocol of the commercial RP-8 SPE method was applied [35]. For the graphite-based SPE methods (G-1, G-2, G-3, and G-5), the manufacturer protocol of the commercial G-5 SPE method was applied. For the graphite+$C_{18}$ based G-4 SPE method, its manufacturer protocol was applied. The protocols for HLB and WAX SPE methods were based on the man-

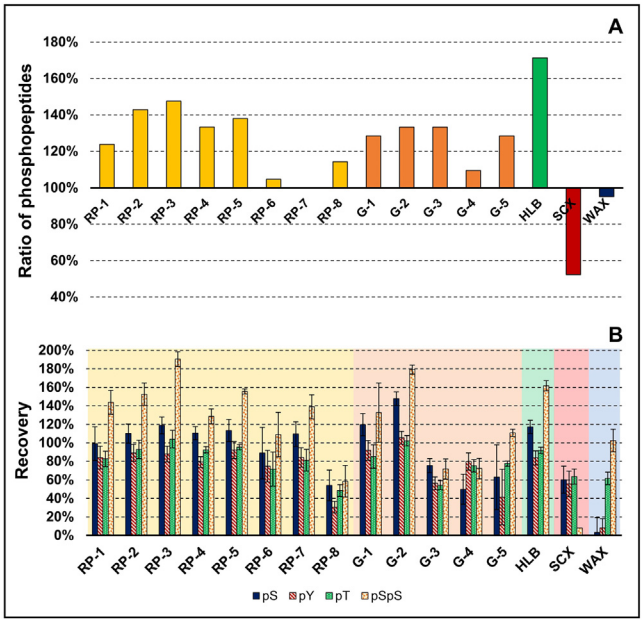


**Fig. 1.** Identification performance and recovery of the investigated SPE methods during the purification of rat digest sample. a) proportion of unique PPs identified in samples prepared by different SPE methods compared to the control sample; b) recovery of the synthetically phosphorylated enolase peptides carrying one pS, pY, pT, and pSpS motifs. For each method, the result of 4 parallel experiments were combined.

ufacturer's recommendations. For the SCX SPE method, one of the University of Washington Proteomics Resource's protocols (Peptide fractionation and Clean-Up Protocols) has been applied [36].

### *3.1. Initial screening of 16 SPE methods with rat smooth muscle sample*

For testing the purification performance of the 13 self-packed centrifugal SPE spin tips and 3 commercial SPE spin tips/cartridges, we used the mixture of 1 µg of rat smooth muscle digested and enriched for PPs and 250 fmol commercially available Enolase tryptic digest containing four synthetically phosphorylated peptides (serine, pS (HLADLpSK); threonine, pT (VNQIGpTLSESIK); tyrosine, pY (NVPLpYK); and double serine phosphorylated, pSpS (VNQIGTLpSEpSIK)).

#### *3.1.1. Identification performance*

The number of unique PPs (PPs identified in at least one out of the four parallel samples) relative to those identified in the unpurified control sample was within a wide range (from -52% to +171%) using the different SPE tips (Fig. 1A). Using the HLB, RP-3, and RP-2 SPE tips, 1.71, 1.48, and 1.43 times more unique PPs were identified compared to the control sample, respectively. The SCX SPE tips and the WAX SPE tips showed the worst performance, 48% and 5% fewer PPs were identified than in the unpurified control sample. One possible explanation is that during the SPE loading step, the phosphorylated peptides bearing a net negative charge could not bind to the negatively charged SCX stationary phase. On the other hand, positively charged PPs could not bind to the positively charged WAX stationary phase. A similar trend was seen for the average number of identified PPs as well, but the repeatability (standard deviation regarding the number of identified PPs) of the RP-2, RP-8, and G-3 methods was superior as compared to the others (Table S2). The ratio of identified PPs in a sample was between 36% and 64% in the case of almost every SPE method. We observed two extremities: the PP ratio was 11%, and 72% in the case of SCX and WAX SPE methods, respectively (Table S2).

