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TUTORIAL

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Optimization of reversed-phase solid-phase extraction for shotgun proteomics analysis

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Abstract

Reversed-phase solid-phase extraction (SPE) is the method of choice for the purification of proteomics samples. Even though the efficacy of SPE methods is sample type-dependent, the manufacturers' protocols are used in most studies. Using an optimized SPE method can lead to a substantial gain in identification and recovery. In this tutorial, we give a brief introduction to the most important parameters influencing SPE performance, and we present a short workflow (16 measurements) for optimizing the SPE procedure. This is complemented by method performance assessment instructions and a short troubleshooting guide to help users further understand and investigate their SPE methods.

KEYWORDS
C18, desalting, HPLC, mass spectrometry, peptide, proteomics, purification, reversed phase, solid-phase extraction

1 | INTRODUCTION

The "bottom-up" approach is a widely used method for proteomic analysis. After protein digestion, several inorganic ions, surfactants, and other unfavorable components are present in the sample that are detrimental to and interfere with the MS analysis and affect the proteome data. Thus, sample preparation removing these contaminants has become essential. Different methods are available for proteomic sample preparation, such as Filter Aided Sample Preparation (FASP) or solid-phase extraction (SPE). FASP facilitates the use of a semi-permeable membrane for combining the digestion and clean-up steps.[1,2] This method gained large popularity during the past decade; however, most workflows use separate digestion and clean-up steps. SPE is the most commonly applied method for peptide purification; even so, it is overlooked in most studies. The most widespread version of SPE is low-pH reversed-phase purification. Several standard manufacturer protocols are available and most are used without further optimization. Detailed optimization can lead to a 20%–30% gain in identification and 30%–50% in the recovery of peptides; therefore, it is crucial to use the best method possible.[3] However, there is no ultimate method for all the sample types (e.g. cell cultures, plasma, tissue, membrane protein extracts, vesicles, glycopeptide enriched samples, histidine enriched materials), because large differences in matrix components and distribution of physicochemical properties of peptides influence the results. Therefore, careful optimization should be performed before analyzing a large sample cohort, considering its properties.

The workflow of SPE can be divided into five main steps (Figure 1). First, the stationary phase is *activated* (usually with medium elution strength solvent) to make the functional groups of the

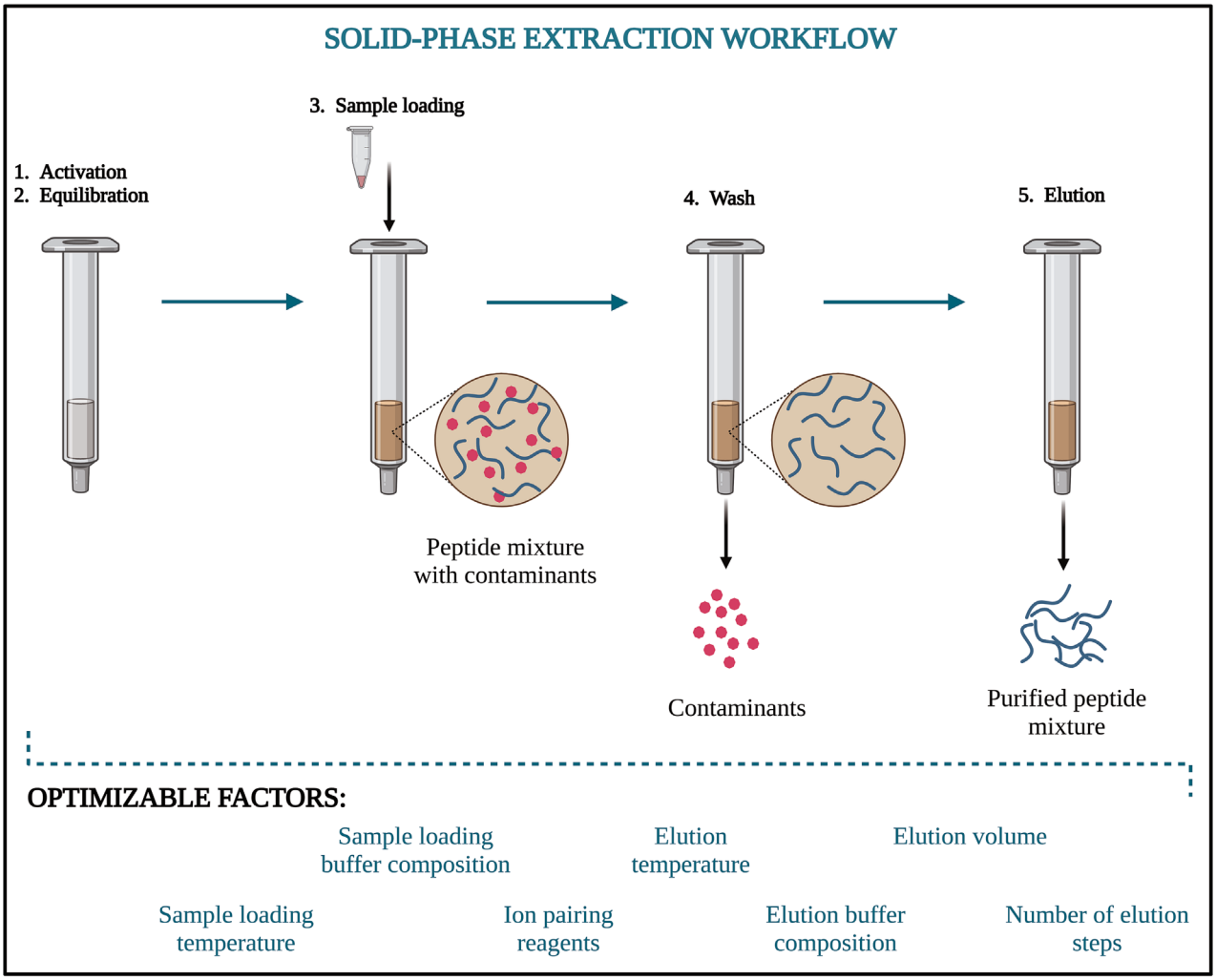

FIGURE 1 A short workflow of the solid-phase extraction steps.

