Proteomic and mass spectroscopic quantitation of protein *S*-nitrosation differentiates NO donors

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Supplementary Information

Synthesis of furoxan 3-(hydroxymethyl)-4-phenyl-1,2,5-oxadiazole 2-oxide (ODZ1)): To glacial acetic acid (8 ml, 8 mmol), cinnamyl alcohol (5.37g, 40mmol) was added and completely dissolved. At which point saturated aqueous $NaNO_2$ (8.25g, 120mmol) was added dropwise for 30 minutes, keeping the reaction temperature below 20 ^{0}C. The reaction was stirred at room temperature for an additional 3 hours, and then quenched with ice water (100 ml), extracted with ethyl acetate (3x100 ml). Combined organic extracts were washed with brine (2x50 ml), dried over Na_2SO_4 and concentrated to give a crude yellow oil. Purified by silica gel chromatography (eluant Hexane/ethyl acetate 3:1) – yield 30%, R_f=0.25 (TLC). ^{1}HNMR: 7.79-7.77 (d,2H); 7.08-7.02 (d,2H); 4.77-4.75 (d,2H); 3.90-3.86 (s,3H); 2.64-2.62 (t,1H). ^{13}CNMR: 161.971, 156.48, 129.281, 118.42, 114.82, 55.45, 53.42.

Detailed methods

GST-P1 expression and purification: Standard recombinant DNA techniques were followed as described by Sambrook et al (*1*). Single colonies of BL21(DE3) cells harboring plasmid pET-HisGST-P1 were used to inoculate 25 ml of overnight culture containing ampicillin (100 μg/ml). These cultures were diluted 1:100 times in LB medium containing same antibiotic and grown at 37 ^{o}C until OD_{600} reached a value of 1.0. Protein expression was induced by the addition of 1 mM IPTG. After an 18 h post induction period, cells were harvested at 4000 g for 15 min. The expressed protein was purified by HPLC using His Trap affinity columns (GE Healthcare).The purity of the protein was assessed by SDS-PAGE and Coomassie blue staining. The protein concentration was measured using BCA protein assay kit (Pierce). Aliquots of purified proteins were stored in 25 mM Tris HCl (pH 8) and 1 mM DTT.

Biotin-switch technique (BST): The Jaffrey et al. BST technique was followed with some modifications (*2*). Purified GST-P1 protein was first exchanged with HEN buffer with Microcon YM-10 filter device to remove storage buffer containing DTT. Amber tubes were used throughout the assay and the whole

procedure was carried out in dim light. The protein solution was treated with indicated concentrations of NO donor and incubated at 37 ^{o}C for 30 min. A vehicle control (only vehicle treated protein) was included in all the experiments. Following nitrosation, the free thiols were blocked with 20 mM NEM and 5% SDS by alternating between sonicating and shaking in 55 ^{o}C water bath for 30 min. The remaining unreacted NEM was removed with Microcon YM-10 filter device. Labeling reaction was performed for 1 hr in the dark at 25 ^{o}C with freshly prepared biotin HPDP (1 mM) and sodium ascorbate (5 mM). The fresh reactions were then resolved by non-reducing SDS-PAGE followed by immunoblotting with HRP-conjugated anti- biotin antibody (1:500). Control experiments with exposure to UV light were conducted as routine checks; photolysis of the RS-NO bond leading to the expected loss of signal for biotinylated protein (data not shown) (*3, 4*).

NO treatment of intact E.coli cells for quantifying nitrosation using d-Switch method: Standard recombinant DNA techniques were followed as described by Sambrook et al (*1*). Single colonies of BL21(DE3) cells harboring plasmid pET-HisGST-P1 were used to inoculate 25 ml of overnight culture containing ampicillin (100 µg/ml). These cultures were diluted 1:100 times in LB medium containing same antibiotic and grown at 37 ^{o}C until OD_{600} reached a value of 1. Protein expression was induced by the addition of 1 mM IPTG. Cells were grown for 2 hours at 37 ^{o}C, divided into 5 ml aliquots and spiked 4 times with NO donor at an interval of 45 mins. After the last spiking, cells were kept for another 45 mins before harvesting by centrifugation at 4000 rpm for 10 mins. The cells were then frozen at -80 ^{o}C. The cells were resuspended in 1 ml of lysis buffer (25 mM Hepes, 50 mM NaCl, 0.1 mM EDTA, 1% NP 40, 0.1 mM neocuproine, DNase (5 µg/ml), lysozyme (100 µg/ml)) and sonicated on ice at 30% amplitude for 9 secs before subjecting to d-Switch analysis.

d-Switch method for quantitation of nitrosation: The analytical column used for the separation was 30 x 2.1mm x 3µ Hypersil BDS C18 column. The flow rate was 0.3 ml/min, solvent system was H_2O/MeCN held for 5 mins at 10% MeCN and ramped to 30% MeCN within 20 mins, and then to 90% MeCN within

15 mins. For the 1% O_2 experiments all buffer and solvents were bubbled with nitrogen before the experiment and were conducted in air tight vials.

Cell culture and extract preparation for MS analysis. SH-SY5Y cells were cultured in an 1:1 mixture of DMEM and Ham F-12 medium containing 5% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml) and 1% non-essential amino acid (NEAA) under an atmosphere of 95% air and 5% CO_2 at 37 ^{o}C medium in a 20 cm dish. Cells were grown up to 80% confluence and treated with CysNO for 20 min. After the treatment, cells were washed two times with PBST, and lysed with 1 ml of lysis buffer (25 mM Hepes, 0.1 mM EDTA, 1% NP-40, 0.1 mM neocuproine, 0.5 mM PMSF, 20 mM NEM and protease inhibitor cocktail tablets, pH 7.4). Cells were scraped and sonicated (10% amplitude for 2sec) before centrifugation (12,000g for 5 min at 4 ^{o}C). Protein concentration was determined using the BCA protein assay kit and adjusted to 1mg/ml.

Cell lysates were treated with 5% SDS and incubated at 55 ^{0}C for 30 min with frequent vortexing. Protein was precipitated with ice -cold acetone at -20 ^{0}C for 30 min and centrifuged at 2000g for 10 min. Pellets were collected after washing 2 times with 70% acetone. The protein pellet was dissolved in 250 μL of 40 mM ammonium bicarbonate, 1% SDS and treated with labeling solution (1mM biotin HPDP/or 2mM d_5-

NEM, 5 mM sodium ascorbate, 1μM CuCl) and incubated at 25 ^{0}C for 60 min. Samples were run on SDS gel and separated protein bands were stained with Coomasie Brilliant Blue. Finally bands containing GST-P1 region (determined by visual inspection) were excised and subjected to in-gel digestion with Pierce digestion kit (cat # 89871).

Mass spectroscopic analysis of tryptic digests. The tryptic digest samples were analyzed by using a hybrid linear ion trap FT-ICR mass spectrometer (LTQ-FT-ICR, Thermo Electron Corp., Bremen, Germany) equipped with a nanospray source, and nano-HPLC with autosampler (Dionex, Sunnyvale, CA): this configuration provides mass accuracy for analytes at 3-5 ppm or greater. Initially the peptide mixture was introduced to the trapping cartridge (Zorbax 300 SB-C18, 5 μm, 5 mm, 0.3 mm) and the cartridge was washed for 10 min by loading pump (95/5 DDI water/MeCN, 0.1% formic acid, 50 μL/min) before introducing the sample to the LC column. Peptide separation was performed using a Zorbax nano-column (Zorbax 300 SB-C18, 3.5 μm, 75 μm i.d. × 150 mm, Agilent Technologies) at 250 nL/min. Initial conditions for the 90 min run were set at 95/5 (v/v) DDI water/MeCN, 0.1 % formic acid (solvent A) and 95/5 (v/v) MeCN/DDI water, 0.1% formic acid (solvent B), held for 10 min before linearly increasing to 45% B over 57 min, and 90% B over 23 min before re-equilibration after a further 5 min. The LTQ- FT-ICR method consisted of six events. The initial event performed a full scan in the ion trap (profile mode) followed by the 5 MS/MS experiments on the 5 most abundant ions in the full-scan MS. The scan range was 100-2000. Dynamic exclusion was used for a duration of 1 min per precursor ion to avoid redundant fragmentation datasets.

Database searching. The .RAW files obtained from LTQ-FT-ICR mass spectrometer were converted to mzXML files by use of ReAdW (http://tools.proteomecenter.org/ ReAdW.php). The mzXML files were

searched by use of MassMatrix (http://www.massmatrix.net) against the IPI human v3.65 database (*5*). The search options were set as follows: i) Variable modification is set to NEM and d_5-NEM; ii) Enzyme: trypsin; iii) Mis-cleavage = 3; Peptide length = 6 to 30 amino acid residues; v) Mass tolerance of 10.0 ppm and 1.0 Da for precursor and product ion.

