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# Impact of Multiple S-Palmitoylation on Peptide Ionization and Fragmentation in Mass Spectrometry

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Lipidation, such as S-palmitoylation, is an important, reversible post-translational modification of proteins determining not only their stability and folding but also their interactions with other proteins or membranes. However, in contrast to other post-translational modifications, lipidation is less explored, and lipidated proteins are underrepresented in large-scale studies. To advance the analysis of S-palmitoylation by mass spectrometry (MS), a model peptide containing four potential modification sites is selected. By selectively introducing S-palmitoylation, a set of multiply modified peptides is generated, differing in the sites as well as the degree of modification. Importantly, the solubility of the peptides decreased tremendously with increasing degree of modification, requiring the use of alternative solvents. Nonetheless, using direct-infusion MS, the ionization and fragmentation behavior of the differently modified peptides is characterized. Lipidation is found to be stable during tandem MS, and the sites of modification can be unambiguously identified. The use of dimethyl sulfoxide during electrospray ionization further improves the signal intensity of multiply modified peptides. In summary, the identification of S-palmitoylation even in multiply modified peptides is possible; however, further improvements are required for large-scale analyses.

## 1. Introduction

Liquid chromatography (LC)-coupled tandem mass spectrometry (MS/MS) is a versatile analytical technique allowing the identification of proteins and their post-translational modifications, even in complex mixtures, and including the sites of modification.[1] While the identification of post-translational modifications such as phosphorylation or acetylation is well-established, lipidation of peptides is less explored.[2] Reasons might be the high mass of the modification or the increased hydrophobicity of the modified peptides; importantly, the hydrophobicity increases even further when more than one amino acid side chain is lipidated.

Lipidation typically includes the covalent modification of proteins by fatty acids, isoprenoids, or phospholipids at nucleophilic side chains (e.g., of cysteine, serine, or lysine) or at the proteins' N-termini. Modifications by sterols and glycosylphosphatidylinositol anchors typically occur at the proteins' C-termini.[3,4] The increase in hydrophobicity of the modified proteins alters their folding, stability, as well as their interactions with other proteins and phospholipid membranes. Consequently, these changes affect the proteins' subcellular localization, signaling networks, and enzymatic activity.[3]

Here, we focus on cysteine palmitoylation (S-palmitoylation), a thioester-linked 16-carbon palmitate group attached to cysteine residues that can be hydrolyzed enzymatically or nonenzymatically, making this modification reversible. S-palmitoylation is usually located within or in proximity of the transmembrane domain of integral membrane proteins, or it anchors soluble proteins to a membrane. Importantly, palmitoylation is not associated with a specific consensus sequence, making its prediction not trivial.[4]

Over the past decades, several methods for the analysis of S-palmitoylated proteins have been established. One of the first methods required the use of radioactive isotope-labeled lipids;[5] however, potential hazards and sensitivity issues due to missing enrichment strategies for radiolabeled proteins and peptides limit its application.[6] To circumvent these issues, bioorthogonal probes containing terminal azido or alkyne derivatives of lipids have been introduced.[7] Nonetheless, both methods are based on metabolic labeling and only provide information on whether a protein is lipidated or not; i.e., information on the modified sites is not accessible. Moreover, structural differences between probes and endogenous lipids might affect cell metabolism, causing off-target effects and, as a consequence, intrinsic lipidation is not fully captured.[6] Due to the reversibility of S-palmitoylation, indirect detection methods such as acyl-biotin exchange, including the replacement of the acyl group with biotin and the enrichment of biotinylated proteins or peptides using avidin-immobilized beads, were implemented.[8,9] However, these approaches include the removal of the initial modification, and it is not possible to differentiate between the types of lipidation, such as myristoylation or palmitoylation.

LC-MS/MS is suitable for capturing both the nature of the lipidation and the modification site. Early studies combined LC-MS/MS with metabolic labeling[10–12] and acyl-biotin exchange,[9,13,14]

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providing proteome-wide insights on protein lipidation; however, due to the limitations discussed above, exact lipid identities still remained elusive. Combining LC-MS/MS with liquid–liquid extraction further enabled the large-scale analysis of lipidated proteins, but showed a clear trend toward the identification of myristoylated peptides, while palmitoylation was clearly underrepresented in this study.[15] As the loss of palmitoyl groups during ionization and fragmentation is problematic for the unambiguous identification of palmitoylation sites by mass spectrometry (MS), a previous study assessed the stability and fragmentation behavior of singly and doubly palmitoylated peptides.[16] Nonetheless, low stoichiometry of the modification and increasing hydrophobicity of multiply modified peptides, which are often observed naturally, remain a challenge for the identification of lipidated peptides by LC-MS/MS.

