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Review Article

Determination of protein carbonyls in plasma, cell extracts, tissue homogenates, isolated proteins: Focus on sample preparation and derivatization conditions

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ABSTRACT

Protein oxidation is involved in regulatory physiological events as well as in damage to tissues and is thought to play a key role in the pathophysiology of diseases and in the aging process. Protein-bound carbonyls represent a marker of global protein oxidation, as they are generated by multiple different reactive oxygen species in blood, tissues and cells. Sample preparation and stabilization are key steps in the accurate quantification of oxidation-related products and examination of physiological/pathological processes. This review therefore focuses on the sample preparation processes used in the most relevant methods to detect protein carbonyls after derivatization with 2,4-dinitrophenylhydrazine with an emphasis on measurement in plasma, cells, organ homogenates, isolated proteins and organelles. Sample preparation, derivatization conditions and protein handling are presented for the spectrophotometric and HPLC method as well as for immunoblotting and ELISA. An extensive overview covering these methods in previously published articles is given for researchers who plan to measure protein carbonyls in different samples.

Contents

Introduction

In recent years, more and more evidence has arisen that oxidative processes play a key role in the pathophysiology of many diseases and in the aging process. Besides regulatory events, a plethora of damaging effects is induced by oxidative processes, one of these damaging processes is protein oxidation [1]. Oxidative damage to proteins results in a multitude of products (for reviews see [2–6]), arising from modification of a wide range of amino acids. These include damage to sulfur-containing, aromatic, and aliphatic amino acids [7–10] (Table 1). Protein carbonyls

Abbreviations: AP, alkaline phosphatase; BAL, bronchoalveolar; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; BHT, butylated hydroxytoluene; DMSO, dimethyl sulfoxide; DNP, 2,4-dinitrophenyl; DNPH, 2,4-dinitrophenylhydrazine; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; HCl, hydrochloric acid; HPLC, high performance liquid chromatography; HRP, horseradish peroxidase; Hsf1, heat shock factor 1; IgG, immunoglobulin G; KLH, keyhole limpet hemocyanin; n.a., not available; NBT, nitro blue tetrazolium; PVDF, polyvinylidene difluoride; RT, room temperature; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; TFA, trifluoracetic acid

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Table 1
Possible oxidation products of amino acid residues resulting in protein carbonyl formation.

Amino acid	Oxidation products
Proline	Glutamic semialdehyde and other ring opened products
Arginine	Glutamic semialdehyde and other side-chain products
Lysine	Aminoadipic semialdehyde and other side-chain products
Threonine	Carbonyls formed at side chain sites
Methionine	Methional
Tryptophan	N-formylkynurenine, kynurenine
Histidine	2-Oxo-histidine and ring-opened species
Alanine	Formaldehyde and carbonyls from methyl group
Valine	Acetone, formaldehyde and carbonyls on side-chain methyl groups
Leucine	Isobutyraldehyde, acetone, formaldehyde, and carbonyls on side-chain
Aspartate	Glyoxylic acid
Isoleucine	Formaldehyde, carbonyls on side-chain

represent an irreversible form of protein modification and have been demonstrated to be relatively stable (degradation/clearance in hours/days) in contrast to lipid peroxidation products that are removed within minutes [11,12]. In addition, protein carbonyls are formed early during oxidative stress conditions and are not a result of one specific oxidant, thus they can be called a marker of overall protein oxidation. Due to the great variety of different modifications [13], one obstacle in the detection of protein-related oxidative stress biomarkers is the requirement of complex procedures for their determination. Furthermore, the instability of some of these products as a result of repair processes (in the case of methionine sulfoxides) [14] and by peroxiredoxins and disulfide reductases [15] can contribute to difficulties in assessing and quantifying oxidation status. It is worth pointing out that some of the formed species resulting from protein oxidation (formaldehyde, acetaldehyde, acetone, etc.) do not remain protein bound but are released. Hence they are not detected by any assays that involve protein separation or precipitation. Depending on the radical treatment used, released carbonyls can however be major products [16].

Various oxidants may attack several amino acids and are thus able to produce both protein-bound and released carbonyl groups (Table 1 and Fig. 1). The yield of these species is however oxidant dependent. Due to the structure of normal amino acids it is expected, that no carbonyl groups are part of a native protein. However, this seems to be a simplifying assumption since many proteins undergo (enzymatic) post-translational modifications where carbonyl groups might be introduced into the native, functional protein. The presence of such structures could be the reason for the high basal (not stress-induced) level of protein carbonyls found in some proteins. Furthermore, inappropriate sample handling might contribute to elevated concentrations observed in some studies.

The most commonly used marker to assess protein oxidation is via the determination of protein-bound carbonyls. Protein carbonyls can be detected by various methods, all relying on the derivatization of the carbonyl group. The reduction with radiolabeled borohydride introduces a measurable radiolabel into the protein, whereas several hydrazine derivatives, most commonly 2,4-dinitrophenylhydrazine (DNPH, Fig. 2) or biotin hydrazine, introduce detectable functional groups into the oxidized protein. So, the most often used procedure to detect protein carbonyls is after their derivatization with DNPH. During the last three decades most of these methods have referred to the basic methods described by Levine et al. [17,18] using the highly-sensitive DNP-modification of protein carbonyls followed by a detection either by spectrophotometric methods, by an HPLC-based technique or using anti-DNP antibodies in immunoblotting [19] or ELISA [20] (see Fig. 3). In addition to this, proteomic techniques have been applied to get a more detailed insight into the mechanism of protein damage, e.g. in blood [21].

In the following we will concentrate on the determination of protein carbonyls in plasma, cell culture, organ homogenate and isolated protein/organelle samples by the methods of Levine et al. [17,18], Shacter et al. [19], Keller et al. [22] and Buss et al. [20].

As described above, DNP-derivatized proteins can be detected by different methods; hence every laboratory should be able to detect carbonyl groups either by the simple spectrophotometric assay or by more complex procedures. The fact that no special equipment is needed for the analysis of DNP-derivatized proteins has led to the application of these methods in numerous publications. The search term “protein carbonyl” leads to more than 15,000 publications on PubMed and “protein carbonyl assay” still leads to around 6000 results (accessed in April 2015).

