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Selective Chemoprecipitation to Enrich Nitropeptides from Complex Proteomes for Mass Spectrometric Analysis

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Abstract

Posttranslational protein nitration has attracted interest due to its involvement in cellular signaling, effects on protein function, and as a potential biomarker of nitroxidative stress. We describe a procedure for enriching nitropeptides for mass spectrometry-based proteomics that is a simple and reliable alternative to immunoaffinity-based methods. The starting material for this procedure is a proteolytic digest. The peptides are reacted with formaldehyde and sodium cyanoborohydride to dimethylate all the N-terminal and side-chain amino groups. Sodium dithionite is added subsequently to reduce the nitro groups to amines; in theory, the only amino groups present will have originally been nitro groups. The peptide sample is then applied to a solid-phase active ester reagent, and those peptides with amino groups will be selectively and covalently captured. Release of the peptides on hydrolysis with trifluoroacetic acid results in peptides that have a 4-formyl-benzamido group where the nitro group used to be.

In qualitative setups, the procedure can be used to identify proteins modified by reactive nitrogen species and to determine the specific sites of their nitration. Quantitative measurements can be performed by stable-isotope labeling of the peptides in the reductive dimethylation step.

Preparation of the solid-phase active ester reagent takes about 1 day. Enrichment of nitropeptides requires about 2 days, and sample preparations need 1 to 30 h varying based on experimental

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Categories

Chemical modification; Proteomics; Protein analysis; Spectroscopy

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design. LC–MS/MS assays take from 4 h to several days and data processing can be done 1 to 7 days.

Keywords

Protein nitration; Nitropeptides; Solid-phase active ester reagent (SPAER) on glass beads; Enrichment; Chemoprecipitation; Mass spectrometry; LC–MS; MS/MS

INTRODUCTION

Protein nitration is a posttranslational modification (PTM) induced by “nitroxidative stress”¹, and has been studied in various biological systems focusing on biomarkers and functional consequences, cellular signal transduction pathways, oxidative attack and cell death^{2–5}. The nitro group (–NO₂) is introduced predominantly to tyrosine (Tyr, Y) residues in *ortho*-position relative to the phenolic hydroxyl group (Figure 1a). Nitration of tryptophan (Trp, W) residues (Figure 1b) has also been reported, but is less common^{6,7}. These modifications can occur through the direct effect of reactive nitrogen species such as peroxynitrite⁸ (ONOO[–]) or nitric oxide (•NO). Peroxynitrite can be formed by a diffusion-controlled reaction between nitric oxide (•NO) and superoxide (O₂^{•–}) radicals; nitrogen dioxide can be formed by hemeperoxidase-catalyzed reduction of nitrite (NO^{2–}) by hydrogen peroxide (H₂O₂)⁶.

Protein Tyr nitration does not occur indiscriminately; both *in vitro* and *in vivo*, a subset of proteins is preferentially targeted¹. Increased levels of nitrated proteins have been detected in diseases impacting various organs and tissues such as the brain^{9,10}, heart¹¹, liver¹², kidneys¹³ and muscles¹⁴, but the consequences of this PTM are poorly understood. In any case, the low abundance of nitroproteins has been a formidable obstacle to reliable proteome-wide explorations focusing on identification of not only the affected proteins, but also on the precise localization of modification sites by tandem mass spectrometry (MS/MS) of proteolytic fragments using the “bottom-up” proteomics approach. Without appropriate enrichment, nitropeptides may remain hidden by the overwhelming number of unmodified (i.e., non-nitrated) species, which could lead to a large number of false positive identifications^{15,16}.

Immunoaffinity-based methods have been developed to enrich peptides containing 3-nitrotyrosine (3NT) residues^{17,18}. However, cross-reactivity and non-specific binding of 3NT-antibodies, combined with bias in MS/MS data analyses and interpretation¹⁹ have invalidated many MS-based nitroproteomics studies using this approach^{15,16}.

The application of reversible covalent chemistry (i.e., capture-and-release enrichment based on chemical selectivity²⁰) holds the promise to address limitations of immunoaffinity-based techniques in this regard. Therefore, we have developed a solid-phase active ester reagent (SPAER) on glass beads (Figure 2) for a highly selective immobilization of nitropeptides as part of a method that requires only a straightforward two-step derivatization prior to their capture on the SPAER glass beads²¹. The procedure, which we call “chemoprecipitation,” affords efficient enrichment and, following an acid-catalyzed release of tagged species, a

significant reduction of sample complexity to facilitate MS analyses focusing on the exploration of the nitroproteome. In this contribution, we describe the detailed simple protocol for the synthesis of SPAER starting from commercially available aminopropyl-functionalized controlled-pore glass beads (CPG-C₃H₇-NH₂) and its application for isolating nitropeptides from biological samples for MS-based identification and localization of nitration sites within the affected proteins.

An advantage of using CPG-C₃H₇-NH₂ as a starting point to create SPAER comes from the fact, that these glass beads (unlike agarose, sepharose and polystyrene solid supports) permit the use of practically any solvent (both inorganic and organic), to wash away all adsorbed (i.e., non-covalently attached) species and synthetic impurities allowing thereby a thorough cleanup and significant reduction of sample complexity for MS analyses.

The procedure also includes the preparation of CPG-hydrazide, which is a synthetic intermediate (Figure 2c), as this has not yet been fully described; references have usually been made to a publication that gave only a brief description on its synthesis²².

Development of the protocol

The SPAER approach was inspired by our previous studies successfully enriching for MS analyses protein carbonyls *via* chemoprecipitation^{23,24}. Using a solid-phase hydrazide reagent assembled on commercially available CPG-C₃H₇-NH₂ beads (“CPG-hydrazide” shown in Figure 2c), carbonylated peptides are immobilized and the interfering “matrix” of unmodified peptides remaining in solution can be removed successfully. The technique is essentially the same practice that has long been used in solid phase peptide synthesis (SPPS) pioneered by Merrifield²⁵, in which the peptide chain is elongated on a solid support while the all soluble reagents are washed away. Purification is simplified to washing-filtration cycles only and, once the desired peptide chain has been assembled, the covalently attached product can be cleaved from the solid support. For peptide carbonyl enrichment, immobilization involves hydrazide chemistry and, thus, the original peptide carbonyls can be released by acid-catalyzed hydrolysis for MS analyses^{22–24}.

We have recognized that anchoring a small-molecule formyl-substituted carboxylic acid or preferably its active ester, *via* its carbonyl group to CPG-hydrazide (Figure 2d) would result in a simple solid phase reagent (i.e., SPAER) that would be capable of selectively capturing nitropeptides from an overwhelming matrix of non-nitrated species according to a procedure summarized schematically in Figure 3. In order to do that, similarly to enrichment methods developed by others and discussed before²¹, we had to convert 3NTs to the corresponding aminotyrosines (3ATs) (Figure 3a)²⁶. Reduction of –NO₂ to –NH₂ is the most trivial choice to create a convenient synthetic handle for covalent immobilization of nitropeptides on a solid support. This modification is necessary, because only a limited number of chemical reactions are known in organic chemistry suitable to manipulate the NO₂-group without requiring special skills and vigorous reaction conditions.