stationary phase well-accessible for the analytes. Then *equilibration* is necessary with the sample loading buffer. Next, the sample is *loaded* onto the column (see Note 1), which retains the target compounds, while most of the contaminants flow through the cartridge. During the *wash* step, the molecules that are non-specifically bound or are in the void volume of the stationary phase are washed away. Last, the *elution* of the bound target compounds to a clean microcentrifuge tube (see Note 2) is performed. A detailed description of the peptide SPE procedure is shown in Figure 2. Solvent strength is determined by the partition coefficient of the given analyte group between the stationary phase and the given solvent. Using a "weak solvent," the analytes have a preference for binding to the stationary phase, whereas using a "strong solvent," analytes spend more time dissolved in the moving phase. A detailed description of solvent strength is out of the scope of this paper; however, it is important to know that the sample loading is performed using weak solvents, and elution from the stationary phase is achieved using strong solvents.

Several stationary phases and formats are available to perform SPE purification; the latter include vacuum and spin cartridges, pipette tips and spin tips, disks, multi-well SPE plates, and so forth. Spin tips and cartridges are designed specifically for micro-scale sample preparation, and they are primarily used in proteomics studies. When the stationary phase is selected, one should always consider the origin and the amount of the samples to be purified. A few examples of the application of reversed-phase SPE through various proteomic studies have been collected in Table 1.[3–22] In these studies, different types of samples (e.g. various tissue specimens, cell lines, different biofluids, isolated exosomes, enriched samples for specific post-translational modifications [PTMs]) from humans, as well as other mammalian origins have been analyzed. Typically, 0.1% trifluoroacetic acid (TFA) aqueous solution was used for sample loading. For elution, however, a wide variety of solvents were used, ranging from 40% to 95% acetonitrile or methanol with or without additives, typically with 0.1% TFA. In most of these studies, the manufacturer protocols were applied without any optimization regardless of the sample type or the chemical/biological modifications of the peptides.

The target audience of this tutorial is young investigators and students with moderate experience in SPE and laboratories working with samples of diverse origins. First, we briefly list the most important parameters that are useful to optimize and briefly explain their effects.