We, therefore, set out to characterize the effects of palmitoylation on solubility, ionization, and fragmentation of tryptic peptides. For this, we chose a model peptide with four cysteine residues that are naturally palmitoylated. We selectively modified specific residues to varying degrees and compared the behavior of the different peptides during MS analysis. We further explored the use of dimethyl sulfoxide (DMSO) to increase ionization efficiency. In summary, we discuss potential pitfalls and challenges when studying lipidated (palmitoylated) peptides and provide reasons why natively lipidated peptides are difficult to identify and are, therefore, often underrepresented in complex peptide mixtures.

## 2. Results and Discussion

### 2.1. Choosing a Model Peptide

To investigate the effects of palmitoylation on ionization and fragmentation during MS analysis, we chose a tryptic model peptide of the synaptosome-associated protein 25 (SNAP25). SNAP25 belongs to the soluble N-ethylmaleimide sensitive factor attachment protein receptor (SNARE) protein family, and is involved in membrane fusion during neurotransmitter release.[17] SNAP25 is anchored to the synaptic plasma membrane through four S-palmitoylated cysteine residues in its linker region.[18,19] For this study, we chose the tryptic peptide containing all four cysteine residues as a model peptide resembling the naturally obtained peptide when following standard proteomic protocols (**Figure 1**A). To gain insights into the effects of palmitoylation during MS analysis, cysteine carbamidomethylation was included during peptide synthesis to block selected lipidation sites, resulting in the modification at specific sites and to a defined degree (Figure 1B). Note that cysteine carbamidomethylation is also the modification that would be introduced during proteomic studies when the peptide is not fully lipidated.

Before introducing palmitoylation, ionization and fragmentation of the unmodified peptide (P1) were assessed. For this, the peptide was dissolved in 70% (v/v) methanol, 25% (v/v) water, 5% (v/v) chloroform, and analyzed by direct-infusion

