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REVIEW ARTICLE

## Analysis of protein carbonylation — pitfalls and promise in commonly used methods

A. Rogowska-Wrzesinska<sup>1</sup>, K. Wojdyla<sup>1</sup>, O. Nedić<sup>2</sup>, C. P. Baron<sup>3</sup> & H. R. Griffiths<sup>4</sup>

<sup>1</sup>Institute of Biochemistry and Molecular Biology, University of Southern Denmark, Odense, Denmark, <sup>2</sup>Institute for the Application of Nuclear Energy, University of Belgrade, Belgrade, Serbia, <sup>3</sup>National Food Institute, Technical University of Denmark, Kgs. Lyngby, Denmark, and <sup>4</sup>School of Life and Health Sciences, Aston University, Birmingham, UK

### Abstract

Oxidation of proteins has received a lot of attention in the last decades due to the fact that they have been shown to accumulate and to be implicated in the progression and the pathophysiology of several diseases such as Alzheimer, coronary heart diseases, etc. This has also resulted in the fact that research scientists are becoming more eager to be able to measure accurately the level of oxidized protein in biological materials, and to determine the precise site of the oxidative attack on the protein, in order to get insights into the molecular mechanisms involved in the progression of diseases. Several methods for measuring protein carbonylation have been implemented in different laboratories around the world. However, to date no methods prevail as the most accurate, reliable, and robust. The present paper aims at giving an overview of the common methods used to determine protein carbonylation in biological material as well as to highlight the limitations and the potential. The ultimate goal is to give quick tips for a rapid decision making when a method has to be selected and taking into consideration the advantage and drawback of the methods.

**Keywords:** carbonylation, immunoaffinity, derivatization, mass spectrometry, standardization

### Nature of carbonylation and oxidizing species

Protein oxidation occurs normally in living organisms. The effects can be both beneficial and harmful. The primary free radical formed in most physiological systems is superoxide anion radical ( $O_2^-$ ) which is in equilibrium with its protonated form, hydroperoxyl radical ( $HO_2$ ) [1].  $O_2^-$  is less potent in protein oxidation than other free radicals and reactive oxygen species (ROS). It undergoes spontaneous dismutation, a process catalyzed by superoxide dismutase, to form non-radical ROS, hydrogen peroxide [2]. Hydrogen peroxide may undergo degradation by catalase or conversion into more reactive radicals.

The major intracellular source of free radicals is leakage from electron transport chains of mitochondria [3]. Certain amounts are produced from other cellular systems, such as peroxisomes [4] and macrophages [5]. ROS can also be generated through the activity of specific enzymes, such as oxidases or tyrosine hydrolase [6,7]. The rate of protein oxidation depends on the formation of ROS capable of modifying biological molecules. In general, increased levels of oxidized proteins are associated with ageing, oxidative stress (hyperoxia, extreme exercise, exposure to UV, X- or  $\gamma$ -radiation, or environmental pollutants) or certain pathologies (Alzheimer's disease, Parkinson's disease, rheumatoid arthritis, atherosclerosis, diabetes) [8–10].

The intracellular levels of ROS are tightly controlled by scavengers and enzymes. These are responsible for

maintaining the balance between ROS production and removal. The enzymes such as superoxide dismutase and catalase remove elevated levels of ROS directly. Metal-binding proteins, such as transferrin, ferritin, lactoferrin, and ceruloplasmin are sinks for ROS formed *in situ* on the protein backbone catalyzed by redox active metal ions [2]. The level of ROS is also dependent on the concentration of vitamins (C, A, and E) [11] and certain metabolites (uric acid, bilirubin) which either directly capture free radicals or assist in the regeneration of metabolites capable to do so [12].

Metal ion-chelator complexes can act both as promoters and suppressors of ROS formation – such complexes may inhibit the ability of metal ions to catalyze ROS formation or their redox potentials can be altered influencing their ability to undergo cyclic conversion between oxidized and reduced states [13]. Finally, cations other than iron ( $Fe^{2+}$ ) and copper ( $Cu^+$ ), such as magnesium ( $Mg^{2+}$ ), manganese ( $Mn^{2+}$ ), and zinc ( $Zn^{2+}$ ) may compete for metal-binding sites on proteins, preventing local formation of free radicals on the protein backbone [2].