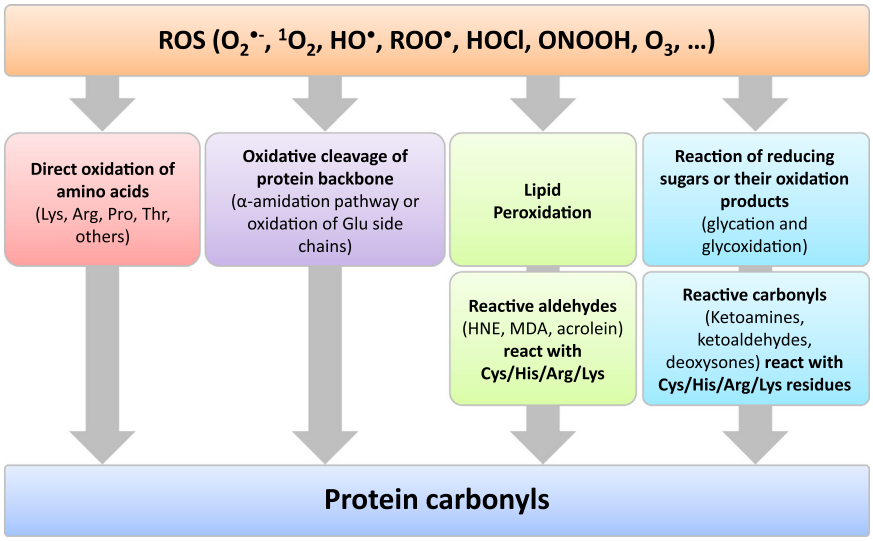

Fig. 1. Protein oxidation resulting in protein carbonyl formation. Reactive oxygen species (ROS) may either react directly with some amino acid residues or lead to oxidative cleavage of the protein backbone. Other possible formation routes of protein carbonyls are via the oxidation of lipids resulting in reactive aldehydes which react with cysteine (Cys), histidine (His), arginine (Arg) and lysine (Lys) residues and thus introduce carbonyl groups and furthermore via the reaction of reducing sugars or their oxidation products with the same residues.

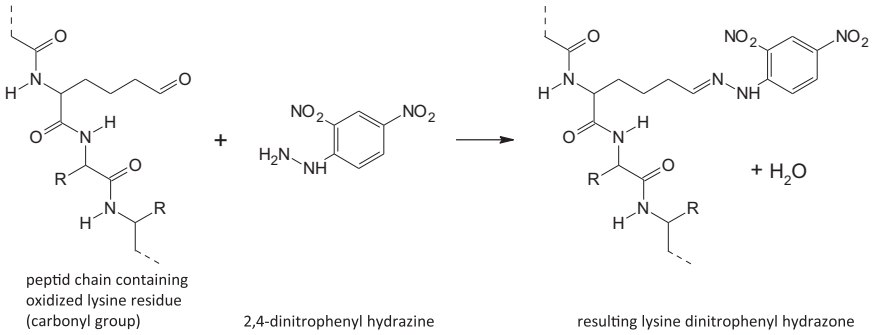

Fig. 2. Reaction of protein carbonyl group with 2,4-dinitrophenylhydrazine. The nucleophilic addition, also called condensation reaction, resulting in a 2,4-dinitrophenyl hydrazone is shown for an oxidized lysine residue (aminoadipic semialdehyde). Note that the reaction is accompanied by the loss of one molecule of water.

Search strategy

PubMed was accessed to view the publications of Levine [17,18], Shacter et al. [19], Keller et al. [22] and Buss et al. [20]. Following this, on the bottom right hand side, the link "Cited by xx PubMed Central articles" was followed to view all publications citing one of these original method publications.

At the time PubMed was accessed in November 2013, the publication of Levine [17] was cited by 130 publications. Forty-four of these were reviews or reported on research carried out with bacteria, plants, yeast, or non-human primates. Seventeen publications referred to the method or the results in general in the introduction or in the discussion of the publication and thus did not apply the method. Five publications described using the commercial kit (OxyBlot). Another 45 publications cited the spectrophotometric, HPLC method or Western blot without any modifications or specific comments on sample preparation and analytical condition; while 19 publications described in more detail the reaction conditions and four publications the sample handling for the spectrophotometric assay and the immunoblotting, respectively (see Table 2 and Fig. 4).

In November 2013, the method description for immunoblotting by Emily Shacter et al. was cited by 38 publications. Fifteen of these publications were reviews or did not relate to human or cell culture studies. Ten publications referred to results but not to the method itself. Three publications applied a commercial kit. Ten publications described the method with or without slight modifications (see Table 3 and Fig. 4).

The method by Keller et al. [22] was cited by 10 publications. Five of these were reviews or used yeast/housefly models. Two publications did not apply the method. Three publications described applying the method of Keller et al. [22] and Shacter et al. [19].

Some authors cited more than one reference/method when describing the immunoblotting technique thus resulting in less relevant publications in Table 3 than observed in Fig. 4.

The method description of Hendrikje Buss et al. [20] was cited by 53 publications when accessed in November 2013. Twenty of these publications only referred to the publication in the introduction or discussion or did not describe using plasma/serum or cell culture samples. Three publications described using a commercial kit, while four did not go into further detail concerning the application of the method. Twenty-six publications described the method in detail with no modifications or only slight changes (see Table 4).

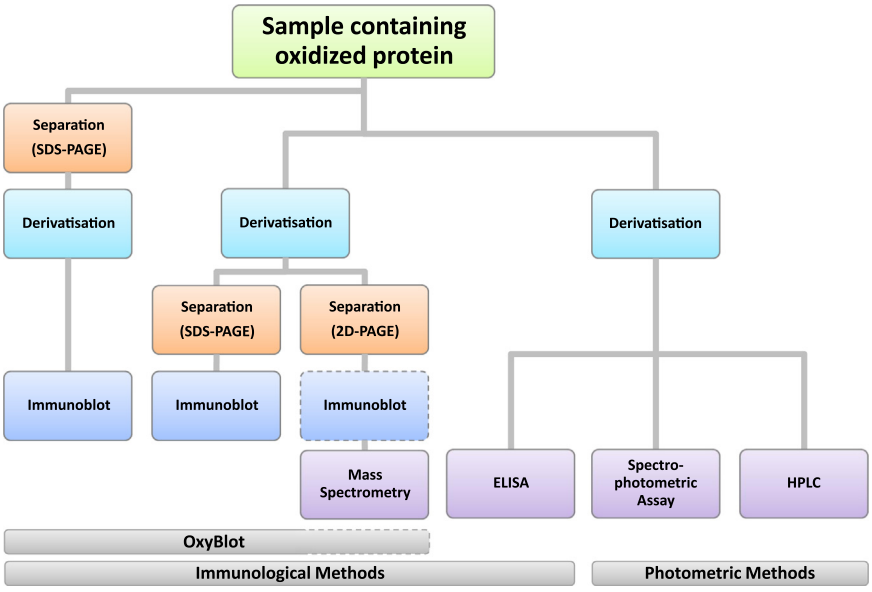

Fig. 3. Flow chart of possibilities to analyze protein carbonyls after DNP derivatization. Several options exist to detect protein carbonyls in a sample, either quantitatively or qualitatively, depending on the research question and laboratory equipment/facilities. The left-hand side shows several ways of the so-called OxyBlot. The proteins can be separated before derivatization or the other way around. Following separation/derivatization, immunoblot is carried out. If separation is performed with 2D PAGE, the immunoblot is not mandatory and is usually followed by mass spectrometry. The right hand side depicts more quantitative methods: the spectrophotometric assay, ELISA and HPLC where standards are commonly used to assess the exact concentration of protein carbonyls in the sample.