To single out amino groups on 3ATs from those at the N-termini and lysine (Lys, K) side chains, the latter amino-groups must be protected (capped) before reduction of 3NTs to 3ATs takes place. This has been mostly achieved by N-acetylation²⁷, because it has been a

well-established protocol in SPPS²⁸. However, besides losing potentially useful ionization sites for MS analyses that affect sensitivity, removal of the O-acyl side products requires treatment with large quantity of an additional reagent or harsh reaction conditions²¹. We therefore chose to use reductive methylation^{29,30} (Figure 3a) for blocking all native $-NH_2$ groups of the proteolytic peptides before 3NT-to-3AT conversion. The usefulness of this straightforward reaction to label peptides for quantitative mass spectrometry experiments has been convincingly shown³⁰. Our protocol takes advantage of two established and validated synthetic steps (Figure 3a) before the covalent capture of the modified peptides by SPAER (Figure 3b)²¹.

SPAER development—We selected a commercially available active ester, 4-formylbenzoic acid N-hydroxysuccinimide ester (4FB-OSu) for the reaction with CPG-hydrazide to create SPAER (Figure 2d). While we used N-hydroxysuccinimide ester ($-OSu$) ester from a practical point of view (i.e., commercial availability), we believe that other active esters (e.g., 1-hydroxybenzotriazole active ester traditionally used in SPPS) would also be suitable²⁸, even when they are generated *in situ* (see Steps 9–10 in Procedure Section).

After thorough washing, the captured species can be released from the glass beads *via* an acid-catalyzed hydrolysis at ambient temperature using a short reaction period. The released peptides will have two modifications: dimethylation on the native aliphatic amino groups and a small tag (i.e., 4-formyl-benzamido) on the Tyr residues, as shown in Figure 3c. This tag seems to have three major advantages²¹: 1) it preserves the fragmentation properties of the parent nitropeptides upon MS/MS analyses using collision-induced dissociation (CID); 2) it eliminates the electron predator effect of $-NO_2$ groups allowing complimentary ionization methods such as electron-capture dissociation (ECD) to improve sequence coverage; 3) the tagged species now carry 4-formyl-benzamido groups instead of the original nitro groups and, therefore, they can be further enriched as peptide carbonyls using the CPG-hydrazide reagent (Figure 2)^{22–24}, if further (“tandem”) enrichment is deemed necessary after having reviewed the results of the SPAER-only protocol.

Applications of the method

The protocol described here enables the specific capture of nitropeptides from proteolytic digests of complex proteomes followed by release of tagged species for mass spectrometric analyses²¹. The method has been applied in a qualitative “shotgun” design using data-dependent LC–MS/MS for the identification of protein targets of posttranslational Tyr nitration including precise identification of the modification sites²¹. With their improved detection sensitivity and increased speed of acquisition, newer mass spectrometers should permit applications to not only test-tube or cell-culture experiments but also to tissue-derived samples in studies focused on this important PTM as a signaling event^{3,31} or as a biomarker of nitroxidative stress^{2,32} affecting a specific subset of proteins in the proteome and, also, localized to specific Tyr residues within these targeted proteins.

Quantitative measurements comparing the relative extent of site-specific protein Tyr nitration using the SPAER-based enrichment method can be achieved through stable-isotope

dimethyl labeling^{30,33}, because the corresponding reductive alkylation is our method of choice to block all native amino groups before the reduction of 3NTs to 3 ATs (Figure 3a). Multiplex peptide stable-isotope dimethyl labeling has been an established method, and users interested in implementing it are referred to its published protocol³⁰. On the other hand, there would be no restriction to use our enrichment procedure in combination with other labeling techniques used in MS-based quantitative proteomics such as stable-isotope labeling with amino acids in cell culture (SILAC)³⁴ or its *in vivo* adaptation³⁵. Selected reaction monitoring (SRM) using labeled (“heavy”) synthetic nitropeptides as internal standards^{36,37} used in combination with the chemoprecipitation protocol described here also has the potential to afford quantification of the nitroproteome including localization of this PTMs. In addition, data-independent acquisitions using sequential windowed data-independent acquisition of the total high-resolution (SWATH-) MS measurements and isotopically labeled heavy peptides³⁸ may be an alternative proteomics approach for integrating targeted quantifications of protein Tyr nitration, when combined with the SPAER-based enrichment.

Comparison with other methods

Although biochemists and biologists may have preference to adopt immunoaffinity-based methods that apply immobilized antibodies for the enrichment of nitropeptides, cross-reactivities have been revealed even upon using custom-made monoclonal anti-3NT and thorough characterization of affinities and binding specificities³⁹. Therefore, the advantage of adhering to a routine practice is outweighed by the procedure’s poor efficiency, lack of selectivity and overall unreliability for discovery-driven nitroproteomics. After pre-enrichment by immunoprecipitation with anti-3NT antibodies, attachment of a tag such as biotin after chemical derivatizations has been developed for the enrichment on an immunoaffinity column with immobilized antibodies (streptavidin) recognizing the introduced affinity tag in proteolytic peptides⁴⁰. Apart from the need for multiple steps in the procedure, naturally biotinylated peptides would also be enriched. This would contaminate the sample and, therefore, could interfere with the identification of low-abundance nitropeptides.

Chemical modifications have been introduced into methods utilizing the attachment of a fluorophore tag after reduction of 3NT residues^{41,42}. These procedures would permit detection of the tagged peptides and proteins by spectrofluorimetry and Western blot, respectively. During online high-performance liquid chromatography (HPLC) separation, elution of a tagged (e.g., dansylated) nitropeptide is detected fluorimetrically, which is then used to trigger MS/MS data acquisition to enable peptide identification⁴¹. Isobaric tags for relative and absolute quantification (iTRAQ) also have been applied to bottom-up exploration of the nitroproteome⁴³. Application of the fluorophore-based and iTRAQ methods without the enrichment of the tagged nitropeptides could raise concerns about suppression effects and undersampling due to the presence of a potentially overwhelming matrix. Additional instrumentation and technical expertise are also necessary to adapt these methods for MS-based nitroproteomics. Therefore, a very simple chemoprecipitation approach would be clearly advantages. This also applies to diagonal chromatography that distinguishes nitropeptides from non-nitro species based on retention time shifts after the

usual sodium dithionite reduction²⁶ in narrow fractions from offline reversed-phase liquid chromatography (RPLC) of the sample⁴⁴.

Other methods also take advantage of the unique chemical properties and reactivity of the nitro-group to facilitate efficient and selective enrichment of nitropeptides. Specifically, reduction of 3NTs to the corresponding 3ATs²⁶ is utilized in combination with other chemical derivatization(s) to ensure selectivity. However, there have been several varieties of the procedures in terms of materials, reagents and, importantly, the number of reaction steps. The SPAER approach uses only two steps of simple and routine derivatization reactions before covalent immobilization, while similar methods employ multi-step chemical conversions^{27,45} to introduce a metal-chelating motif for non-covalent immobilization⁴⁶, which discourages their implementation by the laboratories of non-specialists. Reductive dimethylation furnishes several benefits^{21,33} (essentially quantitative conversion, no hydrophobic shift in RPLC retention, preservation of MS sensitivity with the tagged peptides, commercial availability of the reagent in multiple stable isotope-labeled forms, etc.) over acetylation as capping chemistry^{27,45,46}. In addition, the use of CPG beads in our protocol allows for a more thorough washing to remove non-nitro peptides³³, because alternative methodologies^{27,45,46} use polymer (agarose) support that is not as resistant to organic solvents as CPG.