#### *3.1.2. Recovery*

Recoveries of the synthetic PPs (HLADLpSK, NVPLpYK, VNQIGpTLSESIK, and VNQIGTLpSEpSIK) were calculated as described in Section 2.8 for each method. The G-2 method gave the best recoveries for all four PPs (102-179%). Some other methods, like RP-3, RP-5, RP-2, HLB, and G-1 also showed good performance; a recovery of at least 85% was measured for all the four components using these methods (Fig. 1B). Recovery over 100% is a common phenomenon when working with enriched or purified proteomics samples containing a relatively low number of proteins. This either indicates matrix effect or it is due to removing contaminants or other peptides from the samples causing lower ion suppression, thus a higher recovery. In general, the recovery was the highest for peptides containing pSpS and pS motifs (on average 120% and 98%, respectively), while for peptides containing pT and pY it was significantly lower (on average 80% and 72%, respectively). Besides the pS and pSpS motifs, HLADLpSK and VNQIGTLpSEpSIK peptides contain more apolar amino acids, which might play a key role in their binding to the RP stationary phase. The WAX spin tips performed poorly for pS- and pY-containing PPs (3% and 8%, respectively), but relatively well for pT- and pSpS-containing PPs (62% and 103%, respectively). The unexpectedly high recovery of the peptide carrying a pT motif might appear due to the structure of this peptide, the negatively charged glutamic acid might bind stronger to the stationary phase. The two negatively charged phosphate groups on the doubly phosphorylated peptide ensure strong retention on the positively charged stationary phase resulting in high recovery of the peptide. In contrast, the poor recovery of the doubly phosphorylated peptides (8%) using SCX spin tips reflects that the peptide could not be positively charged enough for retention due to the two negatively charged phosphate groups.

#### *3.1.3. Selectivity*

Different spin tips can show higher selectivity for certain peptides according to their hydrophilic/hydrophobic and acidic properties. The GRAVY score expresses the degree of hydrophobicity of peptides; the more positive the GRAVY score, the more hydrophobic the peptide. The distributions of the hydrophobicity of the unique PPs identified after purification with RP and graphite spin tips were highly similar to those of the unpurified control sample. The number of identified PPs with hydrophobic properties (GRAVY score $> 0$) decreased by 2-10%, and the number of identified PPs with highly hydrophilic properties (GRAVY score $< -2$) increased by up to 12% (Fig. 2A). Using the HLB spin tips, 32% of the identified PPs were highly hydrophilic (GRAVY score $< -2$), while only 4% of identified PPs had hydrophobic properties (GRAVY score $> 0$). This difference is attributed to the surface chemistry of the HLB being developed for stronger retention towards hydrophilic species [16]. Using the SCX spin tips, no PPs were identified with hydrophobic properties (GRAVY score $> 0$). However, the WAX spin tips showed stronger selectivity for highly hydrophobic PPs, 8% of the identified PPs had a GRAVY score over 1.

Using most of the investigated RP and graphite spin tips, the identified PPs had similar acidic distributions to those of the unpurified control sample (Fig. 2B). However, using RP-4, G-1, G-3, G-5, HLB, and SCX spin tips, 69-84% of the identified PPs were in the isoelectric point (pI) range 3–5 and 16–31% of them were in the pI range 5–7, while 62% and 31% of PPs identified in the control sample were in the pI range 3–5 and 5–7, respectively. In contrast, the WAX spin tips had stronger selectivity for PPs with basic properties, 25% of the PPs had a pI greater than 7, while 8% of PPs identified in the control sample had a pI greater than 7.

