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A multi-centre study

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Validation of protein carbonyl measurement: A multi-centre study

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A B S T R A C T

Protein carbonyls are widely analysed as a measure of protein oxidation. Several different methods exist for their determination. A previous study had described orders of magnitude variance that existed when protein carbonyls were analysed in a single laboratory by ELISA using different commercial kits. We have further explored the potential causes of variance in carbonyl analysis in a ring study. A soluble protein fraction was prepared from rat liver and exposed to 0, 5 and 15 min of UV irradiation. Lyophilised preparations were distributed to six different laboratories that routinely undertook protein carbonyl analysis across Europe. ELISA and Western blotting techniques detected an increase in protein carbonyl formation between 0 and 5 min of UV irradiation irrespective of method used. After irradiation for 15 min, less oxidation was detected by half of the laboratories than after 5 min irradiation. Three of the four ELISA carbonyl results fell within 95% confidence intervals. Likely errors in calculating absolute carbonyl values may be attributed to differences in standardisation. Out of up to 88 proteins identified as containing carbonyl groups after tryptic cleavage of irradiated and control liver proteins, only seven were common in all three liver preparations. Lysine and arginine residues modified by carbonyls are likely to be resistant to tryptic proteolysis. Use of a cocktail of proteases may increase the recovery of oxidised peptides. In conclusion, standardisation is critical for carbonyl analysis and heavily oxidised proteins may not be effectively analysed by any existing technique.

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Introduction

Non-enzymatic, free radical-mediated oxidation of proteins is common in biological systems. Some of the earliest work exploring the effects of chemical modification to proteins on function examined the oxidative damage induced by selected radicals on lysozyme, α-1-antitrypsin, and apolipoprotein B in LDL [1,2]. In each of these cases, there was an associated loss of function or null effect. However, the effects of oxidation are not always deleterious. Indeed, the chemical nature of oxidation and the biological consequences of this oxidation are dependent on (1) the primary sequence (2) whether or not the oxidant can gain access to susceptible amino acid residues within that protein i.e. three dimensional structure constraints and (3) the oxidising species [3–5].

Discrete chemical reactions can induce patterns of oxidation that vary according to the initiating radical species. For example, radiolytically-generated hydroxyl radicals will favour oxidation of aromatic amino acids such as tryptophan and tyrosine whereas peroxyl radicals favour formation of hydroperoxides and hydroxides on aliphatic amino acids [6–8]. However, protein carbonyls on both aliphatic and aromatic amino acids are commonly produced by a range of oxidising species [5]. In addition, secondary oxidation of proteins to yield carbonyls is also common. During protein glycation and following lipid peroxidation, aldehydes are formed e.g. glyoxal and 4-hydroxynonenal. These aldehydes form Schiff’s base adducts with primary amine groups present on lysine...
and in the case of dicarbonyls such as glyoxal and malondialdehyde, when cross-linking has not occurred and a free aldehyde group remains, contribute to total carbonylation [9,10]. Protein carbonyl measurements are used to provide an index of global protein oxidation irrespective of the initiating radical species [11].

Recently we have reviewed the existing methods for determining protein carbonyls, considering their strengths and weaknesses [12]. The majority of methods rely on derivatisation of the carbonyl group, most commonly with di-nitrophenol hydrazine (DNPH; Fig. 1) [13,14]. Dinitrophenol hydrazone (DNPH)-carbonyl can be detected quantitatively by immunoassay using high specificity antibodies against DNP or absolutely by spectrophotometric calculation from the absorption measured at 360 nm relative to the extinction coefficient for DNP [15,16]. Mass spectrophotometric methods can also be used for identification as well as relative quantification of carbonylated peptides by label free techniques or using isotopically labelled derivatisation reagents.

Despite the use of common platforms such as ELISA for determination of protein carbonyls, orders of magnitude difference have been reported between different commercial kits [17]. For this reason, it has been hard to compare the data reported in different papers. The analysis of protein oxidation is further complicated by the complexity of tissue matrix which can contribute to differences in oxidised protein extraction according to the methods used.