Experimental design

For the identification of protein targets for posttranslational Tyr nitration with localization of modification sites through discovery-driven proteomics, a qualitative approach is sufficient and we recommend two independent enrichment experiments, when enough sample is available. A typical workflow is done by following Steps 19 to 47 of the protocol using light formaldehyde (HCHO) and NaBH₃CN solutions as dimethylation reagents A and B (see REAGENT SETUP). A control experiment is where Step 27 of the protocol is done without the addition of Na₂S₂O₄, from which no valid nitropeptide identifications should be made. Capture of incompletely dimethylated peptides, if any, could alert users about the need for optimization of the protocol to their samples and about the possibility of misidentifications when using protein database searches with “relaxed” criteria upon actual data analysis. Manual validation should be a vital component of the proper spectral assignments^{19,47}, and Box 1 summarizes the procedure we recommend. Human serum albumin nitrated with tetranitromethane with confirmed presence of several nitropeptides can be used as a positive control sample⁴⁸.

Additional validation beyond MS/MS spectra may be warranted for endogenous 3NT-containing peptides detected from tissue samples⁴⁷. In case of doubts remaining after manual validation (Box 1) or when further confirmation is to be pursued⁴⁷, consider custom-synthesis of the nitropeptide¹⁹ and complete the protocol from Steps 21 to 47 using a 1–5 µg/mL solution of the synthetic nitropeptide in triethylammonium bicarbonate buffer as a reference sample. The MS/MS spectrum from the raw data file of the dimethylated and tagged synthetic peptide should match with the MS/MS spectrum obtained on the putative nitropeptide enriched and analyzed from the biological sample.

Quantitative design relying on multiplex peptide stable-isotope dimethyl labeling should follow its published protocol³⁰. We recommend the use of D¹³CDO and NaBH₃CN as reagents to perform heavy dimethyl labeling in two-way differential quantification experiments³³, while three-way multiplexing should use DCDO and NaBH₃CN for intermediate labeling, and D¹³CDO and NaBD₃CN for heavy labeling to avoid signal interference (“cross-talk”) among the labeled forms of the peptides⁴⁹. A Java program is available for the deconvolution of overlapping isotopic clusters (<https://trac.nbic.nl/opf/>), if required. Rigorous quantitative designs also should consider a pseudo triplex approach even for a two-way comparison (In this design, two identical samples are labeled with light and heavy reagents, respectively; while another comparative sample is labeled with an intermediate forms)⁵⁰.

Limitations

Although reductive dimethylation has been proven essentially quantitative with no side-reactions in proteomics workflows including our nitropeptide chemoprecipitation approach²¹, O-sulfation has been shown for dithionite reduction of 3NTs to 3ATs⁴⁴ to yield by-products. We estimate that the percentage of nitropeptides that results in a predominant formation of O-sulfated 3AT-peptides may reach about 10%. In principle, these by-products can also be enriched by our SPAER-based chemoprecipitation. Protein database searches can be carried out by including Tyr O-sulfation as an additional variable modification, which will reveal the extent of by-product formation through the percentage of O-sulfated nitropeptides within the total nitropeptide identifications obtained. However, including Tyr O-sulfation as variable modification could increase false discovery rate (FDR) among the identified hits due to the added “degree of freedom” (We also note that estimation of FDR in large-scale proteomics studies focused on PTMs may be inherently difficult⁵¹). The enrichment of nitro-Trp-containing peptides by using SPAER can also be presumed⁵². However, the chemistry of dithionite reduction has not been fully tested for the isomeric forms of these nitropeptides.

MATERIALS

!CAUTION Most reagents used in the protocol are toxic and harmful by inhalation, in contact with skin, or if swallowed. Piperidine also has extremely unpleasant odor. Organic solvents are also flammable. Trifluoroacetic acid and acetic acid are corrosive. Ammonia solution is an irritant. Therefore, all solvents and chemicals should be handled in an efficient chemical fume hood and by wearing appropriate personal protective equipment (gloves, lab coat, protective eye glasses and mask). The chemical procedures also must be performed in an efficient fume hood.

REAGENTS

- Acetic acid (AcOH; Sigma-Aldrich, cat. no. 338826)
- Acetonitrile (ACN; EMD, cat. no. AX0145)
- Aminopropyl-modified controlled pore glass particle, size 120-200 mesh, 0.2 mmol/g loading (CPG-C₃H₇-NH₂; Sigma-Aldrich, cat. no. 27791)

- Ammonium bicarbonate (NH_4HCO_3 ; Sigma-Aldrich, cat. no. A6141)
- Ammonium hydroxide solution (NH_4OH ; Sigma-Aldrich, 338818)
- BCA assay (Thermo Scientific, cat. no. 23225)
- Custom-synthesized nitropeptide(s) (Synthetic BioMolecules) – optional, for quality control of SPAER and workflow, as well as hit validation
- D^{13}CDO , (Cambridge Isotope Laboratories, cat. no. CDLM-4599-1) – optional, “heavy” formaldehyde for differential dimethylation in quantitative nitroproteomics
- DCDO (Sigma-Aldrich, cat. no. 492620) – optional, “intermediate” formaldehyde for differential dimethylation in quantitative nitroproteomics
- Dichloromethane (DCM; Sigma-Aldrich, cat. no. 650463)
- N,N-Diisopropylcarbodiimide (DIC; Sigma-Aldrich, cat. no. D125407)
- N-Hydroxysuccinimide (HOSu; Sigma-Aldrich, cat. no. 56480)
- N,N-Dimethylformamide (DMF; Sigma-Aldrich, cat. no. 227056)
- N,N-Diisopropyl ethylamine (DIPEA; Sigma-Aldrich, cat. no. 387649)
- Dithiothreitol (DTT; Sigma-Aldrich, cat. no. D9779)
- 9-Fluorenylmethyl carbazate (Fmoc-NH-NH₂; Sigma-Aldrich, cat. no. 46917)
- Formaldehyde (HCHO; Sigma-Aldrich, cat. no. F8775)
- Formic acid (FA; Sigma-Aldrich, cat. no. 14265)
- 4-Formylbenzoic acid N-hydroxysuccinimide ester (4FB-OSu; Sigma-Aldrich, cat. No. 40923).
- Iodoacetamide (IAA; Sigma-Aldrich, cat. no. I1149)
- Methanol (MeOH; Sigma-Aldrich, cat. no. 34860)
- Phosphate buffer solution (PBS; Sigma-Aldrich, cat. no. P5244)
- Piperidine (Sigma-Aldrich, cat. No. 411027)
- Sequencing-grade modified porcine trypsin (Promega, cat. no. V5111)
- Sodium acetate (Sigma-Aldrich, cat. no. S8750)
- Sodium chloride (NaCl; Sigma-Aldrich, cat. no. S7653)
- Sodium cyanoborodeuteride (NaBD_3CN ; Sigma-Aldrich, cat. no.190020) – optional, “heavy” reductant for differential dimethylation in quantitative proteomics
- Sodium cyanoborohydride (NaBH_3CN ; Sigma-Aldrich, cat. no.156159)
- Sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$; Sigma-Aldrich, cat. no. 157953)
- Sodium hydroxide solution (NaOH; Sigma-Aldrich, cat. no. 415413)