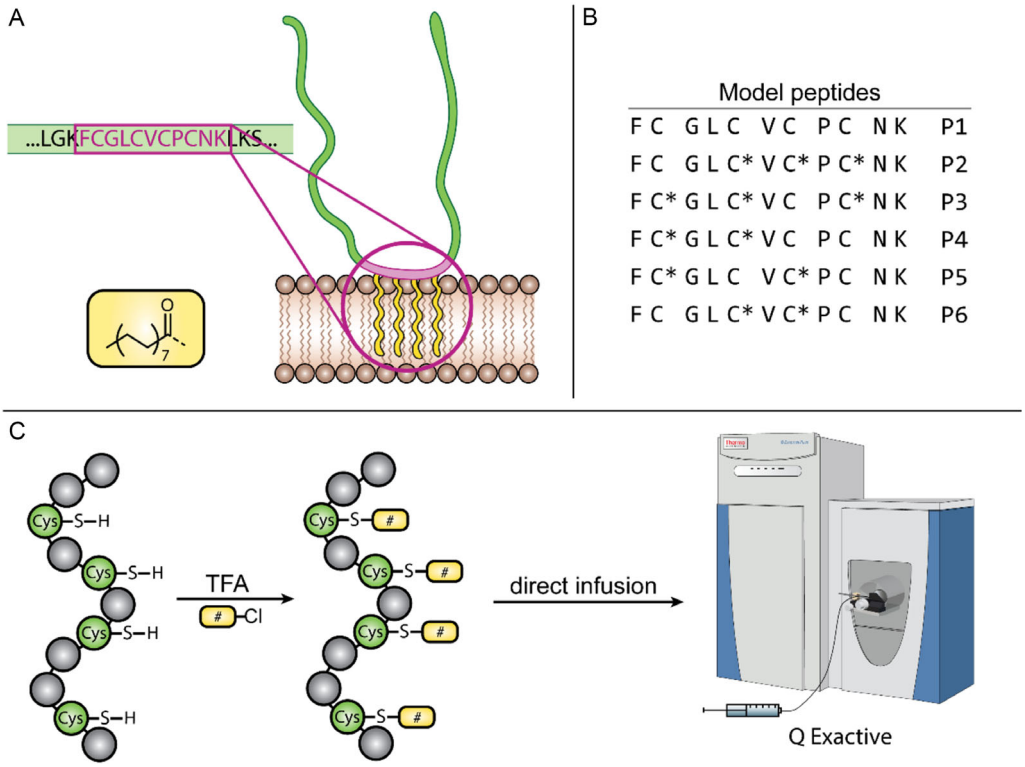


**Figure 1.** Experimental set-up and workflow. A) SNAP25 is anchored to the synaptic plasma membrane through four palmitoyl chains (yellow). The corresponding tryptic peptide is highlighted (purple), and the sequence is given. The chemical structure of the palmitoyl chain is given (yellow box). B) List of model peptides (P1-P6) analyzed in this study. Carbamidomethylation of cysteine residues is indicated (*). C) Model peptides were palmitoylated *in vitro* and analyzed by direct-infusion nanoelectrospray ionization MS.

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nanoelectrospray ionization (ESI) MS as described (see Experimental section for details). The mass spectrum revealed the 1+, 2+, and 3+ charge states. The 2+ charge state showed the highest ion intensity and was, therefore, selected for MS/MS fragmentation. Higher energy collisional dissociation (HCD fragmentation) of the peptide yielded fragment ions covering the full peptide sequence (y2-y10 and b2-b7; see Figure S1, Supporting Information for ion nomenclature and structure). Note that the y4-ion showed comparably high ion intensity due to a proline residue in the peptide sequence. In addition to y- and b-ions, z- and a-ions, as well as internal fragments, were identified. The fully annotated MS/MS spectrum is shown in **Figure 2**.

### 2.2. Introducing S-Palmitoylation into Model Peptides

Having characterized the unmodified peptide, we next introduced S-palmitoylation at specific sites in the peptide sequence. For this, the different peptides (Figure 1B) were dissolved in trifluoracetic acid (TFA) and incubated with palmitoyl chloride (Figure 1C, see Experimental Section for details). Note that the increased hydrophobicity of the modified peptides resulted in solubilization difficulties; the peptides could not be dissolved in water/acetonitrile mixtures, even at higher content of the organic solvent. However, solubilization of the modified peptides could be achieved when using less polar solvents such as chloroform. Note that these solvents are usually not compatible with LC-MS/MS setups for proteomic studies, which often employ mobile phases composed of water and acetonitrile. In addition, the increased hydrophobicity of peptides containing more than one palmitoyl chain could lead to a facile loss of modified peptides during the various steps required for sample preparation. Note that MS is a sensitive technique, and the peptide amounts obtained here yielded high-quality mass spectra of the modified peptides.

### 2.3. MS Analysis of Singly Palmitoylated Peptides

For MS analysis, the modified peptides were dissolved in 90% (v/v) chloroform, 10% (v/v) methanol, and subsequently analyzed by MS/MS (Figure 1C). We first targeted two model peptides (P2 and P3), both containing one free and three carbamidomethylated cysteine residues. The free cysteine residues were successfully palmitoylated, and similar to the unmodified peptide, the most abundant signal intensity observed in the full mass spectrum corresponded to the 2+ charge state. Again, this charge state was selected for HCD fragmentation. The annotated fragmentation spectrum of the palmitoylated peptide (P3$^{\#}$) is shown in **Figure 3**A; this peptide contained the palmitoyl group at an internal modification site (i.e., on the seventh amino acid side chain of the peptide, see Figure 1B). Again, a series of fragment ions confirmed the peptide sequence (y2 to y10 and b2 to b7). Note that fragment ions of the three cysteine residues (y3, y7, and y10) contained carbamidomethylated cysteine residues, resulting in a +57.02 Da mass shift when compared with free cysteine residues of peptide P1. The lipidation at y5 results in an increase of 238.23 Da corresponding to the palmitoyl chain. This mass shift was identified for the y5 fragment ion as well as the following y-ions of the fragment ion series.