In order to understand the reasons underlying the differences between laboratories and methods, we have surveyed the methods used in six different laboratories across Europe. We then undertook a ring study to compare the carbonyl content reported by different methods using a homogenised liver extract with and without UV radiation-induced oxidation. Each participating laboratory received blinded, lyophilised samples and was invited to process according to their protocol and to report their findings. In this manuscript, we compare the results of carbonyl analysis of the same tissue samples that were achieved by each method and highlight the need for improved reference standardisation.

**Materials and methods**

**Preparation of liver lysate samples**

Rat liver tissue (20 g) was homogenised using rotor stator homogeniser in ice cold 1/3 strength phosphate buffered saline (PBS; 45.6 mM NaCl, 0.9 mM KCl, 2.7 mM Na2HPO4 and 0.48 mM KH2PO4 in distilled water, pH adjusted to 7.4 with HCl), in ratio 2:1 (PBS:tissue). Immediately before homogenisation, protease inhibitor phenylmethane-sulphonyl fluoride (Sigma) was added to a final concentration of 1 mM. Homogenate was then centrifuged using a bench top centrifuge for 5 min at 600 g. The supernatant was collected and then re-centrifuged for 20 min at 3000 g. The supernatant was collected again and was finally re-centrifuged using an ultracentrifuge at 100,000 g for 4 h after which clear supernatant containing soluble proteins was collected.

Protein content was measured using bicinchoninic acid (BCA) assay. Protein content of samples was adjusted to 1 mg/ml prior to irradiation. Samples (10 ml aliquots) were irradiated at a distance of approximately 15 cm from the UV lamp (I=1.74 × 20 mW/cm², P=250 W, UV range 280–315 nm, IUV250 UV Curing Flood Lamp 230 V/50 Hz) for 0, 5 and 15 min respectively. After irradiation, protein damage was detected using carbonyl ELISA. Irradiated protein solutions (1 ml) were dried under vacuum centrifuge for 8 h with desferrioxamine added (5 mM) and stored at −80 °C.

**Carbonyl analyses**

**Spectrophotometry method**

Each laboratory that calibrated standards in their laboratory used a spectrophotometric method to ascertain absolute carbonyl
values as follows. Briefly, oxidised and reduced bovine serum albumin (BSA) standards were mixed with 10 mM DNPH in 2 N HCl or 2 N HCl alone and incubated at room temperature for 1 h with vortexing every 15 min. Proteins were precipitated with 20% trichloroacetic acid (w/v), vortexed and centrifuged (13,000g for 3 min). The pellet was washed three times with 1 ml ethanol-ethyl acetate (1:1 v/v) before re-dissolving in 1 ml of 6 M guanidine HCl in 20 mM potassium phosphate adjusted to pH 2.3 with trifluoroacetic acid. The absorbance was measured in the supernatant at 360 nm and carbonyl content was calculated, using the molar absorption coefficient of 22,000 M⁻¹/cm⁻¹ relative to protein concentration [18]. This calculation is shown in Fig. 2.

ELISA method 1

Reduced standards were prepared using sodium borohydride and oxidised standards were prepared using ferrous sulphate as described previously [18]. Unknowns (resuspended in water) and standards were diluted in carbonate buffer (pH 9.6, 0.05 M to 20 μg/ml, and 50 μl) pipetted in triplicate into a Nunc-Immuno plate, Maxisorp. Samples were derivatised directly on the plate using 1 mM DNPH in 2 N HCl (50 μl). Following three washes, the plate was incubated with blocking buffer (Tween 20 (1% v/v) in PBS), overnight at 4 °C to block any non-specific binding. After incubating for 2 h at 37 °C mononclonal mouse anti-DNP (Sigma, UK) antibody diluted 1:2000 in blocking buffer, the bound antibody was detected with peroxidase-conjugated rat anti-mouse IgE antibody (AbD Serotec, diluted 1:5000 in blocking buffer), citrate phosphate buffer pH 5.0, 0.15 M, containing 20 mg tablet o-phenylenediamine and 10 μl of 8.8 M hydrogen peroxide. The reaction was terminated by addition of 2 N H₂SO₄. Absorbance was measured spectrophotometrically at 490 nm, using a BioTek plate reader (BioTek, UK). Carbonyl content was calculated from the standard curve and expressed as nanomol carbonyl per milligram of protein [18].

