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# Proteomic Approaches to Identifying Carbonylated Proteins in Brain Tissue

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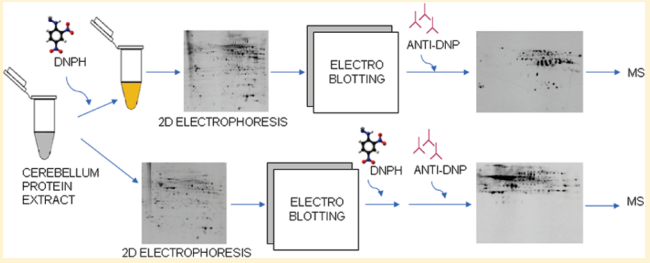
**ABSTRACT:** Oxidative stress plays a critical role in the pathogenesis of a number of diseases. The carbonyl end products of protein oxidation are among the most commonly measured markers of oxidation in biological samples. Protein carbonyl functional groups may be derivatized with 2,4-dinitrophenylhydrazine (DNPH) to render a stable 2,4-dinitrophenylhydrazone-protein (DNP-protein) and the carbonyl contents of individual proteins then determined by two-dimensional electrophoresis followed by immunoblotting using specific anti-DNP antibodies. Unfortunately, derivatization of proteins with DNPH could affect their mass spectrometry (MS) identification. This problem can be overcome using nontreated samples for protein identification. Nevertheless, derivatization could also affect their mobility, which might be solved by performing the derivatization step after the initial electrophoresis. Here, we compare two-dimensional redox proteome maps of mouse cerebellum acquired by performing the DNPH derivatization step before or after electrophoresis and detect differences in protein patterns. When the same approach is used for protein detection and identification, both methods were found to be useful to identify carbonylated proteins. However, whereas pre-DNPH derivatized proteins were successfully analyzed, high background staining complicated the analysis when the DNPH reaction was performed after transblotting. Comparative data on protein identification using both methods are provided.




**KEYWORDS:** Redox proteomics, cerebellum, protein oxidation, carbonylation, 2,4-dinitrophenylhydrazine, 2D oxyblot

Reactive oxygen species (ROS) build up in different physiological and disease conditions as a consequence of an imbalance between their formation and neutralization by antioxidant systems. This situation causes oxidative stress, which in turn promotes cell alterations such as accumulation of oxidized-damaged molecules, increased levels of dysfunctional macromolecules, and severely compromised cell homeostasis.[1−4] Brain tissue is particularly vulnerable to oxidative damage because of its high oxygen requirements, low antioxidant levels, and high polyunsaturated fatty acid and redox transition metal ion contents.[1]

Several types of molecules are directly affected by oxidative damage. However, the oxidation of proteins is of particular concern since it leads to aggregation, polymerization, unfolding, or conformational changes that may confer a loss of structural or functional activity.[1] Although several protein oxidative modifications exist, most oxidized proteins exhibit carbonyl groups (aldehydes and ketones).[2,4,5] These groups are introduced into the proteins through a variety of oxidative pathways.[6] ROS can react directly with the protein (Figure 1A, B) or can react with other molecules, generating products capable of reacting with proteins.[6] A consequence of this modification is that they can react with lysine residues, cross-link, and generate protein aggregates. These oxidized aggregates are not readily degraded in the cell, and their buildup causes cell dysfunction.[2]

Carbonyl groups are good markers of protein oxidation because they are one of the final byproducts of multiple oxidation pathways that occur in the cell.[7] Moreover, because carbonyl groups are chemically stable, they are very useful for laboratory analysis.[3,4,7−9] The carbonyl contents of individual proteins may be assessed using the following procedures, in which the order of steps a and b may be inverted: (a) incorporation of a dinitrophenyl group through reaction of the carbonyl with dinitrophenylhydrazine (DNPH), which leads to the formation of a stable dinitrophenylhydrazone (DNP) product (Figure 1C); (b) two-dimensional (2D) electrophoresis; and (c) immunoblotting using anti-DNP antibodies.[3−5,7,9] A third alternative incorporates the DNPH derivatization step after the isoelectric focusing (IEF).[10,11] Spots detected in the 2D Western blots (2D oxyblots) and 2D stained gels can be matched by computer assisted image analysis. The carbonyl content of each protein is normalized to its total protein quantity through a relation between the chemiluminescence signal intensity detected in the immunoblots and the

