

# Site-Specific Identification of 3-Nitrotyrosine and Nitrosocysteine Residues in Peptides using MALDI Mass Spectrometry

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## Key Words

- Finnigan™ LTQ™
- Finnigan vMALDI™ source
- Labile sample analysis
- Oxidative modifications

## Introduction

Oxidative post-translational modifications are increasingly considered important, particularly for research associated with aging, cardiovascular disease and environmentally associated inflammation. These modifications involve reactive oxygen and nitrogen species with reactions such as nitration or nitrosylation of specific amino acids.

Nitration of tyrosine residues to form 3-nitrotyrosine serves as a fingerprint indicating oxidative stress.<sup>1</sup> S-nitrosylation is the best established example of redox-based specific signaling through the physiological modification of cysteine residues. Like phosphorylation, S-nitrosylation is reversible and plays a role in control of most or all classes of proteins.<sup>2</sup>

It would be valuable to be able to ‘screen’ proteins for these oxidative modifications. Traditional analysis of protein nitrosylation requires multi-step chemical assays. Using proteomics tools such as mass spectrometry, it may be possible to simplify detection and screening of multiple modifications. Identification and localization of 3-nitrotyrosine residues in peptides using electrospray (ESI) mass spectrometry have been reported for some specific proteins, yet their analysis continues to be a challenge due to the labile nature of nitroaromatic compounds and problems with yields and recovery of nitrated peptides, especially from tissue.<sup>1</sup> There are also problems associated with regular high vacuum MALDI-TOF mass spectrometers. 3-Nitrotyrosine containing peptides suffer decomposition from photochemical cleavage reactions thus generating spectra which are difficult to interpret.<sup>3</sup>

These problems can be circumvented by using a “softer” ionization technique, intermediate vacuum MALDI, combined with linear ion trap mass spectrometry to analyze peptides and proteins containing 3-nitrotyrosine or nitrosocysteine residues.

## Goal

This study utilizes the Finnigan LTQ mass spectrometer with vMALDI source (Figure 1) to develop a methodology for the routine, unambiguous identification and localization of 3-nitrotyrosine or S-nitrosocysteine residues in peptides and proteins oxidized *in vitro* by TNM (tetrani-tromethane) or SIN-1 (3-morpholinopyridone).

## Methods

### Protein Nitration

Bovine RNase A was nitrated by reaction with a thirty-fold molar excess of TNM at pH 8 for 20 minutes. The reaction was quenched and covalent dimers removed by size exclusion chromatography. Individually nitrated derivatives were separated from the doubly nitrated derivatives (Tyr 115, 76) by isoelectric focusing. Resultant singly nitrated RNase A was denatured and proteolytically digested for MS analysis.

### Cysteine Oxidation

Oxidation of cysteine was performed according to a previous method,<sup>4</sup> with some modifications. The cysteine-containing synthetic peptide, MSRPACPNCKYE was oxidized using SIN-1 in 10 mM phosphate buffer, pH 7.4. Bovine creatine kinase was denatured in 6 M Guanidine-HCl and reduced using 1 mM TCEP in 100 mM phosphate buffer, pH7.4. The sample was diluted 10 times with Milli-Q® water and then digested by GluC. The digested protein was diluted 10 times again using 10 mM phosphate buffer before being oxidized with SIN-1.

### Intermediate Vacuum MALDI Sample Analysis

One µL of oxidized synthetic peptide or protein digest was spotted directly onto a MALDI plate and 1 µL of CHCA (2.5 mg/mL) or DHB (50 mg/mL) matrix solution was added. Spectra were acquired automatically using the Finnigan LTQ with vMALDI source, under Xcalibur™ software control, using the double play instrument method



Figure 1: The Finnigan LTQ equipped with an intermediate vacuum MALDI source

(1 full MS + 25 MS/MS on the most intense peaks with Dynamic Exclusion™ and with a maximum acquisition time of 15 min). Targeted MS/MS analysis on low abundant peaks was performed to confirm oxidative modifications.

### Database search

BioWorks 3.2, with SEQUEST® database search algorithm, was used to identify the proteins.

### Results

Oxidative modifications of peptides will create expected mass increases of 45 Da for nitration of a tyrosine residue to 3-nitrotyrosine<sup>1</sup> and 30 or 29 Da for nitrosylation of a cysteine residue to S-nitrosocysteine<sup>2</sup> shown in Figure 2).

