Selective Acylation of Primary Amines in Peptides and Proteins

N-hydroxysuccinimide (NHS) esters are derivatizing agents that target primary amine groups. However, even a small molar excess of NHS may lead to acylation of hydroxyl-containing amino acids as a side reaction. We report a straightforward method for the selective removal of ester-linked acyl groups after NHS ester-mediated acylation of peptides and proteins. It is based on incubation in a boiling water bath and does not require a change in pH or the addition of chemicals. It is therefore particularly suited for proteomics samples that are often small in volume and contain low amounts of peptides. The method was optimized and evaluated with two peptides and one protein that were acetylated at a high excess of NHS-acetate. While the large molar excess resulted in complete acylation of all primary amines, hydroxyl-containing amino acids were shown to react as well. By incubating the peptide or protein solutions in a boiling water bath, acetyl-ester bonds were hydrolyzed, whereas acetyl-amide bonds remained stable. The reaction was also performed in 6 M guanidine-HCl, which prevented protein precipitation. In conclusion, the present method allows the selective acylation of primary amines by NHS esters and constitutes a valuable alternative to the treatment with hydroxylamine under alkaline conditions.

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1. **INTRODUCTION**

Derivatization of primary amines in proteins and peptides is widely used for purposes such as fluorescent labeling or biotinylation. Primary amines are found in essentially all proteins and are easily derivatized when non-protonated due to their nucleophilicity. N-hydroxysuccinimide (NHS) esters have been widely used as activated derivatives of carboxylic acid-containing reagents. They are reactive towards primary amines and are generally considered to be amine-specific. NHS ester-mediated derivatization for protein modification has been first described by Becker et al., who studied biotin transport in yeast. Numerous other applications have been developed subsequently, e.g. to enhance amino acid and peptide detection by electrospray ionization mass spectrometry or to do cross-linking experiments. A recently developed, NHS ester-based strategy for isobaric, stable isotope labeling of peptides (commercialized as iTRAQ) is finding more widespread applications in proteomic studies.

Reaction conditions for peptide or protein modification using NHS ester reagents have been well defined. Indeed, techniques aiming at labeling specifically certain classes of amines have been reported (α-amines rather than ε-amines, or aromatic amines rather than aliphatic amines). Other reports have studied the kinetics and the stoichiometry of protein modification with biotinyl NHS ester (NHS-biotin), which is the most commonly used biotinylation reagent.

Despite their widespread use as amine-specific reagents, some reports drew attention to the fact that the phenolic OH-group of tyrosine can also react. Furthermore, Miller et al. have shown that, under aqueous conditions, NHS-ester mediated acylation of hydroxyl-containing amino acid residues (serine, threonine and tyrosine) located two positions at either side next to a histidine residue may be a significant side reaction, which can lead to stable derivatives under conditions normally used for protein modification. Since esterification of aliphatic or phenolic hydroxyl groups cannot be completely avoided using NHS ester reagents, it has been suggested to specifically remove O-linked acyl groups with concentrated hydroxylamine at alkaline pH. However, this method may lead to unwanted side reactions such as the cleavage of peptides bonds and modifications of amides. Furthermore, adding a chemical reagent at high concentrations may introduce contaminants, which can interfere with protein and peptide identification. Finally, adjusting the pH is not an easy task in the small volumes that are generally encountered in proteomics experiments.

To facilitate the workflow of typical proteomics experiments, we therefore sought to simplify the specific removal of O-linked acyl groups from peptides. This can be achieved by heating the sample in a boiling water bath for up to 60 min at the pH and buffer conditions that are widely used for both trypsin digestion and NHS ester-mediated acylation. The effectiveness of ester hydrolysis under these conditions was evaluated by LC-MS and LC-MS/MS under conditions of a high molar excess of NHS-acetate (500-fold) as a “worst case scenario”. To evaluate our method, we used two synthetic peptides that allow to monitor acylation and deacylation reactions of N-terminal α-amines and lysine ε-amines in the presence of amino acids containing “activated” hydroxyl groups, located
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two positions next to a histidine residue. Acylation and deacylation of tyrosine in an “activating” or “non-activating” position was also studied. Finally we applied our technique to a protein in presence of 6 M guanidine-HCl. Our method is particularly suitable for proteomics approaches that involve a proteolytic digestion step prior to labeling and peptide separation (e.g. the shotgun approach or the iTRAQ labeling method).