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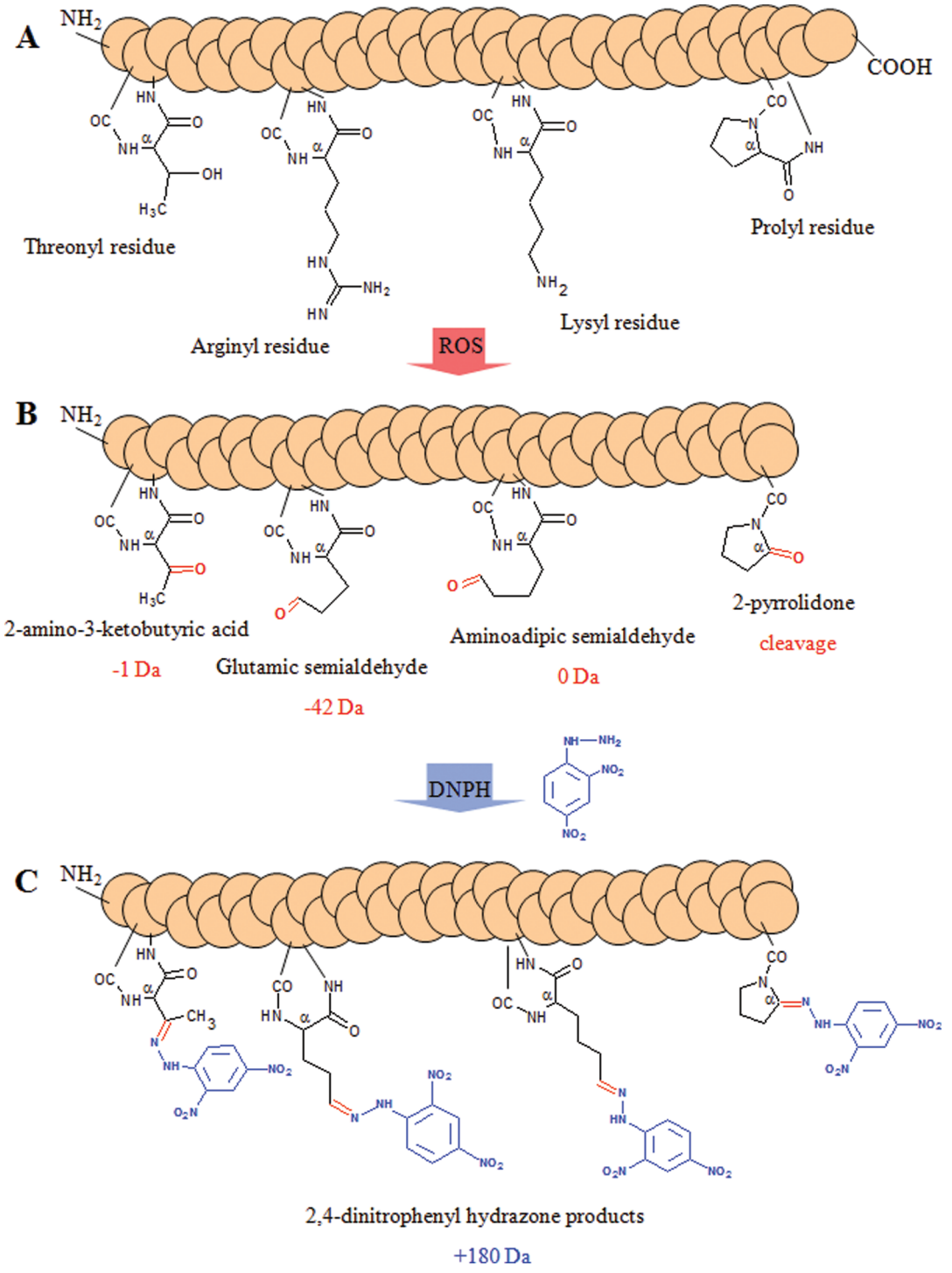


**Figure 1.** Protein carbonyls produced by direct oxidation of amino acid side chains after its reaction with ROS. The structure of several amino acid residues is shown in the backbone of a "hypothetical" protein (A). After its interaction with ROS, oxidation produces a carbonyl derivative that could modify its molecular weight (B). Carbonyl groups and molecular weight modifications are shown in red. Dinitrophenylhydrazine (DNPH) reacts with the carbonyl groups, generating a stable dinitrophenylhydrazone product (C). The molecular weight modification produced by this reaction is indicated in blue.

signal of the same protein in a stained gel. Once a spot is identified as modified by DNPH, it is excised from the gel, digested with trypsin, and then analyzed by mass spectrometry (MS).[12]

Biological samples can be derivatized with DNPH before the 2D electrophoresis step.[7,13−17] However, because this pretreatment could affect subsequent MS identification,[18,19] some authors advocate the use of nontreated samples for protein identification.[18,20,21] DNPH derivatization can alter the molecular weight and/or isoelectric point of proteins.[3,5,22,23] Thus, the use of nontreated proteins in gels for protein identification and DNPH treated proteins for Western blotting could lead to potential mismatching of carbonylated spots in the 2D gel for


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MS. To minimize this, samples can be derivatized with DNPH once the proteins have been transferred to the membrane,[22,24] and this will also improve the low MS scores attributed to molecular weight modifications due to derivatization.

Since the correct MS identification of a protein also depends on the correct "matching" of spots detected in the Western blot with those observed in the gels, we compared the two most contrasting situations, this is, comparison of the use of DNPH treated samples to prepare the 2D gels followed by Western blotting with the use of untreated samples for 2D electrophoresis followed by DNPH derivatization. This is the first side-by-side comparison of two of the most popular oxyproteomics approaches used today.

## MATERIALS AND METHODS

### Materials

3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), decanoyl-*N*-methylglucamide (MEGA 10), L-α-lysophosphatidylcholine (LPC), DNPH, anti-2,4-DNP primary antibody (ref. D9656), and α-cyano-4-hydroxytranscinnamic acid matrix were obtained from Sigma (St. Louis, MO, USA). Immobilized pH gradient (IPG) strips and horseradish peroxidase-conjugated anti-rabbit-Ig secondary antibody were purchased from GE Healthcare (Buckinghamshire, U.K.). Ethylenediaminetetraacetic acid (EDTA) free protease inhibitor cocktail and sequencing grade trypsin were from Roche Diagnostics (Manheim, Germany), polyvinylidene fluoride (PVDF) membranes from Millipore (Bedford, MA, USA) and SuperSignal from Pierce (Rockford, IL, USA).