Table S1. Non-niitrosated peptides detected by d-Switch analysis of ~20 kDa gel band from cell lysates after incubation of SH-SY5Y neuroblastoma cells with CysNO.		
Protein	**Peptides**	**Sequence coverage**
Phosphatidylethanolamine-binding protein 1 (PEBP1)	APVAGTCYQAEWDDYVPK	72%
Peptidyl-prolyl cis-trans isomerase (PPIA; Cyclophilin A)	HTGPGILSMANAGPNTNGSQFFICTAK	67%
Adenine phosphoribosyltransferase (APRT)	LQAEVLECVSLVELTSLK	61%
Cofilin-1 (CFL1)	HELQANCYEEVKDR	59%
Ras-related protein Rab-7a (RAB7A)	GADCCVLVFDVTAPNTFK	55%
Ras-related protein Rap-1b (RAP1B)	QVEVDAQQCMLEILDTAGTEQFTAMR	52%
Isoform 1 of 60S ribosomal protein L12 (RPL12)	EILGTAQSVGCNVDGR	49%
TAGLN3 16 kDa protein	LVDWIILQCAEDIEHPPPGR	48%
Similar to Histone H3.1	FQSSAVMALQEACEAYLVGLFEDTNLCAIHAK	47%
Transforming protein RhoA (RHOA)	IGAFGYMECSAK	43%
TPT1 cDNA FLJ57738, highly similar to translationally-controlled tumor protein	EIADGLCLEVEGK	42%
CDC42 Isoform 2 of Cell division control protein 42 homolog	LRPLSYPQTDVFLVCFSVVSPSSFENVK	40%
ATP synthase subunit O, mitochondrial (ATP5O)	GEVPCTVTSASPLEEATLSELK	39%
Proteasome subunit beta type-6 (PSMB6)	EGMTKEECLQFTANALALAMER	39%
GTPase NRas (NRAS)	QVVIDGETCLLDILDTAGQEEYSAMR	33%
Isoform 1 of Eukaryotic translation initiation factor 5A-1 (EIF5A)	YDCGEEILITVLSAMTEEAAVAIK	31%

Peptidyl-prolyl cis-trans isomerase, mitochondrial (PPIF)	HVGPGVLSMANAGPNTNGSQFFICTIK	31%
40S ribosomal protein S11 (RPS11)	CPFTGNVSIR	24%
Ribosomal protein L10 (Fragment) (RPL10)	VDEFPLCGHMVSDEYEQLSSEALEAAR	22%
Highly similar to 60S ribosomal protein L18a	DLTTAGAVTQCYR	20%

Supplemental Figure 1: Comparison of oxidation ability of NO donors. (A) Non-reducing SDS PAGE analysis of GST-P1 oxidation to dimer by NO donors (500 μM) visualized by Coomassie blue staining. Quantification was by image densitometry from blots. Data show mean and s.d. in arbitrary units, which for oxidation/dimerization were normalized to the untreated control as 0%. ANOVA analysis with Tukey's post test relative to vehicle control: *** $p<0.001$; ** $p<0.01$. (B) Non-reducing SDS PAGE analysis of GST-P1 incubations with IAN visualized by Coomassie blue staining. Data show mean and s.d. in arbitrary units.

Supplemental Figure 2: LC-MS/MS analysis of NEM & d_5-NEM labeled peptides derived from GST-P1. (A) Total ion current chromatogram of GST-P1 (20 μM) subjected to in-gel digestion with trypsin after treatment with NEM (20 mM); the arrows indicate the two stereoisomeric peptides resulting from covalent modification of Cys47 by NEM; x-axis is retention time. (B) Low resolution ESI-MS spectrum of the (K)ASCLYGQLPK(F) peptide fragment containing the NEM-modified Cys47 showing predominantly the +2 charged state (m/z 603.3). (C) Low resolution MS/MS spectrum of ASC(NEM)LYGQLPK(+2) precursor ion fragmented by CID and assigned using Protein Prospector software: matched y ion series is labeled in red and matched b ion series is labeled in blue. (D and E) GST-P1 (20 μM) was treated with IAN at 37^{o} C for 30 min and analyzed by BS and d-Switch with or without Cu(I) catalysis of nitrosthiol reduction. (D) d-Switch analysis showing overlaid extracted ion chromatograms of doubly charged Cys47

peptide ion ASC(NEM)LYGQLPK m/z 603.3 (free thiol labeled with NEM) and m/z 605.6 (nitrosated thiol labeled with d_5-NEM) (E) Blots from BS analysis showing GST-P1 nitrosation. CuCl was added with ascorbic acid in both methods.

Supplemental Figure 3: Concentration and time dependent nitrosation of GST-P1 quantified by d-Switch. The d-Switch method depicted in Figure 1, is a proteomics method adapted from the BS assay, in which NEM is used in place of MMTS for Cys-SH blocking, and d_5-NEM is used in place of biotin-HPDP for Cys-SNO labeling. The method relies upon protein digestion to yield a peptide containing the protein-Cys of interest that is amenable to analysis by LC-MS/MS; in the present study, this is Cys47 of GST-P1. In-gel tryptic digest of GST-P1 monomer followed by LC-MS and ESI-MS analysis revealed the desired decapeptide at approximately 12.8 and 13.4 min in the total ion current chromatogram. Labeling of the peptide by NEM gave 2 separate peaks because a stereogenic center is created by thiol alkylation giving two diastereomeric labeled peptides; this was confirmed by MS/MS analysis of the doubly charged peptides (Supplementary Figures 2,C). Reaction times and temperatures and reagent concentrations were optimized at each step in the d-Switch methodology yielding an assay in which the NEM and d_5-NEM labeled peptides were readily quantified in extracted ion chromatograms (m/z 603.3 (d_0) versus 605.6 (d_5) for z = 2. Both NEM and d_5-NEM labeled peptides have almost identical retention time and ionization efficiency, hence calculating the peak area ratios of the two peptides from the extracted ion chromatogram, allows for quantitative analysis of Cys47 nitrosation. The use of CuCl/ascorbate was chosen for the denitrosation/labeling step, because of the significant increase in signal observed both in the d-Switch methodology and in the traditional BS methodology (Supplementary Figures 2D,E and5). We have also seen that disulfide bonds are not reduced in presence of Cu/ascorbate (Figure 6). Williams originally observed that nitrosothiol decomposition was dependent on trace Cu(I) in the presence of reductants (*6*), and the addition of Cu(I) has been suggested previously as a modification of the BS methodology (*7*). The final output of d-Switch analysis is the peak area ratio of Cys-SNO labeling (d_5) to the summed Cys-SNO

plus Cys-SH labeling ($d_0 + d_5$), reported as percentage nitrosation: $\%[d_5/(d_0 + d_5)]$. GST-P1 (20 μM) was treated with IAN and the reaction was quenched at various time points by addition of NEM. (A) Representative chromatograms showing increasing GST-P1 Cys47 nitrosation by IAN (1 mM) at various reaction time points (Rxn time). (B) Representative chromatograms showing increasing intensity of d_5-NEM labeled peptide from Cys47-SNO versus NEM-labeled peptide from Cys47-SH after 30 min treatment with varying concentrations of IAN. (C) Nitrosation of GST-P1 in *E. coli* treated with NO donor. Growing bacterial cells overexpressing GST-P1 were treated 4 times at intervals of 45 min with vehicle or bolus DEA/NO (1, 2, 5 mM) and subjected to d-Switch analysis after lysis. Representative overlaid extracted ion chromatograms show approximately 8% Cys47-SNO after delivery of a maximum theoretical total dose of 40 mM NO.

Supplemental Figure 4: SDS PAGE analysis of IAN induced GST-P1 oxidation to homodimer: (A) GST-P1 (20 μM) was treated with varying concentration of IAN for 30 min, subjected to BS analysis or (B) treated with IAN (1 mM) for varying times and subjected to d-Switch analysis before analyzing by SDSPAGE and Coomassie blue staining.

Supplemental Figure 5: BS analysis of nitrosation of GST-P1 in the presence of CuCl. GST-P1 (20 μM) was treated with 500 μM of DEA/NO or ODZ1 for 30 min and subjected to biotin switch in the presence and absence of CuCl (1 μM).

Supplemental Figure 6: SDS PAGE analysis of homodimer of GST-P1 in presence of Cu. GST-P1 (20 μM) was allowed to dimerize (by removing storage buffer containing DTT and keeping overnight at 4 ^{o}C) prior to treating with 1 μM CuCl for 30 min and analyzed by SDSPAGE and Coomassie blue staining.

Supplemental Figure 7: MS analysis of nitrosation of proteins in SH-SY5Y cells treated with CysNO. Tandem mass spectrum of the NEM labeled peptide (top), and of d_5-NEM labeled peptide (bottom) in cells treated with 2mM CysNO. (A) GST-P1 (B) CFL1(C) UCHL1 (D) DJ-1. The ions with 5Da difference in mass are marked with arrows.

Supplemental Figure 8: Analysis of protein nitrosation from CysNO treatment of SH-SY5Y cells. (A) Nitrosated proteins in SH-SY5Y cells were visualized by BST analysis after treatment with various concentrations of CysNO. (B) SH-SY5Y cells were subjected to the d-Switch methodology and proteins were visualized by SDS-PAGE and Coomassie blue staining; the GST-P1 region in each lane was identified with molecular weight markers (shown) and excised and digested with trypsin prior to MS analysis. (C) MS spectra of NEM modified peptides from SH-SY5Y cells. The NEM-labeled GST-P1 peptide ASC[NEM]LYGQLPK^{2+} at m/z 602.80, relative to the other peptides in the particular scan number, corresponds to the Cys47-containing doubly charged peptide and eluted from the LC column at retention time 22.0 - 22.5 min.