Oxidation may induce both structural and functional alterations to proteins. ROS can cause oxidation of amino acid side chains and/or polypeptide backbone. Oxidation of the polypeptide backbone results in formation of carbon-centered radical ( $RC\cdot$ ) which may either react with  $O_2$  initiating a chain reaction, including different oxygen-containing free radical intermediates, or (in the absence of oxygen) it may interact with another carbon-centered

radical causing protein cross-links. Transformation of protein alkoxyl radicals may lead to protein fragmentation by diamide or  $\alpha$ -amidation pathways [2]. Polypeptide bond cleavage can occur by other mechanisms as well, the common feature is modification of amino acid residues by ROS [14].

Protein carbonylation is the most frequent irreversible transformation and also the one most often studied [15]. Metal-catalyzed ROS attack on the amino acid side chains of proline, arginine, lysine, and threonine induces formation of carbonyl groups. Carbonylation of lysine, cysteine, and histidine may be caused by their reaction with carbohydrates and lipids having reactive carbonyl groups, produced during glycoxidation (advanced glycation end products, AGE) and lipoxidation (advanced lipid peroxidation end products, ALE). Carbonyl derivatives can also be generated through  $\alpha$ -amidation pathway.

Free radicals and other ROS are highly reactive and short-living species. Modified proteins, on the other hand are more stable and remain longer in a living system. Besides factors that primarily regulate the amount of ROS, the accumulation of oxidized proteins depends on the rate of their clearance. Degradation of modified proteins is influenced by the amount and the activity of specific proteases and the extent of modification. Mildly oxidized proteins are susceptible to degradation, whereas extremely oxidized (carbonylated) proteins form cross-links and aggregates that are poor substrates for proteolysis [16]. Such aggregates may become toxic and they are associated with numerous disorders, such as aging, diabetes mellitus, Alzheimer's disease [10]. ROS-altered proteins may promote autoimmune protein complexes in response to generation of new antigenic epitopes [17].

Determination of physiological concentrations, preferably circulating levels, of the oxidized proteins or their derivatives may serve in assessing the exposure of an organism to oxidizing species and its capacity to overcome the burden. The increase in protein carbonyl content seems to be the most general indicator of protein oxidation [18].

### **Critical appraisal of existing methodology to measure protein carbonylation**

This review takes a step-by-step guide through the analytical processes required for precise and accurate determination of the most frequently used quantitative measure of protein oxidation — carbonyl formation.

We cover published methods, which require a range of equipment from the simplest spectrophotometric analysis to liquid chromatography (LC) and mass spectrometry (MS). The present critical appraisal of existing methodology is intended to improve the quality of data and therefore conclusions arising from protein carbonylation analysis. The overall objective is to provide recommendations for anyone undertaking the most common analyses to avoid the pitfalls. We will consider: 1) Challenges in the analysis of protein carbonylation in general (complexity issue); 2) Limited number of standard materials and

methods; 3) Challenges in sample preparation — from simple to complex biological mixture; 4) Challenges in detection of carbonylated proteins/peptides with currently available methods and technologies.

### *Sample preparation for the analysis of protein carbonylation*

Regardless of the source of material (tissue, cells, or body fluids) biological oxidation events must be preserved and artifactual events minimized during sample preparation. In this section we have addressed issues worth considering prior to any study aiming to determine protein carbonylation levels in biological samples.

Even though the focus of the methods reviewed here are proteins, it is of outmost importance to bear in mind that cells and biological fluids contain a number of other molecules, which might become oxidized. Their presence in a protein extract may cause high background signal, increase sample complexity, and interfere with analysis procedures. Nucleic acids are known to accumulate carbonyl groups and can therefore interfere with some methods of carbonyl detection. Mild extraction strategies may be applied to minimize disruption of nuclei and mitochondria and leakage of nucleic acids. This can be achieved by using hypotonic lysis buffers and avoiding strong detergents and sonication [19].

Reduced carbohydrates may also contain carbonyl groups that can potentially interfere with the protein carbonylation measurements. It is possible to clean protein extracts by selective removal of carbohydrates, e.g., by lectin affinity or by the use of protein specific extraction methods like TCA precipitation following PNGase F treatment [20]. Carbohydrates and lipids are also targets for ROS and may undergo oxidative modifications at an equal rate to proteins. Due to high reactivity oxidation products of carbohydrates or lipids often create hybrid complexes with oxidized proteins — AGEs and ALEs (reviewed in [21]). All of them may interfere with and complicate analysis of oxidized proteins.

Not only may the biological components of cells and body fluids influence the outcome of the measurements of protein oxidation levels, several components of commonly used buffers for cell disruption and protein solubilization may interfere with the analysis or significantly affect the obtained results. Table I presents some of the components of these reagents that may influence the total yield and stability of protein oxidation products.