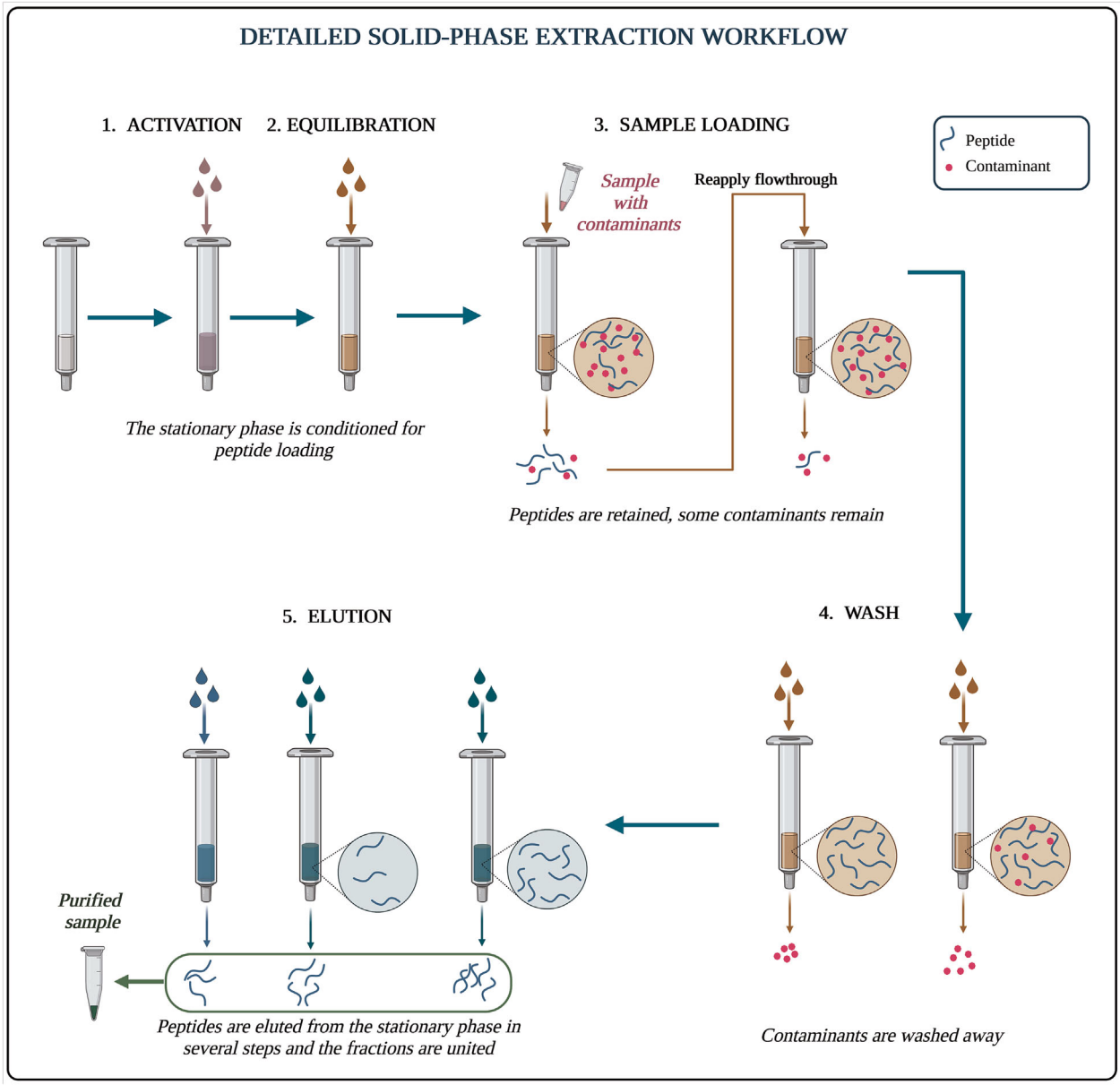

FIGURE 2 Detailed workflow of peptide solid-phase extraction (SPE) purification.

Subsequently, we describe a fast optimization workflow for those four parameters, which have the largest impact on the identification and recovery of peptides. This optimization process is fairly simple and requires only a few hours of laboratory work. For more detailed information, we suggest excellent papers listed among the references.[18,23–29]

2 | MOST IMPORTANT PARAMETERS INFLUENCING SPE PERFORMANCE

During SPE purification, there are several parameters determining the SPE performance. The resin types, as well as the composition of loading and elution solvents fundamentally define the retention characteristics. Whereas, other parameters like resin-to-sample ratio, temperature during sample loading and elution, or the number of elution steps are used to yield the maximum recovery of peptides.

The most important parameters influencing the peptide recovery and identification performance are summarized in Table 2 along with their suggested optimization range and expected effects.

2.1 | Type of resin

The surface chemistry (e.g. C_8, C_{18}, HLB), the exact method of functionalization and end-capping, and the surface coverage all strongly influence the retention characteristics of SPE resins. Therefore, even stationary phases utilizing the same surface chemistry have slightly

TABLE 1 Examples of the application of reversed-phase SPE through various proteomic studies.

Reference	[3]		[4]	[5]	[6]	[7]
Stationary phase	Self-packed C_{18} spin tips, Thermo Pierce C_{18} Columns	Self-packed Oasis HLB spin tips	Thermo Pierce C_{18} Column	C_{18} MicroSpin Columns (The Nest Group)	Thermo Pierce C_{18} Column	Thermo Pierce C_{18} tips
Sample type	Phosphopeptides of rat smooth muscle digest and HeLa tryptic digest		Prostate tissue	Lung tissue samples	A heavily glycosylated fraction of depleted and pooled human plasma	Extracellular vesicles isolated from invasive breast cancer cell lines and plasma samples
Quantity loaded	1 μg protein		200 μg protein	50 μg protein	1 μg protein	N.A.
Manufacturer advised loading	N.A.		0.5 ng to 30 μg	6–60 μg	0.5 ng to 30 μg	0.5 ng to 8 or 80 μg
Loading solvent	0.1% HFBA in H_2O	2% H_3PO_4 in 98% H_2O	N.A. (MP states 0.5% TFA in 5% ACN)	0.1% TFA in H_2O	Reference method: 0.1% TFA in H_2O; Optimized method: 0.1% HFBA in H_2O	N.A. (MP states 0.1%–1.0% TFA in H_2O)
Elution solvent	0.1% TFA in 30:70 v/v% H_2O:ACN, and 0.1% FA in 30:70 v/v% H_2O:ACN	100 v/v% MeOH	N. A. (MP states 30:70 v/v% H_2O:ACN)	0.1% TFA in 20:80 v/v% H_2O:ACN	Reference method: 0.1% TFA in 30:70 v/v% H_2O:ACN; Optimized method: 0.1% TFA in 30:70 v/v% H_2O:ACN, and 0.1% FA in 30:70 v/v% H_2O:ACN	N.A. (MP states 0.1% FA in 50:50 - 25:75 v/v% H_2O:ACN)
Is the method optimized or recovery analyzed?	In-house optimization was carried out, and recovery was analyzed.		NO	NO	In-house optimized method for hydrophilic species, recovery analyzed.	NO
PTM specificity	Phosphorylation		NO	NO	Glycosylation	NO
Additional modification			Tryptic peptides were labeled with 10-plex TMT reagents.			

Reference	[8]	[9]				[10]
Stationary phase	ZipTip C_{18} pipette tips	ZipTip μC_{18} pipette tips	ZipTip C_{18} pipette tips	Glygen TopTip C_{18} pipet tips	Oasis HLB μElution plate	C_{18} Sep-Pak (waters)
Sample type	Bull semen from young and adult bulls	Human healthy saliva				Peptide LKPNM in bonito muscle hydrolysate
Quantity loaded	N.A.	0.75, 1, 2, 4, and 5 μg peptide	1.8, 2.5, 5, 7.5, and 12.5 μg peptide	28, 37.5, 75, 150, and 187.5 μg peptide	15, 20, 40, 80, and 100 μg peptide	125 mg bonito hydrolysate + 0.05–0.3 mg LKPNM
Manufacturer advised loading	1–5 μg	2 μg	5 μg	75 μg	30–50 μg	N.A. (MP states 1%–10% of the total SPE device bed)
Loading solvent	N.A. (MP states 0.1% TFA in H_2O)	1% AA solution				Milli-Q deionized water
Elution solvent	50:50 v/v% H_2O:ACN, and 20:80 v/v% H_2O:ACN					40:60 v/v% MeOH:H_2O

TABLE 1 (Continued)