#### *3.1.4. Summary of initial screening*

Many of the investigated self-packed spin tips proved equally suitable for the purification of rat smooth muscle samples. RP-

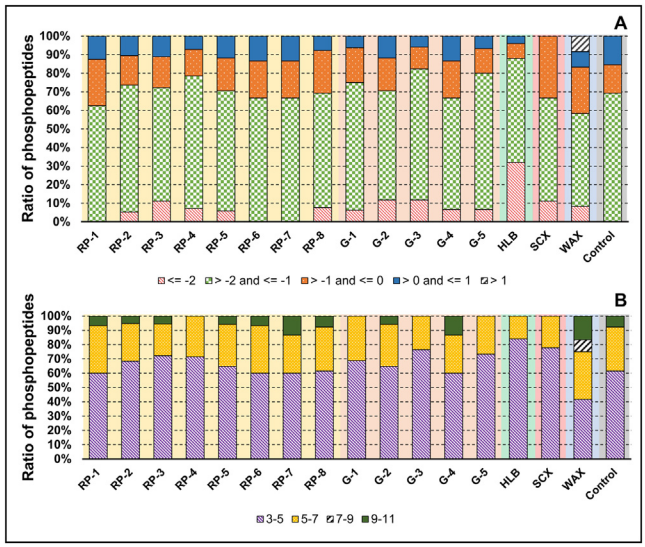


**Fig. 2.** Selectivity of the investigated SPE methods during initial screening. Relative distribution of unique PPs of a) GRAVY score range, b) pI range. For each method, the result of 4 parallel experiments were combined.

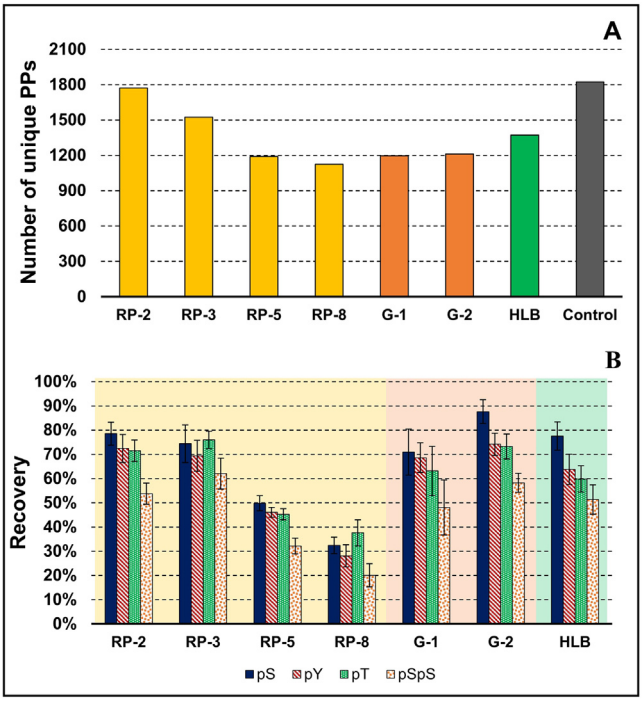


**Fig. 3.** Identification performance and recovery of the investigated SPE methods during the purification of HeLa cell line sample. a) number of unique PPs identified in samples prepared by different SPE methods; b) recovery of the synthetically phosphorylated enolase peptides carrying one pS, pY, pT, and pSpS motifs. For each method, the result of 6 parallel experiments were combined.

2, RP-3, RP-5, G-1, G-2, and HLB centrifugal SPE tips performed outstandingly regarding the identification and/or recovery. Most of these SPE tips were unbiased regarding the hydrophobicity and acidity of PPs, HLB SPE tips showed higher selectivity for hydrophilic peptides and/or peptides with higher acidic properties. The purification performance of these SPE tips was subjected to further investigation. Based on the identification performance and recovery, the tested SCX methodology is not applicable for the purification of PPs. Although WAX spin tips performed well for doubly phosphorylated peptides compared to monophosphorylated peptides, RP and graphite setups proved to be more suitable for the purification of samples containing highly phosphorylated peptides.

### *3.2. Additional performance estimation of 7 selected SPE methods with HeLa cell lysate*

The selected self-packed centrifugal spin tips (RP-2, RP-3, RP-5, G-1, G-2, HLB SPE tips) were further investigated with an alternative sample type: HeLa cell line digest, previously enriched for PPs (Enolase MassPrep Phosphopeptide mix added). RP-8 SPE cartridge was also included for comparison with a commercial setup.