2. MATERIALS AND METHODS

2.1 Materials and reagents
Acetic acid N-hydroxysuccinimide ester (NHS-acetate) was from MP Biomedicals (Illkirch, France), dimethyl sulfoxide (DMSO), sodium dihydrogen phosphate monohydrate and di-sodium hydrogen phosphate dihydrate were from Merck KGaA (Darmstadt, Germany). HPLC-S gradient grade acetonitrile was from Biosolve (Valkenswaard, the Netherlands), angiotensin II human acetate, myoglobin from equine heart, guanidine hydrochloride, PMSF were from Sigma (Zwijndrecht, the Netherlands), endoproteinase Glu-C was from Roche Diagnostics GmbH (Mannheim, Germany) and (D-Lys6)-luteinizing hormone-releasing hormone (K6-LHRH) trifluoroacetate salt was from Bachem (Weil am Rhein, Germany). Ultra-pure water (conductivity: 18.2 MΩ) was obtained from a Maxima System (Elga Labwater, Ede, the Netherlands).

2.2 Acetylation and deacetylation of peptides
A stock solution (100 mM in DMSO) of NHS-acetate was prepared and added at a final concentration of 5 mM to 10-µM solutions of peptide (angiotensin II or K6-LHRH) in 100 mM sodium phosphate buffer, pH 8. Samples were incubated for 2 h at 25°C, 450 rpm in a Thermomixer (Eppendorf, Hamburg, Germany) and subsequently treated for variable time periods (0, 15, 30, 45 or 60 min) in a boiling water bath. Finally they were diluted with an equal volume of 1% (v/v) formic acid and analyzed by liquid chromatography – mass spectrometry (LC-MS).

2.3 Acetylation and deacetylation of myoglobin
Horse heart myoglobin was diluted at 2 mg/mL in 6 M guanidine-HCl, 100 mM sodium phosphate buffer, pH 8 and was subsequently incubated for 5 min in a boiling water bath to insure complete protein denaturation. A stock solution (500 mM in DMSO) of NHS-acetate was prepared and added at a final concentration of 25 mM to the myoglobin-containing solutions. Samples were incubated for 2 h at 25°C, 450 rpm in a Thermomixer and subsequently treated for variable times (0, 15, 30, or 60 min) in a boiling water bath. Samples were subsequently diluted 6-times with 100 mM sodium phosphate, pH 8 and endoproteinase Glu-C was added to a ratio of 1/142 (Glu-C/myoglobin) by weight. Digestion was allowed for 18 h at 25°C, 450 rpm in a Thermomixer. Subsequently 1 mM PMSF and 1% (v/v) formic acid were added to stop the digestion prior to LC-MS analysis.
2.4 LC-MS

The HPLC part of the analytical system consisted of an Agilent series 1100 capillary LC system (Waldbronn, Germany) comprising a degasser, a binary pump with stream splitter and flow controller, a thermostated autosampler (4°C) and a thermostated column compartment (28°C for the analysis of the synthetic peptides and 40°C for the myoglobin digest). Chromatographic separation took place in an Atlantis dC18 column (Waters, Etten-Leur, the Netherlands; 1.0 mm I.D. x 150 mm length, particle size 3 µm) operated at 20-µL × min⁻¹ flow rate. Mobile phase A consisted of 0.1% (v/v) formic acid in ultrapure water. Mobile phase B was 0.1% (v/v) formic acid in acetonitrile.

For the analyses of peptides (angiotensin II and K⁶-LHRH) an injection volume of 4 µL (20 pmol) was used and the separation was performed with an increasing gradient of B (2-60% at 1% × min⁻¹). For myoglobin digests the injection volume was 1 µL (18 pmol), and the gradient slope of B was reduced (2-60% at 0.5% × min⁻¹).

The analytes were detected by an Agilent SL ion-trap mass spectrometer equipped with an electrospray ionization source operated in positive mode. MS data were acquired over a scan range 300-1200 m/z (angiotensin II and K⁶-LHRH) or 200-1200 m/z (myoglobin digest) in MS mode. Peptide analyses in MS/MS mode were exclusively performed on doubly-charged ions with a scan range of 50-1200 m/z.

All extracted ion chromatograms (EICs) were retrieved with a tolerance of ± 0.2 Da based on the major charge states observed after derivatization (i.e. doubly-charged ions for angiotensin II and triply-charged ions for K⁶-LHRH). All displayed chromatograms underwent one cycle of smoothing based on a Gaussian filter (window size: 0.7 s).