- Succinic anhydride (SA; *a.k.a.* dihydro-2,5-furandione, Sigma-Aldrich, cat. no. 239690)
- Tetranitromethane (TNM; Sigma-Aldrich, cat. no. T25003) – optional, for quality control of SPAER and workflow
- Trifluoroacetic acid (TFA; Sigma-Aldrich, cat. no. 302031)
- Urea (Sigma-Aldrich, cat. no. U6504)
- Universal Proteomics Standard Set (Sigma-Aldrich, cat. no. UPS1) – optional, for quality control of SPAER and workflow
- Water (H₂O, HPLC grade; Fisher Scientific, cat. no. W5-4)

EQUIPMENT

- Set of adjustable pipettes (20-200 µL, 200-1000 µL, 500-5000 µL; Eppendorf Research) and tips
- LibraTube G20 with filter and cap (HiPep Laboratories, cat. No. RTG20) fitted with 2-way stop valve. (HiPep Laboratories, cat. No. RTV-SF2)
- Labquake™ Tube Shaker/Rotator
- Digital Vortex Mixer
- C18 solid-phase extraction (SPE) cartridges (SepPak, Waters, or similar)
- SpeedVac (Eppendorf Vacufuge)
- pH paper rolls
- Eppendorf 5702 centrifuge tubes, 1.5 and 2 mL
- Pierce® 69705 spin columns-screw cap kit (Fisher Scientific)
- C18 pipette Ziptips (Millipore)
- Polypropylene autosampler vials (National Scientific Company)
- Laboratory centrifuge
- Nanoflow liquid chromatograph, Nano 2D-HPLC (ABI-Eksigent) or similar
- C18 nanoflow column (PepMap, Dionex)
- Sample trap (IntegraFrit™, New Objective)
- Picotip emitter (New Objective)
- LC-MS/MS system such as a linear ion trap-Fourier transform mass spectrometer hybrid instrument (LTQ-FT, LTQ-Orbitrap or similar; Thermo Scientific) equipped with a nanoelectrospray source
- Vacuum pump (Welch)

SOFTWARE

- LC–MS and MS/MS data reduction (“peak picking”) program such as Bioworks 3.3 (Thermo) or similar
- Database search engine such as Mascot (Matrix Science), Sequest (Thermo), X! Tandem (open source; The Global Proteome Machine Organization), or equivalent, to identify peptides
- National Center for Biotechnology (NCBI) or UniProt (UniProt Consortium) protein sequence database
- Scaffold (Proteome Software), or equivalent, for hit validation, statistical evaluation and visualization of complex MS/MS proteomics experiments (optional)
- MaxQuant (Max Planck Institute of Biochemistry), ProteoSuite (British Biotechnology and Biological Sciences Research Council), or similar program for the evaluation of relative quantitation experiments (optional)

REAGENT SETUP

- Nitropeptides: At least 5–10 mg of total proteins or 1–2 µg of each nitropeptide in order to enrich and identify sufficient number of nitropeptides
- Protein reduction and alkylation solution: PBS buffer containing 8 M urea (urea is freshly added to the buffer)
- Dimethylation solutions A: 4% (v/v) aqueous HCHO, DCDO or D¹³CDO to the sample to be labeled with light, intermediate and heavy dimethyl, respectively. These solutions may be stored in closed vials for up to 1 month in a refrigerator.
- Dimethylation solutions B: Freshly made 50 mM NaBH₃CN to the light and intermediate labeling and 50 mM NaBD₃CN to heavy dimethyl labeling, in 100 mM sodium acetate buffer (pH 5.5), 1% (v/v) aqueous ammonia solution for quenching the dimethylation reaction.
- Quenching solution for the dimethylation reaction: Use 1% (vol/vol) aqueous ammonia solution. This solution may be stored in closed vials for up to 1 month in a refrigerator.
- Quenching solution for the dithionite reduction: Use 50% (vol/vol) aqueous acetic acid solution. This solution may be stored in closed vials for up to 1 month in a refrigerator.
- SPE solutions: loading solution 0.1% (v/v) TFA in H₂O, washing solution 5% (v/v) ACN/0.1% (v/v) TFA, elution solution 80% (v/v) ACN/0.1% (v/v) TFA
- Mobile phase A for RP-HPLC: 0.1% (v/v) FA in H₂O
- Mobile phase B for RP-HPLC: 0.1% (v/v) FA in ACN
- Loading solution for RP-HPLC: 5% (v/v) ACN in H₂O containing 0.1% (v/v) FA

EQUIPMENT SETUP

LC-MS/MS equipment In our setup, a 15 cm $\times$ 75 μ m i.d. PepMap C18 nanoflow column is installed in the Nano-LC-2D system. The peptide solution (5 μ L) is loaded onto a 2.5 cm $\times$ 75 μ m sample trap for desalting and concentrating the sample at a flow rate of 1.5 μ L/min in the HPLC loading solvent. Separation is performed at a constant flow rate of 250 nL/min after 5 min equilibration with 4.8% solvent B, followed by a 90-min gradient to 50% solvent B. Solvent B is held at 50% for 5 min and, then, increased to 90% in 5 min with return to 4.8% solvent B in 10 min. Full-scan FTMS mass spectra are acquired from m/z 350-1500 at mass resolution of 50000 and with an automated gain control (AGC) value of 1×10^6 and data-dependent mode of acquisition is performed with an AGC value of 3×10^4 and the five most intense parent ions selected for MS/MS analysis in the linear ion trap using collision-induced dissociation (CID) with 35% normalized collision energy and helium as the collision gas^{21,33}. Singly-charged precursors and precursors with unassigned charges are excluded, and dynamic exclusion from further MS/MS analysis is applied for 60 s.

PROCEDURE

▲CRITICAL All procedures are done at room temperature (20–25 °C), unless otherwise noted.

Part 1 Synthesis of SPAER: Succinylation of CPG-C₃H₇-NH₂ beads (Figure 2a) • TIMING ~ 5h

1 | Calculate the amount of succinic anhydride (SA) needed that represents 50-fold molar excess over the amino groups on the glass beads. For example, for 0.5 g of CPG-C₃H₇-NH₂ available with 0.2 mmol/g loading (i.e., 0.5 g $\times$ 0.2 mmol/g = 0.1 mmol final loading) use:

a. 0.5 g glass beads $\times$ 0.2 mmol/g loading $\times$ 50 $\times$ 100 mg/mmol SA = 500 mg SA

2 | Weigh 500 mg of CPG-C₃H₇-NH₂ into a 20 mL-LibraTube fitted with 2 way stop valve (reaction vessel).