Table 2
Published sample preparation for spectrophotometric determination of protein carbonyls (n.a.: not available, ratio of sample volume to DNP volume).

Species	Sample preparation	Derivatization conditions	Protein handling (Precipitation, washing, dissolving)	Wave-length	Level[a]	Refs.
n.a.	Oxidatively modified proteins (> 0.5 mg)	10 mM DNPH (in 2 M HCl or in 6 M guanidine-HCl, pH 2.5)ratio n.a.1 hn.a. if under dark conditions	20% TCA	Spectrum (360–390 nm)	0.2–1.0 mol/mol protein	[17,18]
		Ratio n.a. 1 h n.a. if under dark conditions	Washing with ethanol–ethyl acetate (1:1) 6 M guanidine			
Human	Heparin plasma −80 °C Dilution n.a.	DNPH (concentration n.a.) Ratio n.a. Conditions/duration n.a.	n.a. Washing n.a. Guanidine n.a.	360 nm (280 nm)	1.02 nmol/mg	[32]
Human	Serum −80 °C 1:100 dilution Decomplementation at 56 °C for 30 min	10 mM DNPH (in 2.5 M HCl) Ratio n.a. Duration n.a. n.a. if dark	20% TCA Washing with ethanol–ethyl acetate (1:1) 6 M guanidine	n.a. 22,000 M^{-1}/cm	2.4 ± 0.21 nmol/mg	[33]
Human	Plasma −80 °C Concentration n.a.	n.a.	n.a.	n.a. In 96-well plates	0.48–0.7 nmol/mg	[34]
Human	Plasma of children with juvenile chronic arthritis Undiluted plasma	20 mM DNPH Ratio (1:1) 1 h	20% TCA Washing with ethanol–ethyl acetate (1:1) 6 M guanidine	360 nm	Patients vs controls: 1.36 ± 0.68 vs 0.807 ± 0.16 nmol/mg	[35]
Human	Mesenchymal stem cell (hMSCs) suspensions 30% TCA precipitate resuspended in DNPH Concentration n.a.	10 mM DNPH 1 h (37 °C)	20% TCA (v/v) Washing with ethanol–ethyl acetate (1:1) Guanidine in 2 mM phosphate buffer	360 nm	20–70 μmol/10^3 cells	[36,37]
Human	Acute promyelocytic leukemia (APL)-derived NB-4 cells soluble protein fraction with protease inhibitors, sonicated Stored at −20 °C Concentration n.a.	10 mM DNPH Ratio (1:6) 1 h (20 °C) in the dark	20% TCA Washing with ethanol–ethyl acetate (1:1) 6 M guanidine	370 nm	~3.5 nmol/mg	[38]
Mouse	Neutrophil cell lysate in 50 mM potassium phosphate (pH 7.4) Concentration n.a.	High DNPH (in 2 M HCl) Ratio n.a. 1 h in the dark	20% TCA Washing with ethanol–ethyl acetate (1:1) 6 M guanidine	370 nm	10–30 nmol/mg	[28]
Mouse	Peritoneal macrophage cell lysate in 50 mM potassium phosphate (pH 7.4) Concentration n.a.	High DNPH (in 2 M HCl) Ratio n.a. 1 h in the dark Conditions n.a.	20% TCA Washing with ethanol–ethyl acetate (1:1) 6 M guanidine	370 nm	5–12 μmol/mg	[27]
Mouse	Brain cortex homogenate extracts in phosphate buffer (pH 7) with DTT and EDTA Streptomycin substrate (10%) to remove nucleic acids Concentration n.a.	DNPH Ratio, duration and dark conditions n.a.	n.a.	370 nm in 96-well plate	0.2–0.4 nmol/mg	[39]
Mouse	Lysate of primary cortical neurons in phosphate buffer (pH 6.5) Lysis and storage n.a. Concentration n.a.	1 × DNPH (in 2 M HCl) Ratio n.a. 20 min at RT	10% TCA Washing with ethanol–ethyl acetate (1:1) 6 M guanidine in 20 mM sodium phosphate buffer (pH 6.5)	375 nm	Expressed as fold-increase to controls	[40]
Mouse	Serum and kidney homogenate (in PBS, pH 7.4, containing protease inhibitor cocktail) Undiluted serum	10 mM DNPH (in 2.5 M HCl) Ratio (1:4) 1 h at RT in the dark	20% TCA Washing with ethanol–ethyl acetate (1:1) 6 M guanidine	370 nm	3-Fold increase in protein carbonyls in mice treated with tetrachlorethene	[41]

Rat	EDTA plasma of Sprague Dawley rats − 20 °C storage Dilution n.a.	DNPH (concentration n.a.) Ratio n.a. 1 h n.a. if dark	20% TCA Washing n.a. Guanidine n.a.	380 nm	~3 nmol/mg	[42]
Cow	BSA Concentration n.a.	10 mM DNPH Ratio (1:5) 1 h at RT	20% TCA Washing with ethanol-ethyl acetate (1:1) Solubilization in 0.2% (w/v) SDS/20 mM Tris–Cl, pH 6.8	360 nm (scan 320–450 nm)	Method description	[43]
Cow	Aortic endothelial cells 800 μg protein	0.2% DNPH (≙ 10 mM) (in 2 M HCl) ratio n.a.for 1 h in the dark Ratio n.a. For 1 h in the dark	20% TCA Washing with ethanol–ethyl acetate (1:1) 6 M guanidine	370 nm	μmol carbonyl/mg but expressed as percent to control	[44]
Cow	Oxidized BSA 1 mg	10 mM DNPH (in 2 M HCl) Ratio (1:1) 1 h at RT	20% TCA Washing with ethanol–ethyl acetate (1:1) 6 M guanidine	376 nm	Standard curve: 2–27 nmol/mg	[22]
Horse	Heart myoglobin	10 mM DNPH (in 2.5 M HCl)	Centrifugation	400 nm	After TEMPOL incubation the myoglobin N-terminus underwent oxidative deamination in which the Gly N-terminal amino group was replaced with a free carbonyl group	[45]
Hamster	Concentration n.a. Liver supernatant Concentration n.a.	Ratio (1:1) 15 min in the dark 10 mM DNPH (in 2.5 M HCl) Ratio (1:1) 30 min at RT	PD-10 column 20% TCA Washing with ethanol–ethyl acetate (1:1) Dissolved 6% SDS	HPLC analysis 370 nm	2.25–3.74 nmol/mg	[46]

[a] Some levels could only be drawn from figures. The concentration is therefore not the exactly measured one.

