Research Paper

Validation of protein carbonyl measurement: A multi-centre study

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ABSTRACT

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 peroxy 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

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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 (DNP)-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 Na_2HPO_4 and 0.48 mM KH_2PO_4 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 600g. The supernatant was collected and then re-centrifuged for 20 min at 3000g. The supernatant was collected again and was finally re-centrifuged using an ultracentrifuge at 100,000g 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 ($I = 1.74 \times 20$ mW/cm^2, $P = 250$ 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
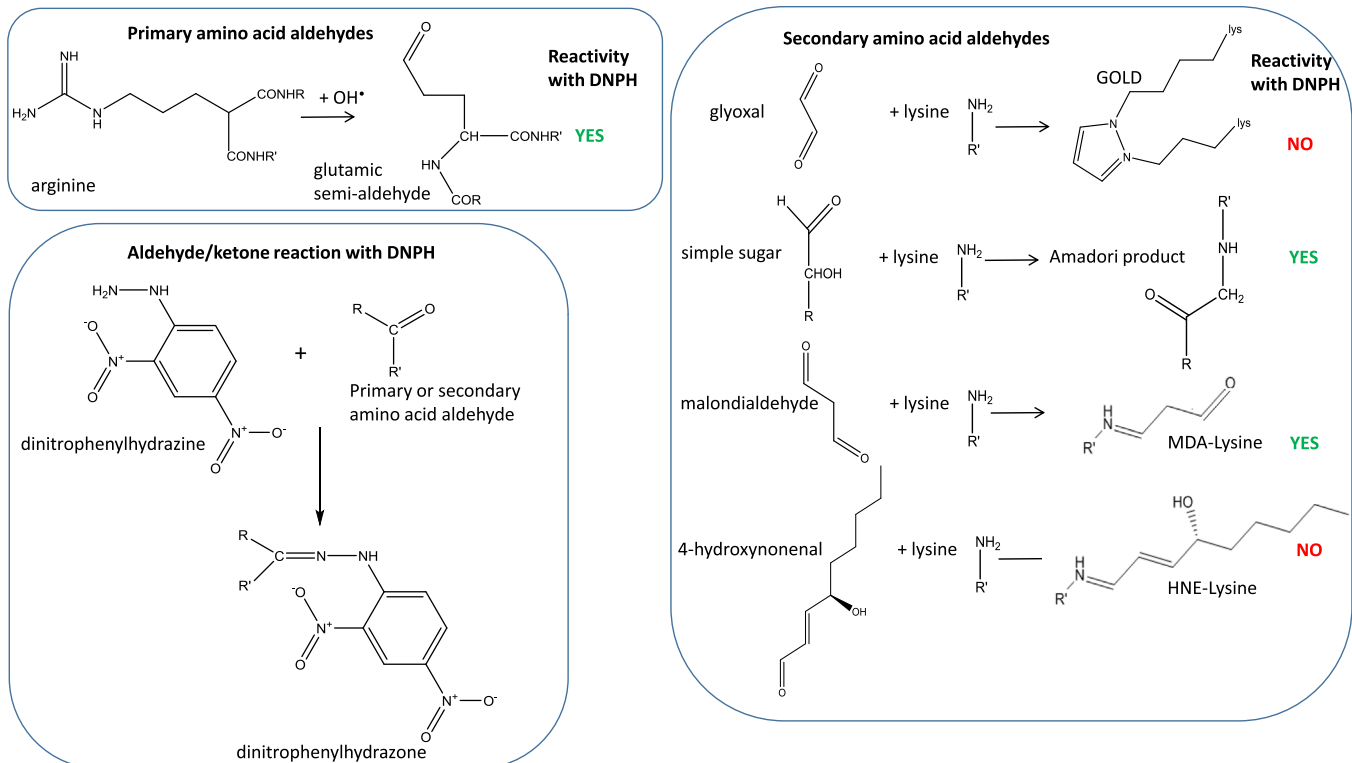