Common chemical components of protein extraction buffers are mild reducing agents such as dithiothreitol (DTT) and  $\beta$ -mercaptoethanol (recommended for sample preparation in the Carbonyl Western Blot kit) but these also interfere with the protein oxidation measurements. During the protein extraction procedure they may reduce some of the protein oxidation products, for example, disulfides, cysteine sulfenic acids [22], or carbonyl groups [23] to the corresponding alcohols, making the modifications unavailable for detection. Paradoxically, they also may have pro-oxidative capacity in the presence of atmospheric

Table I. Lysis buffer components potentially affecting oxidation status of the sample.

Reagent	Primary role	Undesirable effect	Typical concentration	Comments
Buffer	pH stabilization	Buffers containing primary amines (e.g., tris(hydroxymethyl) aminomethane, ammonium bicarbonate) may react with carbonyls	10–100 mM	n/a
Salts (e.g., NaCl)	Control of osmolarity	Problems with gel-based separation	< 140 mM <sup>a</sup>	n/a
Protease inhibitors	Minimize endogenous proteolytic degradation	n/a	n/a	Available with and without EDTA
Urea	Denature and solubilize proteins	Interference with protein concentration determination methods and protein digestion with trypsin	6–8 M	n/a
Detergents	Denature and solubilize proteins	Contamination of protein extracts with nucleic acids from disrupted nuclei and mitochondria. Interference with protein concentration determination methods	Detergent-dependent	n/a
EDTA	Metal ion chelation	n/a	1–5 mM	EGTA and DTPA are alternatives to EDTA
DTT, β-mercaptoethanol	i. Reduction of disulfide bonds, protein unfolding ii. Prevention of protein oxidation <i>in vitro</i>	Introduction of carbonylation via metal-catalyzed protein oxidation [19]	50–100 mM	n/a
Streptomycin sulfate, DNase, RNase	Nucleic acid removal	n/a	Nucleic acid content dependent	Important for spectrophotometric assays, not necessary for gel-based strategies
Guanidine	Prevents aggregation and precipitation of heavily oxidized proteins	n/a	3–6 M	n/a

n/a – not available.

<sup>a</sup>140 mM is a physiological salt concentration.

oxygen and free metal ions [19,24]. Therefore it is recommended to use them with caution and always accompanied by metal ion chelators such as ethylenediaminetetraacetic acid (EDTA) to avoid artifactual oxidation.

In order to measure protein carbonyls, methods involving different derivatization reagents have been developed (for details please see sections below). Due to the high reactivity and transient nature of carbonyl group, derivatization should be performed at the earliest possible stage of sample preparation, either directly during lysis or immediately after protein extraction. This is to ensure that all the existing modifications are captured and stabilized and that new modifications, introduced during further steps of sample preparation, are not contributing to the measured values. Limiting the number of steps in sample preparation lowers the chance of artifactual oxidation.

For those analytical methods, which require free amino acids for identification of oxidative modification, peptide bond cleavage via enzymes or acid is necessary. Both enzymatic and acidic hydrolysis has certain disadvantages. For enzymatic digestion, there will be contamination of sample with degraded enzyme and the recommended proteolysis time is minimum 6 h at 37°C, which increases

the risk of further sample oxidation in oxygenated buffers. Hydrolysis can be carried out before or after derivatization with modification specific reagents. In both cases, care needs to be taken, by using tags that do not interfere with hydrolysis or making sure that the modified amino acid is not changed during hydrolysis.

### Quality control and the importance of standardization of methods

Standardization of laboratory measurements is of high priority in laboratory analysis, aiming to achieve close comparability of results over time and space. Two major components of the standardization procedure are reference materials and reference methods [25].

The reference material should be a well-characterized material that is used as a calibrator for a measurement or as a control to check authenticity of the result [26]. The reference material has a true value (e.g., concentration) and it has to be widely adopted by laboratories involved in analytical testing. A standard or reference analytical method is the way to detect and/or quantify specific

analyte in a specific sample. Reference methods are approved by international agencies or interconnected network of laboratories. The common goal is to obtain consistent results. Sample collection and preparation procedures as well as procedures to remove interfering substances are defined. Each method is characterized by analytical parameters such as sensitivity, precision, reproducibility, measurement interval, possible cross reactivity with related analytes that cannot be removed prior to analysis.