3. RESULTS

In order to investigate the specificity of NHS esters towards primary α-amines and phenolic hydroxyl groups in a potentially reactive position two amino acids next to a histidine residue, angiotensin II (Asp-Arg-Val-Tyr*-Ile-His-Pro-Phe) was acetylated with NHS-acetate at pH 8. A large molar excess of NHS-acetate was used (500-fold) to assure complete acetylation of all primary amines. All derivatives were identified by tandem MS analysis (data are presented under Supporting Information, numbered as Figures S-1, S-2 etc.). LC-MS analysis showed that no residual non-acetylated peptide remained (A-II; Fig. 1A, 2nd panel).

Despite the fact that the expected singly N-acetylated peptide (A-II₁, 31.7 min; Fig. 1A, 3rd panel and Fig. S-1 in Appendix 1 for MS/MS spectra) is clearly visible, most angiotensin II was observed as the doubly, N-,O-diacetylated form (A-II₂, 34.0 min; Fig. 1A, 4th panel). Since angiotensin II does not contain any lysine residues, this second acetylation cannot be amine-specific and the acetyl group was located by MS/MS to reside on the tyrosine residue (Fig. S-2 in Appendix 1). Surprisingly, a triply-acetylated peptide was also observed (A-II₃, 34.6 min; Fig. 1A, 5th panel) and identified by MS/MS as being modified at the arginine residue (Fig. S-3 in Appendix 1) showing for the first time that...
arginine can also react with NHS esters under aqueous conditions. In order to investigate whether the hydrolytically more labile phenol ester and the arginine modification could be removed while maintaining the primary amide bond, the reaction mixture was treated for 15 min in a boiling water bath (Fig. 1B). Under these conditions almost all A-II3 had disappeared (Fig. 1B, 5th panel) and A-II1 was significantly increased (Fig. 1B, 3rd panel). The peak corresponding to A-II2 at 34.0 min (Fig 1B, 4th panel) was also much smaller indicating that hydrolysis of the ester bond was proceeding much more rapidly than hydrolysis of the amide linkage. However, an additional minor earlier eluting peak (32.3 min) with the same m/z value as A-II2 and an indistinguishable MS/MS spectrum was observed (Fig. S-4 in Appendix 1). From these results we deduce that this by-product is an isomer of A-II2, but its identity remains to be established. Increasing the boiling time reduced the intensity of A-II2 and the putative isomer to a level at which they were no longer detectable after 45 min, indicating that the O-acyl group had been completely removed (Fig. 1C-E, 4th panel). Importantly, acetylation of the N-terminus was stable to this thermal treatment, since only a very small amount of non-acetylated peptide (A-II, 28.3 min; Fig. 1E, 2nd panel) was detected after 60 min boiling (the peak observed at 31.2 min is related to a compound with a mass to charge ratio (m/z) that is within ± 0.2-Da of the doubly-charged A-II. MS/MS data and the longer retention time show, however, that it is not A-II. Some spectral similarities indicate that it might be a yet unidentified A-II derivative (data not shown)).

In order to study whether our approach is also functional in the case of aliphatic amino acids with a primary hydroxyl group in an “activating” position, a synthetic agonist of luteinizing hormone-releasing hormone (K6-LHRH; <Glu-His-Trp-Ser*-Tyr-D-Lys-Leu-Arg-Pro-Gly-NH2) was reacted with a 500-fold molar excess of NHS-acetate at pH 8. K6-LHRH contains furthermore a D-lysine residue with a primary ε-amine, a tyrosine residue and an arginine. The N-terminus of the peptide is blocked through formation of pyroglutamic acid and the C-terminal carboxylic acid is amidated. Analysis of the originally obtained K6-LHRH with incubation for 60 min in a boiling water bath showed the apparition of a minor peak eluting about one minute later than the main peak (see for example Fig. 2A, 1st panel) resulting in doublets for all reaction products. The compound corresponding to this smaller peak was one Dalton heavier than the parent compound and MS/MS analysis indicated that it corresponded to K6-LHRH with a free carboxylic acid group at the C-terminus (data not shown). This shows that limited deamidation of the C-terminus occurred when samples were incubated at 100°C for 1 h. After incubation of K6-LHRH with NHS-acetate there was no underivatized peptide detectable (Fig. 2A, 2nd panel). Singly, lysine-(N-)acetylated peptide (K6-LHRH1, 28.2 min; Fig. 2A, 3rd panel) was detected but in negligible quantity. Doubly-acetylated peptide (K6-LHRH2, 29.3 min; Fig. 2A, 4th panel) was observed and by MS/MS (Fig. S-5 in Appendix 1) shown to be modified at the lysine and the serine residues. Most of K6-LHRH was triply-acetylated (K6-LHRH3, 32.0 min; Fig. 2A, 5th panel and Fig S-6 in Appendix 1) on lysine, serine and the tyrosine residue, even though it is not in an activating position two positions next to a histidine. A barely detectable peak corresponding to a 4-times acetylated peptide was also observed (K6-LHRH4, 32.4 min; data not shown). MS/MS data indicated that arginine was again modified under aqueous conditions as observed for angiotensin II (Fig. S-7 in Appendix 1). After 15 min in a boiling water bath (Fig. 2B), K6-LHRH3 disappeared almost completely indicating fast hydrolysis of the phenolic ester bond (Fig. 2B, 5th panel).
Figure 1: LC-MS analysis of acetylated angiotensin II and its treatment in a boiling water bath to remove O-acetyl groups.