### Sample Preparation

All experiments were performed at the Universidad Complutense de Madrid according to the guidelines of the International Council for Laboratory Animal Science. Six adult male C57Bl/6 mice were sacrificed by cervical dislocation and decapitation. The brain was removed, and the cerebellum was dissected on ice and stored at −80 °C until needed. Cerebella were pooled and homogenized in modified RIPA lysis buffer [50 mM Tris-HCl, pH 8 and 50 mM NaCl, supplemented with detergents 3% (w/v) CHAPS; 0.5% (w/v) MEGA 10; 0.5% (w/v) LPC, and EDTA free protease inhibitor cocktail]. For protein extraction, the cerebellum suspension was vortexed for 1 h in intervals of 10 min and centrifuged twice (13,000 × *g*, 30 min). After centrifugation, the pellet was discarded, and the extracted proteins were recovered in the supernatant and quantified using the Bio-Rad Protein Assay (Bio-Rad Laboratories Inc., Munich, Germany) following the manufacturer's instructions.

Four out of 25 mg of protein were derivatized with DNPH as previously described[7] with some modifications. Briefly, proteins were denatured at 100 °C for 3 min at 100 °C in the presence of sodium dodecyl sulfate (SDS) 6%. Derivatization was performed by adding one volume of a solution containing 10 mM DNPH in 10% trifluoroacetic acid and incubating 10 min at 25 °C. The mixture was neutralized by adding one volume of stopping solution (2 M Tris, 30% glycerol and 15% β-mercaptoethanol).

Derivatized and nonderivatized proteins were precipitated using acetone at room temperature and resuspended in a buffer containing 7 M urea, 2 M thiourea, 3% (w/v) CHAPS, 0.5% (w/v) MEGA-10, 0.5% (w/v) LPC, and 10 mM dithioerythritol. Proteins were quantified using the Bio-Rad Protein Assay to adjust the protein loss.

### Two-Dimensional Gel Separation of Cerebellar Proteins

A 200 μg portion of protein was cup loaded onto IPG strips (pH 3−11, 18 cm.) previously hydrated overnight with a buffer containing 7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 100 mM DeStreak, and 2% ampholites at pH 3−11. For first-dimensional separation, IEF was performed using the IPGphor 3 IEF system (GE Healthcare, Buckinghamshire, U.K.) at 20 °C. The voltage was gradually ramped in a step-and-hold manner to 1000 V in three steps: 1 h at 120 V, 1 h at 500 V, and 2 h at 500−1000 V. This voltage was increased to 4000 V in a linear gradient (1000−4000 V) over the next 9 h. The run was terminated after ∼70.000 V h. The focused strips were equilibrated in equilibration solution (100 mM Tris 6.8, 6 M urea, 30% glycerol, 2% SDS) containing 0.5% dithiothreitol (DTT) reducing agent for 12 min and transferred to 4.5% iodoacetamide equilibration solution for a further 5 min. The second-dimension SDS-polyacrilamide gel electrophoresis (SDS-PAGE) was run on homogeneous 12% T and 2.6% C casted polyacrylamide gels. Electrophoresis was carried out at 20 °C, 2 W/gel for 30 min and 20 W/gel for 4 h using an Ettan-Dalt six unit. For each experimental condition, 5 gels were run in parallel under identical 2D electrophoresis conditions. Two gels were stained with colloidal Coomassie brilliant blue[25] to visualize total proteins and for subsequent protein identification.

### Western Blot Analysis

Three of the five gels prepared for each experimental condition were transferred to PVDF membranes. Transfer efficiency was checked to observe that it was similar in both methods. Carbonyl groups in the transferred membranes containing derivatized proteins were immunodetected as previously described.[15] After blocking, membranes were incubated for 2 h with a 1/4000 dilution of the anti-2,4-DNP primary antibody in PBS−0.05% Tween containing 10% skimmed milk powder (w/v). The blots were then washed 3−4 times in PBS−Tween solution for 5 min and one more time in PBS−Tween containing milk (as above) for 15 min. Next, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit-Ig secondary antibody at 1/5000 dilution in PBS−Tween containing milk for 1 h. Membranes were washed twice for 5 min in PBS−Tween containing milk, followed by 2 washes in PBS−Tween solution and finally 2 washes using PBS solution prior to visualization. Membranes containing the nonderivatized samples were derivatized as previously described[22] with minor modifications as follows: membranes were equilibrated in 20% (v/v) methanol−80% TBS for 5 min and incubated in 2 N HCl for 5 min. Next, membranes were incubated in a DNPH solution (0.5 mM) in 2 N HCl for 5 min. Membranes were washed 10 times in 2 N HCl, 30 times in methanol 100%, once in PBS−Tween, and 5 times in PBS. The greater the number of washings, the lower the background induced by DNPH excess. Then, blocking and immunodetection was performed as previously described using a 1/40,000 dilution of the anti-2,4-DNP antibody. SuperSignal was used as the chemiluminescent substrate for visualization.