In practice, calibration based on reference materials and reference methods may be problematic even for very simple analytes. Basically, only methods for determination of simple and small analytes can be reliably standardized. This is because these are mostly robust physicochemical tests. Standardization of methods for determination of complex and large analytes is a challenge, especially if they are in physiological fluids or cell/tissue samples. Analytes such as specific proteins or modifications are often measured by immunochemical methods. Immunochemical reactions, as other reactions based on conformational recognition and affinity - binding, are not based on the clear stoichiometric relation between reactants.

In the case when there are no reference materials, manufacturers of *in vitro* diagnostic tests prepare their own calibrators and standards [27]. They make their own choice of primary substance(s) and methods used for assigning the value to a calibrator/standard. In the field of protein carbonylation there are no reference materials except for glycated hemoglobin, no calibrators or primary standards that are worldwide professionally recognized as such, and no reference method(s). There are, however, commercial

preparations of some oxidized proteins and there are number of companies that produce diagnostic kits for the measurement of some oxidized proteins.

Commercially sourced albumin is already carbonylated and to generate an appropriate range of standards, is reduced using borohydride as detailed by Buss (note that borohydride concentration should be 10 - fold lower than that originally described by [28]). Reduced albumin is mixed with different amounts of oxidized albumin to create a range of carbonyls for which actual carbonyl content is determined using the spectrophotometric method. Although at first glance this may be perceived as a poor approach to prepare a standard curve where the proportion of carbonylated protein is varied rather than the extent of oxidation on each molecule, the evidence that some plasma proteins are oxidized more than others in an apparently stochastic pattern is consistent with this approach. However, a better approach to consider for future development of standards is to vary the time of oxidation to create standards comprising increased level of oxidation in all proteins rather than increased proportion of heavily oxidized proteins.

An overview of commercially available oxidized proteins is given in Table II. Some products are partially characterized and information is given in data sheets. Available data offered to customers are included in Table II. As it can be seen, data supplied by producers are limited, and of the diverse type. Majority of post-translationally modified proteins are produced by "in house" method. Even in the case when a degree of modification is noted, it is not precise (e.g., 1 - 5 mol hexose per 1 mol of albumin or 5000 - 10 000% increase in fluorescence compared to unmodified protein). In some cases proteins

Table II. An overview of commercially available oxidized proteins.

Oxidized protein	Catalog no.	Available data	Producer
Glycated bovine serum albumin	A8426	1-5 mol hexose (as fructosamine)/mol albumin	Sigma-Aldrich
Glycated human serum albumin	A8301	1-5 mol hexose (as fructosamine)/mol albumin	Sigma-Aldrich
Glycated human hemoglobin	IRMM IFCC466 <sup>a</sup>	n/a	Sigma-Aldrich (Fluka)
ProteoProfile™ PTM marker	P1745	A mixture of phosphorylated and glycosylated ovalbumin, β-casein, RNase B and unmodified BSA	Sigma-Aldrich
Advanced glycation end-product bovine serum albumin	121800	Prepared by reacting BSA with glycoaldehyde; 5000 - 10 000% increase in fluorescence as compared to normal BSA	Merck KGaAEMD Chemicals (Calbiochem)
Carboxymethyl-lysine bovine serum albumin	STA-314	CML-BSA immunoblot control for OxiSelect™ CML Immunoblot kit (STA-313)	Cell Biolabs Inc.
Carbonylated bovine serum albumin	STA-309	Oxidized protein immunoblot control; detection limit in ELISA 10 µg/ml	Cell Biolabs Inc.
Glycated bovine serum albumin	2221-10	Prepared by reacting BSA with glycoaldehyde; 7000% increase in fluorescence as compared to normal BSA	Division Inc.
Synthetic glycated human serum albumin	SGA	Prepared by reacting HSA with glucose; 0.3 - 1.5 glyco-groups/mol albumin	Exocell Inc.
Glycated human hemoglobin	glyHb	Prepared from lysed human blood cells by affinity chromatography; the concentration varies with lot	Exocell Inc.
Carboxymethyl-lysine bovine serum albumin	3OP-CML-BS102	n/a	Academy Bio-medical Co.

n/a - not available.

<sup>a</sup>Certified reference material.

are modified in more than one way (e.g., glycosylated and phosphorylated). Taken together, there is a definite need for “true” standards (reference materials, calibrators) that would be precisely and stably (without lot to lot variation) characterized in respect to manufacturing and testing procedures, type of modification, degree and, whenever possible, position of modification, degree of uncertainty in modification (e.g., possible related alterations on secondary residues), application, sensitivity in different assays and stability.