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Supplemental Figure 9: Examination of the effect of DTPA on nitrosation of Cys47 of GST-P1. GST-P1 (20 µM) was treated with CysNO (1 mM) for 30 mins in presence and absence of DTPA (0.5 mM) in the reaction buffer and subjected to d-Switch analysis. The chromatograms show no effect of DTPA in the percentage nitrosation of GST-P1 Cys47.

Supplemental Figure 10: Examination of nitrosation by GTN in SH-SY5Y cells

SH-SY5Y cells were treated with GTN (1 mM) for 30 min (A) and 24 h (B) and subjected to BST analysis. SH-SY5Y cell treatment with CysNO (1 mM) is shown as a positive control in (A). BST analysis by western blot analysis shows negligible nitrosation of SH-SY5Y cells with GTN.

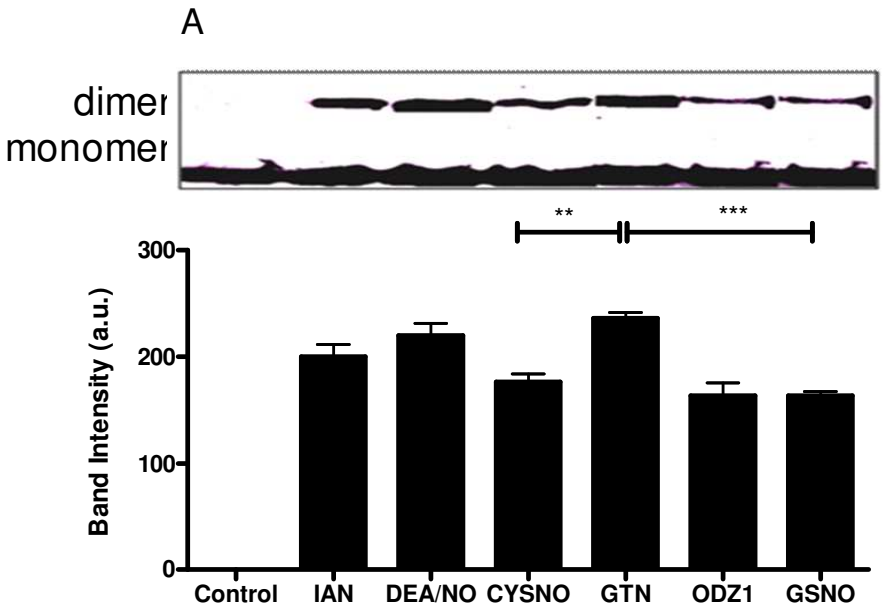
Supplemental Figure 11: Concentration and time dependent study of GST-P1 oxidation and nitrosation by CysNO. GST-P1 (20 μM) was treated with CysNO (1 mM) for varying times and subjected to d-Switch analysis.

Supplemental Figure 12: Nitrosation of GST-P1 by CysNO : GST-P1 (2 μM) was treated with CysNO (0.5 mM, 1mM) for 30 mins and subjected to d-Switch analysis. Figure shows the overlaid extracted ion chromatogram for the two different concentrations.

Supplemental Figure 13: SDS PAGE analysis of CysNO induced GST-P1 oxidation to homodimer: GST-P1 (20 μM) was treated with varying concentration of CysNO for 30 min in duplicate and subjected to analysis by SDSPAGE and Coomassie blue staining. (A) Representative gel showing increase in oxidation with increasing CysNO concentration. (B) Plot from densitometric analysis of the Coomassie blue stained gel; the data are fitted to pseudo-first order and linear (dotted line) curves.

Supplementary Figures

Figure 1



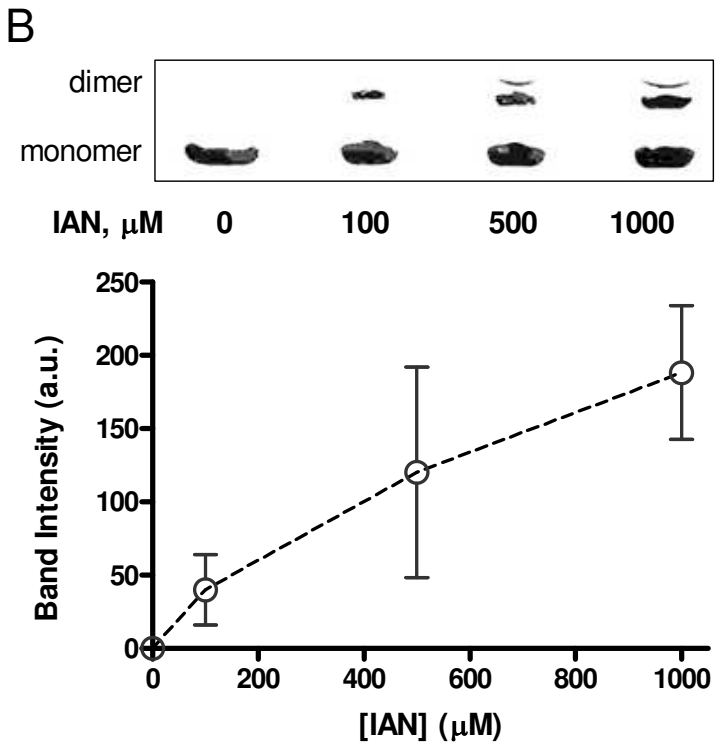
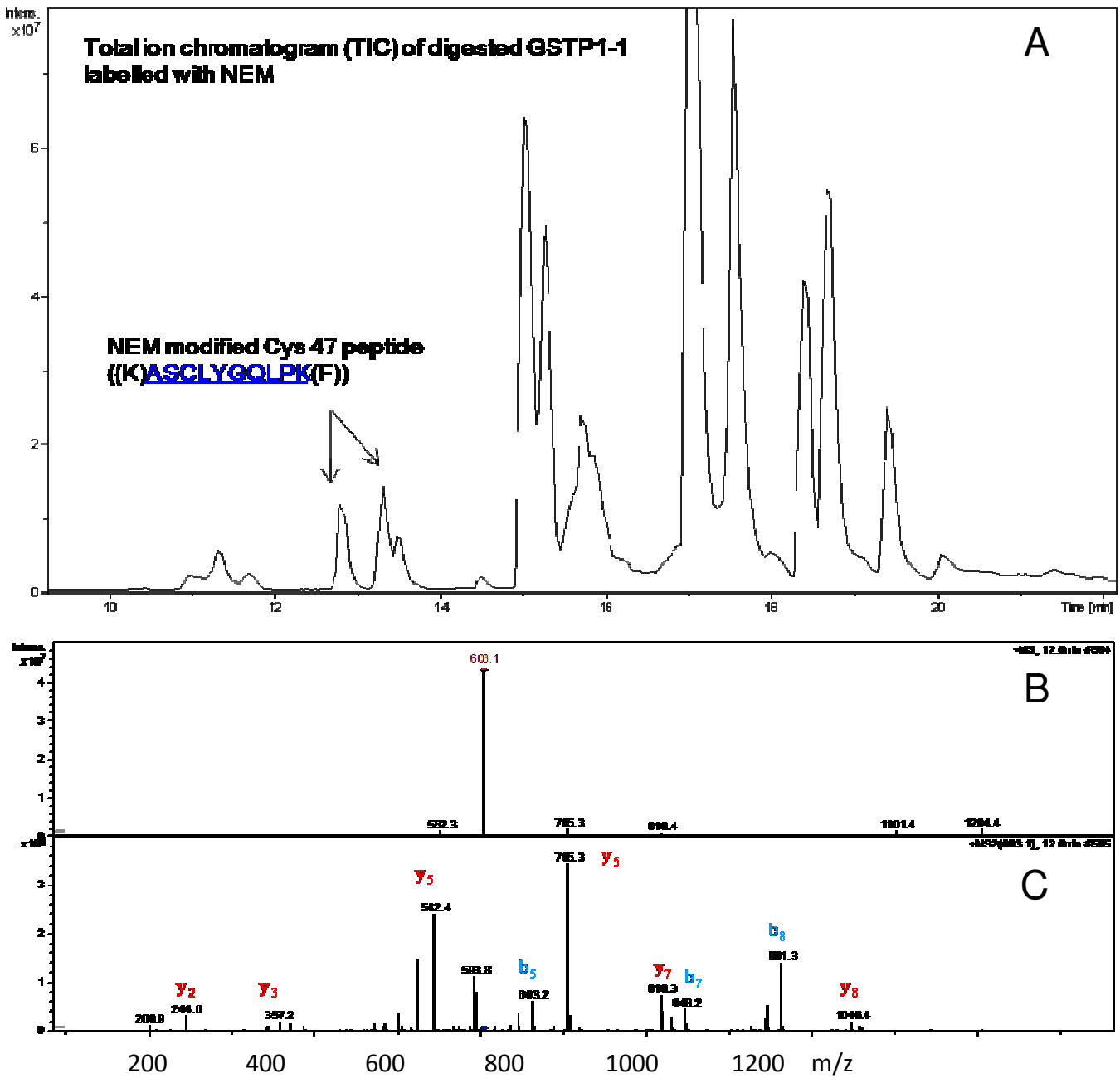