ELISA method 2

The lyophilised liver homogenate samples (nos. 1, 2 and 3) were each suspended in 1 ml PBS and protein content was assessed using Lowry method [19]. All the three samples had the same protein content: 0.5 mg protein/ml. The OxiSelect Protein Carbonyl ELISA kit (STA-310, Cell Biolaboratories) was used. Briefly, samples (10 μg/ml) were allowed to adsorb to wells of a 96-well plate and then reacted with DNPH. The protein carbonyls derivatised to dinitrophenyl hydrazone (DNP) were then probed with an anti-DNP antibody. The standard curve was prepared from commercially prepared reduced and oxidised BSA standards as provided.

ELISA method 3

Oxidised BSA, containing additional carbonyl groups, was prepared by reacting BSA (50 mg/ml in PBS) with hypochlorous acid (final concentration 5 mM). Protein carbonyls were measured with a slightly modified method according to Buss et al. [20]. Proteins were derivatised with DNPH solution (10 mM in 6 mM guanidine hydrochloride, 0.5 mM potassium phosphate buffer, pH 2.5) and incubated at room temperature. Samples and standards (5 μg/ml) were prepared in a coating buffer was added to wells (10 mM sodium phosphate buffer containing 140 mM NaCl, pH 7.0). Plates (Nunc Immuno Plate Maxisorp) were incubated overnight at 4 °C and then washed and 0.1% reduced BSA in PBS (250 μl/well) was added for 1.5 h at room temperature and then 200 μl/well of anti-DNP antibody was added (Molecular Probes Inc., 1:1000, diluted in 0.1% reduced BSA and 0.1% Tween 20 solution) and incubated for 1 h at 37 °C. After this, horseradish peroxidase-conjugated secondary antibody was added and further incubation was for 30 min at 37 °C. Finally, 100 μl of solution containing o-phenylenediamine (0.6 mg/ml) and hydrogen peroxide (4 mM) in 50 mM Na₂HPO₄ plus 24 mM citric acid was added. The development of colour was stopped after 10 min using 50 μl of solution of 2.5 M H₂SO₄ without any washing. Absorbance was read with a 490 nm filter
using a micro plate reader. A blank for DNP reagent in PBS without protein was subtracted from all other absorbances. A 6-point standard curve of reduced and oxidised BSA was included with each plate [21].

ELISA method 4

Reduced BSA was prepared by reacting a 1 g/100 ml solution of BSA in PBS with 2 g/100 ml sodium borohydride for 30 min, followed by neutralising with HCl. Oxidised BSA containing additional carbonyls was prepared for use as a reference by reacting BSA (50 mg/ml in PBS) with hypochlorous acid (5 mM). Protein carbonyls were measured after derivatisation with DNPH according to Buss et al. [20] with modifications carried out by Sitte et al. [22]. Samples were diluted to 1 mg/ml in PBS, and then incubated with 3 volumes of DNPH solution (10 mM DNPH in 6 M guanidine HCl/0.5 M KH₂PO₄) for 45 min at room temperature in the dark before diluting in coating buffer and coating them to the Nunc Immuno 96 Microwell™ MaxiSorp plate (incubation over night at 4 °C). The next day the plate was blocked with 0.1% reduced BSA in PBS for 1.5 h at room temperature, incubated with primary anti-DNP-antibody (Sigma, 1:1000, diluted in blocking solution) for 1 h at 37 °C followed by the secondary antibody (Sigma, 1:10,000) for 1 h at room temperature. The detection was performed with o-phenylenediamine (0.6 mg/ml) and hydrogen peroxide (4 mM) in 50 mM Na₂HPO₄/24 mM citric acid. In between these steps the plate was washed 3 times each with PBS/Tween 20 (0.1%).