Going through scientific literature it becomes evident that majority of the researchers do not use commercial oxidized proteins or tests, but produce their own modified proteins and assay systems. Oxidized bovine, and to a lesser extent human serum albumin are most often employed as standards, preferentially in the form of carbonyl derivatives and AGEs.

Different laboratories prepare their standards using a variety of transforming agents and chemical protocols. Data on how some serum albumin standards are prepared is given in Table III. Only procedures that induce carbonyl modification via primary interaction of serum albumin and ROS are included. The list would be significantly expanded if secondary reactions were also taken into account (modification via interaction with pre-formed reactive carbonyl species). Data in Table III are sufficient to illustrate the variety of protocols used to prepare standards. Therefore standards have different characteristics, which may lead to different interpretation of experimental results. The standards have not been characterized by protein mass spectrometry, which would be a preferred method to identify and quantify the specific types and sites of modifications.

### Analytical approaches to identification and quantitation of carbonylated proteins

Detection and quantitation of protein modifications can be done on different levels. For example, protein

carbonylation can be detected and quantified at the global level in proteins and protein mixtures using derivatization of carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH) followed by spectrophotometric measurements or immunodetection with DNPH-specific antibodies either in gels or in ELISA assay (Figure 1). However, these methods determine only the global level of carbonylation and do not identify which proteins are modified, what type of modification is dominant and which amino acids in the protein are modified. A more detailed analysis of protein modifications can be achieved using proteomics and mass spectrometry approaches. Separation and quantitation of protein modifications can be done by two dimensional gel electrophoresis (2DE) combined with specific detection methods (discussed in more detail in section below). Unfortunately identification of modification sites from proteins separated by 2DE is very difficult due to a low amount of protein isolated and a cross linking effect to polyacrylamide gel matrix. Until now only one study has reported being successful in identifying carbonylated residue from a 2DE spot [29]. Therefore to be able to efficiently identify carbonylation sites in proteins we need to use specifically dedicated proteomics approaches and nanoLC combined with high sensitivity tandem mass spectrometry (MSMS), which are described in further sections.

The first methods for measurement of carbonyl content in biological samples have been developed in the early 1970s. These methods are still applied in many research laboratories today because of their simplicity and low cost. In this section, three of these classical methods will shortly be described and in the later sections newer methods involving 2DE and mass spectrometry will be described.

### DNPH-based spectrophotometric method

The most widely applied method for protein carbonyl determination was established by Fields and Dixon in 1971 [30]. It uses DNPH, also called Brady’s reagent that

Table III. Transforming agents and experimental conditions for the “in house” preparation of carbonylated standards.

Transforming agent 1(M)	Transforming agent 2 (M)	Temperature [°C]	Reaction time	References
Ascorbic acid (25 μM or 25 mM)	FeCl <sub>3</sub> (100 nM or 100 μM)	37°C	2–24 h	[46,124,125]
Ascorbic acid (6 mM or 25 mM)	FeCl <sub>2</sub> or FeSO <sub>4</sub> (24 μM or 100 mM)	RT	1.5–2 h	[28,41,62]
H <sub>2</sub> O <sub>2</sub> (1 mM)	FeSO <sub>4</sub> or CuSO <sub>4</sub> (1 mM)	RT or 37°C	10 min–1.5 h	[40,64]
HOCl (0.3–10 mM)	n/a	37°C	15–24 h	[28,64,126,127]
HOBBr (10 mM)	n/a	37°C	up to 24 h	[127]
2,2’-azobis(2-amidinopropane) HCl (5, 20 mM or 0.5 M)	n/a	37°C	6–24 h	[64,127–129]
Radiolysis (5–1000 Gy, <sup>60</sup> Co or <sup>137</sup> Cs source)	n/a	4–55°C	up to 30 min	[64,127,130–133]
Light illumination (VIS light/345 nm cut off filter or fluorescent light)	n/a	4°C	up to 60 min	[127,131,134,135]

M, molar concentration; Temp, temperature; n/a, not applicable.