Figure 2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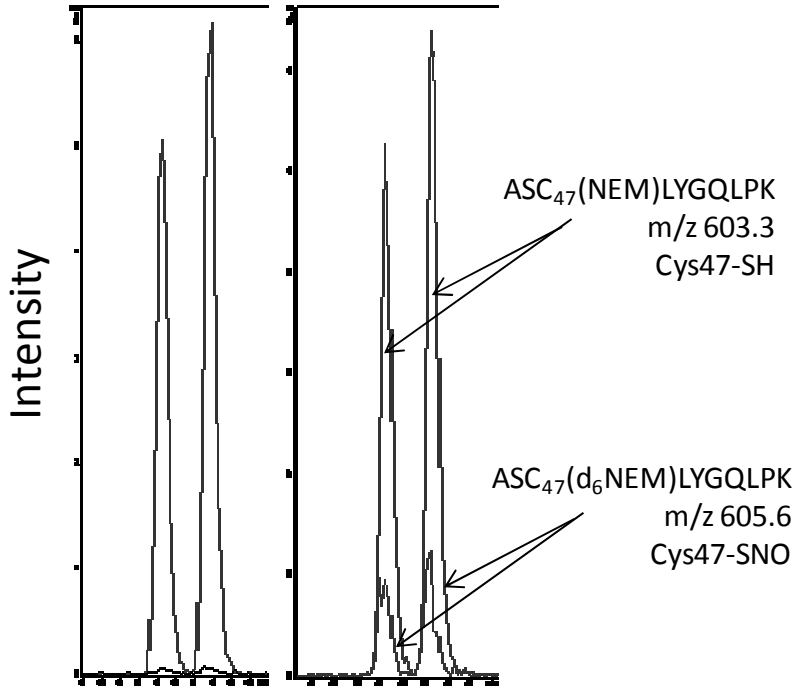
D



IAN, μM	500	500
CuCl (1 μM)	-	+

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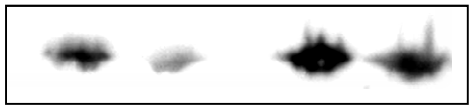

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Figure 3

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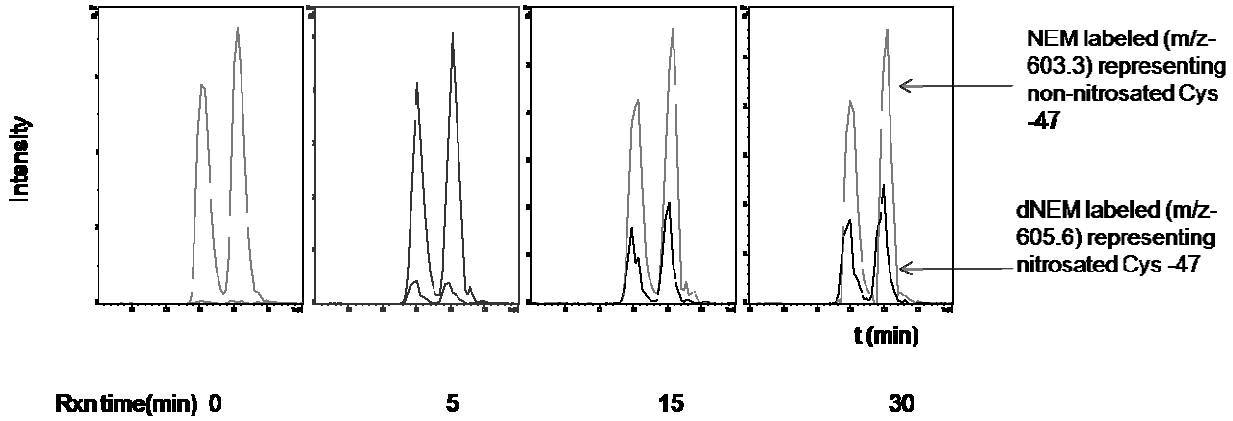
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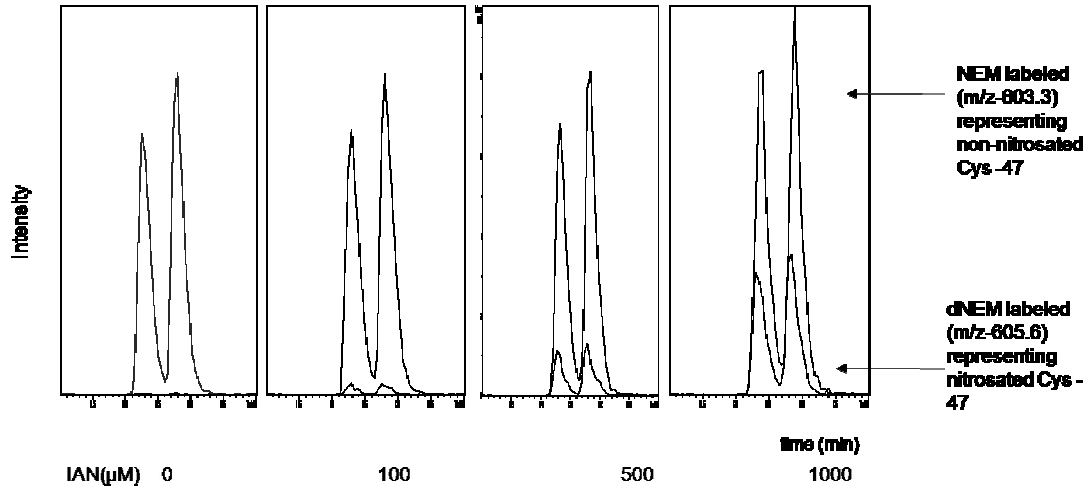
30

B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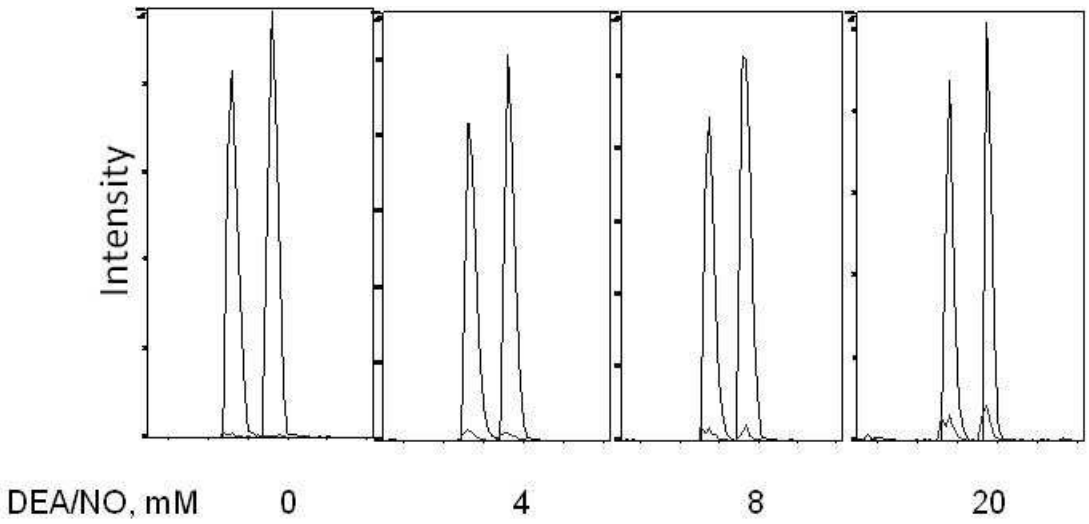
C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8
20

Figure 4

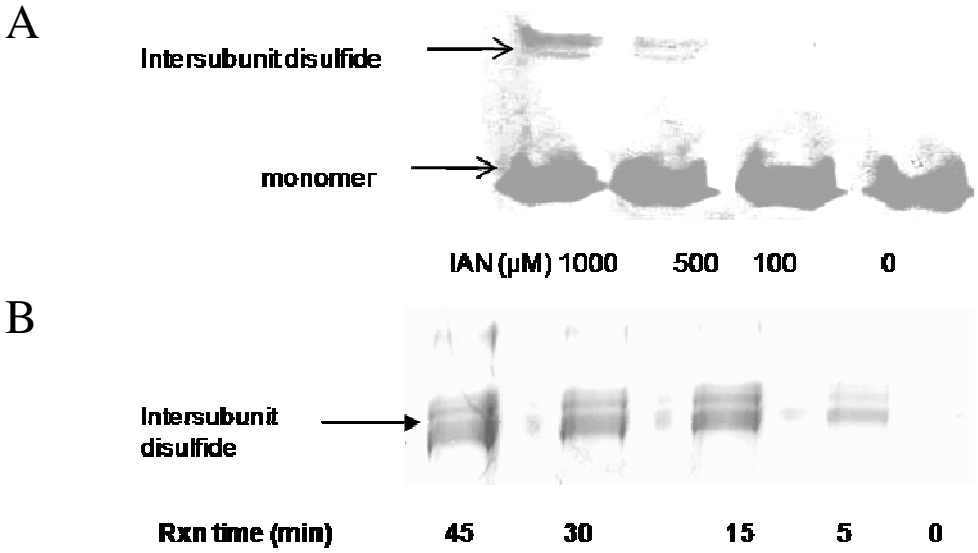


Figure 5

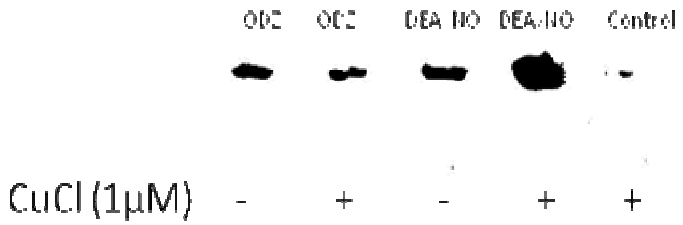
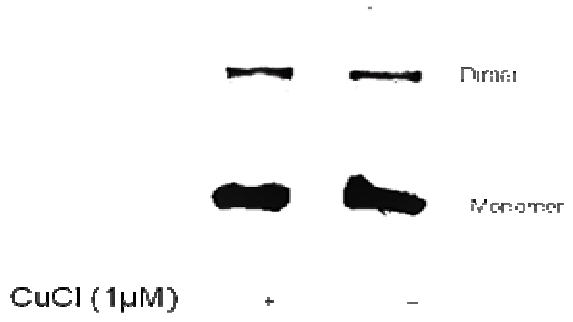