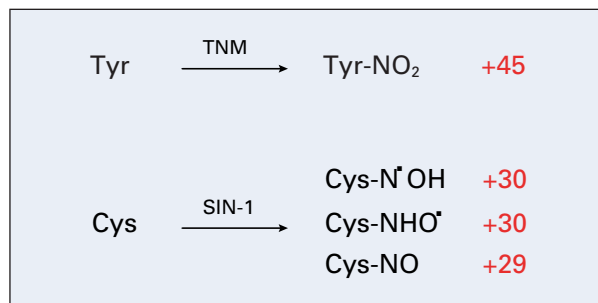


Figure 2: Expected mass increase due to tyrosine nitration and cysteine nitrosylation

### Identification of 3-Nitrotyrosine Residues in RNase A

Two nitrotyrosine-containing peptides were identified in the digest of nitrated bovine RNase A (Figure 3a). These nitrated peptides were of low abundance in full MS spectra. Mapping of nitrotyrosine was achieved by comparing MS/MS spectra of nitrated and unmodified peptides. For nitrated peptides, fragments containing modified tyrosine residues were found to have a mass increase of 45 Da, suggesting tyrosine nitration (Figure 3b and c).

### Identification of S-Nitrosocysteine Residue in Synthetic Peptides

Nitrosylated cysteine was located and identified in the oxidized peptide MSRPACPNDKYE (Figure 4a and 4b).

### Identification of S-Nitrosocysteine Residues in Protein Digests

Using the intermediate vacuum MALDI, nitrosylated cysteine was correctly identified in the oxidized bovine creatine kinase digests (Figure 5a and b).

The abundance of nitrosylated peptides was relatively low in full MS spectra but was detectable with the sensitivity of the Finnigan LTQ. Nitrosylated cysteine was mapped by comparing MS/MS spectra of nitrosylated and unmodified peptides. For nitrosylated peptides, fragments containing cysteine residues were found to have a mass increase of 30 Da, suggesting cysteine nitrosylation (Figure 2), in radical form.

Using the Finnigan LTQ with vMALDI source, these results demonstrate:

- Site specific identification of nitrosylated cysteine from low abundant peaks of unfractionated samples for labile sample analysis and
- A very promising application in the analysis of oxidatively modified proteins for the site-specific identification of oxidative post-translational modifications.

Database: swissprot_139947.fasta		Filter(s): .rspt=5; reference=bovin; consensus score>=15.0		Score	Coverage	MW	Accessio	Peptide (hits)		
File, Score(s)	Reference	Peptide	MH+	z	XC	ΔCn	Sp	RSp	Ions	Count
1	RNP_BOVIN (P00656) Ribonuclease pancreatic precursor (EC 3.1.27.5) R				60.3	54.7	16450.1		6 (6 0 0 0 0)	
	peptidoru_2269	K.HIVAC <sup>o</sup> EGNPYVPVHFDA <sup>o</sup> SV-	2269.09	1	2.701	0.073	231.4	1	1657	12
	ma_2224_C5_1-	K.HIVAC <sup>o</sup> EGNPYVPVHFDA <sup>o</sup> SV-	2224.09	1	3.553	0.123	421.7	1	1857	10
	ma_2130_C4_1-	K.NGQ <sup>o</sup> TNC@YQSY <sup>o</sup> STMSTDC@R.E	2130.92	1	2.665	0.149	113.9	3	1354	7
	peptidoru_2165	R.QHMD <sup>o</sup> STSAAS <sup>o</sup> SNYC <sup>o</sup> NQNMK.S	2164.93	1	3.481	0.415	172.9	1	1550	1
	ma_2517_C1_1-	R.C <sup>o</sup> KPVNTFVHESLADVQAVC <sup>o</sup> BQK.N	2517.22	1	5.068	0.000	456.2	1	2363	8
	ma_2285_C5_1-	K.NGQ <sup>o</sup> TNC@YQSY <sup>o</sup> STMSTDC@R.E	2285.92	1	4.008	0.312	253.5	1	2054	7

Figure 3a: Database search results, using BioWorks 3.2, returned two peptides containing nitrated tyrosine residues RNase A