After stopping the reaction with 2.5 M H₂SO₄, the absorbance was read at 492 nm (reference filter set to 750 nm) using a Microbiology plate reader BioTek Synergy 2 (BioTek Instruments, Friedrichshall, Germany).

An 8-point standard curve of predetermined reduced and oxidised BSA was included with each plate.

Western blot method 1

Liver extracts (5 μg) were mixed in a 1:1 ratio with Laemmli buffer (2x, Sigma, UK), boiled for 5 min at 95 °C and separated by reducing SDS-PAGE using 10% polyacrylamide gels. Each gel included lanes for protein markers (Bio-Rad Precision Plus Protein Standard Kaleidoscope, 10–250 kDa), an oxidised BSA sample (positive control), and liver protein extract. Electrophoresis was at a constant voltage (115 V) for 1 h 45 min or until the gel front had migrated fully. One gel was stained with Flamingo fluorescent reagent (Medicinale, Friedrichshall, Germany).

An 8-point standard curve of predetermined reduced and oxidised BSA was included with each plate.

Western blot method 2

The protein carbonyls were derivatised with DNPH immediately before the electrophoresis, as previously described [24,25]. Protein extracts (20 μg protein/lane) were separated by electrophoresis on 12.5% SDS-PAGE, and Western blot procedures were followed. As primary antibody, rat monoclonal antibody to DNP (Zymed Laboratories, San Francisco, CA) was applied at 1:10,000 dilution overnight at 4 °C. The membranes were incubated with a secondary antibody: goat anti-rat IgG AlexaFluor 680 conjugated secondary antibodies (Life Technologies, Carlsbad, CA, USA) was used at 1:10,000 dilution for 1 h at room temperature. Immunoblots were visualised by Odyssey (Li-Cor Biosciences Inc., Lincoln, NB) and quantified by using Odyssey Software. Equal loading and transfer of the western blot samples were further verified by reversible total protein staining of the nitrocellulose membrane with Ponceau-S reversible membrane staining.

Western blot method 3

Proteins were solubilised in 20 mM Tris EDTA buffer, 4% SDS buffer. Carbonylated proteins were detected and analysed following derivatisation of protein carbonyl groups with DNP, using the OxyBlot kit reagents and conditions (Merck Millipore). Samples (2 μg protein per lane) were separated in a Bolt™ 4–12% Bis-Tris Plus gel using Bolt™ MES SDS Running Buffer (Life Technologies) following manufacturer’s instructions. Separated proteins were electro-transferred onto an Immobilon-P Membrane, PVDF (Merck Millipore). Immunodetection was performed with a primary antibody directed against DNP. Primary antibody binding was detected by incubation with a peroxidase-conjugated secondary antibody and chemiluminescent substrate Luminata Forte (Merck Millipore). Density analysis was performed using Image Studio Light (Li-Cor). Loading control was made using the same amount of samples separated by SDS-PAGE in the same conditions and stained with sensitive Coomassie Blue stain [26].

Mass spectrometry [27]