### Trypsin Digestion and Matrix-Assisted Laser Desorption/Ionization (MALDI) Mass Spectrometry

Spots of interest were manually excised from the 2D gels, in-gel reduced, alkylated, and then digested with trypsin as described elsewhere.[26] Briefly, spots were washed twice in water, shrunk for 15 min with 100% acetonitrile, and dried in a Savant SpeedVac for 30 min. The samples were then reduced using 10 mM DTT in 25 mM ammonium bicarbonate for 30 min at 56 °C

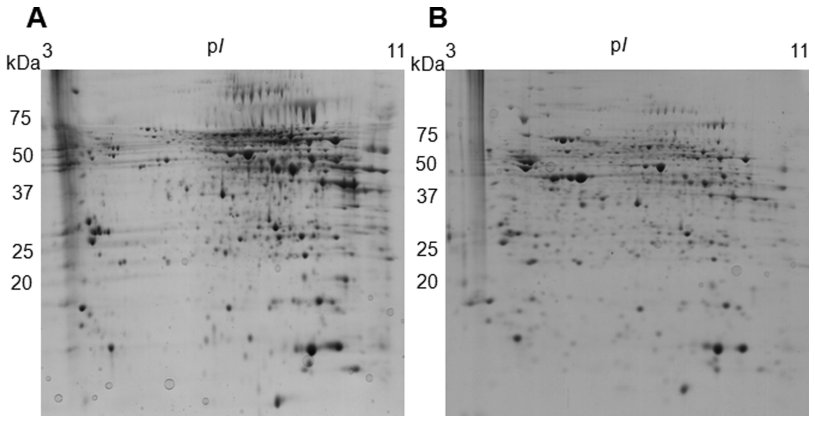


**Figure 2.** Bidimensional SDS-PAGE of mouse cerebellum extracts stained with Coomasie brilliant blue. Samples derivatized with 2,4-dinitrophenylhydrazine before electrophoresis (A) or after transfer (B). In the gels containing pretreated samples, enrichment of the basic range was observed, whereas in those loaded with untreated samples, the acid range was enriched. Gels shown are representative of two independent experiments.

and subsequently alkylated with 55 mM iodoacetamide in 25 mM ammonium bicarbonate for 20 min in the dark. Finally, samples were digested with 12.5 ng/$\mu$L sequencing grade trypsin in 25 mM ammonium bicarbonate (pH 8.5) overnight at 37 °C. After digestion, the supernatant was collected, and 1 $\mu$L was spotted onto a MALDI target plate and allowed to air-dry at room temperature. Next, 0.4 $\mu$L of a 3 mg/mL of α-cyano-4-hydroxytranscinnamic acid matrix in 50% acetonitrile was added to the dried peptide spots and allowed, again, to air-dry at room temperature.

MALDI time-of-flight (TOF) MS analyses were performed in a 4800 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Warrington, U.K.) operated in positive reflector mode, with an accelerating voltage of 20.000 V. Mass spectra were then acquired for peptide mass fingerprinting (PMF).

Proteins were identified by comparing the trypsin-digested peptide masses with the data provided in two databases (NCBI Date 080626 with 6,640,940 sequences, 2,276,975,120 residues and UniprotKB-SwissProt v.56.6) separately using MASCOT 1.9 (http://www.matrixscience.com) through the Global Protein Server v3.5 from Applied Biosystems. The parameters for the PMF search were as follows: modification on cysteine residues by carboxyamidomethylation was set as the fixed modification; methionine oxidation was considered as a variable modification; the maximum number of missed tryptic cleavages was one; peptide mass tolerance was set to 50 ppm and monoisotopic masses were considered. In some cases, taxonomy was restricted to *Mus musculus*. All of the identified proteins fulfilled the criterion of being significant ($P < 0.005$) according to probability based on the Mowse score.

### Image and Statistical Analyses

Gel and immunoblot images were analyzed using PDQuest software, version 8.0.1 (BioRad Laboratories Inc., Munich, Germany). Statistical analyses were performed using the Statgraphics Plus 5.1 software package.

## RESULTS

### Protein Separation

Our comparative analysis of images of Coomasie blue-stained gels obtained using derivatized and nonderivatized samples revealed adequate resolution and spot differentiation across the two dimensions (Figure 2). However, differences were observed in the number of protein spots and their distribution pattern. Pretreatment with DNPH led to a greater number of spots. Automated analysis identified 1157 common spots between gel replicates (out of 1428 ± 75 total spots/gel) when pretreated proteins were used (Figure 2A). In contrast, 802 spots were reproducibly detected in gel replicates containing non-DNPH treated proteins (out of 955 ± 67 total spots/gel) (Figure 2B). When the gel images obtained using the two technical approaches were compared, we detected 577 common spots. For both types of sample, gels exhibited specific areas of significant protein spot enrichment. However, in gels containing DNPH treated proteins, this enrichment was observed in the basic range, whereas in gels loaded with non-DNPH treated samples, protein spot enrichment appeared in the acid range. Vertical streaking was observed for both sample types (Figure 2A, B), and horizontal streaking was reduced in gels containing nontreated proteins (Figure 2B).

### Carbonyl Modified Proteins

In samples prederivatized or derivatized after membrane transfer with DNPH, carbonyl modified proteins were identified by Western blotting using the anti-DNP antibody (Figure 3). The minimal level of protein detection was similar in both methods (0.01−0.05 $\mu$g).