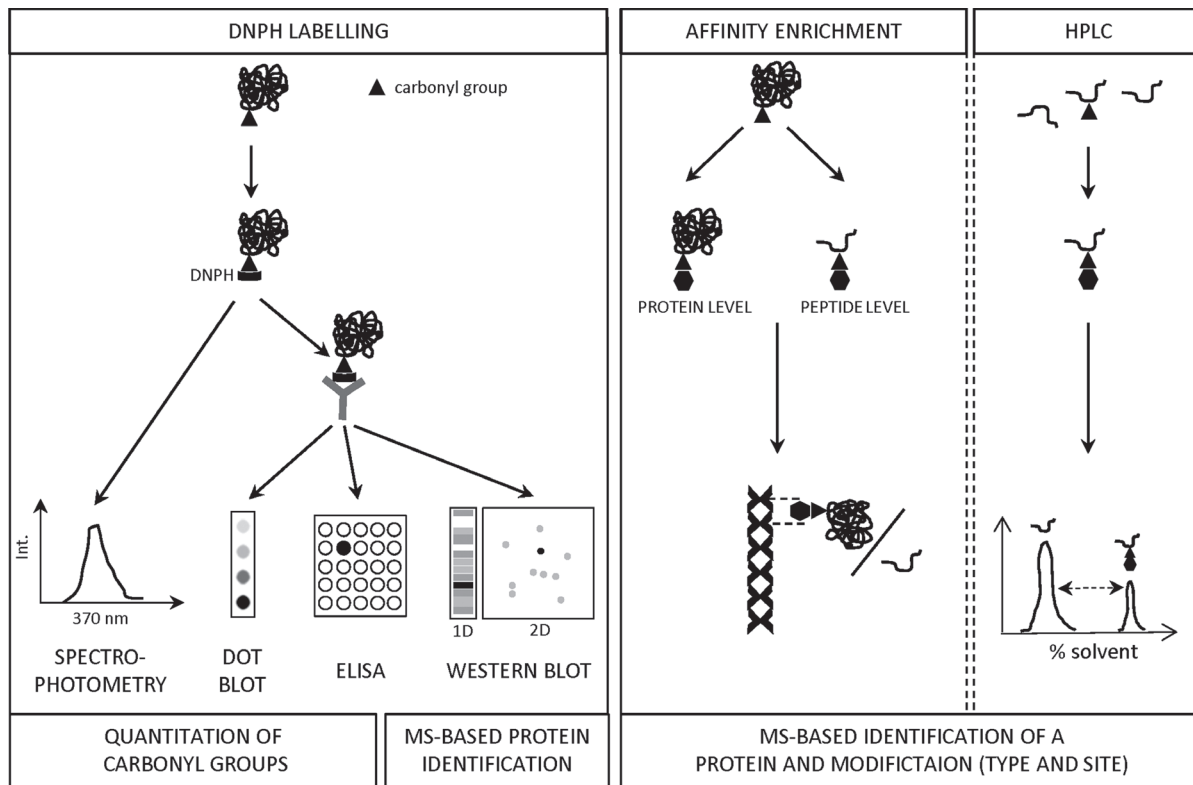


Figure 1. Summary of the selected methods for analysis of protein carbonylation. Depending on the sample type, experimental aims and instrumentation at hand analysis of protein carbonylation may be carried out using Spectrophotometric, Dot Blot, ELISA, Western blot, Affinity enrichment or by HPLC-based methods. Depending on the depth of the analysis, the techniques might be used individually or in combination. Each technique together with respective references is described in more detail in the text.

reacts with the ketone and aldehyde functional groups and produces DNP-hydrazone. The distinct UV absorption of DNP-hydrazone at 370 nm is measured in a spectrophotometer. Quantitation of protein carbonyls after derivatization is achieved by measuring absorbance at 370 nm and calculating hydrazone concentration using the molar extinction coefficient ( $22\,000\text{ M}^{-1}\text{cm}^{-1}$ ) for dinitrophenyl hydrazone per mg of protein. The core principles of the method are derivatization using DNP, which is normally prepared in hydrochloric acid (HCl) with a paired control sample undergoing “mock” derivatization in acid alone. Excess DNP is required to ensure derivatization of all protein carbonyl groups in the sample, but since unbound DNP absorbs at the same wavelength as the protein-bound DNP it is necessary to remove unreacted DNP by extensive washing after the derivatization step. The excess DNP which has not reacted is then washed away by precipitating out the protein using trichloroacetic acid and re-suspending the pellet several times in organic solvents to extract free DNP. After three washes the protein pellet is dissolved in guanidine HCl and the absorbance at 370 nm is measured. Practically speaking it is important to dislodge the pellet with vigorous vortexing between each wash as this releases free DNP and also facilitates redissolving the washed pellet. These washing steps without a doubt result in a loss of protein which has been estimated to be around 10–15% (depending on the protein size). This is a major drawback as it results

in a relatively low reproducibility and in high standard deviation.