Fig. 1. Primary and secondary protein carbonyls and their derivatisation by DNPH.
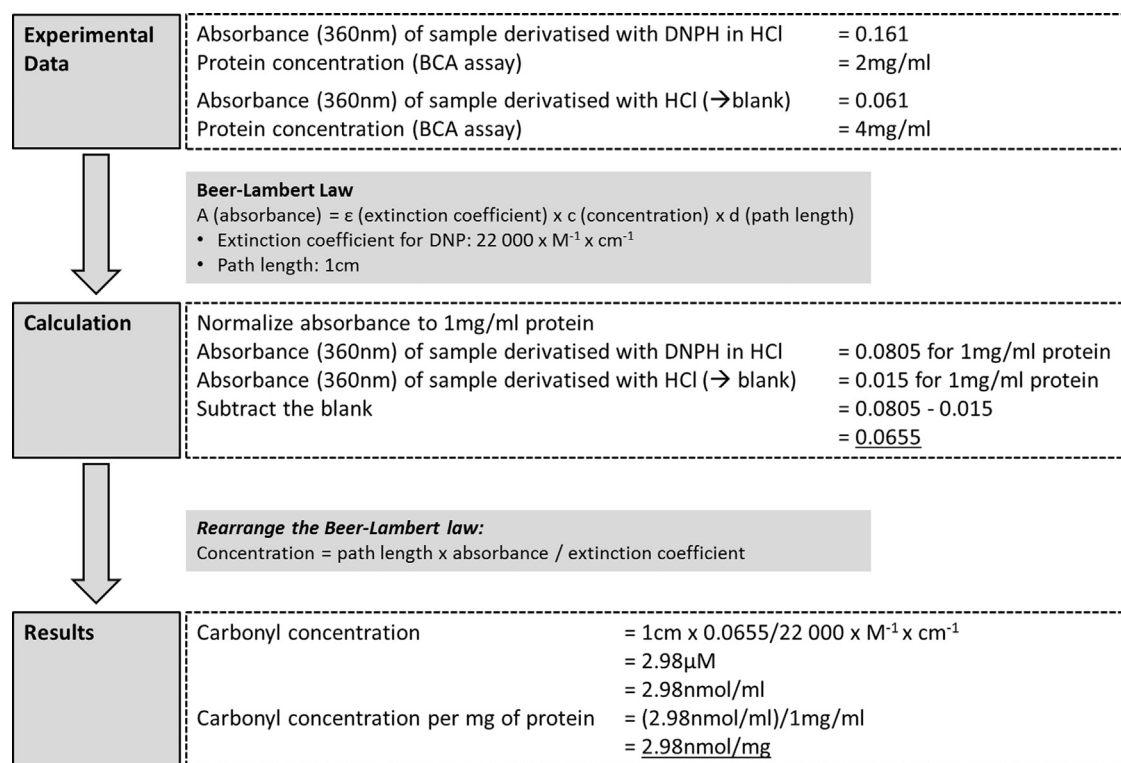
Experimental Data	Absorbance (360nm) of sample derivatised with DNPH in HCl	= 0.161
	Protein concentration (BCA assay)	= 2mg/ml
	Absorbance (360nm) of sample derivatised with HCl (→blank)	= 0.061
	Protein concentration (BCA assay)	= 4mg/ml

Beer-Lambert Law
A (absorbance) = ε (extinction coefficient) x c (concentration) x d (path length)
- Extinction coefficient for DNP: 22 000 x M^{-1} x cm^{-1}
- Path length: 1cm

Calculation	Normalize absorbance to 1mg/ml protein	
	Absorbance (360nm) of sample derivatised with DNPH in HCl	= 0.0805 for 1mg/ml protein
	Absorbance (360nm) of sample derivatised with HCl (→ blank)	= 0.015 for 1mg/ml protein
	Subtract the blank	= 0.0805 - 0.015
		= 0.0655

Rearrange the Beer-Lambert law:
Concentration = path length x absorbance / extinction coefficient

Results	Carbonyl concentration	= 1cm x 0.0655/22 000 x M^{-1} x cm^{-1}
		= 2.98μM
		= 2.98nmol/ml
	Carbonyl concentration per mg of protein	= (2.98nmol/ml)/1mg/ml
		= 2.98nmol/mg

Fig. 2. Calculation for carbonyl quantitation by analysis of DNP adducts.