(A) no treatment; (B) 15 min; (C) 30 min; (D) 45 min; (E) 60 min. From top to bottom: Base Peak Chromatogram (BPC); Extracted Ion Chromatograms (EICs) corresponding to doubly-charged ions of angiotensin II containing respectively 0 (A-II, 28.3 min, 523.9 m/z), 1 (A-II1, 31.7 min, 545.0 m/z), 2 (A-II2, 34.0 min, 566.0 m/z) and 3 (A-II3, 34.6 min, 586.9 m/z) acetyl groups. As shown in A, panels are presented according to different intensity scales. For each panel an identical scale has been used throughout A-E.
Figure 2: LC-MS analysis of acetylated K⁶-LHRH and its treatment in a boiling water bath to remove O-acetyl groups.

Results presented after 0 (A), 15 (B), 30 (C), 45 (D) and 60 (E) min of treatment prior to analysis. For each set of chromatograms, are presented from top to bottom: the BPC and the EICs corresponding to triply-charged ions of K⁶-LHRH containing respectively 0 (K⁶-LHRH, 23.0 min, 418.8 m/z), 1 (K⁶-LHRH₁, 28.2 min, 432.7 m/z), 2 (K⁶-LHRH₂, 29.3 min, 446.8 m/z) and 3 (K⁶-LHRH₃, 32.0 min, 460.8 m/z) acetyl groups. The bottom panel corresponds to the triply-charged ion of singly-acetylated K⁶-LHRH lacking a water molecule (K⁶-LHRH₁*, 29.4 min, 426.7 m/z). For each panel an identical scale has been used throughout A-E.
K⁶-LHRH₁ and K⁶-LHRH₂ were still observed (Fig. 2B, 3rd and 4th panels and Fig. S-8 in Appendix 1), the former being now the major species. Another derivative with the mass of K⁶-LHRH₁ minus a water molecule (K⁶-LHRH₁*, 29.4 min; Fig 2B, 6th panel) appeared during hydrolysis and partially co-eluted with K⁶-LHRH₂. MS/MS analysis (Fig. S-9 in Appendix 1) confirmed that hydrolysis of acetylated serine resulted in the partial β-elimination of water to give dehydroalanine, a reaction that has been described in the literature in conjunction with the analysis of phosphoserine under basic conditions during automated sequence analysis by Edman degradation, during phosphopeptide enrichment or during MS/MS analysis. Beta-elimination has also been used for mass spectrometric determination of O-glycosylation sites in glycoproteins. After 60 min in a boiling water bath (Fig. 2E) the singly-acetylated derivatives K⁶-LHRH₁ and K⁶-LHRH₁* (Fig. 2E, 3rd and 6th panels, respectively) were by far in the majority showing that this treatment does result in specific removal of O-acylation products on serine and tyrosine with only a limited effect on the primary amide at the C-terminus or the amide bond linking the acetyl group to the lysine residue. A very small amount of K⁶-LHRH₂ (less than 1% of the initial amount) was still detectable after 60-min boiling (Fig. 2E, 4th panel) but no non-acetylated peptide was observed (Fig. 2E, 2nd panel). The amount of K⁶-LHRH₁* remained practically unchanged between 15 and 60 min of incubation (Fig. 2B-E, 6th panel) but its occurrence needs to be considered in search algorithms for protein identification.