2D oxyblots obtained from samples prederivatized with DNPH displayed 218 common modified spots among technical replicates (out of 311 ± 86 total spots) (Figure 3A), whereas samples derivatized with DNPH after membrane transfer showed 303 common modified spots among replicates (out of 391 ± 51 total spots) (Figure 3B). Although a higher number of carbonyl modified protein spots was detected when the proteins were treated with DNPH after transfer to the blot, the samples pretreated with DNPH showed improved reproducibility and reduced background staining (Figure 3). To determine the number of spots that could be matched for potential MS identification in the gels, images of the 2D oxyblots and 2D gels stained with Coomasie blue were matched using PDQuest. Since automated matching was possible only for gel-to-gel or blot-to-blot images, and not gel-to-blot images, the manual PDQuest option was used to identify the carbonylated protein spots in the preparative gels. The Coomasie blue-stained gels yielded 155 out of the 218 modified spots (71%) in the 2D oxyblots of pretreated samples. In contrast, only 134 of the 303 modified spots (44%)


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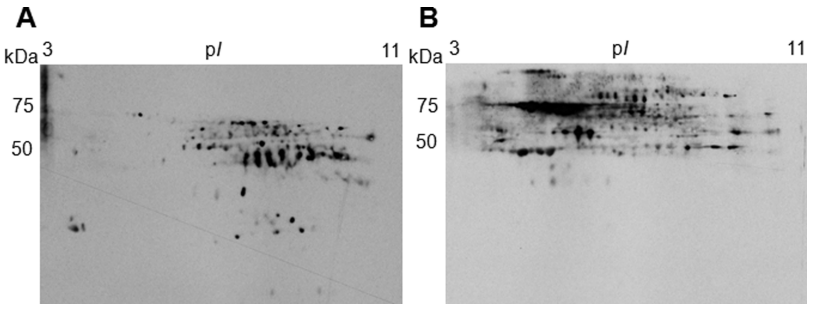


**Figure 3.** Carbonyl modified proteins detected by Western blotting of the gels shown in Figure 2A and B, corresponding to samples previously derivatized with DNPH (A) or samples derivatized after transfer (B). Images are representative of three independent experiments.

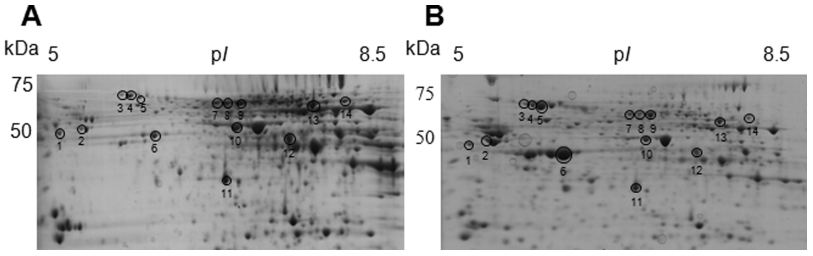


**Figure 4.** Fourteen common carbonyl modified protein spots heterogeneously distributed across the isoelectric point and molecular weight ranges selected for subsequent protein identification by mass spectrometry in prederivatized gels (A) and nontreated gels (B).

could be matched when the samples were treated after transfer to the blot. Computer-assisted image analysis comparing images of the gels and 2D oxyblots obtained using the two approaches revealed only 37 common carbonyl modified protein spots identified by both methods.

To assess whether these 37 common carbonyl modified spots were identified as the same proteins, a selection of 14 protein spots were excised and subjected to MS. These spots were heterogeneously distributed across the isoelectric point, and molecular weight ranges were obtained in the 2D oxyblot (Figure 4A, B). The MS data revealed that all of the excised spots were identified as the same proteins using either method (Table 1). These 14 protein spots were identified as encoded by 11 different mouse genes. Seven of the proteins were related to metabolism and energy processes: $\gamma$ and $\alpha$ enolase and pyruvate kinase (glycolytic enzymes), malate dehydrogenase (citric acid cycle enzyme), long chain specific acyl-CoA dehydrogenase (involved in fatty acid and branched chain amino acid metabolism), glutamine synthetase (essential role in nitrogen metabolism), and creatine kinase (an important energy reservoir). Other proteins identified were structural proteins such as vimentin, an intermediate filament, and dihydropyrimidinase-related protein 2, necessary for remodelling of the cytoskeleton. A further protein identified was the ATPase, H+ transporting, lysosomal V1 subunit A, which is responsible for acidifying the intracellular compartments. The last oxidized protein identified was a hypothetical protein related to heat shock protein 8, which contributes to producing the proper protein conformation.

In our experimental conditions, no significant differences ($p$ = 0.220) were observed between MS scores when DNPH pretreated or post-treated samples were used (Table 1). To clarify whether DNPH pretreatment modifies the isoelectric focusing or relative mobility of proteins during electrophoresis, we compared the isoelectric points and molecular weights calculated by the PDQuest software with the theoretical value predicted in the NCBInr 080626 database (Table 1). Statistical analysis revealed no significant additive effect of DNPH pretreatment causing a molecular weight change ( $p$ = 0.983) or significant differences in isoelectric point ($p$ = 0.249).

The low number of common modified spots detected by both methods could be attributed to slight variations in the migration properties of treated and untreated proteins, which could affect correct matching by the PDQuest software. To address this question, we conducted a MS analysis of some of the carbonyl modified spots in the DNPH pretreated gels that appeared around the nonmodified spots matched by PDQuest analysis to the modified spots in the gels containing non- pretreated samples. This MS analysis indicated that 50% of the surrounding spots excised from gels containing DNPH pretreated samples coincided with the ones identified in the gels containing non-treated samples (Figure 5 and Table 2). These results suggest that minor differences in migration between pretreated and untreated extracts could affect matching of gel images obtained using the two procedures.