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 tri-fluoroacetic 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 monoclonal 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 H_2SO_4. 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 Na_2HPO_4 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 H_2SO_4 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 KH_2PO_4) 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 Na_2HPO_4/24 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 H_2SO_4, 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 stain (Bio-Rad) according to the manufacturer's protocol, while the paired gel was used for western blot analysis. The stained gels were scanned on a Pharos FX Plus Molecular Imager (Bio-Rad).

Analysis of oxidised liver proteins was undertaken as described previously [23]. Proteins were transferred from SDS-PAGE to Hybond-P® PVDF membranes (GE Healthcare, Amersham, UK). Briefly, for analysis of protein carbonyls, the proteins were derivatised using 1 mM DNPH in 2M hydrochloric acid for 1 h. Membranes were washed six times for 5 min in 0.05% Tween 20, in TBS and blocked overnight at room temperature with 0.1% Tween 20 and 3% BSA in TBS. Membranes were further washed six times for 5 min in 0.05% Tween 20, in TBS and incubated for 2 h with monoclonal mouse anti-DNP (Sigma, UK) antibody diluted at 1:1000 with 0.2% BSA in TBS. Membranes were washed a further six times for 5 min with the same washing buffer and then incubated for 1.5 h with peroxidase-conjugated rat anti-mouse IgE antibody (AbD Serotec) diluted at 1:10,000 with 0.2% BSA in TBS. Subsequently, membranes were washed five times for 5 min with 0.05% Tween 20, in TBS and once with TBS only. Oxidised liver proteins were visualised using ECL+chemiluminescence (GE Healthcare, Amersham, UK), and protein bands scanned using a molecular imager GS-800 Calibrated Densitometer (Bio-Rad).

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 1:1000 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% w/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 NaH_2PO_4, 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 Fischer 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 C_{18}, internal diameter 180 μm, length 20 mm, particle diameter 5 μm) at a flow rate of 10 μl/min. Peptides were separated on BEH 130 column (C_{18}-phase, internal diameter 75 μm, length 100 mm, particle diameter 1.7 μm) with a flow rate of 0.4 μl/min. using several linear gradients from 3% to 9% (2.1 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™ on-line 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 μm for product ion scans. Database search included carbamidomethylation on Cys, oxidation of Met, carbonylated and ARP-derivatised Lys (mass shift of 312.08 m/z units), Arg (270.06 m/z units), Thr (311.10 m/z units), and Pro (329.11 m/z units) as variable modifications. The second set of variable modifications in addition to Cys carbamidomethylation and Met oxidation included ARP-derivatised alkenal adducts: 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 2DQuant 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 bands 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
A comparison of the key buffers, antibodies and conditions used in the carbonyl assay by ELISA and Western blot (WB).