Importantly, the modification was stable during the MS/MS experiment, and all y-ions containing the palmitoylated cysteine

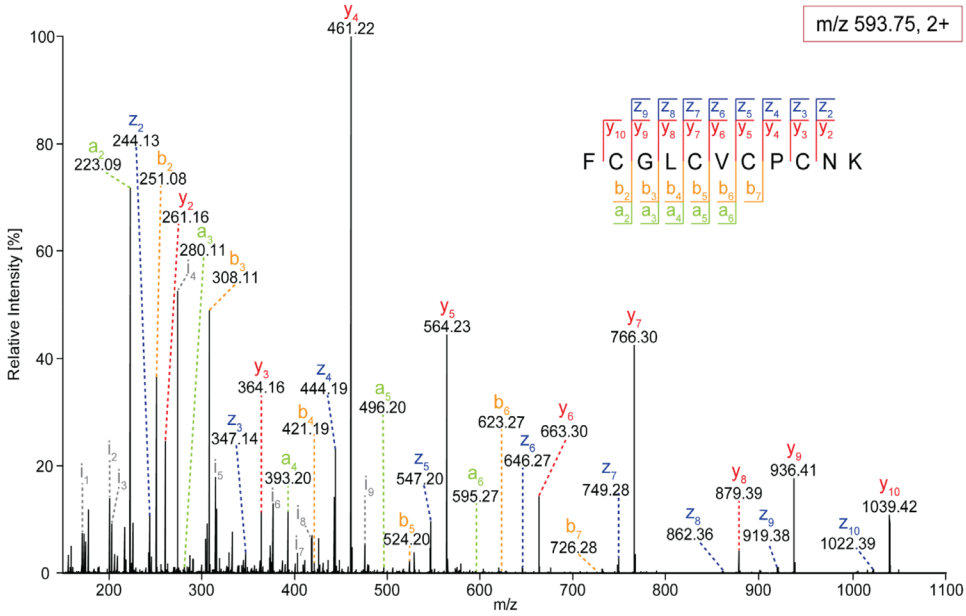


**Figure 2.** Fragment spectrum of the unmodified model peptide FCGLCVCPCNK (P1) acquired in positive ion mode and at a normalized collision energy (NCE) of 30. Y- (red), z- (blue), b- (yellow), and a-ions (green) are annotated. The peptide sequence and the corresponding nomenclature of fragmentation ions are shown. In addition to these fragment ions, internal cleavage ions generated by double backbone fragmentation as a combination of b- and y-fragmentation were observed (labeled i1 to i9). See Figure S1 for details on the ion nomenclature and fragmentation. Fragmentation of the peptide covered the full peptide sequence.

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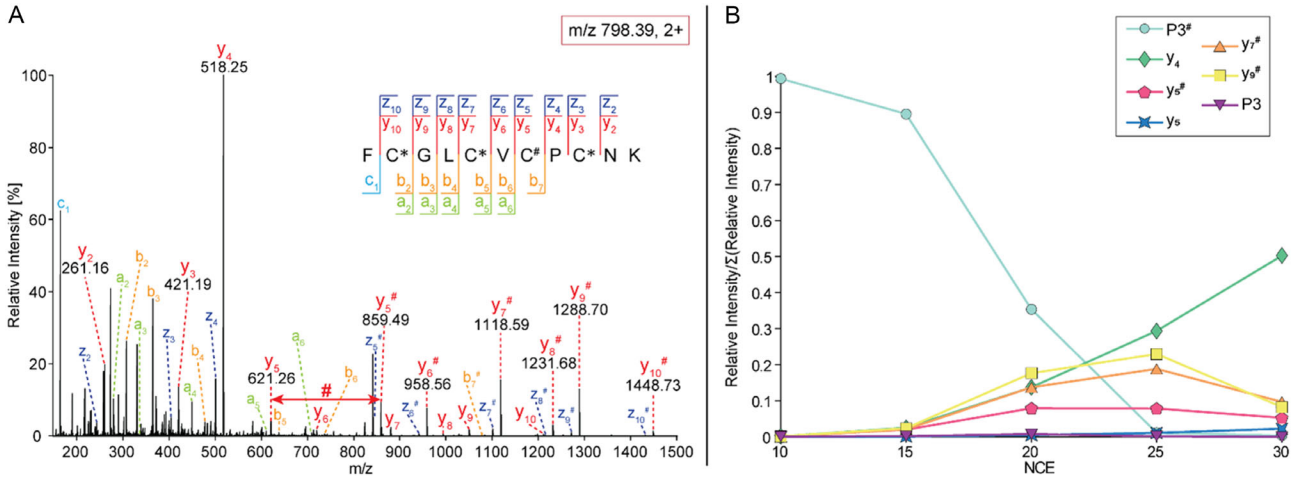