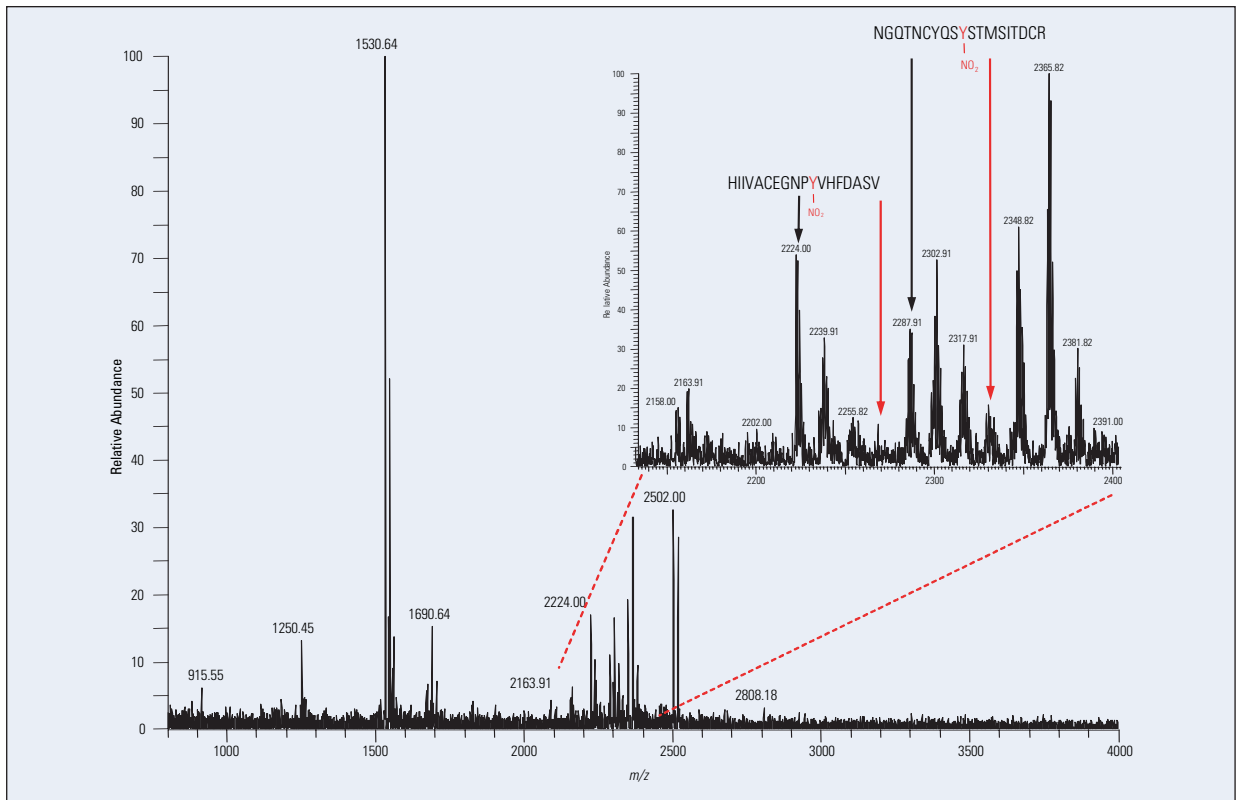


Figure 3b: Full MS spectrum of nitrated, unpurified bovine RNase A digest. The red arrows indicate the peaks of nitrated peptides identified in this study. The black arrows indicate the peaks of unmodified species of the same peptides.