3 | Add 5 mL of DMF with an automatic pipette and gently shake the tube for 2 min on a tube shaker/rotator to “wet” the beads.

▲CRITICAL STEP Instead of DMF, N-methyl-2-pyrrolidinone (Aldrich, cat no. 69116) can also be a solvent of choice. The latter solvent is frequently used in Fmoc-based SPPS and shows enhanced chemical stability compared to that of DMF.

4 | Open the valve to drain the solvent as you would carry out a regular vacuum filtration. For example, a vacuum manifold (e.g., Vac-Man laboratory vacuum manifold from Promega, cat no. A7231) can be used for holding the reaction vessel upon draining the solvents/reaction media. In this case, place the vacuum manifold in a fume hood and connect it to vacuum through a trap. Place LibraTube G20 containing the glass beads onto the vacuum manifold and cap all unused inlets with rubber septa.

5 | Close the valve and add 5 mL of DMF followed by 500 mg (5 mmol) of SA and 200 μ L of DIPEA (4 %, v/v).

6 | Shake the reaction vessel for 3 h using the tube shaker/rotator or similar equipment and, then, open the valve to drain the reaction medium with vacuum filtration.

7 | Wash the beads and the reaction vessel thoroughly three times with DMF (3×5 mL) followed by DCM (3×5 mL) while applying vacuum to remove any residual reaction medium.

8 | Dry the beads by applying vacuum until the solvent evaporates. The succinylated beads are now ready for hydrazide beads (CPG-hydrazide) preparation.

Part 1 Synthesis of SPAER: Preparation of CPG–hydrazide (Figures 2b-c) • TIMING ~ 7h

9 | Calculate the amount of reagents (HOSu, DIC and Fmoc-NH-NH₂) needed that represents 10-fold molar excess over the loading. The values in the steps that follow correspond to the calculation done in Step 1.

10 | Add 5 mL of DMF with an automatic pipette to the succinylated beads followed by adding 115 mg (1 mmol) of HOSu, 254 mg (1 mmol) of Fmoc-NH-NH₂ and 175 μ L (1 mmol) of DIC. Shake the reaction vessel for 3 h using Labquake™ Tube Shaker/Rotator or similar equipment.

▲CRITICAL STEP With Step 10, we activate the carboxylic groups on the succinylated beads *in situ* by using HOSu and DIC to generate –OSu active esters; however, other reagents, such as the widely used 1-hydroxybenzotriazole (HOBt)²⁸ are also suitable for this purpose.

11 | Open the valve to remove the reaction media by vacuum filtration, and then wash the beads and the reaction vessel thoroughly from the top using DMF (3×5 mL), MeOH (3×5 mL) and DCM (3×5 mL) while applying vacuum.

12 | Remove the Fmoc protecting group by adding 4 mL of DMF to the beads followed by 1 mL of piperidine (i.e., 20 % v/v piperidine in DMF). Shake the reaction vessel using Labquake™ Tube Shaker/Rotator or similar equipment for 20 min.

? TROUBLESHOOTING

13 | Open the valve to remove the reaction medium and thoroughly wash the beads and the reaction vessel with DMF (3×5 mL), MeOH (3×5 mL) and finally with DCM (3×5 mL).

■ PAUSE POINT At this point you may store the dried beads –20 °C under nitrogen until further procedures for extended period of time.

▲CRITICAL STEP At this point, you may save an aliquot of the CPG–hydrazide beads if you are also interested in the enrichment of peptide carbonyls based on chemoprecipitation²²⁻²⁴. In step 15 leading to SPAER, the amount of reagent is calculated for the entire starting quantity of CPG-C₃H₇-NH₂ beads (0.5 g). Modify the amount of

reagent according to the amount of CPG-hydrazide beads used for SPAER preparation considering 10-fold molar excess of 4FB-OSu over the original (0.2 mmol/g) loading.

Part 1 Synthesis of SPAER: Condensation of CPG-hydrazide with 4FB-OSu (Figure 2d) • TIMING ~ 8h

14 | Wet CPG-hydrazide with 5 mL of ACN/H₂O (7:3, v/v).

15 | Drain the solvent and, then, treat the beads with 247 mg (1 mmol) of 4FB-OSu in 5 mL of ACN/H₂O (7:3, v/v) containing 0.2% (v/v) AcOH.

16 | Shake the reaction mixture for ~ 6 h on Labquake™ Tube Shaker/Rotator or similar equipment.

17 | Open the valve to drain the reaction medium and wash the beads and the reaction vessel thoroughly first with ACN/H₂O (7:3, v/v) (3 × 5 mL) and, then, with H₂O (3 × 5 mL) followed by ACN (3 × 5 mL) and finally with DCM (3 × 5 mL).

18 | Dry SPAER under vacuum and store the beads under nitrogen at –20 °C.

▲CRITICAL STEP When performing the SPAER protocol for the enrichment of nitropeptides for the first time, it is advisable to evaluate SPAER and getting familiar with the technique by “fishing out” known quantity of a limited number of purified synthetic nitropeptides according to Steps 21 to 47 below. Custom-synthesized peptides with 3NT-residues can be ordered from commercial vendors (e.g., Synthetic Biomolecules, San Diego, CA) by specifying the sequences and required purity (> 95% preferred). Combustion analyses (performed by, e.g., Atlantic Microlab, Norcross, GA) will be necessary to calculate the exact peptide contents. Alternatively, nitration of the universal proteomics standard set USP1 by TNM could be used as a quality control of the protocol, from enrichment to data analysis⁴⁷, considering the user’s MS platform and the detailed supporting information of the cited reference.

Part 2 Sample preparation and SPAER enrichment: Proteolytic digestion of protein samples • TIMING ~1 d

▲CRITICAL All procedures are done at room temperature (20-25 °C), unless otherwise noted.

19 | Determine the protein content of the sample by the Bradford assay⁵³ and perform trypsin or Lys-C/trypsin combination digestion according to previously published procedures⁵⁴.

20 | Dry the sample in a SpeedVac using a 1.5 mL-Eppendorf centrifuge tube.

■ PAUSE POINT At this point, the dried samples can be stored at –20 °C for several weeks.

Part 2 Sample preparation and SPAER enrichment: Pre-enrichment derivatizations (Figure 3a) • TIMING ~2 d

▲**CRITICAL** Cap the N-terminal and side chain native amino groups of the proteolytic peptides via reductive methylation according to the previously published protocol³⁰. Use only formaldehyde if no differential labeling is desired. For multiplex stable-isotope labeling, you may use not only formaldehyde and sodium cyanoborohydride but also DCDO and/or D¹³CDO, as well as sodium cyanoborodeuteride^{30,33} (see Reagent Setup section for dimethylation solutions A and B). In addition, instead of triethylammonium bicarbonate (Sigma-Aldrich, cat. no. T7408), sodium acetate buffer (see 'Dimethylation solution' under Reagent Setup) can also be used.