	ELISA 1	ELISA 2	ELISA 3	ELISA 4	WB 1	WB 2	WB 3
Sample diluent	water, 20 μg/ml	PBS, 10 μg/ml	PBS, 5 μg/ml	PBS, 1.25 μg/ml	Laemmli, 5 μg/well	5% SDS, 20 μg/well	TRIS EDTA 5 μg/well
ELISA coating buffer	carbonate buffer pH 9.6	Cell BioLabs kit	10 mM Na_2PO_4, 140 mM NaCl, pH 7.0	10 mM Na_2PO_4 140 mM NaCl, pH 7.0	Not applicable	Not applicable	Not applicable
DNPH treatment pre or post-coating/separating	1 mM DNPH in 2M HCl for 1 h post-coating	1 mM DNPH in 2 M HCl for 1 h post-coating	1 mM DNPH in 2 M HCl for 1 h pre-coating	10 mM DNPH in 6 M guanidine HCl/0.5 M KH_2PO_4 for 45 min pre-coating	1 mM DNPH in 2 M HCl for 1 h post-separating	1 mM DNPH in 2 M HCl for 1 h pre-separating	1 mM DNPH in 2 M HCl for 1 h pre-separating
% Gel and acrylamide	Not applicable	Not applicable	Not applicable	Not applicable	10% Acrylamide	12.5% Acrylamide	4–12% bis-tris acrylamide
Blocking buffer	0.5% Tween	Not reported	0.1% Reduced BSA in PBS	0.1% Reduced BSA in PBS	0.1% Tween 20 and 3% BSA in TBS	0.05% Tween 20 and 5% fat-free milk in TBS	Not reported
Primary antibody	Sigma	Not reported	Zymed Laboratories, San Francisco, CA	Sigma	Sigma	Zymed Laboratories, San Francisco, CA	Not reported
Secondary antibody	AbD Serotec	Not reported	Life Technologies	Sigma	AbD Serotec	Life Technologies	Not reported
Membrane	Not applicable	Not applicable	Not applicable	Not applicable	PVDF	Nitrocellulose	PVDF
Substrate	OPD, hydrogen peroxide	Not reported	OPD, hydrogen peroxide	OPD, hydrogen peroxide	ECL	infrared dyes - no substrate	Luminata Forte
Within 95% CI	Yes	Yes	No	Yes	Yes	Yes	Yes
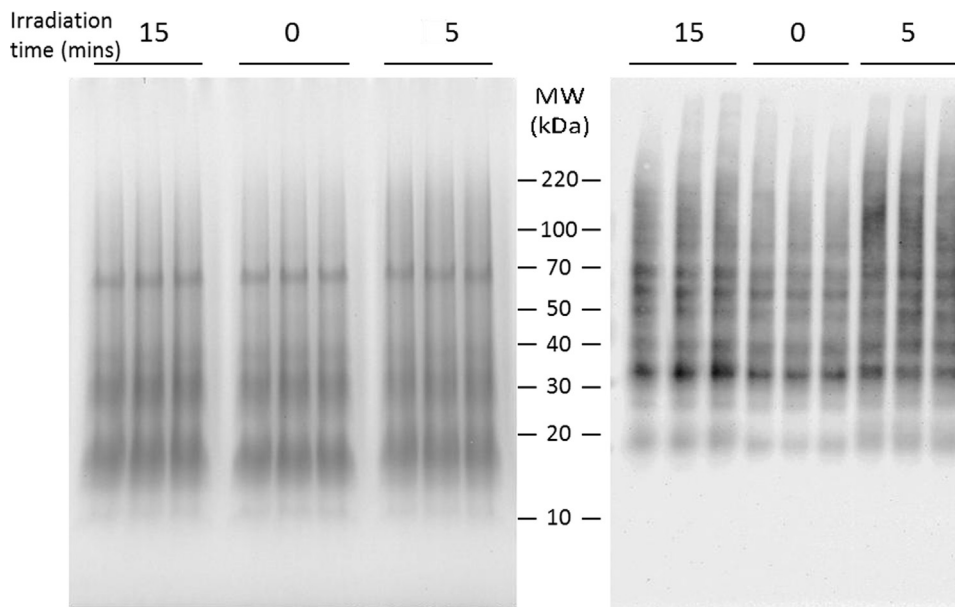
Fig. 3. Semi-quantitative soluble liver protein carbonyl content following 0–15 min UV irradiation. (A) Coomassie stain and (B) protein carbonyl analysis by SDS-PAGE using a gradient gel followed by western blotting (method 3).