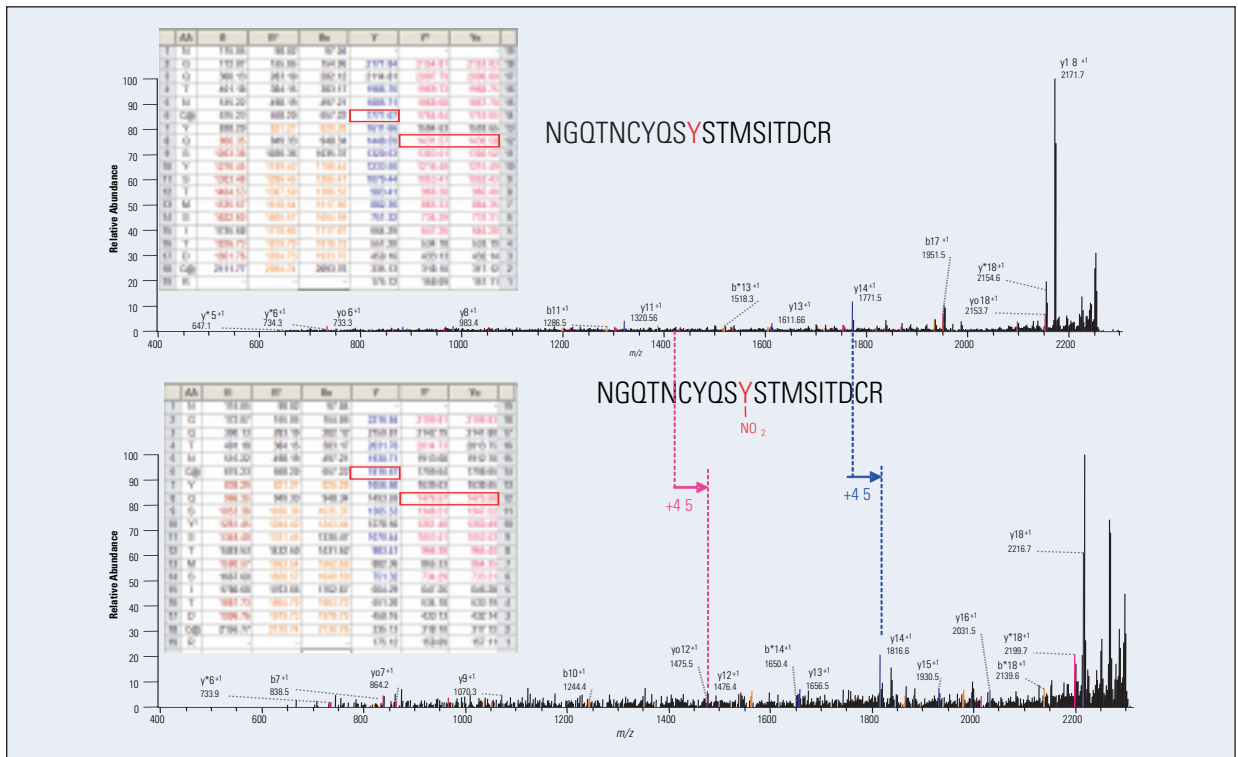


Figure 3c: MS/MS spectra of nitrated and unmodified peptide NGQTNCYQSYSTMSITDCR. The inserted tables list the b and y fragment ions found (in color) in the spectra.