21| Reconstitute the digested samples from Step 20 in 100 µl of 100 mM triethylammonium bicarbonate (Fig. 3a).

▲**CRITICAL STEP** Do not use any buffer or other reagents with free aliphatic amino groups, as they will also be participating in the reductive dimethylation process. Add 4 µl of 4% (vol/vol) of dimethylation solution A (heavy, intermediate or light formaldehyde solution, according to the required labeling), and then vortex the solution for 30 s.

22| Add 50 µl of dimethylation solution B (sodium cyanoborohydride or sodium cyanoborodeuteride solutions, depending on whether light, intermediate or heavy labeling is required), and then vortex for 30 s.

23| Mix the reaction for 1 h at room temperature by using a laboratory tube shaker/rotator.

24| Stop the reaction by adding 16 µl of quenching solution (1% (vol/vol) aqueous ammonia solution) and vortexing for 1 min.

25| Add 8 µl of FA to further quench the reaction and to acidify the sample for the subsequent SPE.

? TROUBLESHOOTING

26 | Clean up the dimethylated samples with SPE by using C18 Sep-Pak cartridge according to the manufacturer instruction. Condition the column with 3 × 1 mL of ACN and 3 × 1 mL of SPE loading solution. Load samples to the column. Wash samples with 4 column volume of SPE washing solution. Elute the peptides with 1 mL of SPE elution solution. Dry the samples by using SpeedVac in a 1.5 mL Eppendorf centrifuge tube.

▲**CRITICAL STEP** Different volumes of conditioning or washing buffer may be applied based on the size of C18 cartridge used in the experiments. Select the correct size of the cartridge according to the manufacturer's recommendation.

■ **PAUSE POINT** Store the dried samples at -20 °C until further usage.

27 | Reconstitute the dimethylated sample in 300 µL of 100 mM phosphate buffer (pH 7.5) using 1.5-mL Eppendorf centrifuge tube and add approximately ~500-fold molar excess of Na₂S₂O₄ (in 300 µL aqueous solution) over the protein content determined in Step 19.

Adjust the pH to about 8 by adding 5% (w/v) of aqueous NaOH solution. Rotate the mixture for 30 min on Labquake™ Tube Shaker/Rotator or similar equipment.

? TROUBLESHOOTING

28 | Quench the reaction by acidifying the solution with 5% AcOH (v/v) to pH 3 while incubating the reaction mixture for 30 min.

Part 2 - Sample preparation and SPAER enrichment: Chemoprecipitation by SPAER and subsequent release of the tagged species (Figure 3b and 3c) • TIMING ~ 1 d

29 | Increase the pH of the solution from pH 3 to ~5.5 with aqueous NaOH (5%, w/v) solution.

▲CRITICAL STEP The pH around 5 is optimal for the reaction between 3ATs and the immobilized 4FB-OSu that is stable at this pH.

30 | Measure out ~10 mg of SPAER (representing approximately 0.2 mmol/g loading) directly into a screw-cap spin column equipped with the larger frit at the bottom of the column. Make sure that the column plug for the spin column is in place.

▲CRITICAL STEP Follow the manufacturer's instruction to assemble the spin column set.

Figure 4 summarizes schematically the capture and release procedure described below.

31 | Transfer the reaction mixture with an automatic pipette from Step 29 (i.e., from the of the dimethylated 3AT-containing peptides) into the spin column containing SPAER. Close the spin column with the screw cap.

32 | Rotate the resultant slurry for 6–8 h by using Labquake™ Tube Shaker/Rotator or similar equipment.

33 | Remove the column plug and place the spin column into a 1.5-mL Eppendorf centrifuge tube and pellet SPAER by centrifugation at 1,500 g for 90 sec to collect the supernatant as the flow-through.

▲CRITICAL STEP Analyze the flow-through by LC–MS/MS. If the results reveal the presence of 3AT-containing peptides, perform a secondary enrichment (i.e., repeat Steps 29 to 32 above). In this case, use 4–6 h reaction time and, after pelleting SPAER, combine SPEAR beads from the primary and secondary enrichments.

34 | Replace the screw cap with Luer™-lok and remove the column plug. Thoroughly wash the beads and the spin column by slowly pushing thorough the washing solution, while gently applying vacuum (optional), to elute non-binding peptides and other synthetic impurities. Use the following solvents in the given order: 8 M freshly made urea in PBS; 2 M NaCl; 80% (v/v) ACN/H₂O containing 0.1% AcOH; ACN; DMF; DCM; MeOH and, finally, H₂O. For each washing solvent, use 4 × 5 mL.

▲CRITICAL STEP By slowly pushing the washing solvent through the beads, make sure to gently generate a slurry of the beads to thoroughly clean them.

35 | Replace the screw cap or close the Luer™-lok adaptor and dry the beads by placing the spin column on a stand connected to vacuum.

■ PAUSE POINT At this point, SPAER containing the immobilized aminotyrosine-containing peptides can be stored under nitrogen at -20°C for up to one week.

36 | Carefully transfer the dried SPAER into a 1.5-mL Eppendorf centrifuge tube.

37 | Treat the beads with 300 μL of 95% (v/v) TFA/ H_2O to facilitate the release of the captured peptides of interest.

38 | Rotate the resultant slurry by using Labquake™ Tube Shaker/Rotator or similar equipment for 30 min.

39 | Pellet the beads at 1,500 g for 90 s and collect the supernatant.

40 | Add another aliquot of 300 μL of 95% (v/v) TFA/ H_2O and repeat Steps 38 to 39.

▲CRITICAL STEP Keep the beads in case of insufficient cleavage.

41 | Combine the collected supernatants and evaporate the solvent under a gentle nitrogen stream to almost dry.

▲CRITICAL STEP Evaporation of the sample to complete dryness may lead to sample loss.

▲CRITICAL STEP While direct treatment of the beads with the acidic medium directly in the spin column (Step 37) to release of the tagged peptides is possible, we found strong polymer contamination (probably originating from the frit material) upon LC–MS/MS analysis.

! CAUTION TFA is corrosive; proceed with extra caution and use a fume hood. Also handle the flow-through carefully, because it contains the tagged peptides of interest.

■ PAUSE POINT The sample can be stored at -20°C until LC–MS/MS analysis.

? TROUBLESHOOTING

Part 3 LC-MS/MS analysis of the enriched and tagged nitropeptides • TIMING variable (dependent on the number of samples): Sample preparation

42 | Add 20 μL of H_2O to the sample from Step 41.

43 | Use C18 pipette ZipTip to clean up the sample. Condition the C18 ZipTip with 6 cycles of aspiration and dispensing 10 μL ($6 \times 10 \mu\text{L}$) ACN and, then, $6 \times 10 \mu\text{L}$ of 0.1% (v/v) TFA/ H_2O .

44 | Load the samples to ZipTip by $15 \times 10 \mu\text{L}$ aspiration and dispensing.

45 | Wash samples with $10 \times 10 \mu\text{L}$ of aspiration and dispensing using 5% (v/v) ACN/0.1% (v/v) TFA.