In order to extend our approach to whole proteins, it was necessary to evaluate whether denaturating reagents that prevent protein precipitation upon boiling, such as 6 M guanidine-HCl would interfere with the reaction. Horse heart myoglobin was dissolved in 6 M guanidine-HCl, 100 mM sodium phosphate, pH 8 followed by addition of NHS-acetate to a final concentration of 25 mM (molar ratio NHS-acetate/protein: 232; molar ratio NHS-acetate/primary amines: 12). Horse heart myoglobin contains 20 primary amines (19 lysines and the N-terminus), 4 hydroxyl-containing residues located at two positions next to a histidine residue, which are putative targets for NHS-acetate (T₃₅,G₃₆,H₇₇, H₈₅,G₆₆,T₉₇, H₉₄,A₉₅,T₉₆,K₉₇,H₉₈, S₁₈,K₁₁₉,H₁₂₀), and 2 tyrosines (Y₁₀₄ and Y₁₄₇). After incubation at 25°C for 2h and subsequently in a boiling water bath for variable lengths of time, myoglobin was digested with endopeptidase Glu-C, since acetylated lysines are no longer recognized by trypsin, which would result in very large peptide fragments. The digest was analyzed by LC-MS (Fig. 3) and the identity of a selected number of modified peptides was confirmed by tandem MS (Appendix 1).

All lysines were acetylated (results not shown), but several peptides containing sensitive residues (tyrosines and/or hydroxyl-containing amino acids located at two positions next to a histidine residue) reacted as well, as indicated by a number of additional peaks. Most of these peaks diminished considerably or disappeared completely after 15 min of incubation in a boiling water bath. Some peaks increased due to the removal of ester-bonded acetyl groups.

Extending the boiling step to 30 or 60 min further hydrolyzed remaining acetyester bonds and there was only a very small amount of one peptide containing an acetyester left after 60 min boiling (Fig. 3D, 58 min). MS/MS analysis showed that this peptide was acetylated at a tyrosine residue that was not located two residues next to a histidine residue (Fig. S-11 in Appendix 1). Boiling for 60 min resulted in an increase in
intensity of 3 peptides due to the oxidation of a methionine residue as confirmed by their MS/MS spectra (Fig. 3D, 61.5, 62.6 and 68.6 min, respectively Fig. S-12 to S-14 in Appendix 1). Of note is that one of them had a mass corresponding to one remaining acetylation (located between D$_{110}$ and A$_{128}$, likely on S$_{118}$ since this residue is at two positions next to H$_{120}$) despite the extensive boiling time (Fig. 3D, 62.6 min, Fig. S-13 in Appendix 1). This result, together with the previous observation, raises the possibility that the rate of ester hydrolysis is somewhat lower in presence of guanidine-HCl. While deamidations of glutamine or asparagine residues may be difficult to detect due to the limited mass resolution of the ion trap, especially in the case of multiply-charged ions, the observation that the peptide eluting at 81.3 min (Fig. 3) is stable in terms of retention time, mass and intensity, despite the fact that it contains 2 glutamine and 1 asparagine residues, indicates that deamidation is not a general phenomenon under the reaction conditions (Fig. S-15 in Appendix 1).

**Figure 3:** LC-MS analysis of a Glu-C digest of acetylated horse heart myoglobin followed by treatment in a boiling water bath to remove O-acetyl groups. Results after 0 (A), 15 (B), 30 (C), and 60 (D) min boiling prior to Glu-C digestion and analysis are shown. In panel A, peaks of which the intensity increased or decreased after 15-min in a boiling water bath are depicted by arrows (↑ and ↓, respectively). In panel D, peaks of which the intensity increased or decreased between 15 and 60 min of incubation in a boiling water bath are depicted by arrows (↑ and ↓, respectively). Chromatograms are represented as Base Peak Chromatograms (BPCs) on an identical intensity scale.
4. DISCUSSION