## DISCUSSION

In this study, we compared the two most commonly used 2D PAGE oxyproteomic methods for identifying carbonylated proteins: (1) the widely used method of derivatizating samples with DNPH followed by 2D electrophoresis, in which carbonyl groups are subsequently detected in corresponding 2D Western blots using specific anti-DNP antibodies[7,13−17] and (2) derivatization of the blot with DNPH after 2D electrophoresis.[22,24]

Because of the complexity of this methodology, we ensured that optimal conditions were fulfilled for all the gels/blots analyzed. First, we focused on sample preparation, trying to solubilize most proteins in the sample. To do this, we modified the RIPA buffer by adding the zwitterionic detergent MEGA 10 and the zwitterionic lipid LPC. These zwitterionic detergents


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**Table 1. Identification of Carbonylated Proteins in Mouse Cerebellum**

| | | | no. of peptides matched/ searched[d] | | % coverage[e] | | score[f] | | | MW (kDa)/ p*I* predicted (PDQuest)[h] | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| spot no.[a] | protein ID[b] | accession no.[c] | preblot derivatized | post- blot derivatized | preblot | postblot | preblot | postblot | MW (kDa)/p*I*[g] | preblot | postblot |
| 1[k] | vimentin | sp\|P20152\| VIME_MOUSE | 34/65 | 40/65 | 64 | 67 | 337 | 382 | 53.7/5.1 | 48.5/5.2 | 50.3/5.3 |
| 2[k] | γ-enolase | sp\|P17183\| ENOG_MOUSE | 21/37 | 28/65 | 49 | 55 | 237 | 215 | 47.6/5.0 | 49.8/5.4 | 52.4/5.4 |
| 3 | unnamed protein product | gi\|74211333 | 32/65 | 39/65 | 48 | 53 | 299 | 363 | 68.2/5.3 | 68.4/5.8 | 73.6/5.9 |
| 4 | unnamed protein product | gi\|74211333 | 37/65 | 44/65 | 53 | 55 | 387 | 448 | 68.2/5.3 | 67.7/5.9 | 72.9/6 |
| 5[k] | ATPase, H+ transporting, lysosomal V1 subunit A | gi\|31560731 | 36/65 | 32/65 | 56 | 46 | 293 | 221 | 68.6/5.4 | 66.5/6 | 70.4/6 |
| 6 | creatine kinase, brain | gi\|10946574 | 30/65 | 38/65 | 70 | 71 | 330 | 356 | 43.0/5.4 | 47.9/6.1 | 46.0/6.3 |
| 7[k] | dihydropyrimidinase-related protein 2 | sp\|O08553\| DPYL2_MOUSE | 19/54 | 38/65 | 36 | 69 | 150 | 398 | 62.6/6.0 | 63.3/6.8 | 66.8/7.0 |
| 8[k] | dihydropyrimidinase-related protein 2 | sp\|O08553\| DPYL2_MOUSE | 35/65 | 37/65 | 68 | 65 | 375 | 359 | 62.6/6.0 | 63.1/6.9 | 66.9/7.1 |
| 9[k] | dihydropyrimidinase-related protein 2 | sp\|O08553\| DPYL2_MOUSE | 35/65 | 38/65 | 65 | 72 | 375 | 411 | 62.6/6.0 | 62.6/7.1 | 66.2/7.3 |
| 10[k] | α-enolase | sp\|P17182\| ENOA_MOUSE | 33/65 | 32/65 | 72 | 73 | 343 | 329 | 47.5/6.4 | 50.0/7.0 | 52.5/7.2 |
| 11[k] | malate dehydrogenase, cytoplasmic | sp\|P14152\| MDHC_MOUSE | 23/65 | 24/65 | 49 | 52 | 209 | 222 | 36.7/6.2 | 37.3/6.9 | 37.6/7.1 |
| 12[l] | glutamine synthetase | sp\|P15105\| GLNA_MOUSE | 27/65 | 20/65 | 56 | 35 | 347[i] 275[j] | 309[i] 166[j] | 42.8/6.6 | 46.9/7.6 | 46.9/7.8 |
| | long-chain specific acyl-CoA dehydrogenase, mitochondrial | sp\|P51174\| ACADL_MOUSE | 14/65 | 20/65 | 36 | 40 | 347[i] 94[j] | 309[i] 145[j] | 48.3/8.5 | | |
| 13[k] | pyruvate kinase isozymes M1/M2 | sp\|P52480\| KPYM_MOUSE | 18/65 | 31/65 | 38 | 49 | 120 | 262 | 58.4/7.2 | 61.8/7.9 | 62.3/8.0 |
| 14[k] | pyruvate kinase isozymes M1/M2 | sp\|P52480\| KPYM_MOUSE | 36/65 | 39/65 | 61 | 63 | 358 | 383 | 58.4/7.2 | 64.4/8.2 | 64.5/8.3 |

[a] Spot number as indicated in Figure 4. [b] Protein ID from NCBInr database. [c] Accession numbers from NCBInr database. [d] Number of matched peptides versus total number of peptides. [e] Coverage of the matched peptides in relation to the full-length sequence. [f] Probability-based MOWSE score. [g] Theoretical molecular mass (MW) (kDa) and isoelectric point (p*I*) from NCBInr database. [h] Theoretical molecular mass (MW) (kDa) and p*I* from PDQuest analysis. [i] Probability-based MOWSE score for the protein mix. [j] Probability-based MOWSE score for the indicated protein. [k] Identification confirmed by MS/MS sequencing. [l] Spot 12 was identified as a mix of two different proteins. This spot was not used for statistical analyses.

improve the resolution of the membrane protein fraction in the sample.[27] After extraction, samples were differentially derivatized or not with DNPH and then processed in the same electrophoretic and transfer conditions.