46 | Elute the peptides with $30 \mu\text{L}$ of aspiration and dispensing using 80% (v/v) ACN/0.1% (v/v) FA.

47 | Dry the cleaned-up sample under vacuum using SpeedVac to approximately $3 \mu\text{L}$ volume.

■ PAUSE POINT Add $\sim 10 \mu\text{L}$ of 0.1% (v/v) FA/ H_2O to the sample, and store it at -20°C until LC-MS analysis.

Part 3 LC-MS/MS analysis of the enriched and tagged nitropeptides: LC-MS/MS

48 | Run the samples according to the complexity and sensitivity of the enriched peptides. We recommend a longer gradient (e.g. 90–120 min for 0–50 % HPLC mobile phase B) after enrichment from complex samples (e.g., non-fractionated human plasma digest), or a short gradient (45 min) after enrichment from less complex samples (e.g., digest of a single nitrated protein).

Part 3 - LC-MS/MS analysis of the enriched and tagged nitropeptides: Data analysis • TIMING variable (dependent on the number of raw data files)

49 | Explore the LC-MS spectra using the Xcalibur's Qual Browser program to check quality of data. Extract peak lists with Bioworks (version 3.3, Thermo) for database search.

50 | Use search engines such as Mascot (Matrix Science), Sequest (Thermo), X!Tandem, or equivalent, to identify peptides by searching the National Center for Biotechnology (NCBI) or UniProt protein sequence database for the species of interest. Apply the following settings and options, when performing the searches: specify trypsin as the digesting enzyme with one missed cleavage allowed.

51 | Set parent-ion and fragment-ion mass tolerances to 25 ppm and 0.80 Da, respectively, when using a linear ion trap–FTMS hybrid instrument with MS/MS performed in the linear ion trap (Adjust these tolerances according to precursor- and product-ion mass accuracy attainable with the instrument and chosen MS/MS method).

52 | Set carbamidomethylation of cysteine (57.0215 Da), dimethylation of the N-terminus and Lys (28.0313 Da) and monomethylation of N-terminal proline, Pro (14.0157), as fixed modifications in the searches.

53 | Specify oxidation of methionine (15.99492 Da) and tagging of tyrosine (147.0320 Da) as variable modifications.

54 | For searching raw data files of discovery-driven control experiments, allow dimethylation, monomethylation and tagging (132.0211 Da) of the N-terminus and the Lys

side-chain as variable modifications; then, inspect hits for plausible matches to incomplete reaction products of the procedure.

55 | When searching the results from experiments utilizing heavy or intermediate reagent(s) in Steps 21 and 22, also permit the algorithm to consider dimethylation (monomethylation for Pro, P) along with the intermediate formaldehyde/NaBH₃CN (32.0564), heavy formaldehyde/NaBH₃CN (34.0631), and heavy formaldehyde/NaBD₃CN (36.0757) combinations, whichever applies to your chosen differential labeling scheme, as variable modifications.

56 | Use Scaffold (Proteome Software) or similar proteomics software suite to validate and filter MS/MS-based peptide and protein identifications. Accept peptide identifications at >95% probability or FDR of <1%.

? TROUBLESHOOTING

57 | Manually annotate the MS/MS spectra from enriched peptides to confirm the identification using theoretical CID-MS/MS fragment ion masses obtained from the MS-Product program (<http://prospector.ucsf.edu/>). Validation can be based on criteria we recommended previously¹⁹ (**Box 1**).

58 | For differentially dimethyl-labeled samples, obtain the relative quantitation directly from the comparison of extracted-ion chromatograms (XICs) for identified or target peptides^{33,33}. Alternatively, use software (e.g., MaxQuant, ProteoSuite, or similar) to perform bottom-up differential nitroproteomics in an automated fashion.

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 1.

ANTICIPATED RESULTS

The protocol utilizing SPAER-based chemoprecipitation is expected to remove or significantly diminish overwhelming amounts of non-nitrated species from complex biological samples, which would enable in-depth mass spectrometric analyses and the exploration of the nitroproteome. Hundreds of nitroproteins and modification sites could be identified by using today's mass spectrometers after *in vitro* protein nitration. The example summarized in Figure 5 shows the identification of Tyr-227 of hemopexin as a target for modification¹⁹, which we could not identify without the use of the enrichment procedure reported in this protocol. Optimization has included the introduction of hardware (screw-cap spin column; Figure 4) that facilitated a thorough washing of the SPAER after the immobilization of the dimethylated 3AT-containing peptides, which minimizes carryover. Anticipated enrichment factors estimating the degree of retaining the specifically modified over the removal of unmodified peptides are, depending on protein abundances and the extent of the targeted PTM, at least several hundred but may exceed ten thousand by the use of chemoprecipitation²⁴. A typical result showing relative quantification of a nitroprotein from two human plasma samples is shown in Figure 6³³.

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Box 1**Manual validation of MS/MS-based peptide identifications**

We recommend the use of MS-Product tool of ProteinProspector (available at <http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msproduct>) to annotate the CID-MS/MS spectra of dimethylated and tagged 3AT peptides. Enter “Dimethyl” in the **N Term** box of the web-form, and type the sequence from a search hit into the **Sequence** box using one-letter abbreviations for the amino acid residues. Include the mass of the tag (147.032) and side-chain modifications by appropriate names in parentheses (click the [±] sign above the entry boxes to see the available modifications, and enter exactly as shown in the list). For example, the sequence of #DY FMPC@PGR identified in Figure 5b should be entered as: DY(147.032)FMPC(Carbamidomethyl)PGR. Should the tryptic peptide contain carboxy-terminal lysine (K), type “(Dimethyl)” after K in the **Sequence** entry box. Leave the **C Term** box empty. Check the box after the Use instrument specific defaults to override ion types item, and set **Max charge** under the “Induce Fragmentation” button to the charge of the precursor, if known, and enter 3 if unknown. Select the appropriate type of mass spectrometer from the **Instrument** menu, below and, then, click the “Induce Fragmentation” button. Fragments are summarized in the Main Sequence Ions, All Sequence Ions and Theoretical Peak Table items. Click the [±] sign before the **Theoretical Peak Table** and match the list to the m/z of the fragments observed in the MS/MS spectrum queried (within production mass accuracy of the instrument used). You may consider the peptide identification valid, if it meets the following criteria¹⁹:

- (A) The modified peptide has more than seven amino acid residues in the sequence.
- (B) Theoretical precursor m/z is within mass accuracy attainable by the instrument.
- (C) MS/MS peaks with >20% relative abundance and six of top ten peaks are annotated.
- (D) The dimethyl-labeled non-nitrated peptide is identified from the first flow-through (supernatant) of Step 30.
- (E) The annotated MS/MS peaks conform to rules of peptide fragmentation using CID. Although no rules have been established for the fragmentation of dimethylated and tagged 3AT-containing peptide sequences, several can be adapted from those applied to automated expert validation of phosphopeptide MS/MS-spectrum matches (ProPhosSI)⁵⁵:
 - (i) Four sequential b- or y-ions are observed.
 - (ii) Five of six sequential ions are present.
 - (iii) Distinctive fragmentation pattern around Pro, if found in the sequence, are observed (If there is no Pro, this test automatically passes). This rule recognizes that cleaving the imino bond N-terminal to the residue should be one of the major peaks in the CID-MS/MS spectrum, while fragmentation of the C-terminal amino bond of Pro should be very weak. With more than one Pro in the sequence, this

criterion is met if at least one of the Pro residues has the expected fragment-intensity distribution.