Reference	[8]	[9]				[10]
Stationary phase	**ZipTip C_{18} pipette tips**	**ZipTip μC_{18} pipette tips**	**ZipTip C_{18} pipette tips**	**Glygen TopTip C_{18} pipet tips**	**Oasis HLB μElution plate**	**C_{18} Sep-Pak (waters)**
	N.A. (MP states 0.1% FA in 50:50 v/v% H_2O:ACN)					
Is the method optimized or recovery analyzed?	NO	Comparison was performed, different amounts of loading material were tested. Recovery analyzed.				NO
PTM specificity	NO	NO				NO
Additional modification						

Reference	[11]	[12]	[13]			[14]
Stationary phase	**C_{18} StageTips**	**In-house made C_{18} StageTips**	**ZipTip C_{18}, microbed ZipTipU-C_{18}, ZipTip C_4 pipette tips**	**OMIX C_{18} and microbed OMIX C_{18} pipette tips (Agilent Technologies)**	**C_{18} tips (Eppendorf PerfectPure)**	**Oasis HLB μElution plate (2 mg HLB sorbent), Oasis HLB 5 mg, Oasis HLB 10 mg, Oasis HLB 30 mg, Sep-Pak C_{18} 50 mg, Sep-Pak tC_{18} 50 mg, and Sep-Pak C_{18} 100 mg**
Sample type	Lysine-acetylated peptides	Histone digest of HEK293 cells	Unlabeled BSA and ^{14}C-methylated BSA samples			Pooled human plasma
Quantity loaded	5 mg protein before enrichment	N.A.	4 μg samples			150 μg protein
Manufacturer advised loading	2–4 μg	N.A.	1–5 μg	75 μg	N.A.	N.A. (MP states 1%–10% of the total SPE device bed)
Loading solvent	0.15% TFA in H_2O	N.A. (MP states 0.1% TFA in 2% ACN)	N.A. (MP states 0.1% TFA in H_2O)	N.A. (MP states 0.5%-1.0% TFA in H_2O)	N.A.	H_2O
Elution solvent	0.2% FA in 49.8:50 v/v % H_2O:ACN	N.A. (MP states 0.1% TFA in 40:60 v/v% H_2O:ACN)	N.A. (MP states 0.1% FA in 50:50 v/v% H_2O:ACN)	N.A. (MP states 0.1% FA or 0.1% AA in 50:50–25:75 v/v% H_2O:ACN or H_2O:MeOH)	N.A.	20:80 v/v% H_2O:ACN
Is the method optimized or recovery analyzed?	NO	NO	Binding, recovery, and desalting efficiency were analyzed.			Three sorbents with different sorbent sizes were compared, and recovery was analyzed.
PTM specificity	Acetylation	NO				NO
Additional modification		Histones and histone variants were derivatized by treatment with propionyl anhydride reagent.				

(Continues)

TABLE 1 (Continued)

Reference	[15]	[16]	[17]	[18]	[19]	
Stationary phase	**Glygen TopTip C_{18} pipet tips**	**Primed oligo R3 reversed-phase SPE microcolumn**	**In-house-made RP microcolumns packed with oligo R3 RP resin**	**Empore™ SDB-XC extraction disks**	**SOLAμ™ HRP SPE spin plates**	**ZipTip C_{18} pipette tips**
Sample type	Exosomes of breast cell line	Enriched glycopeptides from fresh prostate tissue	Lysine-acetylated peptides of triple-negative breast cancer cells	Phosphopeptides from HeLa cell line	Porcine retinal tissues	
Quantity loaded	N.A.	250 μg peptides before enrichment	100 μg of peptides before enrichment	100 μg of peptides before enrichment	50 μg protein before SDS Page and digestion	
Manufacturer advised loading	75 μg	N.A.	N.A.	N.A.	N.A.	1–5 μg
Loading solvent	N.A. (MP states 0.1% FA or 0.05% TFA in H_2O)	N.A.	0.1% TFA in H_2O	0.1% TFA in 0%–4% ACN	0.1% TFA in H_2O	
Elution solvent	N.A. (MP states 0.1% FA or 0.05% TFA in 40:60 v/v% H_2O:ACN)	N.A.	0.1% TFA in 40:60 v/v% H_2O:ACN	0.1% TFA in 20:80 v/v% H_2O:ACN	100 v/v% MeOH	0.1% TFA in 50:50 v/v% H_2O:ACN
Is the method optimized or recovery analyzed?	NO	NO	N.A.	In-house optimization was carried out, recovery analyzed.	NO	
PTM specificity	NO	Glycosylation	Acetylation	Phosphorylation	NO	
Additional modification		Tryptic peptides were labeled with 10-plex TMT reagents.	Peptides were labeled with the TMT 11 plex kit.			

Reference	[20]	[21]			[22]
Stationary phase	**OMIX C_{18} pipette tips (Agilent Technologies)**	**Thermo Pierce C_{18} Columns**	**Waters Oasis HLB**	**Glygen TopTip C_{18} + graphite pipet tips**	**Waters Sep-Pak C_{18} 96-well plate**
Sample type	*Rhipicephalus sanguineus* salivary gland and midgut	Labeled HL60 cell-surface proteins			Mouse adipose tissue
Quantity loaded	150 μg protein	50–100 μg protein before enrichment			80 μg peptide before SCX fractionation (15 fractions)
Manufacturer advised loading	75 μg	0.5 ng to 30 μg	N.A. (MP states 1%–10% of the total SPE device bed)	75 μg	N.A. (MP states 1%–10% of the total SPE device bed)
Loading solvent	N.A. (MP states 0.5%–1.0% TFA in H_2O)	0.1% HFBA in H_2O	2% H_3PO_4 in 98% H_2O	H_2O	Mobile phase of SCX fractionation (that contained 500 mM KCl, 10 mM KH_2PO_4 in 25% ACN)