These relevant publications were further examined for sample origin and derivatization conditions etc. to allow better comparison of results as well as for finding an appropriate protocol for scientists planning future experiments.

Spectrophotometric and HPLC determination of protein carbonyls after dinitrophenyl hydrazine modification

The most-cited (original) method for derivatizing carbonyl groups with different agents to determine the carbonyl content was described by Levine in 1990 [17]. The publication discusses the reaction of carbonyls with borohydride, DNPH, fluorescein thiosemicarbazide and fluorescein amine.

In this protocol different oxidatively modified proteins (> 0.5 mg protein) are treated with 10 mM DNPH for 1 h. The ratio of sample volume to DNPH volume is not stated. The protein is precipitated with 20% TCA followed by a washing step with ethanol–ethyl acetate (1:1) and dissolving in 6 M guanidine. The protocol for DNP derivatization is completed by the spectrum measurement from 360 to 390 nm.

Another publication of Levine, published 4 years later (1994), also describes the derivatization with DNP followed by HPLC measurement and/or immunoblotting [18].

The general drawbacks of the spectrophotometric assay are that the method is rather work-intensive, time-consuming and high throughput measurement is not possible. Additionally, the requirement of protein and volume is relatively high, accompanied by the fact that the loss of acid-soluble proteins during washing steps (about 10–15%) must be considered and the results adjusted to the actual protein concentration. Furthermore additional carbonyl groups may also be introduced due to acidic conditions, DNP may be trapped in the protein pellet and the resolubilization may be incomplete, thus falsifying the results [23].

Another general problem is that nucleic acids may interfere as they also contain carbonyl groups, and in addition other biological compounds such as hemoglobin, myoglobin and retinoids absorb at 370 nm which result in high background readings.

Since there are no established commercially available protein standards of reduced and oxidized BSA to include as controls it is difficult to compare results obtained with the spectrophotometric assay (and other methods) in different laboratories.

Some of these drawbacks can be overcome by HPLC analysis which provides the advantages that DNP absorbance can be monitored at 366 nm in parallel to protein absorbance at 280 nm. Here DNP derivatization should be carried out in sodium dodecyl sulfate (SDS) since guanidine–HCl is not suitable for most columns.

Detection of protein carbonyls by immunoblotting

In 1994 Shacter and Levine et al. published the immunoblotting method to detect carbonyls of plasma proteins [19]. Here they describe the preparation of standards with iron and ascorbate as oxidizing agent. Heparin plasma was then treated with 10 mM DNPH (in 10% TCA) for 15–30 min at room temperature. The separation was performed with an SDS-PAGE (4–12% gradient) followed by blotting onto a nitrocellulose membrane by standard procedures. A monoclonal anti-DNP IgE, a secondary biotinylated rat anti-mouse IgE and biotin–avidin-peroxidase complex were used to detect DNP-carbonyl epitopes.

The publication is a general method description which uses different oxidized proteins. Fibrinogen was identified as a major oxidized protein in plasma. The amount of carbonyls present in untreated plasma samples was 0.6 nmol/mg. Native, as well as oxidized glutamine synthetase were run to assess the sensitivity of the assay which was found to be 30 ng of protein.

Keller et al. developed an independent immunoblotting

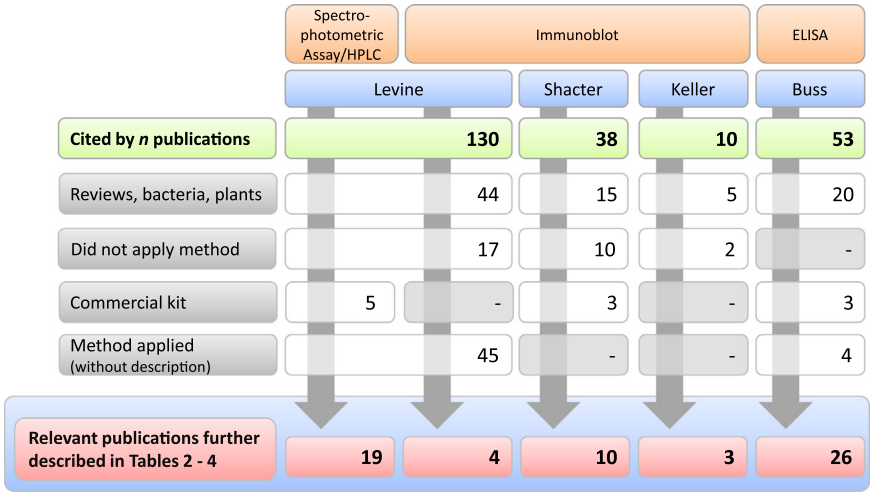

Fig. 4. Final number of publications highlighted in Tables 2–4. As described in the search strategy, PubMed was accessed in November 2013 to view the publications of Levine, Shacter et al., Keller et al., and Buss et al. as well as all publications citing one of these original method publications. The extracted publications were grouped into categories (review, bacteria, plants; did not apply method; commercial kit; method applied but not described; relevant publication describing sample preparation etc.).

protocol relying on the DNP-reaction [22]. Here, the authors oxidize BSA with H_2O_2/vanadyl and radiolysis and derivatize the samples with an equal volume of DNP for 1 h. SDS-PAGE is followed by blotting on nitrocellulose and detection with BCIP and NBT. The results show that the oxidation results in a linear increase in carbonyl concentration in the albumin molecule and the method is clearly more sensitive than the spectrophotometric assay.

Optimizing the immunoblotting method can be quite time-consuming. One must decide whether to perform pre- or post-electrophoresis derivatization and consider the different types of membranes. In terms of membrane choice, nitrocellulose membranes should only be used when derivatization is carried out before SDS-PAGE and immunoblotting because the membrane is not suitable for incubation in strong acids. The derivatization time is also crucial and varies significantly between laboratories (see Table 3). Concerning post-electrophoresis derivatization; Robinson et al. note that the membrane should be treated with DNP for exactly 5 min at RT [24], which is also our recommendation.