**Figure 3.** Fragmentation of the model peptide FC*GLC*VC#PC*NK (P3#). A) Fragment spectrum acquired in positive ion mode and at an NCE of 30. Y- (red), z- (blue), b- (yellow), and a-ions (green) are annotated. Cysteine carbamidomethylation (*) and cysteine palmitoylation (#) are indicated. The sequence of the peptide and observed fragmentation ions are shown. The full sequence of the peptide was covered, allowing identification of the modified site. B) Relative intensity of fragment ions at increasing NCE. The intensity values were averaged from 30 scans and are plotted as relative intensity of the ion, divided by the sum of the relative intensity of the ions considered in this diagram (see legend). The y5#- and P3#-ions show higher intensities than their unmodified counterparts, indicating stability of S-palmitoylation during HCD fragmentation.

residue are observed in the mass spectrum. Interestingly, a series of y-ions that lost the palmitoyl chain was also observed, albeit at very low intensity (Figure 3A). In order to assess the stability of the modification during tandem MS, and to examine whether the modification dissociates without fragmentation of the peptide backbone, a range of collisional voltages was employed (Figure S2). Plotting the relative intensities of specific fragment ions (i.e., y4, y5, y7, and y9) and the precursor ion against increasing collisional voltages shows an increase in fragmentation, while the precursor ion intensity decreases (Figure 3B). More precisely, when applying a normalized collision energy (NCE) of 10, only the intact precursor ion is observed with high intensity. At an NCE of 15, all fragment ions emerge; their intensities increase at higher collisional voltages of NCE 20–30. Again, the y4-ion is prevalent, showing the highest intensity already at an NCE of 20. Note that the abundant y5- to y10-ions include the palmitoyl moiety; corresponding fragment ions that lost the palmitoyl chain were only observed at very low intensities. Comparing the fragmentation behavior of this peptide (P3#) with that of P2#, the model peptide (P2) that carries the palmitoyl group at a terminal position (i.e., on the second amino acid side chain of the peptide, see Figure 1B), a similar pattern was observed when varying the NCE values (Figure S3, Supporting Information). Notably, peptide P2 shows a higher degree of dissociation of the palmitoyl chain, likely due to the location of the modification being at the terminal side of the peptide (Figure S3 and S4, Supporting Information). These experiments confirm the stability of S-palmitoylation during HCD fragmentation making the assignment of palmitoylation sites within the peptide sequence possible. Note that it was not possible to induce dissociation of the modification alone, i.e., independently from peptide backbone fragmentation. Importantly, the palmitoyl modification represents an aliphatic fatty acyl chain, and HCD fragmentation is sufficient to identify the correct type of lipidation. However, in the case that the modifications include double bonds or saccharolipids, advanced fragmentation techniques, such as ultraviolet photodissociation,[20] are useful to identify the structure of the modification.

### 2.4. MS Analysis of Multiply Palmitoylated Peptides

Next, we analyzed peptides including more than one palmitoylation, mimicking the natural occurrence of palmitoylated peptides. For this, we employed model peptides with two free cysteine residues for S-palmitoylation (P4, P5, and P6). The positions of the accessible cysteine residues included one peptide with two free cysteine residues close to the N- and C-termini (P6, amino acids two and nine), one peptide with two free cysteine residues located in the C-terminal half of the peptide (P4, amino acids seven and nine) as well as one peptide with two free cysteine residues that are separated by one potential modification site (P5, amino acids five and nine). In vitro palmitoylation was performed as described (see above and Experimental Section), resulting in singly and doubly modified peptides. Note that the introduction of two palmitoyl chains further increased the hydrophobicity of the peptides and hampered ESI. Nonetheless, in all cases, dissociation of the palmitoyl moiety was only observed at very low intensities, allowing unambiguous assignment of the presence and positions of the two palmitoyl chains (Figure S5, Supporting Information).

