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Misidentification of nitrated peptides: Comments on Hong, S.J., Gokulrangan, G., Schöneich, C., 2007. Proteomic analysis of age dependent nitration of rat cardiac proteins by solution isoelectric focusing coupled to nanoHPLC tandem mass spectrometry. *Exp. Gerontol.* 42, 639–651

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Aging tissue has shown elevated 3-nitrotyrosine (3-NT) levels associated with posttranslational protein nitration caused by various reactive nitrogen species (Greenacre and Ischiropoulos, 2001). The extent of biological nitration is low, reaching merely up to five 3-NT residues per 10,000 tyrosine residues under inflammatory conditions (Radi, 2004). Therefore, the major challenge in the proteomic analysis of nitroproteins is the need to distinguish modified proteins from large amounts of unmodified proteins. Thus far, an overwhelming majority of publications addressing this posttranslational modification *in vivo* have relied on two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) in combination with Western blot detection of 3-NT immunoreactive spots (Butterfield and Sultana, 2008). Because the technique relies on a precise alignment of Western blot analyses and 2D-PAGE gel images and presumes the presence of a single protein per gel spot, the identification of nitroproteins by these investigations should be considered putative, when no information is sought and obtained to localize specific nitration sites in the proteins (Amoresan et al., 2007). By a gel-free methodology and through the use of electrospray ionization (ESI) and tandem mass spectrometry (MS/MS), Hong et al. (2007) have attempted an accurate localization of modification sites required for unequivocal identification of nitration-sensitive proteins in the rat cardiac muscle, and subsequently implicated the effect of this age-associated posttranslational modification on protein functions. Their data was generated on a low-resolution ion trap mass spectrometer and, most probably, employing a high-tolerance protein database search to find potential nitropeptides. Also, “zoom” scans that permit determination of the number of charges on the precursor peptide ions (Davis and Lee, 1997) were not part of the data-dependent acquisition method used for data collection. Without charge state information, database search programs such as SEQUEST try to match both doubly- and triply-charged precursors to the submitted tandem mass spectra, and the higher charge state matches usually get higher scores. Although specific details about protein database search were missing from the paper (Hong et al., 2007), default precursor-ion and MS/MS product-ion mass tolerances are 1.5 Da and 1.0 Da, respectively, when one selects “LCQ” from the “Instrument

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type.” menu of the search parameters window in the Bioworks (SEQUEST) software employed. Because of charge-state ambiguities and high mass tolerance in protein database search, emphasis on validation of the identified hits was highly justified.

The authors apparently accepted nitropeptide identifications based on annotating their MS/MS spectra from theoretical fragmentation obtained using MS-Product, a web-based application incorporated into Protein Prospector (<http://prospector.ucsf.edu>; Chalkley et al., 2005). MS-Product offers options of displaying instrument-dependent MS/MS fragmentations, including an “ESI-ION-TRAP-low-res” option whose pre-set parameters reflect the features of a quadrupole ion trap used by Hong et al. (2007) to perform their MS/MS acquisitions. The pre-set parameters presume that b- and y-type fragment ions are the most abundant ones, and internal ions as well as multiple neutral losses are not present among the product ions of collision-induced dissociation (CID) in this type of instrument. However, Hong et al. (2007) disregarded such characteristics of CID experiments in an ion trap mass spectrometer (Sleno and Volkmer, 2004), and overrode default parameters set for this type of instruments by permitting multiple neutral losses and internal fragment ions to appear and actually considered these nonsensical fragment ions for annotation of the reported MS/MS spectra.

Specifically, Fig. 2 presented as EYRKDLEESIR (where amino acid residues were abbreviated with their one-letter code and the underlined residue indicated posttranslational modification of tyrosine to 3-NT) displayed annotation of the most intense fragment (m/z 619.4) to $b_{10}^{2+}-2H_2O-2NH_3$ ion and, besides several additional irrational assignments of similar kind such as $b_{10}-2H_2O-NH_3$, b_6-2NH_3 and a_7-2NH_3 , even an internal fragment showing multiple neutral loss (RKDLE- $2H_2O$, m/z 606.1). In the m/z 200 to 1500 Th range covered by the MS/MS spectrum in Fig. 2, MS-product may have given Hong et al. (2007) 677 possibilities in a “Theoretical Peak Table” to annotate fragment ions by changing default settings for “ESI-ION-TRAP” to include multiple losses and internal fragment ions. However, a senseless matching of the observed fragment ions to all listed possibilities arising from such manipulations of program options is by no means justifiable. The formation of intense product ions due to multiple neutral losses and due to y- and b-type cleavages at two peptide bonds (internal fragment ions) plus neutral losses should be considered practically impossible in the instrument employed by Hong et al. (2006) in their nitroproteome-focused study, which is supported by a large-scale statistical analysis of ion trap data from doubly charged tryptic peptides (Tabb et al., 2003). It has also been commonly accepted that further fragmentation of b-type ions does not readily take place in an ion trap mass spectrometer, because the fragment ions fall out of resonance with the excitation frequency (Barton and Whittaker, 2009).