Both approaches, the pre- and post-electrophoresis derivatization have limitations. They both involve numerous washing steps making the approaches very time-consuming. The post-electrophoresis derivatization requires several pre-treatment steps (methanol, HCl) of the membrane. The pre-electrophoresis derivatization is not recommended for samples with a low protein concentration which will result in problems with loading the pockets and in separating/visualizing low-molecular weight proteins.

Because carbonyl groups are present in every molecule there are problems with the reproducibility and the analysis of the bands, furthermore the membranes often show a high background. The acidic derivatization affects the p*I* and hence complicates the identification of proteins in 2D PAGE.

An accurate determination of the carbonyl concentration is not possible with immunoblotting. Therefore the results should always be expressed in relation to appropriate controls/samples and theoretically, a control which has not been treated with DNP must be included in every assay.

Using ELISA to determine protein carbonyls

Buss et al. described the use of an anti-DNP antibody in an ELISA for the first time in 1997. Standards of HOCl-oxidized and sodium borohydride-reduced BSA are prepared for a standard curve. It is important to note that the blocking step is carried out with reduced BSA (original publication) and only with PBS in the original publication and in the erratum, respectively. The original publication contained errors concerning the preparation of reduced BSA [20], i.e. the ten-fold amount of $NaBH_4$ and the four-fold amount of BSA are described in the original publication.

Plasma from healthy and critically ill patients was diluted to 4 mg/ml and treated with 10 mM DNPH for 45 min at RT. The ratio of sample volume to DNPH volume was 1:4. Samples were coated onto MaxiSorp plate and detected with a biotinylated anti-DNP and streptavidin-biotinylated-HRP. The fully reduced BSA showed a carbonyl concentration of 0.6 nmol/mg, this concentration has also been stated by Shacter et al. [19]. There was a linear correlation ($r=0.70$) between the absorbance of the spectrophotometric assay (375 nm) and the ELISA for plasma samples ($n=26$). After subtracting the absorbance of the blank (reduced BSA), plasma protein carbonyl concentration of healthy controls was in the range of 0.06 nmol/mg whereas that of patients was around 0.75 nmol/mg.

A modification of the protocol described by Buss et al. is the method by Alamdari [25]. Here the samples are adsorbed onto the plate prior to derivatization. This is especially interesting for samples with low protein concentrations (5 μg/ml).

Of the immunological methods, ELISA methods allow the simultaneous quantification of a great number of samples and require only small sample volumes. They can be used for the measurement of carbonyl concentrations not only in plasma, but also in tissues and cell culture samples.

Limitations of this approach include that the determination of the protein concentration is mandatory before carrying out the assay and the assay takes 2 days because the samples are usually adsorbed to the plate overnight. In addition the in-between washing steps promote the risk for loss of sample. Many companies offer ELISA plates with different binding capacities for different requirements, e.g. for molecules with hydrophobic, hydrophilic or mixed domains.

The available different monoclonal as well as polyclonal antibodies also represent a problem since they all potentially react with different epitopes. However, when the carbonyl groups of a protein are chemically reduced, e.g. by borohydride, there is less binding of DNP and hence also less antibody binding resulting in low signal intensity [26] so antibody bias is of limited relevance.

As every laboratory uses its own standards accompanied by the lack of available uniform and accepted protein standards resulting

Table 3
Measurements of protein carbonyls by immunoblotting.

Species	Sample preparation	Derivatization Ratio (sample/DNP) Conditions	Gel, PAGE, membrane proteins amount, transfer/blot	Antibody	Level/result	Refs.
Human	Heparin plasma	10 mM DNPH (in 10% TCA) Nitrocellulose membrane	SDS-PAGE (4–12% gradient) (Southern Biotechnologies), and biotin–avidin-peroxidase complex (Vector Laboratories) Blotting by standard procedures	Monoclonal anti-DNP IgE (Sigma), biotinylated rat anti-mouse IgE	Method description, different oxidized proteins	[19]
Human	Serum	Post-electrophoresis derivatization 0.5 mM DNPH	10% SDS-PAGE Protein amount n.a. Nitrocellulose and PVDF membranes	Anti-DNP (Molecular Probes) Goat anti-rabbit HRP-conjugate (Sigma)	To compare pre- and post-electrophoresis derivatization	[47]
Human	Lysed SH-SY5Y cell supernatant	20 mM DNPH (in 2 M HCl) sample in 12% SDS (1:1) Ratio (1:1) For 45 min at 25 °C Precipitation of protein in chloroform/methanol	SDS-PAGE (10% and 15%) 30 μg soluble and 5 μg purified protein PVDF membrane	Polyclonal rabbit anti-DNP (Invitrogen) Anti-rabbit HRP-conjugate	Exposure of Sphingosine Kinase 1 to TNFα caused substantial carbonylation	[48]
Rabbit	Purified muscle actin, total protein of SH-SY5Y cell extracts, and actin immune-precipitated from SH-SY5Y cells centrifuged soluble protein from cell extracts	20 mM DNPH (in 2 M HCl) Sample in 12% SDS (1:1) (30 min, 25 °C) Ratio (1:1)	SDS-PAGE and 2D Nu-PAGE (4–12%) 30–50 μg soluble and 5 μg purified protein PVDF membrane	Polyclonal rabbit anti-DNP (Invitrogen) Secondary Ab n.a.	Considerable actin carbonylation in SH-SY5Y cells acutely exposed to TNFα or IL-1β	[49]
Human	Acute promyelocytic leukemia (APL)-derived NB-4 cells soluble protein fraction Sample in lysis buffer containing 6% SDS (pH 7.2)	10 mM DNPH Ratio (1:1, 15 μl:14 μl) For 10 min at RT	12% gel Nu-PAGE 25 μg protein Nitrocellulose membrane	Anti-DNP AP-conjugated anti-rabbit IgG	Treatment of NB4 cells with increasing concentrations (0.5–6 M) of As_2O_3 leads to increased protein carbonyls	[38]
Human	HeLa cell extract 1 μg/μl	10 mM DNPH Ratio (1:4) 30 min at RT	Dot-Blot microfiltration apparatus and TransBlot transfer membrane (Bio-Rad)	Anti-DNP (Dako) AP-conjugated anti-rabbit (Dako)	Slightly increased carbonylation in cells lacking glutaredoxin 1	[50]
Human	Alzheimer's disease brain homogenates	20 mM DNPH Ratio (1:4) 20 min at 25 °C	% n.a. SDS-PAGE 150 μg protein Nitrocellulose membrane Semi-dry transfer	Rabbit anti-DNP (Millipore) Goat anti-rabbit IgG (Sigma)	Different proteins carbonylated in AD on comparison to age-matched controls	[51]
Human	Bronchoalveolar lavage (BAL) fluid	OxyBlot kit (Chemicon Inc.)	10% SDS-PAGE	Anti-DNP	Amount of carbonylated albumin per mg total albumin in BAL fluid was four times higher in older current smokers and three times higher in older former smokers than in age matched non-smokers	[52]
Human	Colon cells (SW620)	Post-electrophoresis	5 μg protein/slot	Anti-DNP-KLH, rabbit IgG (H&L) fraction	Development of an immunochemical	[24]