Peptides containing several cysteine residues may or may not be fully palmitoylated, posing another challenge because multiple combinations of modified and unmodified residues are possible, and some of these incompletely, but multiply modified peptides have the same mass. Model peptide P1 constitutes one such example; four sites are available for palmitoylation, but only a subset of them might be modified. To assess partial palmitoylation of this peptide, we selected the doubly modified

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peptide for MS/MS fragmentation for which six combinations with the same precursor *m/z* value are possible. The fully annotated spectrum is shown in **Figure 4**. The MS/MS spectrum shows many fragment ions of the different combinations; in most cases, these fragment ions belong to more than one peptide species. However, two y-ions (i.e., y5 and y6 of the peptide that contains two palmitoylated cysteine residues at amino acids seven and nine) are unique. Two additional b-ions (i.e., b5 and b6 of the peptide containing two palmitoylated cysteine residues at amino acids two and nine) would also be unique; however, these fragments were not observed in the fragment spectrum. Remarkably, all possible combinations of doubly palmitoylated peptides can be confirmed in this mass spectrum through the combination of y-ions of the corresponding fragmentation series. In addition to the partially modified peptide, the fragment spectrum of fully modified P1$^{\#}$ clearly showed mass shifts for all palmitoylated cysteine residues (Figure S6, Supporting Information).

### 2.5. Increasing Signal Intensities of Lipidated Peptides by the Addition of DMSO

In addition to the increasing hydrophobicity of (multiply) palmitoylated peptides, the addition of palmitoyl chains results in a decreased ESI efficiency. Various attempts were previously made to increase the ESI response of proteins or peptides. These include modifications of the instrumental setup, e.g., improved nanoemitters[21,22] or modified ionization sources,[23–25] as well as the addition of ethylene glycol[26] or DMSO[27,28] to the solvents. As DMSO was introduced to enhance the ESI signal of proteins, peptides, and hydrophobic compounds, and ethylene glycol might be immiscible with the solvents required for dissolving palmitoylated peptides, we explored the effect of DMSO in different concentrations and at different ionization potentials for a doubly palmitoylated peptide (P5$^{\#}$, **Figure 5**).

Note that the use of borosilicate emitters produced in-house might have contributed to the variability between the different replicates, and the position of the emitter might affect the ionization;[29] nonetheless, we maintained the position of the emitter during the measurements, and a clear trend was noticed. While the signal intensity appears to be consistent throughout the different ionization potentials in the absence of DMSO, the addition of DMSO resulted in an increase in the peptide signal at low ionization voltages. More precisely, a signal increase of approximately twofold was observed in the presence of 10% DMSO and at an ionization voltage of 1.0 kV. Notably, for all percentages of DMSO, the signal intensity decreased at higher voltages. The fact that DMSO resulted in high signal intensity at low ionization voltages is an advantage, as potential in-source fragmentation of the palmitoyl group is decreased. Note that we did not observe facile loss of the modification here; however, this observation might be advantageous when employing a different instrument set-up or when studying other reversible and labile lipid modifications. As previously proposed,[27] DMSO leads to an efficient production of ions due to its low surface tension, resulting in the production of smaller charged droplets during ESI. Importantly, depending on the flow rates, solvents and emitters employed during ESI and the resulting droplet sizes, ESI conditions require optimization, including parameters such as the capillary voltage.[30] We, therefore, assume that smaller droplets require lower voltages for efficient ionization while higher voltages might cause electric discharge or other effects that reduce the spray current. In the case of hydrophobic peptides as analyzed here, DMSO further supports the solution of the peptides.