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 ($p = 0.031$; 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 standarising 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
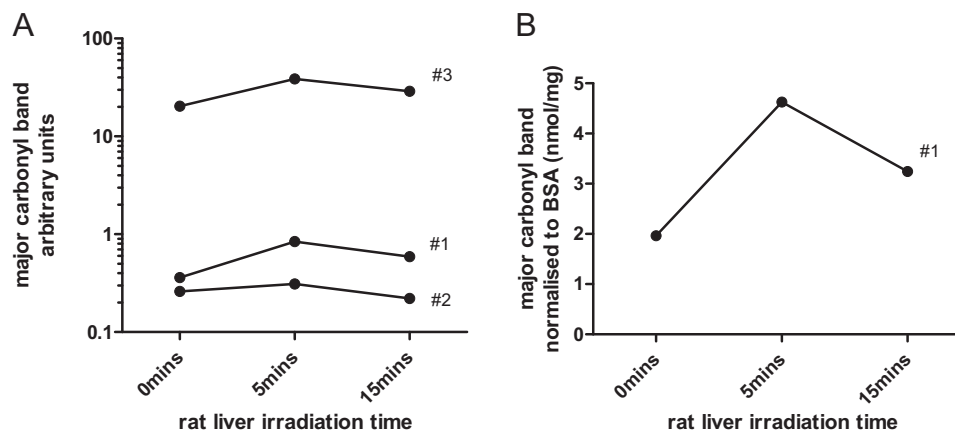
Fig. 4. Quantitation of the protein carbonyls in soluble protein rat liver extract. Densitometric analysis using arbitrary units (A); and (B) after calibrating the major oxidised band at 66 kDa against oxidised bovine serum albumin of known carbonyl content that had been separated on the same gel.
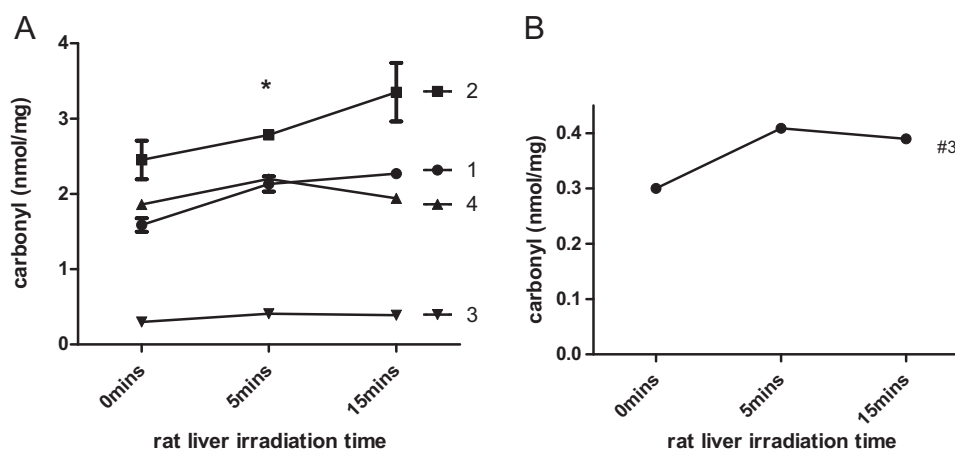
Fig. 5. Protein carbonylation in rat liver soluble protein fraction following increase in irradiation by UV light, determined by ELISA. (A) Mean carbonyl+SEM determined by four laboratories and expressed in nmol/mg. * Represents $p=0.031$ compared to control by paired comparison analysis; and (B) expanded scale to show the trend for the data reported by laboratory method # 3.

assay is not recommended for metal ion containing samples. Here the presence of haem in the soluble protein fraction may cause interference in protein determination of the rat liver samples when using BCA.

Considering the many differences in methods used between the laboratories, the results obtained were remarkably homogeneous. There seems to be no major difference in detection of protein carbonyls whether samples are derivatised before coating onto a plate or separating by SDS-PAGE or if DNPH derivatisation is performed afterwards. It is widely accepted that exposure of proteins to UV irradiation induces oxidation, aggregation and resistance to proteolysis [28,30]. We did not detect any obvious aggregates in the SDS-PAGE gels, however, if greater than 250 kDa these may be excluded from the gel by limiting pore size.