(iv) Tag-specific mass differences between the series ions on either side of the modified Tyr are confirmed (which reflects that the tag is presumed not labile under low-energy CID conditions; caution: high-energy CID has not been tested).

The CID-MS/MS spectrum of #DY FMPC®PGR (from the doubly-protonated precursor, m/z 659.2767) shown in Figure 5 is an excellent example for meeting all criteria (i)–(iv) above.

Figure 1. Posttranslational protein nitration

Posttranslational nitration modifies (a) tyrosine¹, forming 3NT, and (b) tryptophan residues (sites of possible modifications are numbered⁷). RNS, reactive nitrogen species

Figure 2.

Reaction scheme for the preparation of SPAER. Steps: **(a)** Succinylation of aminopropyl-modified controlled pore glass (CPG-C₃H₇-NH₂) with succinic anhydride (SA); **(b)** Fmoc-hydrazide formation *via* N-hydroxysuccinimide active ester *in situ* generated from N-hydroxysuccinimide (HOSu) and N,N-diisopropylcarbodiimide (DIC) followed by coupling with 9-fluorenylmethyl carbazate (Fmoc-NH-NH₂); **(c)** Removal of Fmoc protecting group with piperidine generates CPG-hydrazide; **(d)** Condensation of this immobilized hydrazide reagent with 4-formylbenzoic acid N-hydroxysuccinimide active ester (4FB-OSu) produces SPAER. The red-colored group is the active ester (AE) function, while the reaction of magenta-colored groups results in the immobilization of the AE on the surface of the CPG as hydrazide.

Figure 3.

Schematic illustration of the SPAER-based enrichment procedure. This involves **(a)** pre-enrichment derivatizations, **(b)** chemoprecipitation by covalent capture of the dimethylated and reduced nitropeptides and removal of non-nitro species, and **(c)** producing a solution of tagged peptides by acid-catalyzed hydrolysis.

Figure 4.

Scheme illustrating Steps 30 to 41 of the protocol. (Red arrow: addition of SPAER; blue arrow: transfer of solution or suspension; thin blue arrow: remove or replace; straight block arrow: move to next procedure; curved block arrow: vortex or rotate; curved and double-headed block arrow: shake.)

Figure 5.

Example of anticipated results²¹: LC-ESI-MS base-peak chromatograms of tryptic digest from *in vitro* nitrated human plasma proteins (a) before and (b) after SPAER-based enrichment. The latter shows significantly reduced sample complexity thereby permitting the identification of, e.g., Tyr-227 of hemopexin as a target for nitration. Without enrichment, DY*FMPC@PGR (where Y* represents 3NT and C@ denotes carbamidomethylated Cys) was most likely suppressed and/or not selected for data-dependent CID-MS/MS acquisition. This was due to overwhelming matrix of non-nitro peptides burdening the instrument's productive working cycles thereby eluding identification of this low-abundance nitropeptide. On the other hand, the enriched peptide #DY FMPC@PGR (where Y denotes the tagged 3AT obtained from 3NT after enrichment and # indicates dimethylation) was the major constituent of the chromatographic peak eluting at 51.8 min, when post-run extracted-ion chromatogram (XIC) was retrieved to determine its retention time (RT). This resulted in the acquisition of CID-MS/MS spectrum from the doubly-protonated precursor m/z 659.2767, which was matched to the dimethylated and tagged peptide upon searching a human protein database using both Mascot and SEQUEST algorithms. Annotation and inspection of the MS/MS spectrum validated the identification of #DY FMPC@PGR.

Figure 6.

Example for relative quantification by light and heavy dimethyl labeling via the SPAER-based nitropeptide enrichment³³: Nitroubiquitin⁵⁰ was added to human plasma and, after tryptic digestion, the peptides were labeled in two samples differentially by reductive dimethylation using HCHO/NaBH₃CN and D¹³CDO/NaBD₃CN, respectively, as reagents. After enrichment, the relative concentrations of the tagged light (blue shading and line) and heavy dimethyl-labeled #TLSDY NIQK# (red shading and line) reflected nitroubiquitin concentrations accurately both in the (a) XICs and in the (b) narrow-range ESI-FTMS spectrum obtained through averaging over their elution period. No chromatographic isotope effect was observed.

Table 1

Troubleshooting table.

Step	Problem	Possible Reason	Solution
18	SPAER synthesis was unsuccessful.	Forgot to remove the Fmoc protecting group in Step 12 after coupling Fmoc-NH-NH ₂ to the succinylated beads	Perform Fmoc removal with freshly made piperidine /DMF (20% v/v) solution for 20 min. Then repeat cleavage with a fresh aliquot of the cleavage solution.
25	Dimethylation is not complete.	Residual iodoacetamide is present in the sample Other contaminants containing primary amino groups are present in the sample	Perform SPE after protein digestion. Increase the concentration of the reagents.
27	Reduction of 3NTs is not complete.	The pH varies after adding Na ₂ S ₂ O ₄	Add Na ₂ S ₂ O ₄ in three aliquots (3 × 100 µL) while maintaining (adjusting) the pH at (to) ~8 each time.
56	No tagged peptides detected.	The pH was not optimal for the covalent capture of the dimethylated 3ATs on the SPEAR (Step 32) The washing process was insufficient before removing the tagged species from SPAER TFA cleavage is not complete. During evaporation of the sample too strong nitrogen stream was used that “blew away” the sample.	Adjust the pH of the reaction medium around 5 during covalent capture of 3ATs (Step 29) Follow carefully the washing protocol as described under Step 34 TFA cleavage should be at least 30 min. Freshly make 95% TFA/H ₂ O solution. Repeat the cleavage with another aliquot of cleavage solution Be careful when you remove the solvent with a gentle stream of nitrogen to prevent sample loss.
56	Very small number of modified peptides identified after enrichment.	Low abundance of nitropeptides in the sample.	Increase sample quantity for Step 21

• TIMING

Part 1 (Steps 1-18), synthesis of SPAER: ~ 1d

Part 2 (Steps 19 and 20), proteolytic digestion: ~1 d (several samples can be digested in parallel)

Part 2 (Steps 21–28), pre-enrichment derivatization: 2 d (several samples can be processed in parallel)

Part 2 (Steps 29-41), chemoprecipitation and enrichment: ~ 1.5–2 d

Part 3 (Steps 42–47), sample preparation for LC–MS analyses: approximately 1–30 h (depending on qualitative or quantitative experimental design were used)

Part 3 (Step 48)), LC–MS analyses: approximately 4 h – several days (depending on qualitative or quantitative experimental design were used)

Part 3 (Steps 49–58), data analysis: 1 d to 1 week (depending on the purpose and scale of the experiments, as well as computer hardware and the software package used)