#### *3.2.1. Identification performance*

The number of unique PPs identified was the highest using RP-2, RP-3, and HLB spin tips, 1774, 1525, and 1373 PPs, respectively (Fig. 3A). The average number of identified PPs were the highest using the RP-2 (1052 ± 159), RP-3 (915 ± 88), and G-2 (803 ± 56) spin tips (Table S3). The fewest PPs were identified using the RP-8 SPE cartridge (706 ± 32 on average, and 1124 unique PPs), however, the standard deviation of the number of identified PPs was one of the lowest. The ratio of the identified PPs in a sample was 137-147% in the case of the spin tips and the control sample, and it was 114% using the commercial RP-8 SPE cartridges (Table S3). This slight decrease in the ratio of the PPs may be attributed to a loss of PPs with hydrophilic character during sample loading.

#### *3.2.2. Recovery*

The recovery of the selected spin tips for the four synthetically phosphorylated Enolase peptides was similar to those of the rat smooth muscle sample (Fig. 3B). G-2, RP-2, and RP-3 spin tips performed well, the recovery was 58-88% for G-2 spin tips, 54-79% for RP-2 spin tips, and 62-76% for RP-3 spin tips. Commercial RP-8 SPE cartridges and self-packed RP-5 spin tips showed the lowest recovery, 20-38%, and 32-50%, respectively. The overall recovery of the Enolase peptides showed a different distribution than in the experiments with the rat smooth muscle sample. This difference is mainly attributed to the different origins of the sample resulting in altered quantity and physicochemical properties of the peptides. The recovery was the highest for the pS-containing peptide, on average 67%. However, the pY- and pT-containing peptides had also relatively high recovery values, on average 60% and 61%, respectively. The pSpS-containing peptide had the lowest recovery, on average 47%. The retention of the doubly phosphorylated peptides was weaker than the retention of mono-phosphorylated peptides, thus during the sample loading step, more doubly phosphorylated peptides might be lost.

#### *3.2.3. Selectivity*

The selectivity of the investigated centrifugal spin tips and cartridges was unbiased in terms of the hydrophobicity and acidity of the identified PPs compared to the unpurified control sample (Fig. S1A and Fig. S1B). However, large differences in the identified individual peptides were observed. Altogether 2630 unique PPs were identified in the samples prepared with RP-2, RP-3, G-1, HLB spin tips, and in the control sample (Fig. 4). 710 unique PPs (27%) were identified in the case of all 4 spin tips, and the unpurified control sample. Nearly 30% of PPs were identified using only one method (116, 207, 80, 123 PPs using RP-3, RP-2, G-2, and HLB spin tips, respectively, and 268 PPs in the control sample), and nearly 50% of the PPs were identified in the case of at least 3 methods. It is in correlation with recently published data, 10-37% of the identified proteins are unique for different SPE methods during peptide clean-up [13,14]. The different selectivity of these self-packed centrifugal spin tips originates from the slight differences in the surface chemistry of the stationary phases.

**Table 2**
Summary of the performance of the investigated SPE methods.

| Rat smooth muscle sample | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| ID | | RP-2 | RP-3 | RP-5 | RP-8 | G-1 | G-2 | HLB |
| Identification | Number of unique PPs | 30 | 31 | 29 | 24 | 27 | 28 | 36 |
| | Ratio of PPs | 65% | 52% | 56% | 64% | 53% | 45% | 52% |
| Recovery (n = 4) | HLADLpSK | 110% ± 21% | 119% ± 18% | 113% ± 24% | 54% ± 33% | 120% ± 24% | 148% ± 15% | 117% ± 14% |
| | NVPLpYK | 89% ± 18% | 88% ± 17% | 92% ± 18% | 31% ± 13% | 92% ± 21% | 106% ± 14% | 84% ± 15% |
| | VNQIGpTLSESIK | 93% ± 20% | 104% ± 19% | 95% ± 6% | 49% ± 13% | 85% ± 25% | 102% ± 11% | 92% ± 7% |
| | VNQIGTLpSEpSIK | 153% ± 24% | 191% ± 16% | 156% ± 5% | 59% ± 34% | 133% ± 64% | 179% ± 10% | 162% ± 11% |
| Selectivity | Hydrophobicity | unbiased | unbiased | unbiased | unbiased | unbiased | unbiased | Higher selectivity for hydrophilic peptides |
| | Acidity | unbiased | unbiased | unbiased | unbiased | unbiased | unbiased | Higher selectivity for acidic peptides |