Samples were diluted with ammonium bicarbonate (25 mmol/l) to a final protein concentration of 1 g/l. Sodium deoxycholate was added (1% v/v). Disulphide bridges were reduced with tris (2-carboxyethyl) phosphine (5 mmol/l, 60 °C, 30 min) and the thiols were alkylated with iodoacetamide (10 mmol/l, 37 °C, 30 min, dark). Excess of iodoacetamide was quenched with dithiothreitol (10 mmol/l, 37 °C, 30 min). Proteins were digested by trypsin (50:1 enzyme to protein ratio, 25 mmol/l ammonium bicarbonate; 37 °C, 16 h). The digest was terminated by adding formic acid (0.5% v/v) and the precipitated sodium deoxycholate was removed by centrifugation. The tryptic digest (150 μl) was acidified with formic acid (1% v/v) and incubated with aldehyde reactive probe (ARP; 100 μl, 25 mmol/l in water) at room temperature for 2 h. Excess of ARP was removed by solid phase extraction using Waters Oasis HLB 1 cc (10 mg) cartridges (Waters GmbH, Eschborn, Germany). The eluates were vacuum concentrated and reconstituted in PBS (0.1 ml, 20 mmol/l NaH₂PO₄, 0.3 mol/l NaCl) and enriched by avidin affinity chromatography (Pierce™ monomeric avidin agarose). Enriched ARP labelled peptides were vacuum concentrated and stored at −80 °C. Prior mass spectrometric analysis samples were dissolved in 50 μl of 0.1% formic acid in 3% aqueous acetonitrile.

A nano-Acquity UPLC (Waters GmbH, Eschborn, Germany) was coupled on-line to an LTQ Orbitrap XL ETD mass spectrometer equipped with a nano-ESI source (Thermo Fisher Scientific, Bremen, Germany). Eluent A was aqueous formic acid (0.1% v/v) and eluent B was formic acid (0.1% v/v) in acetonitrile. Affinity enriched peptides (1.5 μl) were loaded onto the trap column (nano-Acquity
symmetry C18, internal diameter 180 μm, length 20 mm, particle diameter 1.7 μm) with a flow rate of 0.4 μl/min, using several linear gradients from 3% to 9% (2 min), 9.9% (1.9 min), 17.1% (10 min), 18% (0.5 min); 20.7% (0.2 min), 22.5% (3.1 min), 25.6% (3 min), 30.6% (5 min), 37.8% (2.8 min), and finally to 81% eluent B (2 min). Together with an equilibration time of 12 min the samples were injected every 46 min. The transfer capillary temperature was set to 200 °C and tube lens voltage to 120 V. An ion spray voltage of 1.5 kV was applied to a PicoTip™ online nano-ESI emitter (New Objective, Berlin, Germany). The precursor ion survey scans were acquired at an orbitrap (resolution of 60,000 at m/z 400) for an m/z-range from 400 to 2000. The CID-tandem mass spectra (isolation width 2, activation Q=0.25, normalised collision energy 35%, activation time 30 ms) were recorded by data dependent acquisition (DDA) for the top six most abundant ions in each survey scan with dynamic exclusion for 60 s using Xcalibur software (Version 2.0.7).

The acquired tandem mass spectra were searched using Sequest search engine (Proteome Discoverer 1.1, Thermo, Fisher). The setting allowed up to two missed cleavage sites and a mass tolerance of 10 ppm for precursor and 0.8 ppm for product ion scans. Database search included carbamidomethylation on Cys, oxidation of Met, carbonylated and ARP-derivatised Lys (mass shift of 10 ppm for precursor and 0.8 ppm for product ion scans). Variable modifications in addition to Cys carbamidomethylation and Met oxidation included ARP-derivatised alkendic acids: HNE (469.23 m/z units), HHE (427.18 m/z units), ONE (467.22 m/z units), and OHE (425.17 m/z units) adducts at Cys-, His- and Lys-residues.

Results and discussion

Carbonyl analyses were undertaken at least in triplicate in different European Laboratories using seven ELISA and Western blot methods. Three laboratories used commercial kits for derivatisation and detection and one of the ELISA kits included commercial standards. Table 1 compares the similarities and differences between assays except where commercial kits were used. Specific antibodies, buffers and dilutions were not available from kits. The amount of protein that was loaded for analysis varied four-fold between methods. However, different laboratories also used different methods to determine protein concentration of the lyophilised samples after re-suspending into 1 ml of diluent; protein concentration was reported as 0.5 mg/ml by ZDQuant and 0.5 mg/ml by Lowry. However, a third laboratory used the method according to Bradford and determined the protein content to be between 9.9 and 1 mg/ml. Some derivatised before coating onto an ELISA plate or loading onto the gel whereas other laboratories performed derivatisation afterwards. A range of antibodies and detection methods were used.