The use of gels loaded with untreated samples for protein identification and DNPH treated samples run in parallel for 2D oxyblot analysis has been reported to solve the potential problems caused by the DNP moiety in MS identification.[18−21,28] To investigate how protein matching and identification could be affected by these procedures, direct comparisons were made between 2D Coomasie blue-stained gels containing untreated and DNPH treated samples. In our hands, both sample treatments led to adequate separation of membrane proteins from the cerebellum, a tissue rich in these proteins. However, differences were detected in the protein patterns produced in the 2D gels. Thus, the different sample treatments led to differential areas of protein distribution enrichment, and this precluded matching of 2D oxyblots of pretreated samples with the 2D Coomasie-stained gels of untreated samples, as previously suggested.[22] Some authors[11] claim that differences in protein patterns produced by 2D electrophoresis are the consequence of differential protein precipitation or protein mobility (see below). A loss of proteins


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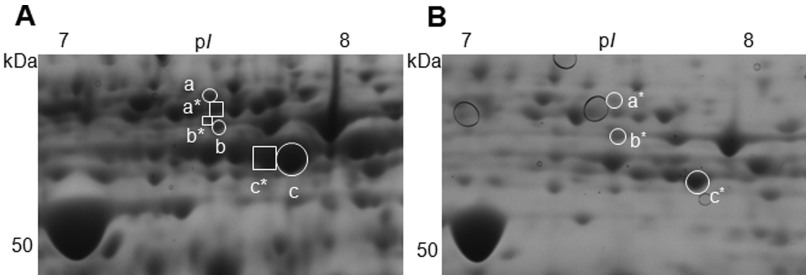


**Figure 5.** Carbonyl modified spots in pretreated gels not matching in the PDQuest analysis but close to a carbonyl modified spot in nontreated gels that were identified as the same protein. (A) DNPH pretreated gel. Spots matched by PDQuest analysis with spots in the untreated gel are indicated by circles. Spots identified as the same as spots in the nontreated gel are indicated by squares. (B) Spots in the nontreated gel. Carbonyl modified spots are indicated with an asterisk in both images. Spots with the same letter represent each pair of PDQuest-matched or coinciding spots.

**Table 2. Identification of Non-PDQuest Matched Carbonylated Spots in Both Types of Gel**

| spot[a] | protein ID[b] | accession no.[c] | no. of peptides matched/searched[d] | % coverage[e] | score[f] | MW (kDa)/p*I*[g] | MW (kDa)/p*I* predicted (PDQuest)[h] |
|---|---|---|---|---|---|---|---|
| a* (post) | dihydropyrimidinase-related protein 1 | sp\|P97427\|DPYL1_MOUSE | 15/65 | 24 | 199[i] 96[j] | 62.5/6.6 | 68.3/7.7 |
| | electron transfer flavoprotein-ubiquinone oxidoreductase, mitochondrial | sp\|Q921G7\|ETFD_MOUSE | 14/65 | 27 | 199[i] 90[j] | 68.9/7.3 | |
| a* (Pre) | dihydropyrimidinase-related protein 1 | sp\|P97427\|DPYL1_MOUSE | 19/65 | 32 | 327[i] 139[j] | 62.5/6.6 | 64.0/7.6 |
| | dihydropyrimidinase-related protein 4 | sp\|O35098\|DPYL4_MOUSE | 15/65 | 34 | 327[i] 104[j] | 62.5/6.5 | |
| | NAD-dependent malic enzyme, mitochondrial | sp\|Q99KE1\|MAOM_MOUSE | 16/65 | 29 | 327[i] 100[j] | 66.4/7.5 | |
| b* (post) | pyruvate kinase isozymes M1/M2 | sp\|P52480\|KPYM_MOUSE | 24/65 | 43 | 186 | 58.4/7.2 | 63.5/7.7 |
| b* (pre) | pyruvate kinase isozymes M1/M2 | sp\|P52480\|KPYM_MOUSE | 14/65 | 26 | 402[i] 82[j] | 58.4/7.2 | 62.7/7.6 |
| | dihydropyrimidinase-related protein 1 | sp\|P97427\|DPYL1_MOUSE | 20/65 | 37 | 402[i] 164[j] | 62.5/6.6 | |
| | tyrosyl-tRNA synthetase, cytoplasmic | sp\|Q91WQ3\|SYYC_MOUSE | 17/65 | 32 | 402[i] 117[j] | 59.4/6.6 | |
| c* (post) | glutamate dehydrogenase 1, mitochondrial | sp\|P26443\|DHE3_MOUSE | 42/65 | 53 | 365 | 61.6/8.1 | 57.9/7.9 |
| c* (pre) | glutamate dehydrogenase 1, mitochondrial | sp\|P26443\|DHE3_MOUSE | 14/65 | 29 | 379[i] 79[j] | 61.6/8.1 | 57.7/7.7 |
| | ATP synthase subunit alpha, mitochondrial | sp\|Q03265\|ATPA_MOUSE | 23/65 | 43 | 379[i] 172[j] | 59.8/9.2 | |
| | succinyl-CoA:3-ketoacid-coenzyme A transferase 1, mitochondrial | sp\|Q9D0K2\|SCOT_MOUSE | 15/65 | 39 | 379[i] 97[j] | 56.4/8.7 | |

[a] Spot letter as indicated in Figure 5. The type of gel is indicated in brackets: preblot DNPH treatment (pre) and postblot DNPH treatment (post). [b] Protein ID from NCBInr database. [c] Accession numbers from NCBInr database. [d] Number of matched peptides versus total number of peptides. [e] Coverage of the matched peptides in relation to the full-length sequenc. [f] Probability-based MOWSE score. [g] Theoretical molecular mass (MW) (kDa) and isoelectric point (p*I*) from NCBInr database. [h] Theoretical molecular mass (MW) (kDa) and isoelectric point (p*I*) from PDQuest analysis. [i] Probability-based MOWSE score of the protein mix. [j] Probability-based MOWSE score of the indicated protein.

due to sample processing during DNPH derivatization has also been reported,[11] though this contrasts with our results in that we detected more spots using the pre-DNPH derivatized samples. Since 50% of these spots were also present in the gels prepared using nontreated samples, it could be that the derivatization step might differentially precipitate proteins.