| HeLa cell line sample | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| ID | | RP-2 | RP-3 | RP-5 | RP-8 | G-1 | G-2 | HLB |
| Identification | Number of unique PPs | 1774 | 1524 | 1192 | 1124 | 1198 | 1213 | 1373 |
| | Ratio of PPs | 142% | 137% | 139% | 114% | 144% | 142% | 147% |
| Recovery (n = 6) | HLADLpSK | 79% ± 10% | 74% ± 16% | 50% ± 6% | 32% ± 7% | 71% ± 19% | 88% ± 10% | 78% ± 12% |
| | NVPLpYK | 72% ± 12% | 69% ± 13% | 46% ± 4% | 28% ± 9% | 69% ± 12% | 74% ± 9% | 64% ± 13% |
| | VNQIGpTLSESIK | 71% ± 9% | 76% ± 7% | 45% ± 4% | 38% ± 11% | 63% ± 20% | 73% ± 10% | 60% ± 11% |
| | VNQIGTLpSEpSIK | 54% ± 9% | 62% ± 13% | 32% ± 6% | 20% ± 10% | 48% ± 23% | 58% ± 8% | 51% ± 12% |
| Selectivity | Hydrophobicity | unbiased | unbiased | unbiased | unbiased | unbiased | unbiased | unbiased |
| | Acidity | unbiased | unbiased | unbiased | unbiased | unbiased | unbiased | unbiased |
| | Individual unique PPs | 207 | 116 | - | - | - | 80 | 123 |

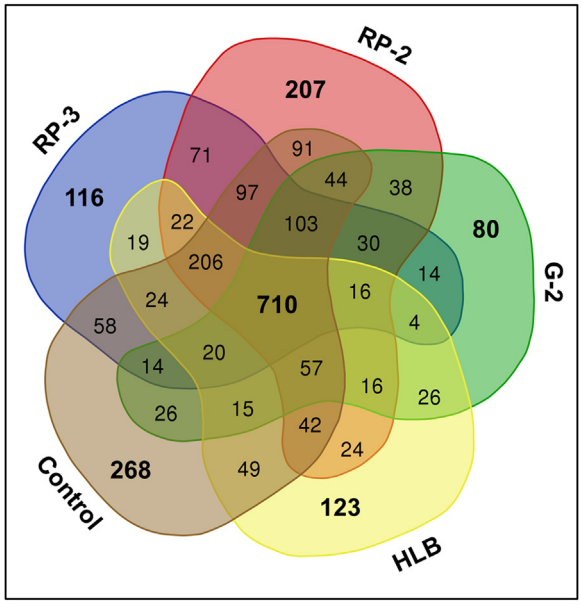


**Fig. 4.** Venn-diagram of the identified individual PPs during the purification of HeLa cell line sample. For each method, the result of 6 parallel experiments were combined.