Fig. 3 (annotated as ERYAAWMIYTYSGLFCVTVNPYK, where the underlined residues indicate modifications; i.e., oxidation of M and tyrosine nitration, respectively) also displayed numerous ions labeled as y-type ions with multiple neutral losses and an internal fragment ion with water loss. In Fig. 4 (presented as YEEEEIK), there was a b_5-2H_2O annotation, in addition to an unlabeled fragment ion (m/z 580) whose relative abundance was around 57%. The latter clearly begs for an explanation. In Fig. 5 (annotated as SYKYLLLSMVK), Hong et al. (2007) labeled the second most intense fragment ion (m/z 1063.5, relative abundance approximately 66%) as $a_9-NH_3-2H_2O$. Because a-type ions are believed to form from b-type ions by the loss of carbon monoxide (Tabb et al., 2003), this annotation may represents a quadruple neutral loss. Taken together, such improper annotations of all four MS/MS spectra attributed to nitrated peptides and implicated by Hong et al. (2007) as markers of age-associated nitrosative stress in the rat cardiac proteome were indicative of false positives. Indeed, when fragment ion m/z values given in the MS/MS spectra of Fig. 2 to Fig. 5 were input into the MS-Tag program of Protein Prospector, it returned non-nitrated peptide hits with high scores. In addition, peptide syntheses followed by ESI-MS/MS analysis on an ion trap mass spectrometer identical to those employed by Hong et al. (2007) unequivocally confirmed that

their findings on age-dependent nitration of nebulin-related anchoring protein, myosine heavy chain polypeptide 6, tropomyosin and neurofibromin were, indeed, false positives (Stevens et al., 2008). Specifically, none of the nitropeptides synthesized based on the report (Hong et al., 2007) behaved unusually, such as yielding multiple neutral losses or internal fragments, when their molecular ions were subjected to CID. Moreover, all four peptides incorrectly identified as nitrated species were matched with high confidence based on their reported MS/MS spectra to tryptic peptides of abundant cardiac proteins, as summarized in Table 1, and could be annotated considering ordinary y- and b-type of ions. These matches were also confirmed by peptide synthesis followed by ESI-MS/MS analysis (Stevens et al., 2008). Therefore, discussion about the biological significance of age-dependent nitration of nebulin-related anchoring protein, myosine heavy chain polypeptide 6, tropomyosin and neurofibromin in Hong et al. (2007) should be disregarded, because they have been proven false positives without any doubt. Consequently, identification of nitrated FAESKLIYTYSGILVAMNPYK, EPCENYMRCVSVLR, QLTAYMK and LLIKEGQYK should also be considered invalid until MS/MS spectra are available for inspection.

Finally, there also is a strong case for disputing the evidence for the presence of a nitrated peptide in the young rat heart claimed to be within the sequence Thr¹⁰¹–Lys¹¹² of NADH dehydrogenase (ubiquinone) Fe–S. According to section 2.5 of Hong et al. (2007), the protein fractions were reduced by dithiothreitol and alkylated by treatment with iodoacetamide prior to precipitation and proteolytic digestion, followed by nanoHPLC–MS/MS. Moreover, section 2.8 specified that their protein identification was carried out with “alkylation of cysteine (+57 amu)” as an amino acid modification. When, according to the program's syntax, “TGTC (Carbamidomethyl)AY(Nitro)C(Carbamidomethyl)GLQFK” was used as an input string for MS-Product of Protein Prospector, no match to the spectrum reported in Fig. 6 could be obtained. The authors apparently have left the reader to figure out by the process of elimination that their matched sequence was actually TGTCAYC@GLQFK, where C indicates free cysteine and C@ denotes carbamidomethylated cysteine. There was no explanation about the incomplete alkylation in the Result section that discussed Fig. 6, nor any indication was given on the figure that one cysteine (Cys¹⁰⁴) residue was not alkylated while the other (Cys¹⁰⁷) was. Nevertheless, there were several noteworthy discrepancies between predicted and actual *m/z* values of fragment ions, even when TGTCAY(Nitro)C(Carbamidomethyl)GLQFK was used as an input for MS-Product, in addition to the nonsensical labeling a pair of intense fragment ions as CAY(NO₂)CGL–28 (*m/z* 686.2) and TCAY(NO₂)CGLQ–28 (*m/z* 916.5). Detecting the most intense fragment ion presumed to be y₆ at *m/z* 753.3 instead of the predicted *m/z* 752.4 was remarkable. Although Hong et al. (2007) did not specify deamidation as a possible modification, TGTCAY(Nitro)C(Carbamidomethyl)GLEFK (i.e., deamidation of Q to E) was also tested. Although the *m/z* value of the presumed y₆ ion was matched correctly to the resultant MS-Product output, several discrepancies between predicted and actual *m/z* values of fragment ions have remained in the spectrum annotated as TGTCAYC@GLQ#FK (where # indicates deamidation). Taken together, the nitropeptide shown in Fig. 6 Hong et al. (2007) should also be considered false positive. Unfortunately, *m/z* values indicated in Fig. 6 were not sufficient to match to any peptide and protein with high confidence by MS-Product, which again calls for the release of raw MS/MS data for detailed inspection.