Figure 6



A

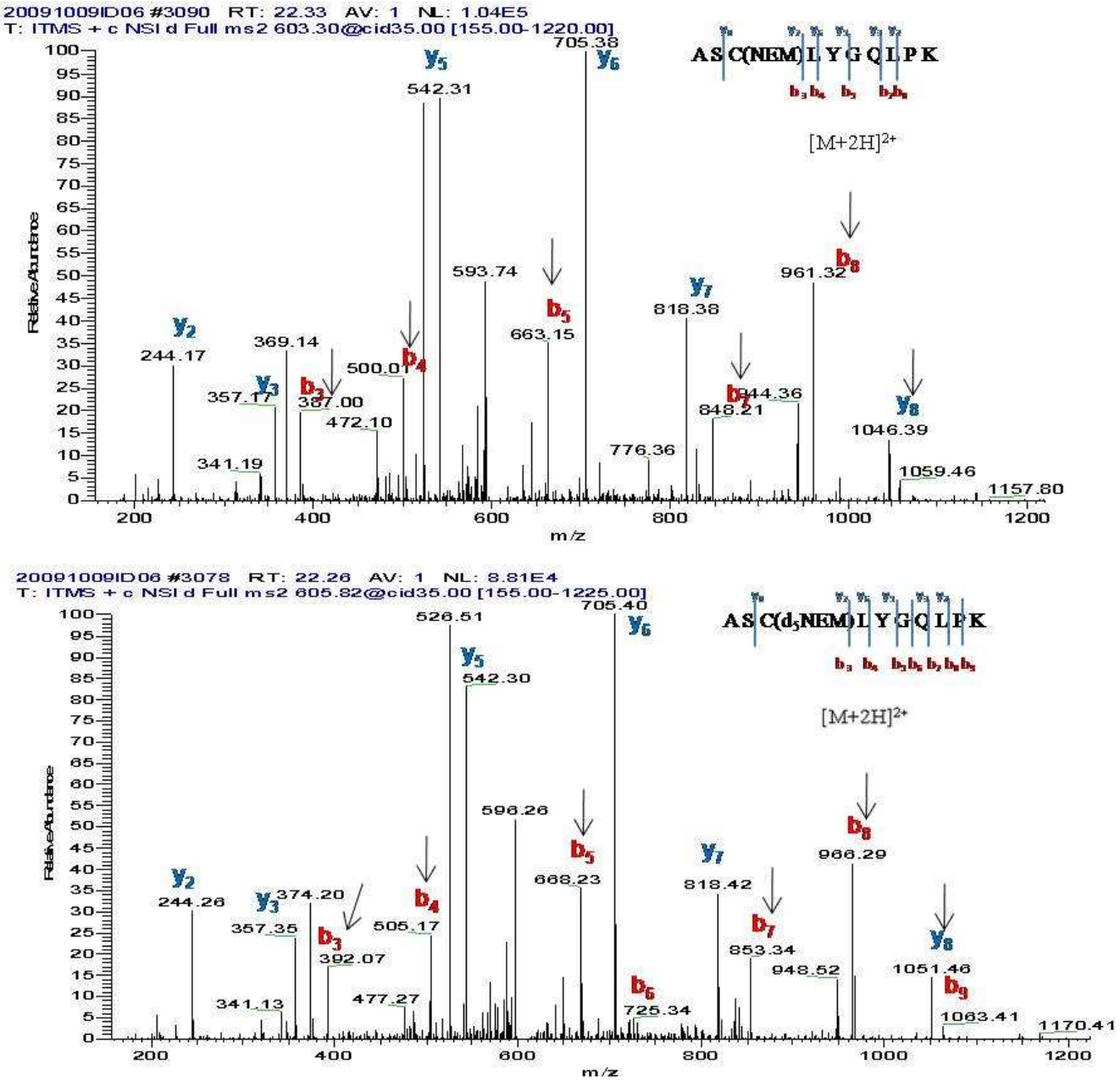

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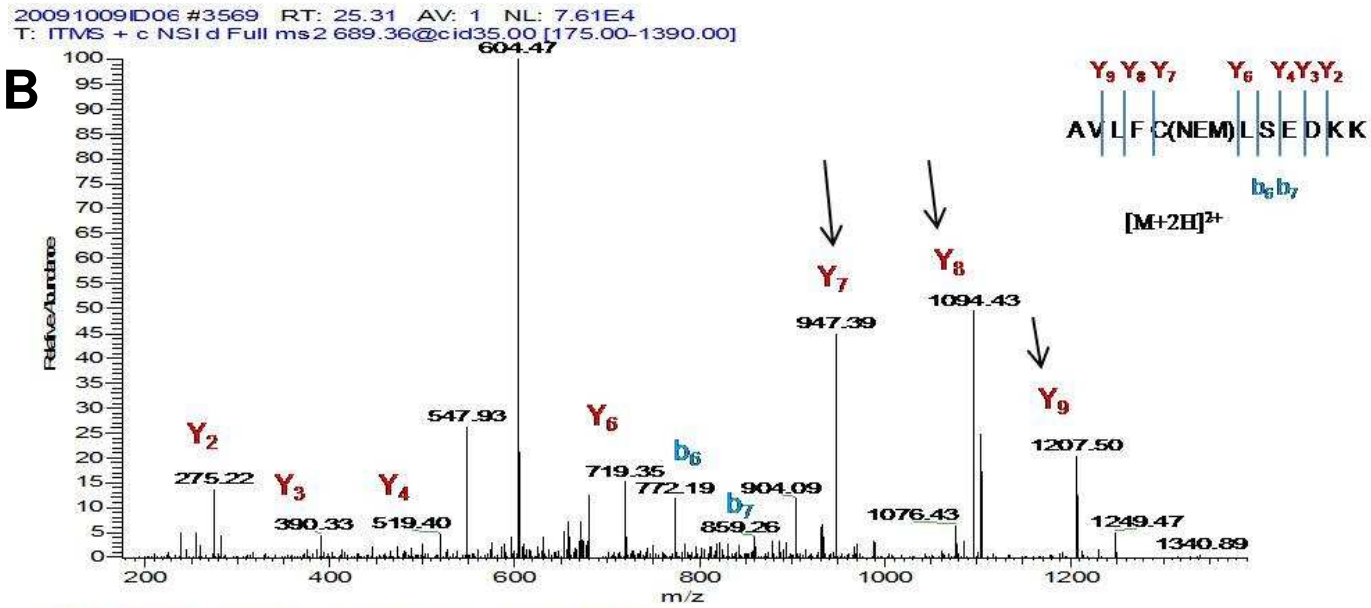
Figure 7