TABLE 1 (Continued)

Reference	[20]	[21]			[22]
Stationary phase	**OMIX C_{18} pipette tips (Agilent Technologies)**	**Thermo Pierce C_{18} Columns**	**Waters Oasis HLB**	**Glygen TopTip C_{18} + graphite pipet tips**	**Waters Sep-Pak C_{18} 96-well plate**
Elution solvent	N.A. (MP states 0.1% FA or 0.1% AA in 50:50–25:75 v/v% H_2O:ACN or H_2O:MeOH)	0.1% TFA in 30:70 v/v% H_2O:ACN, and 0.1% FA in 30:70 v/v % H_2O:ACN	100 v/v% MeOH	0.05% TFA in 60:40 v/v% H_2O:ACN	0.1% TFA in 30:70 v/v% H_2O:ACN
Is the method optimized or recovery analyzed?	NO	Comparison was performed, recovery was analyzed.			NO
PTM specificity	NO	NO			NO
Additional modification		Cell-surface proteins were covalently biotinylated.			

Abbreviations: AA, acetic acid; ACN, acetonitrile; BSA, bovine serum albumin; FA, formic acid; HFBA, heptafluorobutyric acid; HLB, hydrophilic-lipophilic balance; MeOH, methanol; MP, manufacturer's protocol; N.A., information not given in the article; PTM, post-translational modification; RP, reversed-phase; SCX, strong cation exchange; SPE, solid-phase extraction; TFA, trifluoroacetic acid; TMT, Tandem Mass Tag.

TABLE 2 Parameters to be optimized during the development of an SPE method. The solvent system is inherent to the stationary phase chosen for the sample clean-up; however, as it is seen in the examples in Table 1, most of the solvent systems use mainly highly aqueous loading buffers and organic elution solvents.

Parameters	Optimization range	Expected effect	Difficulty of optimization	Importance of optimization
Resin chemistry, particle size and porosity, vendor	C_{18} stationary phases are used for general applications. Lower binding chemistries can be used for hydrophobic peptides and special hydrophilic properties might be of use for PTM analysis.	Defines all the characteristics from qualitative annotations to quantitative aspects.	Optimization is limited only by resources and time. However, these three characteristics are linked together by the available phases on the market.	Careful selection of resin should be addressed. This is the most important factor for a successful cleanup procedure.
Resin-to-sample ratio	Based on the manufacturer's instructions.	Analyte breakthrough should be avoided at high loading, while permanent retention of the compounds can be avoided at low loading levels.	It can be addressed in a few steps with different loadings of a test sample.	An important factor for repeatability and accuracy. The maximum loading capacity is often overestimated in protocols. This should be addressed before the detailed optimization of the method.
Solvent composition of sample loading buffer	0% - 20% organic solvent content	Proper binding avoiding analyte breakthrough.	It can be addressed in a few steps.	Important to optimize.
Solvent composition and volume of washing buffer	0.5–2 times the loading volume. Absolute range inherent to the stationary phase used.	Proper desalting efficiency along with minimized sample loss on hydrophilic species.	It can be addressed in a few steps.	Moderate effect in general used, high importance for hydrophilic species.
Solvent composition of elution solvent	Organic solvent content: 70%–100% ACN or MeOH	An increase in recovery and repeatability can be expected.	It can be addressed in a few steps.	Moderate effect in general use, high importance for hydrophobic species.
Ion pairing reagents	0.01%–1% FA/TFA/HFBA	Ion pairing reagents are especially useful for providing sufficient retention of analytes. Too strong ion pairing causes permanent retention, and too weak ion pairing causes analyte breakthrough. Proper ion pairing results in good binding, thus good repeatability.	Moderately difficult, since ion pairing reagents also influence the pH of buffers.	Important to optimize.
Volume of elution	0.5–2 times as stated in the manufacturer's instructions.	An increase in recovery and repeatability can be expected.	It can be addressed in a few steps and should be optimized together.	Important to optimize.
Number of elution steps	1–5	Optimum can be found between hands-on time and the efficiency of elution.		
Contact time between solvents and the stationary phase	From seconds to a couple of minutes. Controlled by centrifugal speed/vacuum/incubation with solvents.	Most of the manufacturer protocols give a good indication for this parameter. A minor increase in recovery and repeatability can be expected with further optimization.	Easy to optimize.	Moderately important to optimize.
Bead suspension and incubation after loading or elution	No mixing/mixing 0–2 min incubation before solvent flow	A minor increase in recovery and repeatability can be expected.	Feasible only with a classic cartridge setup, and not with pipet tip SPE systems.	Moderately important to optimize.
Loading and wash solvent temperature	24–0°C	A minor increase in recovery and repeatability can be expected.	It can be addressed in a few steps; however, incubation of solvents and stationary phases takes a longer time.	Moderately important to optimize.
Cartridge temperature	24–4°C			
Elution solvent temperature	24–50°C			

Abbreviations: ACN, acetonitrile; FA, formic acid; HFBA, heptafluorobutyric acid; MeOH, methanol; PTM, post-translational modification; SPE, solid-phase extraction; TFA, trifluoroacetic acid.

different retention characteristics. Several stationary phases have been developed for special applications, for example, Waters HLB reversed-phase media for samples with hydrophilic characteristics.[30] Further important parameters are particle size and the porosity of the material. These characteristics are inherent to the type of SPE used, so have to be carefully selected in the beginning. To help this process, most vendors provide sheets for selecting the appropriate resin for the compounds in interest. The most commonly used stationary phase is C_{18}-functionalized silica.