Multiple proteins bands were detected in the soluble protein fraction from liver by SDS-PAGE and western blotting (Fig. 3A). Oxidised proteins were used for quantitative analysis by densitometry. Following UV irradiation, some groups reported that the protein carbonyl content was increased at the upper edge of bands i.e. migration appears slower than in un-irradiated control samples. This suggests a post-translational modification occurs after irradiation that affects protein conformation and impacts on its ability to unwind in SDS, slowing its subsequent rate of migration in the gel.

Irrespective of the method adopted to analyse the protein carbonyls, three laboratories that had analysed carbonylation by western blot reported lower protein carbonyls in samples that had been irradiated for the longest time by UV light. This corresponded with lower Coomassie protein staining in the heavily oxidised (15 min irradiation) paired gels (Fig. 3B). We and others have previously reported that extensive irradiation causes protein aggregation [28,29]. The gels used in these studies can only resolve proteins up to 250 kDa and one possible explanation for the apparent lower level of protein oxidation in the samples following the longest time of irradiation is that the protein itself has not entered the gel and therefore only partial soluble protein oxidation is being analysed.

Semi-quantitative analysis of the protein carbonyl bands visualised by gel electrophoresis confirmed the same trend of increasing protein carbonyl content between 0 and 5 min of irradiation. All laboratories reported a decrease in overall band intensity for oxidised bands between 5 and 15 min (Fig. 4A).

Table 1

<table>
<thead>
<tr>
<th>Sample diluent</th>
<th>ELISA 1</th>
<th>ELISA 2</th>
<th>ELISA 3</th>
<th>ELISA 4</th>
<th>WB 1</th>
<th>WB 2</th>
<th>WB 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water, 20 μg/ml</td>
<td>PBS, 10 μg/ml</td>
<td>PBS, 5 μg/ml</td>
<td>PBS, 1.25 μg/ml</td>
<td>Laemmli, 5 μg/well</td>
<td>5% SDS, 20 μg/well</td>
<td>TRIS EDTA 5 μg/well</td>
<td>Not applicable</td>
</tr>
<tr>
<td>DNP1 treatment</td>
<td>1 mM DNP1 in 2 M HCl for 1 h post-coating</td>
<td>1 mM DNP1 in 2 M HCl for 1 h post-coating</td>
<td>1 mM DNP1 in 2 M HCl for 1 h pre-coating</td>
<td>10 mM DNP1 in 6 M guanidine HCl pre-coating</td>
<td>Not applicable</td>
<td>10% Acrylamide</td>
<td>12.5% Acrylamide</td>
</tr>
<tr>
<td>% Gel and acrylamide blocking buffer</td>
<td>Not applicable</td>
<td>Not applicable</td>
<td>0.1% Reduced BSA in PBS</td>
<td>0.1% Reduced BSA in PBS</td>
<td>Not applicable</td>
<td>0.1% Tween 20 and 3% BSA in TBS</td>
<td>4–12% bis-tris acrylamide</td>
</tr>
<tr>
<td>Primary antibody</td>
<td>Sigma</td>
<td>Not reported</td>
<td>Zymed Laboratories, San Francisco, CA</td>
<td>Sigma</td>
<td>Sigma</td>
<td>Sigma</td>
<td>Zymed Laboratories, San Francisco, CA</td>
</tr>
<tr>
<td>Secondary antibody</td>
<td>AbD Serotec</td>
<td>Not reported</td>
<td>AbD Serotec</td>
<td>Life Technologies</td>
<td>AbD Serotec</td>
<td>Life Technologies</td>
<td>Not reported</td>
</tr>
<tr>
<td>Membrane substrate</td>
<td>Not applicable, OPD, hydrogen peroxide</td>
<td>Not applicable, OPD, hydrogen peroxide</td>
<td>No</td>
<td>Not applicable, OPD, hydrogen peroxide</td>
<td>Yes</td>
<td>PVDF</td>
<td>PVDF</td>
</tr>
<tr>
<td>Within 95% CI</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>
One laboratory also included bovine serum albumin standard in the gel and when the band intensity was normalised to the carbonyl content of albumin also run on the gel in parallel (carbonyl content previously determined by spectrophotometry), protein oxidation in the rat liver fraction was estimated between 2 and 4.5 nmol/mg, depending on irradiation time (Fig. 4B). These values are consistent with the range of carbonyl content determined by ELISA and suggest that western blotting can be used in a quantitative manner if appropriate standardisation is available.