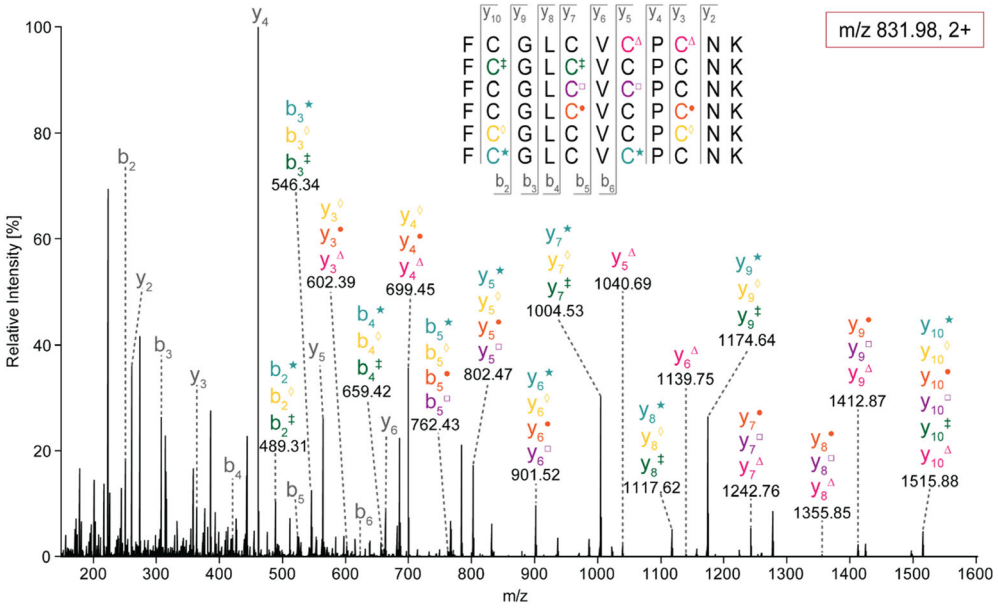


**Figure 4.** Fragment spectrum of the doubly palmitoylated model peptide FCGLCVCPCNK (P1) acquired in positive ion mode and at an NCE of 30. Y- and b-ions are annotated. Cysteine palmitoylation (#) is indicated. The sequence of the peptide, the combination of different modification species, and the observed fragment ions are given. As the differently palmitoylated peptides have the same precursor mass, their fragmentation patterns overlap.

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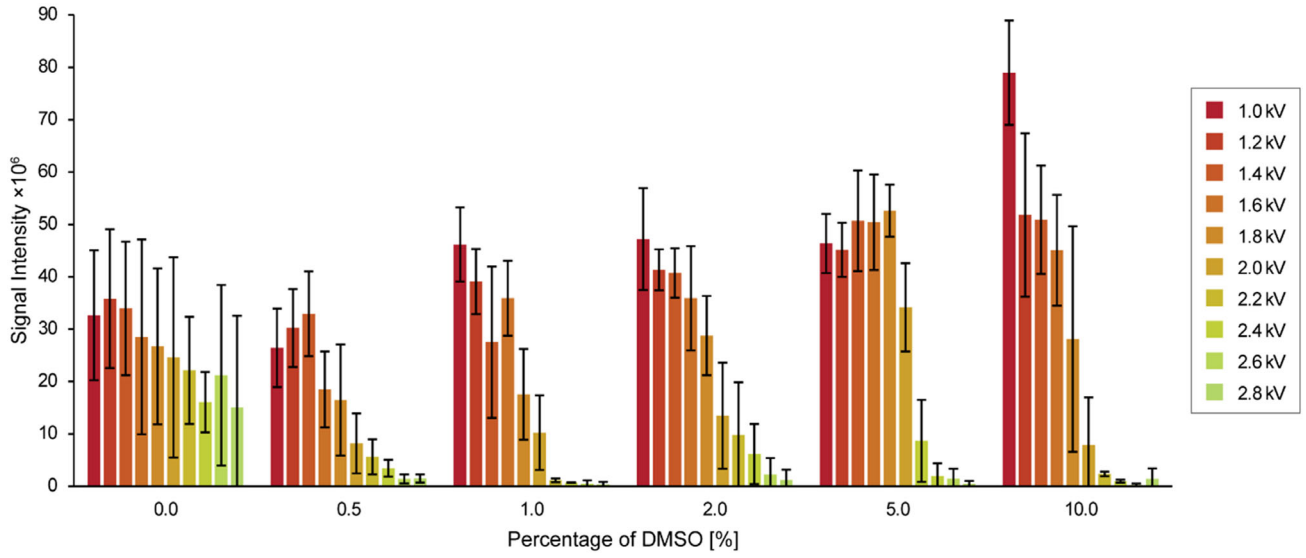


**Figure 5.** Effect of DMSO on signal intensities of palmitoylated peptides. The signal intensity of FC*GLC$^{\#}$VC*PC$^{\#}$NK (P5$^{\#}$) with two palmitoyl chains was monitored (*m/z* 889.00, acquired in positive ion mode). The signal intensity is plotted for a range of DMSO percentages. For each percentage, increasing ionization voltages (1.0 to 2.8 kV) were applied to the emitter (see color legend). For each DMSO percentage, three different replicates using different borosilicate emitters were analyzed. Note that ionization voltages were increased using the same emitter. The intensity values were averaged from 60 scans. Error bars represent the standard deviation of the mean; negative intensity values of error bars were cut as they extend into the lower order of magnitude.

## 3. Conclusion

In this study, we investigated the behavior of a range of (multiply) palmitoylated peptides during MS analysis. After initial characterization of the fragmentation profile of the unmodified model peptide, *in vitro* S-palmitoylation and subsequent MS/MS analysis were performed.