The Western blot images of the oxidized proteins rendered by the two oxyproteomic approaches were also compared. Thus, although more carbonyl modified spots were detected when the proteins were derivatized with DNPH after electrophoresis, an improved background enhancing reproducibility between replicates was observed in the samples treated with DNPH before electrophoresis. Moreover, when Coomasie blue-stained gels were compared with Western blots for each method, more matching spots were detected when samples were treated before electrophoresis, consistent with reports that postelectrophoresis derivatization might produce high background staining,[29] which highlights the importance of clear backgrounds to define and identify oxidized proteins. High background staining not only hinders comparisons between 2D oxyblots but also complicates spot matching between Western-blot spots and preparative gels. However, the solvents used to remove background staining tend to wash off the proteins, and several washings in aggressive chemicals were required in our experiments to decrease background staining.


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When we compared the number of common spots detected by the two methods, it was found that only 37 common spots appeared oxidized. MS analysis of a representative number of these spots confirmed that the same proteins were identified by both methods. Although some authors suggest that pretreatment of proteins with DNPH gives rise to MS misidentification or unresolved data,[18,19] in our experimental conditions, these protein spots were successfully identified by MS fingerprinting as described by others.[23] The fact that both methods yielded a sufficient number of peptides guarantees good protein identification, which could depend on the protein size and its level of oxidation. High oxidation in small proteins is expected to generate a lower amount of nonmodified peptides. Conversely, protein modification and derivatization could make difficult MS identification in the cases where the complete genome is not still annotated in the databases.

Several authors claim that DNPH treatment modifies isoelectric focusing and/or molecular weight, which produces a change in protein mobility.[11,22,23,29] However, we observed no major differences between isoelectric points or molecular weights predicted automatically by the PDQuest software and the real values predicted after MS identification. This is in agreement with the views of other authors, who also fail to observe major differences in protein electrophoretic positions after DNPH treatment.[18,19] Thus, although we cannot exclude minor differences in the positions of DNPH treated and nontreated proteins, major changes in the molecular weight isoelectric point did not seem to affect protein matching in our brain tissue samples.

The low level of correspondence between the number of spots detected by the two methods could justify a combined approach to redox proteomics. In each method, missing spots could perhaps be attributed to their different capacity for carbonyl detection or to the different patterns observed in the preparative gels. This low correspondence could also be explained by a protein positioning effect that prevents correct matching by the PDQuest software in some areas. To address this potential effect, carbonylated spots rendered by both methods that were not matched by PDQuest analysis but appeared in nearby positions in the gel were subjected to mass spectrometry (MS). Because some of the spots (50%) were identified as the same proteins by both methods, it is possible that minor differences between pretreated and nontreated extracts could affect matching of the gel images. As indicated above, although no major effects on molecular weight and isoelectric point were observed, differences in protein patterns have been suggested by us and other authors.[11] Considering that (i) oxidative modifications *per se* often slightly alter a protein's electrophoretic properties such that it migrates as a satellite of the unmodified protein and (ii) a single protein can undergo more than one type of oxidative modification,[5] small differences between DNP spots and their unmodified protein counterparts could hinder comparisons between the modified spots detected by both methods.

## CONCLUSIONS

In conclusion, although more horizontal streaking was produced when brain tissue extracts were DNPH treated before their 2D electrophoretic separation, a larger number of spots was obtained. In addition, because a highly biased pattern was observed for the two methods, the use of nontreated extracts for identifying modified protein spots in DNPH pretreated 2D oxyblots is not recommended for routine analysis. The DNPH pretreated 2D oxyblots showed low background staining and improved reproducibility, which made it easier to compare the modified spots in the 2D oxyblots with the Coomasie-stained gels. This comparison yielded the largest number of matching spots. Given that the use of pretreated samples rendered similar MS fingerprinting identification scores, in our opinion, this approach seems the most appropriate for redox proteomics of brain tissue. Moreover, this method prevents further oxidative processes during the storage and manipulation, the washing is less aggressive and its handling shortened. However, before selecting any procedure for a given tissue, we would suggest a comparative analysis such as the present. Moreover, because these two methods do not show 100% efficiency, whenever possible, the combined use of the two redox-proteomics approaches guarantee identification of carbonylated proteins that match in both methods.

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## ABBREVIATIONS

CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; 2D, two-dimensional; DNP, 2,4-dinitrophenylhydrazone; DNPH, 2,4-dinitrophenylhydrazine; 2D oxyblot, 2D Western blot; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; IEF, isoelectric focusing; IPG, immobilized pH gradient; LPC, L-α-lysophosphatidylcholine; MALDI, matrix-assisted laser desorption/ionization; MEGA 10, decanoyl-*N*-methylglucamide; MS, mass spectrometry; SDS-PAGE, SDS-polyacrilamide gel electrophoresis; PVDF, polyvinylidene fluoride; PMF, peptide mass fingerprinting; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; TOF, time-of-flight

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