2.2 | Resin-to-sample ratio

The amount of peptides retained is strongly determined by the amount of available active functional groups on the stationary phase. If too much material is loaded, a substantial part of the peptides will not be retained and thus will be lost in the flow-through fraction. On the other hand, if the amount of peptides is much less than ideal, some of them may be permanently bound and cannot be eluted from the cartridge. Therefore, manufacturer instructions on the loading capacity of the given phase should be taken into consideration and checked before starting the optimization workflow.

2.3 | Incubation and mixing steps

Incubating and suspending the resin in the sample loading or elution solvents can help to reach the binding sites, thus increasing binding or elution performance, respectively. However, these steps are only feasible with a classic cartridge setup, and not with pipet tip SPE systems. For additional tips, see Notes 3 and 4.

2.4 | The temperature during sample loading and elution

Binding to the reversed-phase stationary phase is strongly influenced by the temperature. In general, lower temperatures (e.g. 10°C) facilitate stronger binding, while higher temperatures (e.g. 50°C) weaken the binding, due to weakened interactions between the analyte and the stationary phase. As a result, cooled sample loading increases the binding efficiency, while heated elution may help in maximizing recovery from the cartridge. This is especially important when performing quantitative proteomics analyses. However, the optimal temperature depends on the stationary phase applied, thus it has to be optimized. For additional tips, see Note 5.

2.5 | Composition of sample loading buffer with emphasis on ion pairing reagents (IPRs)

The ultimate goal during sample loading is to bind as many peptides as possible to the stationary phase. Most peptides have acidic properties, their isoelectric points are in the range of 2–5. The use of appropriate pH (e.g. pH < 2) allows peptides to be positively charged, thus forming ion pairs with, for example, TFA. Ion pairs are well retained on the reversed-phase media. Usually, perfluorinated carboxylic acids are used for IPRs, and the strength of the binding increases with the number of carbon atoms (thus increasing hydrophobicity). The concentration of the IPR influences both the pH and the peptide/IPR ratio, thus careful optimization is necessary.

2.6 | The volume of elution solvent and number of elution steps

In general, increasing the volume of the elution solvent increases the recovery from the stationary phase. However, SPE purification is typically followed by solvent evaporation (to concentrate the sample), and in this case, larger elution volumes may cause higher sample loss. This is attributed mainly to two things: (i) a larger interaction surface with the walls of the microcentrifuge tubes; and (ii) an increased possibility of sample droplets escaping the tube. Another important factor to maximize recovery is the number of elution steps: using more—smaller volume—elution steps instead of using one-step elution with a large volume is desirable (e.g. 3 × 50µL is more efficient than 1 × 150µL).

2.7 | Composition of elution buffer

The optimal organic solvent ratio during elution relies on the hydrophobic-hydrophilic nature of the sample. When working with especially hydrophobic samples (e.g. membrane proteins), high organic solvent content may be needed for optimal elution, while hydrophilic samples (e.g. highly glycosylated plasma fractions) require lower organic solvent content. In the elution step, it is important to weaken the ion paring effect with the stationary phase, thus the use of less hydrophobic carboxylic acids, such as formic acid (FA), may facilitate complete elution.

3 | ASSESSMENT OF METHOD PERFORMANCE

The performance naturally can be assessed by applying the FDA Bioanalytical guidelines.[31] However, this can easily become complicated when trying to apply it to tens of thousands of peptides. Thus, for a general utility assessment for proteomics, we advise addressing three more general parameters according to the aim of the study: recovery, peptide/protein identification performance, and distribution of chemical properties of the detected peptides/proteins. Along with the performance, the repeatability of the method should also be checked based on the abovementioned three parameters (see Note 9).

Recovery is an essential parameter for quantitative proteomics experiments; it can be addressed in two ways. When a respective control is available (see Notes 10–11) and can be injected into the HPLC-MS system without significant damage, it is advisable to perform the

comparison based on the protein intensity values of all commonly quantified proteins. When a control sample is not available (this is mostly the case), labeled or synthetic peptides may be spiked in the sample before cleanup and recovery can be calculated the traditional way (see Notes 12–13). Calculating recovery in proteomics experiments is inherently biased by the ion suppression effects of co-eluting components during the HPLC-MS run. Therefore, it is common to observe recoveries over 100% when an effective SPE cleanup is performed (the investigated peptide is suppressed more by matrix components in the control sample than in the purified sample). The target range for recovery is 70%–130%.

Peptide/protein identification performance is the most straightforward parameter to compare. The higher the performance for removing matrix components (and the recovery of the peptides), the lower the ion suppression during HPLC-MS measurements, thus the higher the identification performance will be.

It is important to ensure that the chemical distribution of the analytes does not bias the findings when using the clean-up methods. Non-complete binding/elution of the peptides or excessive washing may cause a partial loss of the analytes. Therefore, it is advised to check the sanity of the results based on chemical parameters, such as peptide length (number of amino acids per peptide), hydrophobicity (Grand Average of Hydropathy, GRAVY score[32]), and IEP. Peptide GRAVY scores can be calculated by averaging the contributions of individual amino acids; an Excel sheet for this is available from the authors upon request. For the calculation of protein GRAVY scores, we recommend the web application developed by Stephan Fuchs.[33] IEP calculations can easily be performed by using the Isoelectric Point Calculator by Kozlowski.[34] The distribution acquired by using the tested methods can be compared visually or by mathematical correlations.

4 | FAST OPTIMIZATION WORKFLOW

The fast optimization workflow presented here provides time-efficient optimization of the SPE procedure for a given sample type. It requires 16 measurements on a test sample and only a couple of hours of laboratory work. This workflow is only given as an example; the exact chemical compositions and parameter settings can be adjusted based on the manufacturer's recommendations and the reader's previous expertise.