We found that the palmitoyl modification was stable during ionization and HCD fragmentation, allowing the unambiguous identification of modification sites. However, the palmitoylation of cysteine residues results in an increased hydrophobicity and low solubility in solvent mixtures commonly employed during proteomic analyses. In addition, palmitoylated peptides show a lower ionization efficiency, constituting additional challenges when analyzing peptide mixtures. Importantly, the addition of 10% DMSO when employing low ionization voltages increased the signal intensity notably. Nonetheless, the discussed challenges and the commonly low stoichiometry of palmitoylation make the identification of natively lipidated peptides in complex mixtures, when LC separation is required, difficult. An experimental set-up without LC separation (e.g., using matrix-assisted laser/desorption ionization) might be appropriate, although this is not sufficient for complex peptide mixtures obtained from cell lysate or other sources. In addition, top-down MS in combination with native MS was successfully employed to study palmitoylation of intact proteins;[31,32] however, this approach is only applicable to purified proteins or samples of low complexity. In summary, we found that MS is capable of unambiguously identifying (multiple) lipidation, e.g., S-palmitoylation; however, difficulties with solubility and ionization efficiency render the analysis of multiply modified peptides from complex samples without further improvements very difficult.

## 4. Experimental Section

### Materials

The following model peptides without and with (*) cysteine-carbamidomethylation, corresponding to the sequence of a tryptic peptide of SNAP25 (*rattus norvegicus*) were acquired from Sigma–Aldrich (St. Louis, Missouri, USA; >96% purity): FCGLCVCPCNK (P1), FCGLC*VC*PC*NK (P2), FC*GLC*VCPC*NK (P3), FC*GLC*VCPCNK (P4), FC*GLCVC*PCNK (P5), FCGLC*VC*PCNK (P6). TFA (≥99.9% purity, LC-MS grade) was purchased from Carl Roth (Karlsruhe, Germany), palmitoyl chloride (98% purity), and DMSO (≥99.7% purity) were purchased from Sigma–Aldrich (St. Louis, Missouri, USA). Chloroform (≥99.5% purity, HPLC grade) and methanol (UHPLC-MS grade) were purchased from Thermo Scientific (Waltham, Massachusetts, USA), water (Optima LC-MS grade), and acetonitrile (≥99.9% purity, HPLC gradient grade) were purchased from Fisher Scientific (Hampton, Virginia, USA).

### Peptide Modification

The model peptides (see above) were palmitoylated according to a previously published protocol[33] with some modifications. Briefly, 200 μg of each peptide were solubilized in 40 μL TFA. 1 μL of palmitoyl chloride was added, followed by incubation at room temperature for 10 min. TFA was then evaporated under a flow of nitrogen. Modified peptides were dissolved in 90% (v/v) chloroform, 10% (v/v) methanol.

### Peptide Analysis

Peptides were analyzed by direct-infusion MS using a Q Exactive Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a Nanospray Flex ion source (Thermo Fisher Scientific, Bremen, Germany). For this,

unmodified peptides were dissolved in 70% (v/v) methanol, 25% (v/v) water, 5% (v/v) chloroform and modified peptides were initially dissolved in 90% (v/v) chloroform, 10% (v/v) methanol and then diluted in 70% (v/v) methanol, 25% (v/v) water, 5% (v/v) chloroform. Peptides were loaded into gold-coated borosilicate offline emitters produced in-house.[34]

The following instrument settings were used for MS analysis: capillary voltage, 1.0–2.8 kV; capillary temperature, 250 °C; target resolution, 70.000; RF-lens level, 50; ion mode, positive; max. injection time, 200 ms; automatic gain control, $2{\cdot}10^6$; microscans, 2. Tandem MS settings were as follows: max. injection time, 200 ms; automatic gain control, $2{\cdot}10^6$; microscans, 2; target resolution, 70,000; fragmentation type, HCD; NCE, 10–30 (charge state 2); mass selection window, 1 *m/z*. Acquired mass spectra were annotated manually.[35,36]

## Supporting Information

Additional figures are available in the Supporting Information. The authors have cited additional references within the Supporting Information.[35,36]

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## Conflict of Interest

The authors declare no conflict of interest

## Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Keywords:** fragmentation · ionization · lipidation · mass spectrometry · palmitoylation

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