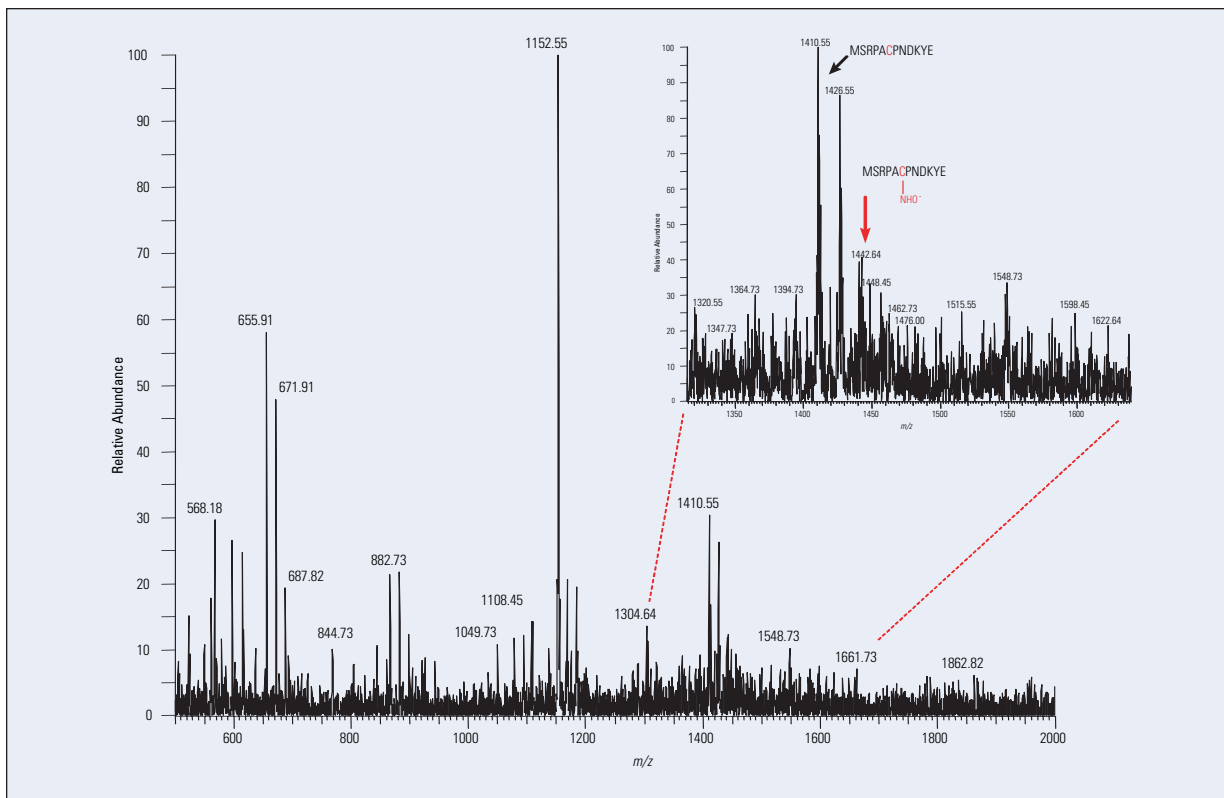
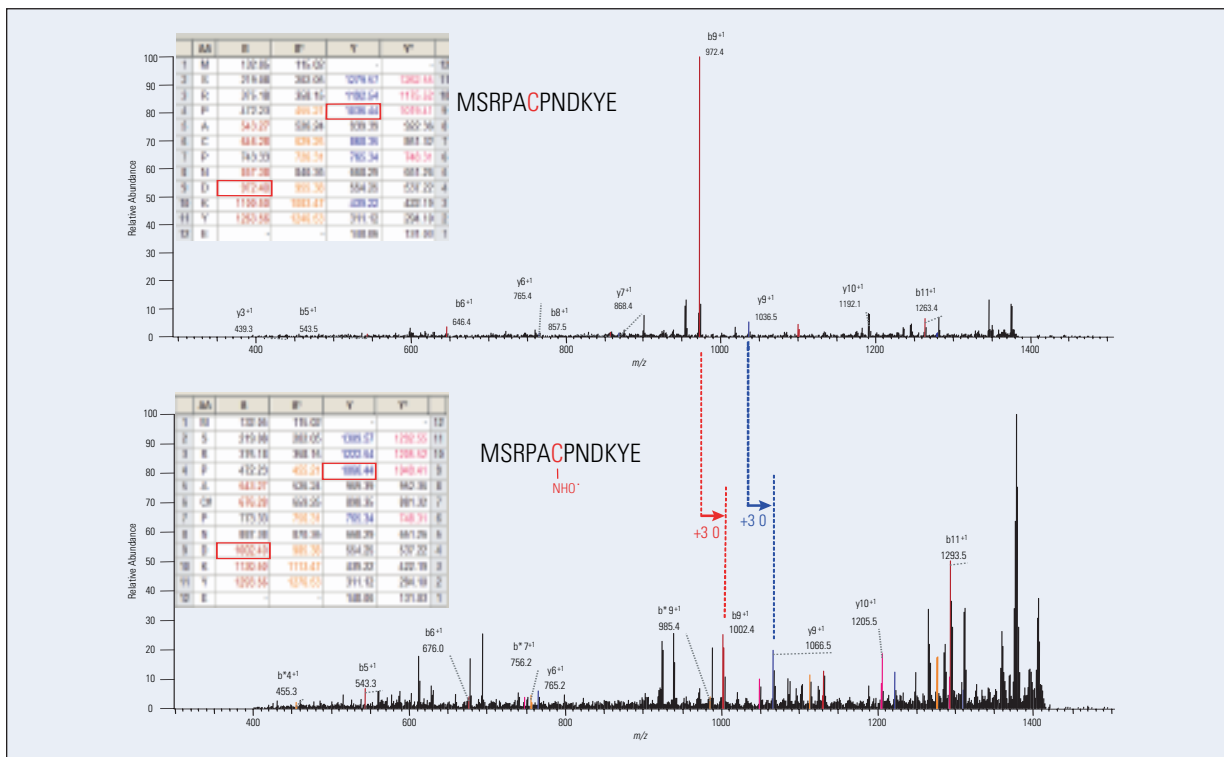


Figure 4a: Full MS Spectrum of SIN-Treated unpurified peptide MSRPACPNCKYE. Red arrow indicates precursor ion peak of nitrosylated peptide. Black arrow indicates peak of unmodified species of the same peptide.