B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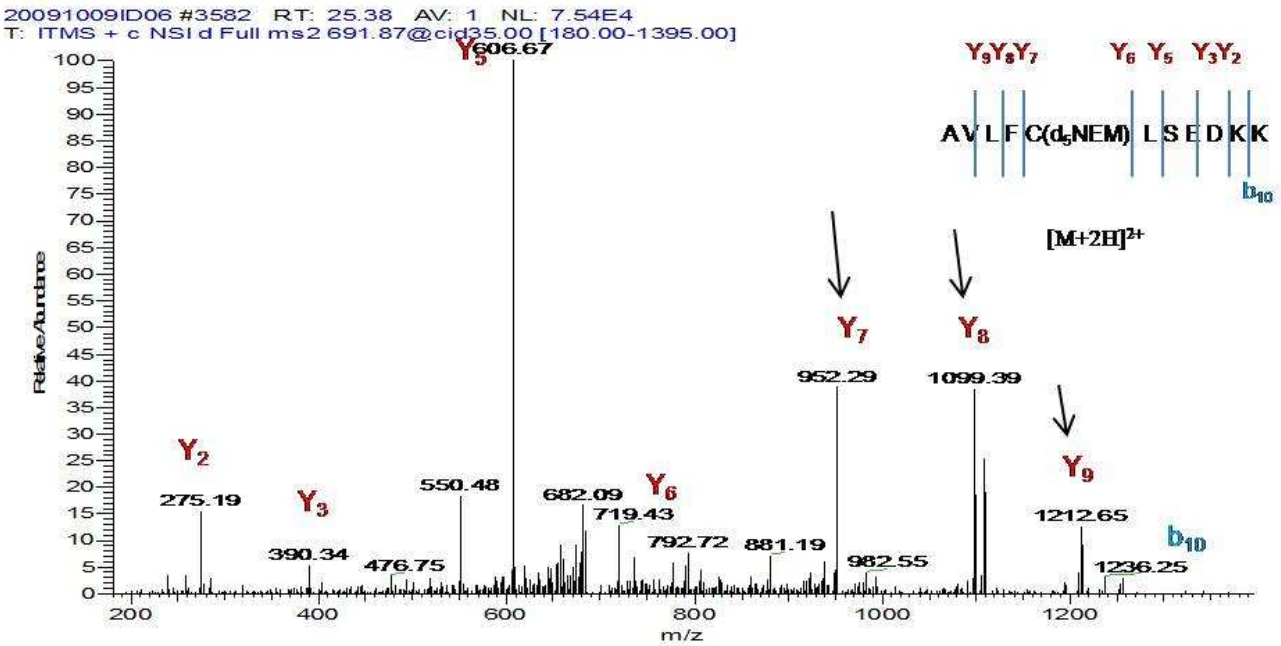
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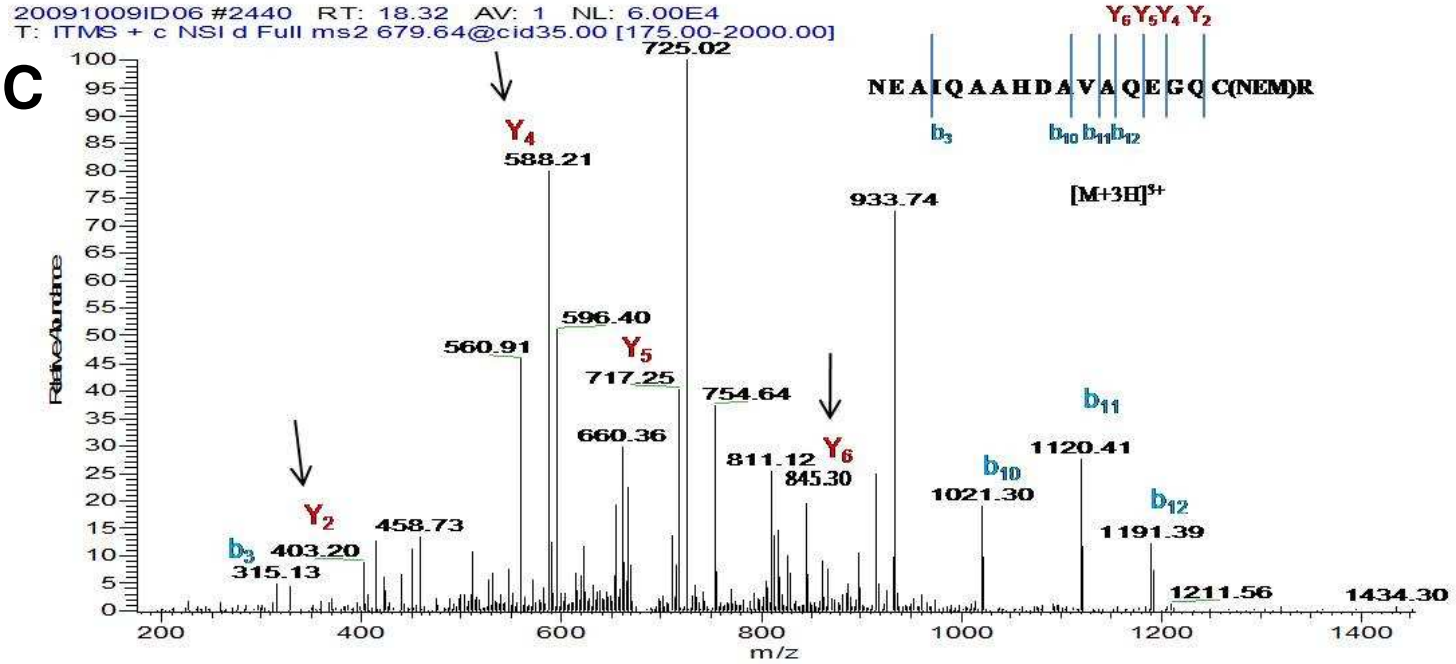
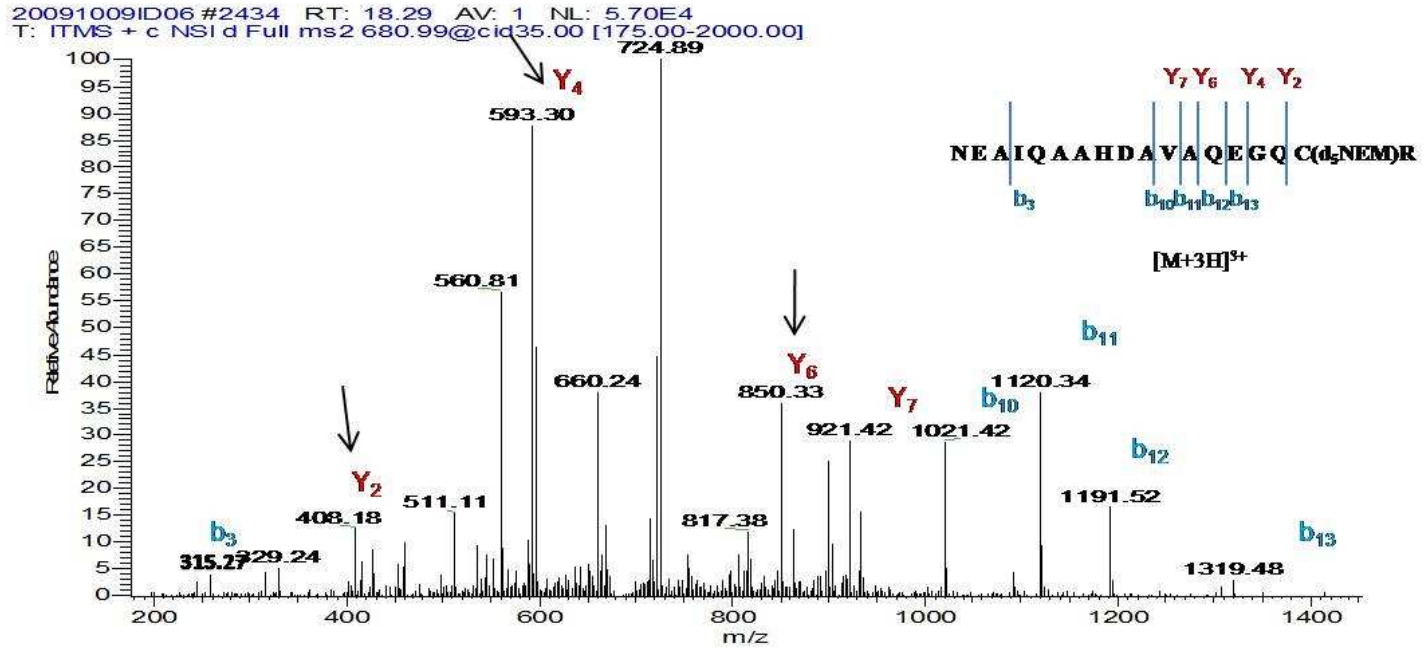
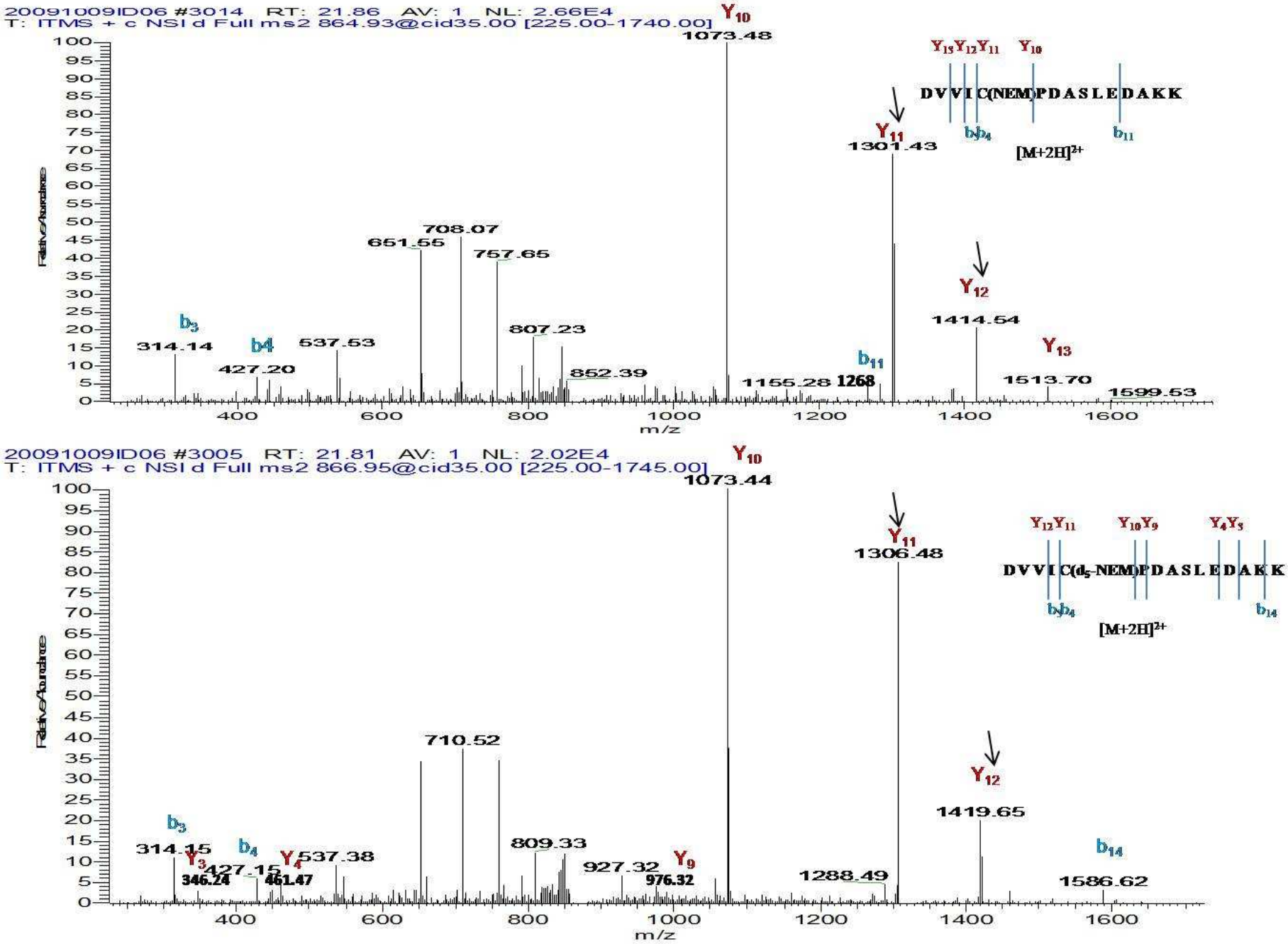
Y2
Y3
Y5
Y6
Y7
Y8
Y9
b10
275.19
390.34
476.75
550.48
606.67
682.09
719.43
792.72
881.19
952.29
982.55
1099.39
1212.65
1236.25