In conclusion, protein nitration in rat heart by Hong et al. (2007) presented in their Fig. 2–6 has been found to be unequivocally false positives. Researchers engaged in analyzing posttranslational protein modifications by tandem mass spectrometry should be cautious and refrain from being overly “creative” in attempts to validate their hits emanating from high-tolerance database searches by questionable spectrum annotation practices that defy the physical principles of their instrumentation. A new validation procedure has become available recently to guide their effort in nitroproteomics (Stevens et al., 2008).

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Table 1

List of nitrated peptides and proteins misidentified, as well as incorrectly implicated in the aging process by Hong et al. (2007), together with the actual peptides and proteins matched to the published MS/MS spectra (Stevens et al., 2008).

Misidentified nitrated peptide (<u>Y</u> indicates nitrotyrosine)	Protein reported to correspond misidentified peptide ^a	Actual peptide	Protein(s) corresponding to actual peptide ^a
EYRKDLEESIR (Fig. 2 of Hong et al., 2007)	Nebulin-related anchoring protein isoform C, N-RAP (gi:109468170)	KVQHELDEAEER	Alpha myosin, heavy chain 6, cardiac muscle (gi:127741 and gi:186659510) Beta myosin heavy chain (gi:32493127) Beta myosin, heavy chain 7, cardiac muscle (gi:8393807) rCG23467, isoform CRA_a (gi:149063941) rCG23467, isoform CRA_b (gi:149063942) rCG23609, isoform CRA_b (gi:149063939)
ERYAAWMIYTYSGLFCVTNPNYK (Fig. 3 of Hong et al., 2007) ^b	Myosin heavy chain polypeptide 6 (gi:8393804)	DQEGQDVLLFIDNIFR	ATP synthase, H ⁺ transporting, mitochondrial F1 complex, beta subunit (gi:54792127 and gi:89574017) ATP synthase, H ⁺ transporting, mitochondrial F1 complex, beta subunit, isoform CRA_a (gi:149029718)
YEEIEIK (Fig. 4 of Hong et al., 2007)	Tropomyosin 1, α isoform e (gi:78000196)	SIHELEK	Beta myosin, heavy chain 7, cardiac muscle (gi:8393807) Myosin, heavy chain 3, skeletal muscle, embryonic (gi:6981234) rCG34382, isoform CRA_a (gi:149052965) rCG34382, isoform CRA_b (gi:149052966) rCG23467, isoform CRA_a (gi:149063941) rCG23467, isoform CRA_b (gi:149063942)
SYKYLLLSMVK (Fig. 5 of Hong et al., 2007)	Neurofibromatosis 1, neurofibromin (gi:6981264)	NLVHIITHGEEK	Myosin regulatory light chain 2, ventricular/cardiac muscle isoform (gi:127167) Myosin, regulatory light polypeptide 2, cardiac, slow (gi:78214305) Myosin regulatory light chain 2, ventricular/cardiac muscle isoform, isoform CRA_a (gi:149063386)

^a gi: Gene info number (<http://www.ncbi.nlm.nih.gov/>)

^b This misidentification was an example for the database search program (SEQUEST) incorrectly matching, due to the unavailability of explicit charge-state information from the submitted data, a triply-charged (3+) precursor to the tandem mass spectrum, while the actual peptide (DQEGQDVLLFIDNIFR) was doubly-charged (2+). (M indicates oxidized methionine.)