Step 1:

Choose a stationary phase for your purpose. Take the manufacturer's protocol as a starting step.

Step 2:

For optimization purposes, prepare a test sample for 16 experiments. The parameters to be optimized are the following:

a. Composition of sample loading buffer

0.1% TFA *versus* 0.5% TFA *versus* 0.1% heptafluorobutyric acid (HFBA) *versus* 0.5% HFBA—four conditions, leave the other steps of the original protocol as they are

b. Temperature of sample loading

Cooled cartridge and solvents *versus* room temperature (RT) cartridge and solvents—two conditions, leave the other steps of the original protocol as they are or use the parameter optimized in the previous step.

c. Temperature of elution

Elution solvent heated to 40°C *versus* at RT—two conditions, leave the other steps of the original protocol as they are or use the parameters optimized in the previous steps.

d. Composition of elution solvent

- Organic solvent content: for hydrophilic samples, use 10% lower organic solvent content; for hydrophobic samples, use 10% higher organic solvent content than suggested in the manufacturer's protocol *versus* using the original composition. For samples without any distinction in hydrophobicity, this step can be left out.
- Acid type: compare TFA and FA, and keep acid concentration constant (see Note 6).

Four conditions, leave the other steps of the original protocol as they are or use the parameters optimized in the previous steps.

e. Elution volume

Add one more step of elution to the manufacturer's protocol—two conditions, leave the other steps of the original protocol as they are or use the parameters optimized in the previous steps.

Altogether, only 14 experiments should be performed when a step-by-step optimization is done. As a rule of thumb, the effects of the various parameters discussed above can be considered additive. The number of samples analyzed can be reduced to 10 when every experiment of the *Fast Optimization Workflow* is performed at the same time.

Step 3:

Evaluate the acquired data and choose the best-performing method. Depending on the nature of the planned study, evaluation may be based on the number of peptides/proteins identified or on a quantitative measure (e.g. the average area of the quantified peaks). Naturally, both aspects can be considered when choosing the optimal parameters. For detailed instructions, see Section 3.

Step 4:

Compare the method with the optimized parameters to the manufacturer's protocol in two additional experiments.

5 | TROUBLESHOOTING

The most common consequences of problems occurring are the following: (i) poor recovery of analytes, (ii) a low number of detected

peptides, and (iii) an unexpectedly skewed distribution of physical–chemical parameters of the identified peptides. These problems usually go hand-in-hand, and there is a multitude of reasons that can cause problems. Peptides can be found in the loading/wash flow-through, in the elution fraction, or stuck on the stationary phase. Decomposition of peptides under the generally applied experimental conditions is not expected, mainly loss of PTMs can cause performance loss. Here, we list the most common reasons, explanations where necessary, and possible solutions. Problems occurring only at the SPE cleanup will be discussed, troubleshooting during other sample preparation steps or the HPLC-MS measurement is out of the scope of this tutorial.

OBSERVATION A: Generally poor identification and quantitation performance (possibly along with non-normal distribution of chemical properties).

Reason 1: The resin is not activated/wetted properly before sample loading; therefore, most of the peptides are lost during loading.

Most of the resins on the market need an activation step, as indicated in this tutorial as well. If the stationary phase is not prepared for the binding properly, secondary interactions between the analytes and the chemical bonding of the particles are weaker than they should be. As a result, peptides will be weakly retained (or not at all) and flow through the column/spin tip. Thus, they end up in the loading/wash fractions that are usually discarded.

Reason 2: The sample or the sample loading buffer has too high solvent strength.

Solvent strength is defined by the partition coefficient of the analytes between the stationary phase and the solvent. During sample loading, the strong retention of the analytes is required on the column/spin tip; therefore, a sufficiently weak solvent has to be used. Usually, 0%–10% organic solvent is used during loading on C_{18} materials, for example, 20% acetonitrile content can cause a substantial sample loss.

Reason 3: The sample loading solvent did not contain a sufficient concentration of ion-pairing reagents.

When ion pairs are formed by the deprotonated IPR ion and the positively charged peptide, a larger and more hydrophobic compound is produced. This increases retention during sample loading. If the concentration of the IPR is too low, then not all the peptides will form ion pairs, and a part of the population will have weaker retention. The IPRs are usually used for controlling the pH as well (see Reason 4).

Reason 4: The sample loading/wash solvents have incorrect pH or are not buffered well.

As peptides are ionizable compounds (weak acids or bases), solvent pH is one of the most important factors in controlling retention on reversed-phase materials. Low pH (<3) is required when acidic peptides are of interest (most cases), and high pH (>10) is required for basic peptides. The rationale behind this is to keep all the copies of a given peptide in ionized/non-ionized form. This can be achieved by adjusting the pH to at least two units above or below the peptide's pK_a/pK_b values. Unfortunately, this is not ubiquitously achievable, because we work with a large population of different peptides. If using the digestion buffer as the sample loading solvent, one should be particularly careful.

Reason 5: Washing was too extensive.

Even when using sufficiently weak solvent for sample loading and washing, weakly retained peptides can still be eluted. This is particularly true when the stationary phase is operated near the upper limit of its capacity.

Reason 6: The solvent flow rate is too fast.

The time of binding to the stationary phase is insufficient because the speed of centrifugation or the applied vacuum was too high. A high solvent flow rate also decreases the time of interactions needed for eluting the peptides. To address this issue, consider reducing the solvent flow rate by lowering the centrifuge rotation speed or the vacuum. Introducing a reapplication step at loading, and/or an additional elution step may also help overcome this issue.

Reason 7: The elution was incomplete and peptides are stuck on the stationary phase.