C


20091009ID06 #2440 RT: 18.32 AV: 1 NL: 6.00E4
T: ITMS + c NSI d Full ms2 679.64@cid35.00 [175.00-2000.00]
Y6 Y5 Y4 Y2
NEAIQAAHDAVAQEGQC(NEM)R
b3 b10 b11 b12
[M+3H]3+
725.02
Y4
588.21
933.74
596.40
560.91
Y5
717.25
754.64
660.36
811.12
Y6
845.30
b11
1120.41
b10
1021.30
b12
1191.39
Y2
458.73
b3
403.20
315.13
1211.56
1434.30
Relative Abundance
m/z


20091009ID06 #2434 RT: 18.29 AV: 1 NL: 5.70E4
T: ITMS + c NSI d Full ms2 680.99@cid35.00 [175.00-2000.00]
Y7 Y6 Y4 Y2
NEAIQAAHDAVAQEGQC(d5NEM)R
b3 b10 b11 b12 b13
[M+3H]3+
724.89
Y4
593.30
560.81
Y6
850.33
b11
660.24
Y7
921.42
1021.42
b10
1120.34
b12
Y2
408.18
511.11
817.38
1191.52
b3
315.27
329.24
b13
1319.48
Relative Abundance
m/z

D


20091009ID06 #3014 RT: 21.86 AV: 1 NL: 2.66E4
T: ITMS + c NSI d Full ms2 864.93@cid35.00 [225.00-1740.00]
Y10
1073.48
Y15Y12Y11 Y10
DVVIC(NEM)PDASLEDAKK
bjb4
b11
[M+2H]2+
Y11
1301.43
708.07
651.55
757.65
Y12
1414.54
b3
314.14
b4
537.53
807.23
Y13
427.20
852.39
b11
1155.28
1268
1513.70
1599.53
Relative Abundance
m/z
20091009ID06 #3005 RT: 21.81 AV: 1 NL: 2.02E4
T: ITMS + c NSI d Full ms2 866.95@cid35.00 [225.00-1745.00]
Y10
1073.44
Y11
1306.48
Y12Y11 Y10Y9 Y4Y3
DVVIC(d5-NEM)PDASLEDAKK
bjb4
b14
[M+2H]2+
710.52
Y12
1419.65
b3
314.15
b4
Y3
346.24
427.15
Y4
461.47
537.38
809.33
927.32
Y9
976.32
b14
1288.49
1586.62
Relative Abundance
m/z

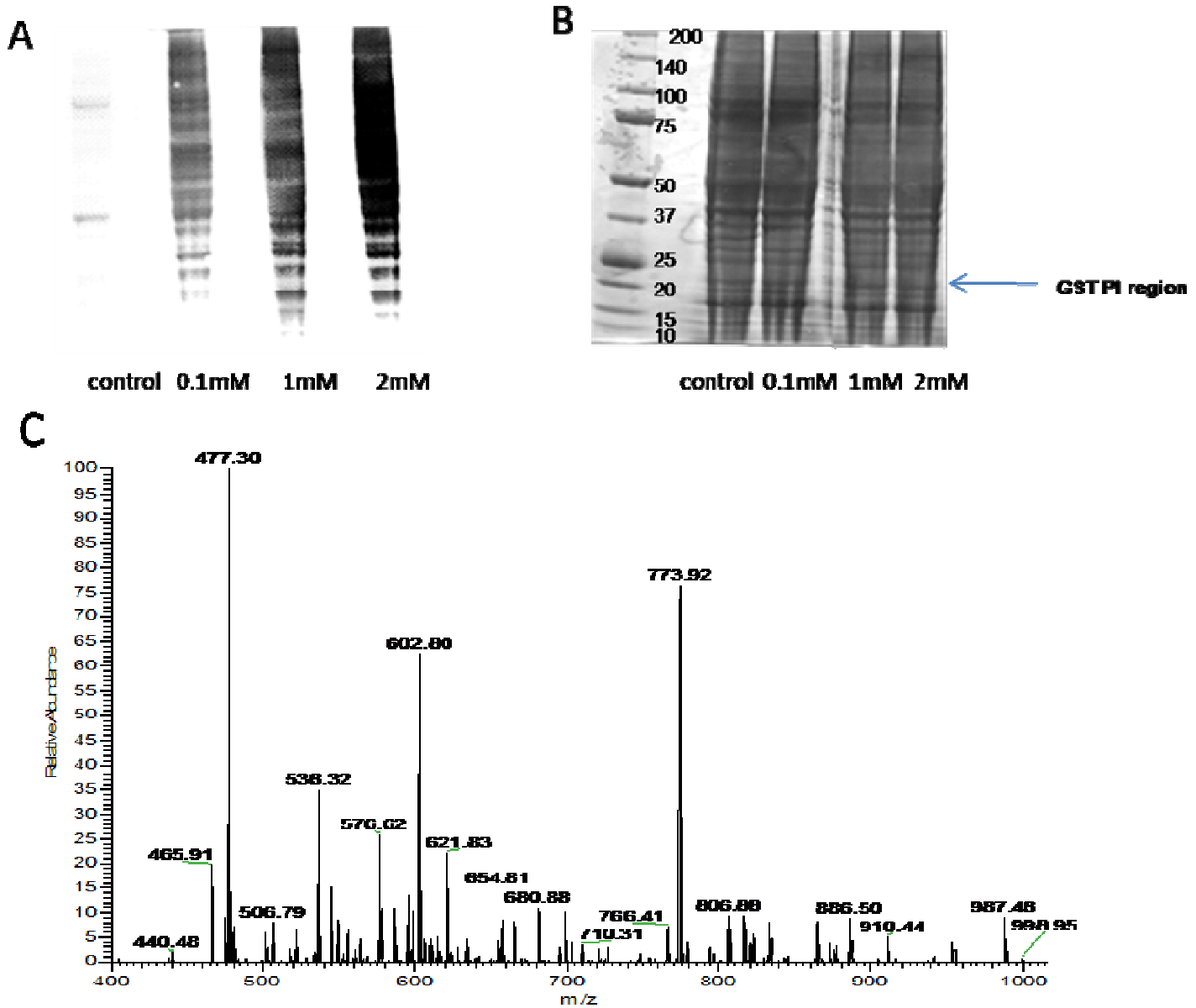

A
control 0.1mM 1mM 2mM
B
200
140
100
75
50
37
25
20
15
10
CSTPI region
control 0.1mM 1mM 2mM
C
RelativeAbundance
m/z
477.30
773.92
602.80
536.32
570.62
621.83
465.91
654.61
680.58
506.79
766.41
806.88
886.50
987.46
910.44
440.48