Table 3 (*continued*)

Species	Sample preparation	Derivatization Ratio (sample/DNP) Conditions	Gel, PAGE, membrane proteins amount, transfer/blot	Antibody	Level/result	Refs.
		derivatization: 10 μg/ml DNPH (≙0.5 mM) in 2 M HCl min at RT		(Molecular Probes)	technique for the quantification of carbonyl groups in protein samples from small tissue samples and cell cultures	
			PVDF membrane	Peroxidase-conjugated $F(ab')_2$ fragment donkey anti-rabbit IgG (H&L) fraction (Jackson Immunoresearch Laboratories)		
			Slot-blot			
Mouse and human	C57BL mouse tissue and human fibroblasts	DNPH/TFA from OxyBlot Kit (Oncor) Ratio (1:1) 15 min at RT	% n.a. SDS-PAGE Protein amount n.a. Nitrocellulose and PVDF membrane Semi-dry blotting	From the OxyBlot Kit	Improve methods	[53]
Mouse	Cortical neuron homogenate and lysate 20 μg	n.a.	12.5% SDS-PAGE 20 μg Nitrocellulose membrane	Anti-DNP kit (Chemicon Inc.)	Relative to control	[40]
Mouse	Isolated collagen	0.5 mM DNPH	6% slab gel	Rabbit anti-DNP (Sigma)	Induced damage to collagen after treatment with pefloxacin	[54,55]
		Ratio (1:1) 1 h at RT	SDS-PAGE 50, 15 or 7 μg collagen PVDF membrane (Sigma) Trans-Blot apparatus	AP-conjugated goat anti-rabbit IgG (Sigma)		
Mouse	Mitochondria from Heat Shock factor 1 (Hsf1) knockout mice	10 mM DNPH	% n.a.	Rabbit anti-DNP (Sigma)	Higher extent of carbonylation in mitochondrial proteins of *Hsf1* knockout mice	[56]
		Ratio n.a. 60 min at RT	SDS-PAGE Protein amount n.a. PVDF membrane Mini trans-blot electrophoretic transfer cell (Bio-Rad)	HRP-labeled goat anti-rabbit IgG		
Rat	Serum	20 mM DNPH (in 10% TFA)	% n.a.	Goat anti-DNP (Bethyl Laboratories Inc.)	Changes in serum levels of total protein carbonylation correspond to cardio-protective activity	[57]
		Ratio (1:1) 10 min at RT	SDS-PAGE 5 μg protein PVDF membrane	Donkey anti-goat IRDye 800CW (Li-COR)		
Rat	Mitochondria from head homogenate (male, weanling Sprague-Dawley rats)	OxyBlot Kit	10% SDS-PAGE	Anti-DNP (OxyBlot Kit)	Comparison between rats with copper deficient diets (reduced carbonyls in copper deficiency)	[58]
			6 μg protein PVDF membrane Semi-dry transfer	HRP-coupled anti-sheep IgG (Amersham)		
Rat	Ovary tissue sections	10 mM DNPH (in 10% TFA)	12% SDS-PAGE	Rabbit anti-DNP	Increased protein carbonyls were detected in ovaries of rats exposed to tetrachlorethylene water for 2 weeks compared to controls	[59]
		Ratio n.a. 45 min	25 μg protein PVDF membrane Tank blot	Biotinylated anti-rabbit (Vectastain ABC-AP kit)		
Rat and cow	Histones from PC12 cells (rat) and thymus, liver, spleen (bovine)	10 mM DNPH (in 10% TFA)	% n.a.	Anti-DNP (Dako)	Higher carbonylation in untreated histone H1 in comparison to core histone	[60]
		Ratio (1:1)	SDS-PAGE	Goat anti-rabbit conjugated with HRP (Jackson Immunoresearch Laboratories)		
		20 min at RT	5 μg protein Nitrocellulose membrane			

Cow	Oxidized BSA	0.5 mM DNPH Ratio (1:1) 1 h at RT	Semi-dry blot % n.a. SDS-PAGE Protein amount n.a. Nitrocellulose membrane	Anti-DNP antisera (Dako) Mouse anti-rabbit IgG conjugated with AP (Jackson Immunoresearch Laboratories)	Development of an immunochemical assay	[22]
E. coli	Glutamine synthetase	20 mM DNPH and 0.5% TFA in 92.5% DMSO Ratio n.a. 15 min at RT	Dot-blot and Western blot (14% and 8–16%) PVDF membrane	Goat anti-DNP (Bethyl Laboratories Inc.) Donkey anti-goat IRDye 800CW (Li-COR)	Comparison between dot blot and Western blot	[61]

in insufficient comparability must be mentioned.

Discussion and conclusions

The described assays require no specific equipment and can be adapted to actual laboratory facilities and any methods which are applicable in the respective laboratory can be used.

As mentioned above, each method has its limitations.

Besides these, there are some other facts that must be considered. As shown in Tables 2–4, the applied protocols differ considerably in important details such as the protein concentration, ratio of sample volume to DNP volume, DNP concentration and incubation duration, temperature and conditions.

The biggest difference between these various assays lies in the derivatization step. The DNPH concentration for the spectrophotometric assay is often used according to Levine, i.e. 10 mM, equal to 0.2%, but 20 mM has also been used. Two groups state excessively high DNP derivatization concentrations [27,28]. For ELISA 10 mM seems to be the most practical concentration, while for immunoblotting 10 and 20 mM are most frequently used. For the case that the derivatization is carried out after SDS-PAGE or adsorption of samples onto ELISA plate the DNP concentration is significantly lower: 0.5 and 0.05 mM DNPH, respectively. Besides the concentration, the ratio of sample volume to DNP volume is not consistent as it varies between equal volumes, 1:3, 1:4, 1:5 and 1:6. However, often this information is not stated at all. Additionally the protein concentration or dilution of the sample is often not stated, nevertheless this should be considered as the protein concentration and DNP concentration should be adjusted/correspond.