In addition incomplete re-solubilization of the protein in guanidine may also result in underestimation of the protein carbonyl content and any turbidity in the solution due to incomplete solubilization in detergent can interfere with spectrophotometric analysis. Due to the insolubility of the pellet, it is important to analyze the protein content of the acid treated and washed protein pellets using an appropriate protein determination assay. Spectrophotometric protein determination at 276 nm is frequently used but other, compatible with guanidine HCl assays such as amino acid composition analysis can also be used.

Using this approach to measure plasma protein carbonyls, the normal range of molar carbonyl content per mg of protein is reported between 2–3 nmol/mg. In a variety of chronic diseases plasma protein carbonyl content has been described between 3.5 and 10 nmol/mg [31].

This method is widely used to estimate carbonyl content in biological samples in many different contexts. Therefore several drawbacks and pitfalls of the method have been identified over the years. Some of the most important ones are mentioned below.

It has been reported that commercially supplied 10 times concentrated DNP stock solution in 2 N HCl is not stable and is subject to degradation. It is not clear whether this is true for 10 mM DNP but it has been suggested that fresh solutions have to be prepared every 30 days [24].

The acidic conditions used for derivatization may also promote further carbonyl formation from existing hydroperoxides within any given mixture. Reduction of the hydroperoxides with triphenylphosphine (PPh<sub>3</sub>) eliminates this problem, giving more accurate carbonyl levels [32]. It has been shown that DNPH can also react with oxidized thiols (sulfenic acid) [33]. Sample pretreatment with a mild reductant such as PPh<sub>3</sub> or tri-butyl phosphine (TBP) that can reduce mildly oxidized thiols will reduce the contribution of the thio-aldehydes to the DNPH assay results. The presence of other chromophores absorbing at 370 nm such as myoglobin or retinoids may result in an overestimation of the protein carbonyl content, and therefore an extra washing step with acetone to remove the chromophores is recommended [34].

Very recently, an alternative strategy was developed which seems to overcome limitations of classical DNPH-based spectrophotometric assay [35]. Protein samples after DNPH derivatization in acid are neutralized with NaOH prior to spectrophotometric detection. Neutralization shifts the absorbance of protein-conjugated hydrazone to 450 nm [35]. This eliminates interference at 370 nm from both unbound DNPH and intrinsic protein absorbance increasing robustness and throughput of the analysis.

Despite the criticism, the DNPH-based approach is considered the standard method for quantifying protein carbonyls and has been applied in a variety of studies in a wide range of tissues from healthy to disease states. Based on this method it was possible to accumulate evidences of increase in carbonyl content during aging and in age-related diseases [36,37].

### Tritiated sodium borohydride method

Mild reducing agents can reduce carbonyls to alcohols. This principle has been used in a method based on the reduction of carbonyls with tritiated sodium borohydride [38]. The conversion of the carbonyl to an alcohol introduces a tritium (radioactive hydrogen) that can be detected and quantified by liquid scintillation. This method is the most sensitive among the classical methods for analysis of carbonyls [39]. However, it is mainly suitable for purified proteins, due to high level of background and poor specificity. Tritiated sodium borohydride can also react with Schiff bases. This made the method less suitable for applications to non-fractionated tissue supernatants [40]. Additionally, the use of radioactive labeling probably contributed to the lack of interest for this method compared to, for example, the DNPH-based method.

### DNPH-based Enzyme-Linked Immunosorbent Assay

The principles of protein carbonyl determination by immunoassay are founded on detecting DNPH using DNPH specific antibody. DNPH-modified proteins have been known for over 50 years to be potent immunogens with the antibody specificity directed against the hapteno-

moiety. In 1997 Buss and collaborators developed DNPH-ELISA method and showed that carbonyl levels were significantly elevated in critically ill patients [28]. This method has been modified to increase sensitivity for analysis of samples with low protein concentration [41]. Subsequent studies showed that the ELISA method is very sensitive for analysis of purified proteins, however, the method is not recommended for complex mixtures [42,43]. The DNPH-ELISA assay is available as a commercial kit.