Figure 8

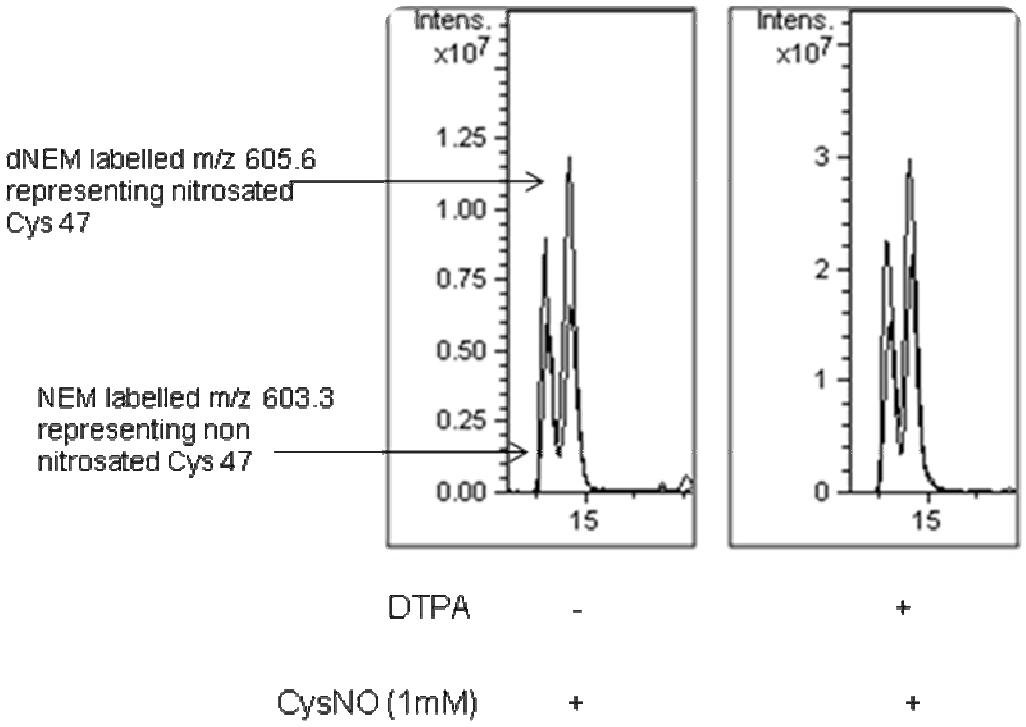

dNEM labelled m/z 605.6
representing nitrosated
Cys 47
NEM labelled m/z 603.3
representing non
nitrosated Cys 47
Intens.
x10^7
1.25
1.00
0.75
0.50
0.25
0.00
15
Intens.
x10^7
3
2
1
0
15
DTPA - +
CysNO (1mM) + +

Figure 9

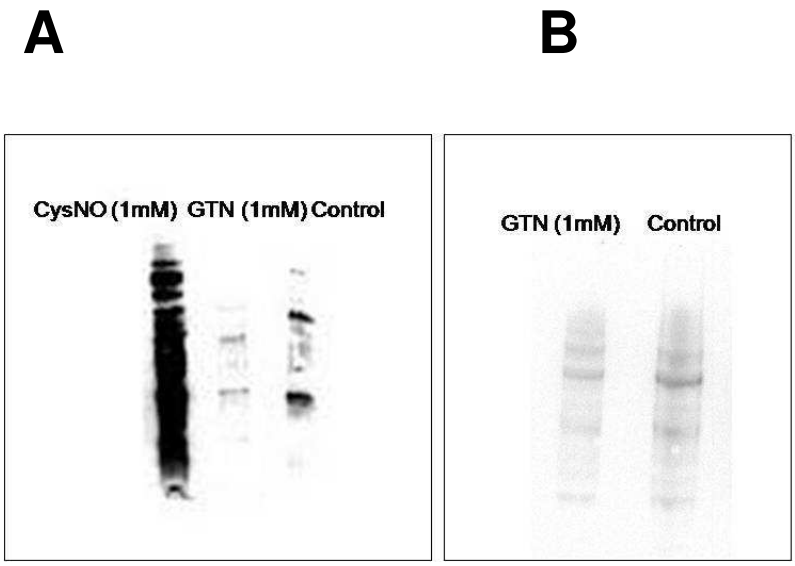


Figure 10

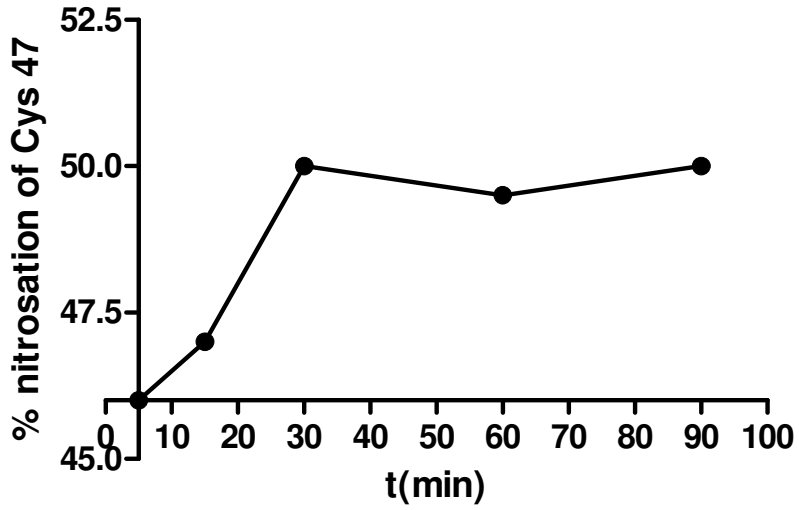


Figure 11

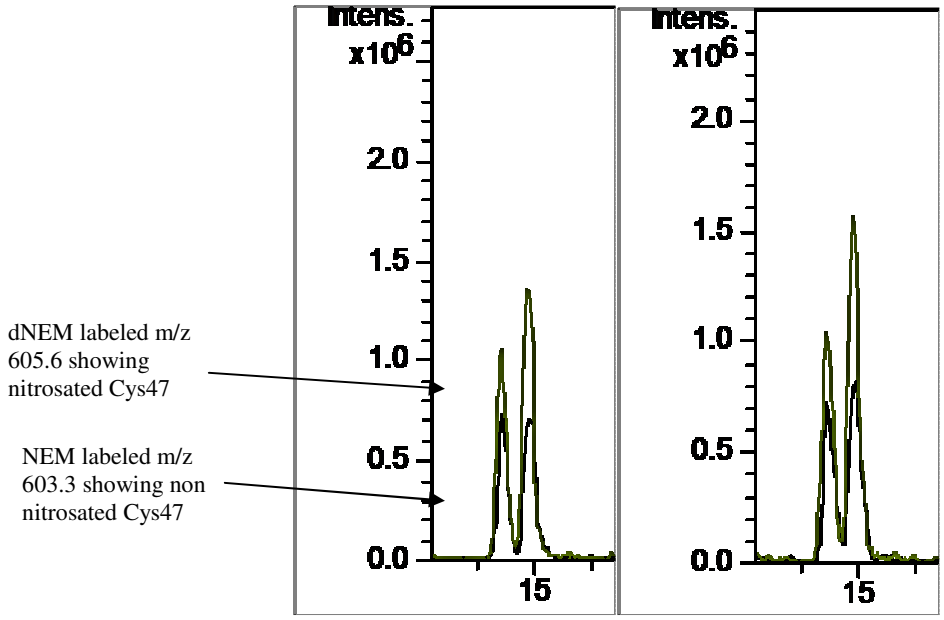
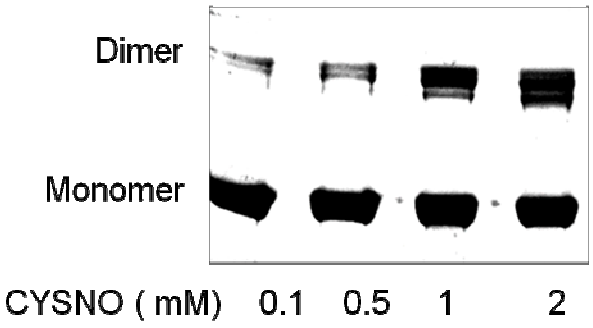

dNEM labeled m/z 605.6 showing nitrosated Cys47
NEM labeled m/z 603.3 showing non nitrosated Cys47
Intens.
x10^6
2.0
1.5
1.0
0.5
0.0
15
Intens.
x10^6
2.0
1.5
1.0
0.5
0.0
15
1mM CysNO
0.5 mM CysNO

Figure 12

A


Dimer
Monomer
CYSNO (mM) 0.1 0.5 1 2

B

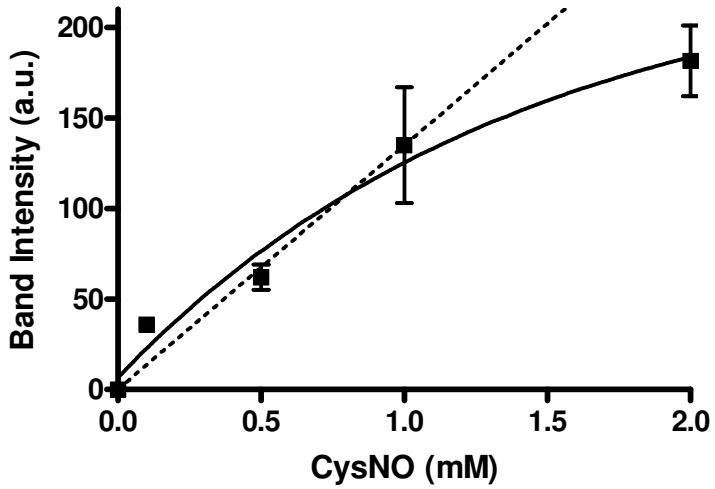

Band Intensity (a.u.)
200
150
100
50
0
0.0
0.5
1.0
1.5
2.0
CysNO (mM)

Figure 13

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