Furthermore the influence of the incubation temperature should be considered. While some authors do not state any temperature, most authors keep their samples at RT (or close to RT: 20 °C, 25 °C) but 37 °C is also frequently used, perhaps because it is easier to keep this temperature constant. The next critical point is the duration of incubation: Levine suggests incubating the sample for 1 h. However, other authors apply incubation times of as short as 5 min. For ELISA, most authors incubate their samples between 45 and 60 min. This seem to be relatively comparable, however samples for the spectrophotometric assay are incubated between 15 and 60 min, and those for immunoblotting even differ between 5 and 60 min. We suggest incubating the samples for ELISA and spectrophotometric assay for 45–60 min and mixing the samples every 15 min (or applying constant agitation) to help minimize differences in protocols.

The results, even if theoretically measured by the same protocol, are difficult to compare, as the levels, shown in Tables 2–4, are often not given in the same concentration.

The immunoblotting method does not of course give quantitative data (i.e. absolute concentrations). The results of the spectrophotometric method, which could easily be given in as mol/mg (depending on the concentration of a native or treated sample) are frequently displayed in different units, e.g. mol/mol protein, nmol/mg, μmol/mg or μmol/10^3 cells. Some authors only present their results expressed as fold-increase or percentage to controls. The units for ELISA results are given as nmol/mg, pmol/mg, nmol, nmol/ml, nmol/g, nmol carbonyl groups fibrinogen/mg of plasma proteins or carbonyl residues/HSA molecule. Some authors state their results as arbitrary units, relation to control, and relative amount. This makes it especially difficult to compare results and is also odd since the original protocol uses the unit pmol/mg and this should be easily achieved by diluting all samples to the same protein concentration and preparing standards of known carbonyl concentrations.

The protein carbonyl concentration of a given sample reflects a

Table 4
Determination of protein carbonyls by ELISA.

Species	Sample preparation	Derivatization	Antibody	Plate producer	Level*	Ref
Human	Plasma from healthy and critically ill patientsdiluted to 4 mg/ml	10 mM DNPH (in 6 M guanidine HCl, 0.5 M potassium phosphate buffer, pH 2.5) Ratio sample/DNPH (1:4) 45 min at RT	Biotinylated anti-DNP (Molecular Probes) Streptavidin-biotinylated-HRP (Amersham)	MaxiSorp (Nunc)	0.06–0.75 nmol/mg	[20,62]
Human	Plasma from sepsis patients vs controls Dilution n.a.	n.a.	Biotin-conjugated polyclonal anti-DNP IgG Streptavidin-conjugated HRP	n.a.	0.32–0.45 nmol/mg	[63]
Human	Citrate-plasma from schizophrenia patients and healthy volunteers Dilution n.a	n.a.	n.a.	n.a.	0.178–0.482 nmol/mg	[64]
Human	Heparin and EDTA plasma and urine Dilution n.a.	n.a.	n.a.	n.a.	Significantly higher concentration in blood of subway workers and bus drivers than in office workers (17 and 18 vs. 15 nmol/ml)	[65]
Human	Plasma from 71 hepatocellular carcinoma patients and 694 controls Dilution n.a.	n.a.	n.a.	n.a.	0.11–1.41 nmol/mg	[66]
Human	Plasma of chronic obstructive pulmonary disease (COPD) patients 4 mg/ml	n.a.	Polyclonal rabbit anti-DNP (Molecular Probes) HRP-conjugate (Amersham)	MaxiSorp (Nunc)	17.9 ± 2.9 nmol/mg	[67]
Human	Plasma from participants of the New York Early Lung Cancer Action Project 4 mg/ml	n.a.	Polyclonal rabbit anti-DNP (Molecular Probes) HRP-conjugate (Amersham)	n.a.	~17 nmol/ml	[68]
Human	Plasma 4 mg/ml	10 mM DNPH (in 6 M guanidine–HCl, 0.5 M potassium phosphate, pH 2.5) Ratio and conditions n.a.	Biotinylated anti-DNP (Molecular Probes) Streptavidin–biotin HRP (Amersham)	ELISA (Corning Costar)	~11–15.3 nmol/mg	[69]
Human	Serum from middle-aged obese subjects 4 mg/ml	Concentration and conditions n.a. Ratio (1:4)	n.a.	n.a.	0.12 arb. u.	[70]
Human	Heparin plasma 5 μg/ml	0.05 mM DNPH (in H_3PO_4, pH 6.2) 45 min at RT in the dark *NOTE*: derivatization after samples have adsorbed to the plate!	Anti-DNP (Sigma) Anti-rabbit HRP-linked IgG (H&L, Upstate Cell Signaling)	n.a.	Method for measuring protein carbonyl in samples with low amounts of protein	[25]
Human	Plasma and LDL Dilution n.a.	n.a.	Anti-DNP (Dako) Goat anti-rabbit IgG peroxidase conjugate (Sigma)	n.a.	Modification of method	[71]
Human	Plasma Dilution n.a.	n.a.	n.a.	n.a.	0.1–0 5 nmol carbonyl groups fibrinogen/mg of plasma proteins	[72]
Human	Plasma from women with preeclampsia and controls Dilution n.a.	45 min at RT	Rabbit anti-DNP-KLH (Invitrogen) HRP-conjugated porcine anti-rabbit IgG (Dako A/S)	n.a.	Significantly higher concentration in cases than in controls	[73]
Human	Plasma 4 mg/ml	n.a.	Biotinylated anti-DNP (Molecular Probes) Streptavidin-biotinylated HRP-conjugate (Amersham)	n.a.	< 11.5 nmol/ml up to > 19.2 nmol/ml	[74]
Human	K562 cell lysates (human chronic myelogenous leukemia)	n.a.	Rabbit anti-DNP IgG antiserum (Sigma)	n.a.	Relation to control	[75]