Despite using four different variants of protein carbonyl ELISA assay here, all laboratories recognised a significant increase in protein oxidation between 0 and 5 min UV irradiation \((p=0.031; \text{ Fig. 5A and B})\). Two of the four laboratories failed to observe a further increase protein oxidation by carbonyl ELISA in samples after 15 min irradiation. This may be either due to assay differences or more likely due to protein aggregation and loss of the most heavily oxidised proteins from the analyses. One of the four laboratories used a commercial kit standard. Three of the four laboratories that were using the protein carbonyl ELISA assay synthesised their own standards for calibration purposes. Of these, laboratories 3 and 4 both used the same approach with HOCl as the oxidant according to the method of Buss et al. [20]. Laboratories 1–3 used different methods for standardising e.g. with commercial or self-oxidised BSA and were within the 95% confidence interval for the mean value. Each laboratory calculated the carbonyl content of their standard by the spectrophotometric DNPH assay.

Considering the data in Fig. 5A, three of the laboratories analysed the time zero sample with less than two-fold difference, within the 95% confidence interval. One of these laboratories was using a commercially prepared standard from a kit and the other two were using self-prepared standards. This significant variance in absolute carbonyl content determined by ELISA in the time 0 samples, suggests that an error exists in calibration of the synthesised standards. The fourth laboratory, using their own standard, calculated the carbonyl content to be ten time lower than the other three laboratories. Nevertheless, the trend for irradiation effect was to detect an increase in carbonyls with irradiation time (Fig. 5B).

Calculation of the carbonyl content by spectrophotometry requires both accurate calculation of the DNP adduct formed and also accurate analysis of protein concentration. For samples that contain high quantities of detergents and metal ions the protein determination assays may be susceptible to interference. The BCA

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**Fig. 3.** Semi-quantitative soluble liver protein carbonyl content following 0–15 min UV irradiation. (A) Coomassie stain and (B) protein carbonyl analysis by SDS-PAGE using a gradient gel followed by western blotting (method 3).

**Fig. 4.** Quantitation of the protein carbonyls in soluble protein rat liver extract. Densitometric analysis using arbitrary units (A); and (B) after calibrating the major oxidised band at 66 kDa against oxidised bovine serum albumin of known carbonyl content that had been separated on the same gel.
LPP carbonylated proteins (digested after 5 min of UV irradiation showed a higher number of spectrally, were identified and reactions with lipid peroxidation products (LPP), respectively, were identified in control sample (Table 2). The sample digested after 5 min of UV irradiation showed a higher number of LPP carbonylated proteins \(n=88\), however, the number of proteins carbonylated by direct oxidation did not change \(n=70\). In the samples obtained after 15 min of UV treatment lowest number of carbonylated proteins was identified – 35 and 29 proteins modified by direct oxidation and reaction with LPP, respectively (Table 2). The number of unique proteins identified as being carbonylated by MS did not follow any trend with irradiation time. However, it is important to note that LC–MS/MS method used here does not provide any quantitative information and directed only to identification of carbonylated proteins.