This can especially be the case when a small amount of sample is introduced on a high-capacity resin. As a result, many binding sites are available for each peptide. This can either be caused by a high solvent flow rate (see Reason 6) or insufficient elution conditions. Increasing the organic solvent content of the elution solvent (and/or adding isopropanol) and removing ion-pairing reagents can facilitate elution. Raising the elution temperature can also be effective, and multiple elution steps can also be beneficial (see Note 6).

Reason 8: The resin is insufficiently packed or is too old.

Degradation of the silica and bleeding of the stationary phase chemistry are more likely to occur when shelf-life is exceeded. Unfortunately, this cannot easily be investigated. However, insufficient packing of the material is easily visible, and can also be indicated by restricted solvent flow through the stationary phase.

Investigation points for Reasons 1–7:

When developing a method, it is always advisable to collect the Load, Wash, and Elution fractions separately and store them for further analysis. If an appropriate control sample is used, one can decipher the distribution of peptides among the three fractions and determine the ratio that is still retained on the stationary phase.

OBSERVATION B: Hydrophilic peptides, glycopeptides, and phosphopeptides have poor recovery. This is mainly in connection with the loading and wash steps; peptides end up in the discarded flow-through.

Reason 1: The sample loading/wash solvent strength was too high.

Reason 2: The washing step was too extensive.

Reason 3: The chosen stationary phase is not suitable for this group of peptides.

Explanations for Reasons 1 and 2 can be found under Observation A. If the stationary phase is deemed to be unsuitable, the user should try specific stationary phase types, such as HLB, HILIC, or mixed-mode resins capable of retaining polar compounds.

OBSERVATION C: Long and/or hydrophobic peptides have poor recovery. This is mainly due to the elution step, where peptides end up being retained on the stationary phase.

Reason 1: The elution solvent strength was too low.

To address this issue, one should consider using fully-organic elution solvents and adding isopropanol to the elution solvents. Another possible solution is to increase the temperature (e.g. 50–60°C) of the elution solvent to weaken the bonds and facilitate elution.

Reason 2: Elution was not extensive enough, and peptides are still retained on the stationary phase. The issue can be solved by adding more elution steps.

Reason 3: The chosen stationary phase is not suitable for this group. The reader should try lower-binding chemistries, such as C_8.

OBSERVATION D: Repeatability issues occur.

Reason 1: Uncontrolled sample loading pH or ion pairing. Special attention is needed when using the digestion solution directly for sample loading.

Reason 2: Solvents do not go through all the SPE columns. This is usually the case for centrifugal spin tip systems. If the centrifugation is insufficient, for example, not long enough or the speed of the centrifuge is too low (centrifugal force is not enough to compensate for the back pressure), some solvent may be retained on the top of the stationary phase.

Reason 3: The microcentrifuge tubes used for sample collection are not "protein low-bind" leading to undiscovered recovery issues.

Reason 4: Elution volume is too high, and the repeatability issues are mainly caused by subsequent dry-down steps. As solvent volumes increase, the possibility of escaping solvent droplets during dry-down also increases.

Reason 5: The column dries before the sample is added. This obliterates all the column activation and conditioning efforts. This is common in vacuum-operated SPE systems, requiring activation and conditioning to be started over.

Reason 6: The binding capacity of the column is exceeded, resulting in an uncontrolled breakthrough of the analytes during the loading step. The sample amount should be decreased to match the capacity. As a rule of thumb, manufacturer recommendations should be followed but critical assessment should also be taken.

OBSERVATION E: Salt contamination is observed during sample dry-down or the HPLC-MS runs.

Reason: The washing was insufficient. To resolve this issue, increase the wash volume.

6 | ADDITIONAL NOTES

Note 1. Reapplying the flow-through to the stationary phase once or twice may help in the complete binding of the target compounds. The peptides not retained in the first step of sample loading may go through a different flow path in the second step reaching empty binding sites.

Note 2. Always use protein low-bind microcentrifuge tubes in all SPE steps.

Note 3. A 1-min incubation may help by providing enough time for the analytes to reach binding equilibrium with the stationary phase.

Note 4. Suspending the resin in the applied solvent before centrifugation may increase the efficiency of elution.

Note 5. Cooling the cartridge may require up to 24 h to reach equilibrium.

Note 6. It might be beneficial to use FA for the last 1–2 steps of elution and keep the original buffer component for the previous steps. This way, the ion pairing is weakened and two solvents with slightly different selectivity can be used for optimal elution.

Note 7. It is useful to keep the effluents of the loading and the wash step for future analysis if troubleshooting needs to be done.

Note 8. Generalized (stringent) database search and quantitation parameters should be used for all the measurements when they are compared. However, in some cases, it can be useful to perform the assessment with looser criteria (for all the samples in question) to determine smaller differences that may otherwise be hidden.

Note 9. When assessing the repeatability of methods, at least five technical replicates should be used.

Note 10. You may consider using an already purified sample as the control. However, it is advised only at the initial step of the method development. The matrix components in a real sample (digestion buffers, enzymes, small metabolites, etc.) can dramatically change the binding characteristics, thus altering method performance. This is the main reason why SPE methods should be optimized for all the distinct sample types.

Note 11. An unpurified sample may only be used as the control if it does not contain large quantities of surfactants and urea.

Note 12. When spiking peptides in the sample, they should cover the whole range of chemical diversity to be analyzed. This includes peptide length and hydrophobicity, different phosphorylation positions, different types of glycans attached, and so forth. It is advised to use at least six different peptides. Additionally, they should match the quantities in the real sample to provide viable information on the method's performance.

Note 13. Spiking exogenous proteins in the sample before the digestion is not advisable, because it does not only reflect the SPE performance.

CONFLICT OF INTEREST STATEMENT

All the authors declare no financial or non-financial conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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