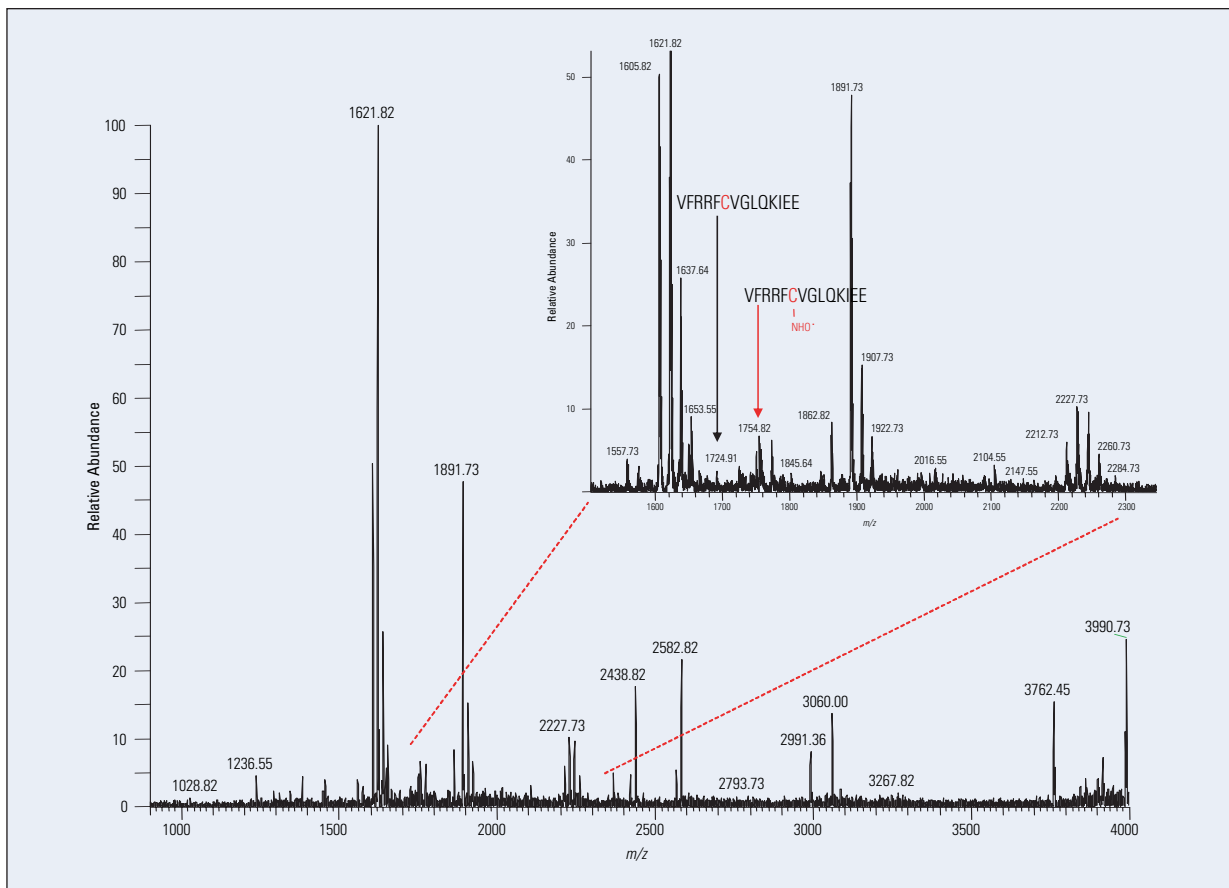


Figure 5a: Full MS of SIN-1 Treated Unpurified Bovine Creatine Kinase Digest. The red arrows indicate the peak for nitrosylated peptide: VFRRCVGLQKIEE. The black arrows indicate the unmodified version of the same peptide.