Despite the fact that NHS esters have been previously described as primary amine-specific reagents,^{4-7} Miller et al. showed early on (i.e. with the Bolton-Hunter reagent$^{27}$ or NHS-biotin and its derivatives$^{28-31}$) that NHS ester-mediated acylation may also occur on hydroxyl-containing amino acid residues (serine, threonine, and tyrosine) under reaction conditions normally employed for the acylation of primary amines. Significant O-acylation was even observed within 10 min with only a 4-fold molar excess of NHS ester over primary amine groups,$^{29}$ whereas a 10-fold molar excess is recommended for quantitative acylation of all amino groups.$^{40}$ OH-groups were shown to exhibit an increased reactivity towards NHS esters when located two positions next to a histidine residue most likely due to hydrogen bonding between the histidine imidazole nitrogen and the hydroxyl hydrogen thereby increasing the nucleophilicity of the oxygen atom.$^{30}$ Based on the natural occurrence of individual amino acids in proteins,$^{41}$ a statistical estimate can be calculated showing that such a constellation may occur once every 142 amino acids (see note S-10 in Appendix 1). Moreover, tyrosine residues (average occurrence in proteins: 3.2%)$^{41}$ have been shown to be intrinsically nucleophilic enough to react with NHS esters, even when not located in an activated position.$^{10, 23-26}$ We have recently observed with a pentafluorophenyl active ester (data not shown) that O-acylation is a quite general phenomenon when working with activated esters to derivatize primary amines. Thus O-acylation is by no means a rare event and its selective removal is critical if amine-specificity is intended.

Since it is impossible to completely prevent O-acylation when using NHS ester reagents, it is critical to be able to remove such modifications selectively, if amine-specificity is required. Hydroxylamine has been shown to react with short chain carboxylic acid esters under alkaline conditions through the reaction: $\text{RCOOR'} + \text{NH}_2\text{OH} \rightarrow \text{RCONHOH} + \text{R'}\text{OH}$.$^{42}$ It has consequently been used for the hydrolysis of O-linked acyl groups resulting in restoration of the free alcohol.$^{43}$ It was shown that, in the presence of hydroxylamine, esters of tyrosine could be hydrolyzed at neutral pH but that the esters of serine and threonine required a strongly alkaline pH.$^{32}$ Riordan and Vallee$^{32}$ advised a pH above 10.5, yet Miller et al.$^{28, 31}$ showed that milder pH conditions could be used for all esters (pH 9.2) though at the expense of longer hydrolysis times (4-5 h). Disadvantages of hydrolysing aliphatic and phenolic esters when applying this method are (i) the use of marked alkaline pH conditions, (ii) a fairly high concentration (in the molar range) of hydroxylamine hydrochloride, (iii) a relatively long reaction time, and (iv) the fact that hydroxylamine is a harmful chemical, which may promote further protein modifications such as the non-enzymatic cleavage of asparagine-glycine bonds$^{44-46}$ or of peptide bonds involving proline.$^{46}$ Beside the possible spontaneous, non-enzymatic deamidation of asparagine and glutamine to aspartatic or glutamic acid, or an isoform under alkaline conditions (that we experienced ourselves with our approach), hydroxylamine can further promote the conversion of these amino acids into hydroxamic acids.$^{45-46}$

Our experiments studying the acylation of tyrosine in its free amino acid form (data not shown) emphasized the importance of the pH and of the acyl leaving group on both the reactivity of the NHS esters and the subsequent hydrolysis kinetics. For instance
lowering the pH to 7.2 or using NHS-biotin instead of NHS-acetate slowed down both O-acetylation and O-acetyl ester hydrolysis. However, complete deacylation was achieved in each case using the described method.

Our method can be applied to peptides in a simple phosphate buffer, since peptides will not precipitate upon incubation in a boiling water bath. A process involving: i) NHS-mediated acylation of whole proteins, ii) protein digestion with an endoprotease such as trypsin or Glu-C and iii) removal of O-acylation following the method presented in the current report is feasible, since all steps share the same optimal buffer conditions (phosphate buffer, pH 8). The described approach is also applicable to whole, non-digested proteins, although 6 M guanidine-HCl has to be added to prevent precipitation during boiling. Denaturing proteins prior to acylation has the additional advantage that all residues are available for complete acylation, prior to selective deacylation.

In summary, we describe a simple and straightforward method to render the labeling of peptides and proteins with NHS ester-activated reagents primary-amine specific. Our method requires neither addition of chemicals nor readjustment of pH. The reaction sequence of acylation and hydrolysis in a boiling water bath under acylation conditions ("one-pot reaction") is particularly amenable to the small volumes and low amounts of peptides encountered in proteomics studies. Some side reactions, that may also be expected when peptides are incubated under alkaline conditions (i.e. β-elimination on substituted serine, limited deamidation), were observed upon treatment in a boiling water bath but they will not affect protein identifications if taken into account during database searching.
5. REFERENCES