### 3.3. Summary of the performance of SPE spin tips

The investigated self-packed centrifugal RP-2, RP-3, G-2, and HLB SPE spin tips were found to be excellent for the purification of small amounts of complex phosphopeptide mixtures (Table 2). The identification rate and recovery were the highest in the case of these methods; 1.1–1.6 times more unique PPs were identified and 33–43% higher recovery was achieved compared to the commercial SPE cartridges (e.g. RP-8). However, we observed small differences in the performance characteristics when working with different sample types. Analyzing the rat sample, the numbers of identified unique PPs were significantly higher than it was in the unpurified control sample, and the recoveries of the enolase PPs were extremely high. However, when analyzing the HeLa cell line sample, only the RP-2 SPE method reached the levels of the control sample regarding the identification performance and recovery. This difference is attributed to the different complexity of the samples. The phosphopeptide-enriched rat smooth muscle sample contained relatively few components, thus most of the interfering components were removed during purification, and a small number of co-eluting PPs and peptides were observed. On the other hand, the phosphopeptide-enriched HeLa cell line digest contained almost 2000 PPs and peptides resulting in a vast number of co-eluting components in the purified sample, thus influencing the ionization efficiency and identification.

Sample loss during a sample preparation step is inevitable in the case of highly complex samples, however, these losses can be minimized using appropriate methods. Excluding the purification step after PP enrichment seems reasonable; the highest number of unique PPs were identified in the unpurified control sample in the case of the HeLa cell line sample. However, residual reagents after PP enrichment (like hydroxy acids, glycerin, citric acid) cause poor chromatographic performance, clogging of the emitter, and ion suppression during the HPLC-MS measurements, therefore, purification is inevitable on the long run.

The results obtained with the selected SPE methods (presented in section 3.2.) were unbiased regarding the hydrophobicity and acidity of the PPs, but, a different selectivity for individual PPs was observed. Hence, splitting the sample, and purifying it with different SPE methods seems to be an option, when an extended profiling of PPs is the main goal. However, this requires a larger amount of sample and multiplies the analysis time.

The implementation of these SPE methods into a routine phosphoproteomic workflow is straightforward, and in our experience, it is necessary to perform purification both before and after phosphopeptide enrichment. The exact method should always be tested and partially optimized for the given sample type and matrix. The preparation of the presented self-packed SPE spin tips is fast, and the overall time required for the purification with these self-

packed SPE spin tips is similar to those of the commercial SPE cartridges.

## 4. Conclusion

In this study, we investigated the purification performance of 13 self-packed centrifugal SPE spin tips as well as 3 commercial SPE cartridges/spin tips to improve the analysis of PPs. We performed an initial screening using 1 µg rat smooth muscle sample, and additional experiments on the SPE methods considered suitable for PP purification using 1 µg HeLa cell line sample. RP-2, RP-3, G-1, and HLB self-packed centrifugal SPE spin tips were found to be excellent choices for the efficient purification of low amounts of PP-enriched biological samples. The sample loss during purification is minimized (3-33% in unique PPs and 30-37% in recovery). Furthermore, the methods are unbiased regarding the hydrophobic and acidic characteristics of the sample, however, their different selectivity towards individual PPs should not be excluded.

## Appendices

Appendix A

Table S1. Purification protocols for the investigated SPE spin tips/cartridges.

Table S2. Average number and ratio of identified PPs during the initial screening. For each method, 4 parallel experiments were performed.

Table S3. Average number and ratio of identified PPs during the purification of HeLa cell line sample. For each method, 6 parallel experiments were performed.

Appendix B

Figure S1. Selectivity of the investigated SPE methods during the purification of HeLa cell line sample. Relative distribution of unique PPs of a) GRAVY score range, b) pI range. For each method, the result of 6 parallel experiments were combined.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## CRediT authorship contribution statement

**Fanni Bugyi:** Conceptualization, Methodology, Investigation, Data curation, Visualization, Writing – original draft. **Gábor Tóth:** Conceptualization, Methodology, Investigation, Data curation, Visualization, Writing – original draft. **Kinga Bernadett Kovács:** Resources, Writing – original draft. **László Drahos:** Writing – original draft, Funding acquisition, Project administration, Supervision. **Lilla Turiák:** Writing – original draft, Funding acquisition, Project administration, Supervision.

## Data availability

Data will be made available on request.

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## Supplementary materials

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