	1 mg/ml in lysis buffer with 1 mM BHT		Monoclonal anti-rabbit peroxidase-conjugated IgG (Sigma)			
Human	Parenchymal lung tissue of current smokers with chronic obstructive pulmonary disease Dilution n.a.	n.a.	Plate was pre-incubated with mouse anti-HSA before addition of samples rabbit anti-DNP Anti-rabbit HRP-conjugate	n.a.	0.5–5 carbonyl residues/HSA molecule	[76]
Human	MRC-5-fibroblast cell lysates (fetal lung) 4 mg/ml	n.a.	Anti-DNP rabbit-IgG-antiserum (Sigma) Monoclonal anti-rabbit-IgG-peroxidase conjugated (Sigma)	n.a.	~1.5–3.2 nmol/mg	[77]
Mouse	Brain, liver, heart and spleen homogenates from 6-week-old male DDY mice 1 mg/ml	10 mM DNPH (in 2 M HCl) For 1 h at RT Ratio n.a.	n.a.	n.a.	Relation to control	[78]
Mouse	Heart tissue homogenate of SAMP8 mice Dilution n.a.	n.a.	Rabbit anti-DNP IgG-antiserum (Sigma Aldrich) Monoclonal anti-rabbit IgG peroxidase conjugate (Sigma Aldrich)	n.a.	664 ± 37 nmol/g (high-polyphenol diet) and 958 ± 70 (low-polyphenol diet) nmol/g	[79]
Mouse	Mesenchymal stem cells derived from adipose tissue of C57/Black6 mice Dilution n.a.	n.a.	n.a.	n.a.	4–32 Carbonyls/mg protein	[80]
Mouse	Brain homogenate from ApoD-knockout mice Dilution n.a.	n.a.	Biotinylated anti-DNP (Molecular Probes) Streptavidin-biotinylated HRP (Amersham)	n.a.	Relation to control	[81]
Mouse	Isolated mitochondria from livers of Bcs1lG/G mice Dilution n.a.	Concentration and ratio n.a. 45 min at RT	Anti-DNP (Invitrogen) Swine anti-rabbit IgG-HRP (Dako A/S)	n.a.	1.09 ± 0.36 relative amount	[82]
Mouse	Retinal pigmented epithelial cell lysates 4 mg/ml	10 mM DNPH (in 6 M guanidine–HCl, 0.5 M potassium phosphate, pH 2.5) Ratio (1:4) 45 min at RT	Polyclonal rabbit anti-DNP (Molecular Probes) HRP-conjugate (Amersham)	MaxiSorp (Nunc)	0.6–1.2 nmol	[83]
Mouse	RAW264.7 murine macrophage-like cells Dilution n.a.	Kit from Cell Biolabs	Kit from Cell Biolabs	Kit from Cell Biolabs	Results not significant	[84]
Mouse	HT22 cell lysates4 mg/ml in lysis buffer with 1 mM BHT	n.a.	Rabbit anti-DNP IgG antiserum (Sigma) Monoclonal peroxidase-conjugated anti-rabbit IgG (Sigma)	n.a.	6.5–9.0 pmol/mg	[85]
Rat	Ileal mucosa of salmonella infected rats 4 mg/ml	n.a.	Biotinylated anti-DNP (Molecular Probes), streptavidin-biotinylated HRP (Amersham)	MaxiSorp (Nunc)	0.1–0.2 nmol/mg	[86]
n.a.	Ferritin Dilution n.a.	(10 mM in 6 M guanidine HCl, 0.5 M potassium phosphate, pH 2.5) Ratio (1:3) 45 min at RT	Biotinylated anti-DNP Anti-rabbit-IgG-peroxidase (γ-chain specific)	MaxiSorp(Nunc)	0.55–1.0 nmol/mg	[87]

snapshot of generated and removed carbonyls. Protein carbonyls are not only a result of amino acid oxidation but can also arise from secondary reactions by nucleophilic addition of aldehydes or reducing sugars and their oxidation products. Scientists must evaluate whether they are interested in the oxidation of a specific cellular or circulating protein or want to compare the carbonyl content in blood of many samples, e.g. in clinical studies.

Theoretically, due to steric reasons the DNP molecule should not be able to attack all carbonyl moieties in a similar way. However, the derivatization of carbonyl compounds with DNP has been used since the late 1950s [29]. We are not aware of any publication studying the differences in terms of yield and rate of the reaction between DNP and different carbonyl moieties.

The so-called OxyBlot is semi-quantitative but has shown the best sensitivity and specificity [30], and is especially applicable in cell culture studies. On the other hand ELISA seems to be best-suited for clinical studies and the inclusion of external standards allows standardization, better comparison, as well as high-throughput. Nevertheless this decision depends on the laboratory equipment and research question.

MS-based methods are inevitable when the aim is to identify specific carbonylated residues of single proteins. However, not every laboratory is able to apply MS and for some research questions it is also not practicable, e.g. in clinical/epidemiological studies where high-throughput of complex protein mixtures such as plasma is necessary. In these cases ELISA is still the method of choice. For further information on MS-based techniques to identify and quantify oxidative protein modifications on proteins and peptides see Rogowska-Wrzesinska et al. [23].

Conclusion

When immunoblotting is applied, the results cannot be expressed in a certain concentration since the method is semi-quantitative. In this case appropriate controls must be included (such as untreated cells, etc.). Every researcher should act according to good laboratory practice and include appropriate controls.

When using the spectrophotometric assay or ELISA, most authors present their results in pmol/mg. However, as you can see in Tables 2 and 4, some authors only express their results in relation to controls or do not take the protein concentration into consideration. In our opinion, it is inevitable to measure the protein concentration when assessing protein-bound carbonyls since the protein concentration is the measure which best corresponds to the number of modifiable residues. Since carbonyl groups on proteins can arise from a multitude of chemical, thermal and radiation processes it is impossible to link carbonyl groups to a specific mechanism. It has been demonstrated that the carbonyl groups increased linearly in samples treated with iron/ascorbate [19]. Hence, when measured in complex mixtures such as plasma, protein carbonyls serve as a biomarker of global protein oxidation.

In a multi-center ring study by Augustyniak et al. [31], six European laboratories were invited to measure protein carbonyls according to their own protocol in homogenized liver samples (UV-radiated and not radiated). Four laboratories used ELISA, while three used immunoblotting techniques. Unexpectedly, the concentrations measured by ELISA were quite similar and results from immunoblotting were also homogenous, indicating that ELISA techniques (self-prepared standards, quantification of standards by spectrophotometry; commercial kits) represent the best available method to quantify protein carbonyl concentration, whereas immunoblotting allows the comparable detection of the molecular weight of oxidized proteins.

The ring study relies on a review by Rogowska-Wrzesinska et al. [23] which covers advantages and pitfalls of the most commonly used methods and examines commercially available oxidized proteins, oxidants used and lysis buffers. This review, together with the previous one will help researchers to make a decision which method to use and may lead to a consensus on common protocols, hence allowing better comparability of results.

Therefore, we are convinced that also in future the detection of protein carbonyls will be a valuable tool in the detection of oxidative damage, although further improvements in sample stabilization, method robustness and standard design have to be performed.

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