<table>
<thead>
<tr>
<th>Irradiation time</th>
<th>0 min</th>
<th>5 min</th>
<th>15 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of proteins with carbonyls (direct oxidation)</td>
<td>69</td>
<td>70</td>
<td>35</td>
</tr>
<tr>
<td>Number of proteins with carbonyls (from lipid peroxidation products)</td>
<td>68</td>
<td>88</td>
<td>29</td>
</tr>
</tbody>
</table>

There can be several explanations to the low number of carbonylated proteins identified in the sample after 15 min irradiation. Previous works demonstrated that heavily oxidised proteins are more resistant to trypic digestion. There is a risk for underestimating protein carbonyls if protein digestion is inhibited for example by Schiff base formation between lysine residues and reactive carbonyls that are formed as the proteins become oxidised. However, main limitations might result from the bioinformatics solutions currently available for high-throughput proteomics. Data obtained from LC/MS/MS of complex biological samples are analysed using conventional search engines for database search and identification. One limitation of such database search, if de novo sequencing in not considered, is that a set of possible modifications (e.g. different types of protein carbonylation) should be selected before performing protein identification. Therefore, peptides (and proteins) carrying disregarded or unknown modifications cannot be identified. Additionally, combinations of different modifications in a single peptide are usually hard to be resolved. In case of harsh oxidative stress (such as 15 min UV irradiation), numerous oxidation events can occur on different amino acid residues and it is difficult to predict possible combinations of modifications which can be used for the database search.

The pattern of carbonylated proteins that were identified by MS differed markedly between treatment conditions. Seven proteins were reproducibly identified as carbonylated in the three rat liver samples which were identical apart from irradiation time (Table 3). Molecular weights of carbonylated proteins indicate that they correspond to the bands on Western blot described above (Fig. 3). For instance, serum albumin, which most probably represented by the band around 66 kDa on Western blots, was identified as carbonylation target in all three samples. Using MS to analyse specific protein carbonyl formation may offer an important oxidation-target discovery tool.

**Conclusions**

This multi-centre ring study has shown a greater degree of robustness in determining protein carbonyls by ELISA than has previously been reported in a comparison of commercial kits. The self-preparation of standards and lack of internal quality control material is likely to underpin the variance between the data shown here and highlights the need for improved standardisation. The analysis of protein concentration should be undertaken with
care, to avoid the interference of buffers and proteins with colo-

Table 3
Summary of carbonylated proteins identified in more than one sample.

<table>
<thead>
<tr>
<th>UniProt ID</th>
<th>Protein name</th>
<th>MW (kDa)</th>
<th>Carboxylation type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Identified in all three samples</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q8PQ9Q</td>
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<td>94.4</td>
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<td>P20770</td>
<td>Serum albumin</td>
<td>68.7</td>
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<tr>
<td>Q99P55</td>
<td>Sphingosine-1-phosphate phosphatase 1</td>
<td>47.6</td>
<td>DO</td>
</tr>
<tr>
<td>O70444</td>
<td>Serine/threonine-protein kinase pm-3</td>
<td>36</td>
<td>DO</td>
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<tr>
<td>Q5M883</td>
<td>Chloride intracellular channel protein 2</td>
<td>28.1</td>
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<tr>
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<td>Haemoglobin subunit beta-1</td>
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</tr>
<tr>
<td>P38371</td>
<td>PDH finger-like domain-containing protein 5A</td>
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<tr>
<td>Identified in 0 and 5 min</td>
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<td>P42346</td>
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<td>Q92583</td>
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<td>Kinesin-like protein KIFC1</td>
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<td>ATP-binding cassette sub-family G member 2</td>
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<td>P02770</td>
<td>Serum albumin</td>
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<td>LPP</td>
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<td>DNA primase large subunit</td>
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<td>PDZ and LIM domain protein 3</td>
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<td>60S ribosomal protein L18</td>
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<td>Lipid phosphate phosphatase-related protein type 1</td>
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</table>

oxidised or the nature of carbonylation i.e. primary or secondary. Oxyblotting and related Western blotting is less quantitative but provides insight into the molecular mass of oxidised protein targets. Finally, MS methods provide targets and sites of carbonyl modifications, but quantitative applications for protein carbo-

References

[1] W.A. Pryor, M.M. Dooley, D.F. Church, Inactivation of human alpha-1-protei-