One laboratory undertook LC–MS/MS analysis to identify carbonylated proteins. Analysis was based on protein tryptic digestion, derivatisation of carbonylated peptides with ARP, affinity enrichment and LC–MS/MS using data-dependent acquisition. LC–MS data were used for database search using two sets of variable modifications – direct oxidation (on Lys, Arg, Pro and Thr) and carbonylation via Michael addition of reactive lipid peroxidation products (hydroxy-, oxo-hexanal and hydroxy-, oxo-nonenal on Lys, Cys and His). 69 and 68 proteins carbonylated via direct oxidation 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 2

Number of identified proteins in liver samples after 0, 5 and 15 min of UV irradiation.

Irradiation time	0 min	5 min	15 min
Number of proteins with carbonyls (direct oxidation)	69	70	35
Number of proteins with carbonyls (from lipid peroxidation products)	68	88	29

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 tryptic 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 bioinformatic 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 can 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

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

UniProt ID	Protein name	MW (kDa)	Carbonylation type
Identified in all three samples			
Q5PQQ9	Centrosomal protein of 70 kDa	94.4	DO
P02770	Serum albumin	68.7	DO
Q99P55	Sphingosine-1-phosphate phosphatase 1	47.6	DO
O70444	Serine/threonine-protein kinase pim-3	36	DO
Q5M883	Chloride intracellular channel protein 2	28.1	DO
P02091	haemoglobin subunit beta-1	16	DO
P83871	PHD finger-like domain-containing protein 5A	12.4	DO
Identified in 0 and 5 min			
P42346	Serine/threonine-protein kinase mTOR	288.8	DO
Q925B3	Transient receptor potential cation channel subfamily M member 7	212.4	LPP
P05197	Elongation factor 2	95.3	LPP
Q7TMB7	Lipid phosphate phosphatase-related protein type 4	83.4	LPP
Q5XI63	Kinesin-like protein KIFC1	76.1	LPP
Q80W57	ATP-binding cassette sub-family G member 2	73	LPP
P02770	Serum albumin	68.7	LPP
Q5XIR8	Clathrin heavy chain linker domain-containing protein 1	67.5	LPP
O89044	DNA primase large subunit	58.6	LPP
Q66HS7	PDZ and LIM domain protein 3	39.1	LPP
Identified in 0 and 15 min			
Q62976	Calcium-activated potassium channel subunit alpha-1	134.4	DO
P0C1X8	AP2-associated protein kinase 1	103.8	LPP
O54861	Sortilin	91.2	LPP
Q8R512	UBX domain-containing protein 11	54.7	DO
P12001	60S ribosomal protein L18	21.7	LPP
Identified in 5 and 15 min			
P51111	Huntingtin	343.8	DO
P29994	Inositol 1,4,5-trisphosphate receptor type 1	313.3	DO
Q63796	Mitogen-activated protein kinase kinase kinase 12	96.3	LPP
Q6WAY2	Lipid phosphate phosphatase-related protein type 1	35.9	DO

care, to avoid the interference of buffers and proteins with colourimetric reagents. A method for mathematical calculation of carbonyl content in DNP-derivatised proteins has been outlined to encourage common ways of working. The inclusion of a commercial protein e.g. BSA, is recommended as an internal control in every analysis batch. BSA has ∼3.5 nmol/mg protein carbonyls in its native form and confirming this in every assay will further improve standardisation of analyses. In studies of heavily oxidised proteins, there is a risk that the most heavily oxidised proteins are lost to analysis in both MS and carbonyl western blot if aggregation occurs. Prior to MS analysis, digestion using a combination of peptidases may increase the probability of finding more oxidised proteins in a sample, if lysine residues have been modified by Schiff's bases. Improvement of bioinformatics tools for analysis of high-throughput proteomics in respect of protein post-translational modification data are required. The MS method offers an important tool for oxidation target discovery and should be further extended for quantitative application using standard carbonylated peptides.

ELISA is the best available method for quantification of protein carbonyls, but does not give any information about the molecules

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 carbonylation are very poorly developed at this time.

Acknowledgements

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