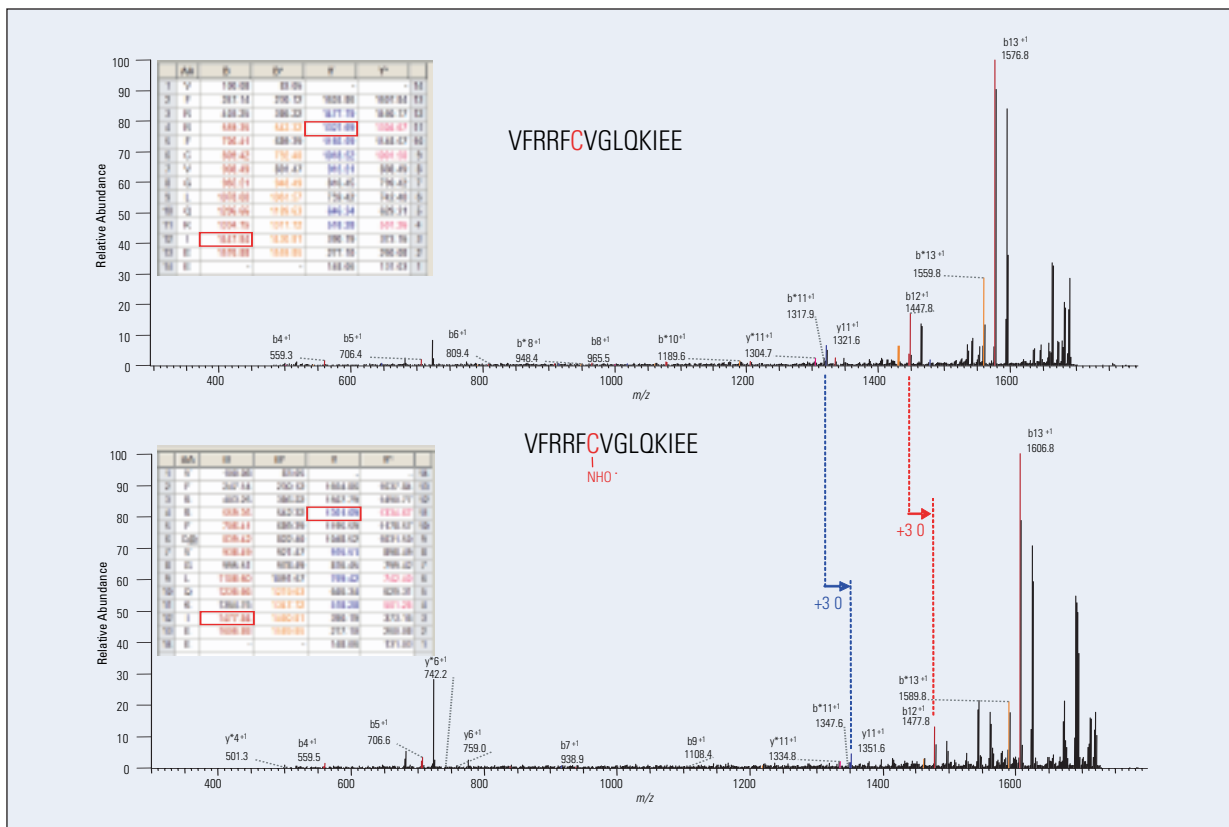


Figure 5b: MS/MS of Nitrosylated and Unmodified Peptide VFRRCVGLQKIEE

## Conclusions

- “Softer ionization” using the Finnigan vMALDI source with the superior sensitivity of LTQ linear ion trap mass spectrometry enabled:
  - i. Site-specific identification of both 3-nitrotyrosine and S-nitrosylated cysteine residues in *in vitro* oxidized peptides and proteins
  - ii. Potential high-throughput screening of oxidative post-translational modifications. Samples did not require pre-fractionation or clean-up, yet sufficient information was obtained to identify site specific nitrotyrosine and nitrosylated cysteine residues in peptides.
- Capability for MS/MS analysis allows confirmation of the presence of 3-nitrotyrosine or S-nitrosylated cysteine residues in the investigated peptides or proteins. Oxidation on other amino acid residues, such as tryptophan or lysine, was also observed (data not shown).
- With purified or fractionated samples, there is a potential improvement in precise localization of 3-nitrotyrosine and S-nitrosocysteine. Application of this method to *in vivo* oxidized proteins is under evaluation.

## References

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