The procedure consists of three major steps; immobilization of sample on the ELISA plate, DNPH derivatization, and antibody-based detection. The ELISA is developed by standard methods using enzyme-conjugated secondary antibody and enzyme-specific substrate. Two variations exist in derivatizing approaches for “home-made” standards and samples for ELISA. One approach is to derivatize in solution, as described for the spectrophotometric assay, then coat onto the ELISA plate. The second is to coat standards and proteins onto the ELISA plate using alkaline buffer to charge the protein and improve its binding. Derivatization on the plate proceeds using a 10 - fold lower concentration of DNPH. There are perceived strengths and limitations to each approach (summarized in Table IV); however, to date no direct comparison has been undertaken.

Standards for DNPH-ELISA are available in several kits. Unfortunately these standards are not standardized to a common reference and therefore the apparent concentration of carbonyl estimated in identical samples varies depending on the kit used. For example, Mohanty and colleagues have reported that analysis of plasma protein carbonyl content using two ELISA methods gave very different values for protein carbonyls that were both different from the spectrophotometric method [44]. No studies have been undertaken to explain the differences between different DNPH-ELISA assays, the possible contributing factors can be preferential adsorption of certain pools of protein carbonyls to the plate, difficulties in removing

Table IV. Summary of the strengths and weaknesses of derivatization methods for DNPH-ELISA assay.

In solution derivatization	On plate derivatization
<b>Strengths</b>	
<ul style="list-style-type: none"> <li>• Carbonyls are trapped quickly</li> </ul>	<ul style="list-style-type: none"> <li>• Quick and easy—reduced sample handling</li> </ul>
<b>Weaknesses</b>	
<ul style="list-style-type: none"> <li>• Requires more material in order to produce a pellet of protein after acid precipitation</li> <li>• Dissolution of protein into guanidine-HCl is variable and increases inter-sample variation</li> <li>• Protein concentration must be accurately determined after derivatization and acidic sample made alkaline to promote binding to ELISA plate plastic</li> <li>• Effect of hydrazone presence on ability to bind to ELISA plate is unknown</li> </ul>	<ul style="list-style-type: none"> <li>• Potential for further protein oxidation at alkaline pH during coating onto ELISA plate</li> </ul>



unreacted DNPH, selective reaction with antibodies and HRP linkage for certain types of adsorbed DNPH-reacted proteins [41,45].

Many commercial antibodies with high affinity and specificity are available for detection of the DNPH-hydrazone. Monoclonal antibodies should be preferred as they produce results with lower probability of nonspecific binding. While performing the assay it is of outmost importance to include controls containing no antigen, no DNPH, and no primary antibody, with Tween-20 being the preferred blocking agent.

### Gel electrophoresis based detection of carbonyls

Polyacrylamide gel electrophoresis can resolve proteins and remove low molecular mass impurities. Since most of the problems related to the global quantitation of carbonyls were associated with the presence of unreacted DNPH and non-protein carbonyls [42,43] the adaptation of gel electrophoresis in the carbonyl measurement was very suitable.

Levine's group has adapted the western blot technique and the high specificity of the anti-DNPH antibodies for the detection of carbonylated proteins in gels [46]. Today Carbonyl Western Blot (western blot detection of carbonylated proteins popularly named after the trade name of OxyBlot™ Protein Oxidation Detection Kit supplied by Millipore™) is widely used in academic research. The procedure consists of four major steps: 1) DNPH derivatization of carbonyl groups at acidic pH (1M HCl); 2) gel electrophoresis; 3) electrotransfer to PVDF membrane, and 4) antibody-based detection. In order to maximize labeling efficiency proteins are denatured prior to derivatization and excess DNPH is used for labeling. It is crucial to control reaction time (no longer than 30 min is recommended by the OxyBlot™ manual) to prevent formation of side products [33]. After derivatization pH is neutralized and protein samples are separated on 1D or 2D polyacrylamide gels and electrotransferred onto PVDF membrane. Once unspecific binding sites are blocked, the membrane is incubated with anti-DNPH antibody followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody or fluorescent antibody. Differentially oxidized proteins are then detected using chemiluminescent substrate and visualized on photographic film or by digital camera or fluorescent scanner, respectively.

Combining carbonyl specific detection method (e.g., Carbonyl Western Blot principle) with 2DElectrophoresis opens up a possibility not only to isolate and identify carbonylated proteins, but also to quantify the degree of carbonylation of each protein in relation to its overall quantity. Different chemical probes for detection of protein carbonyls in polyacrylamide gels have been developed including DNPH, tritiated sodium borohydride, biotin hydrazide-containing probes, and fluorescent probes. The far most commonly used approach for detecting carbonylated proteins on 2D gels is based on DNPH

derivatization and immunodetection with anti-DNPH antibody (Carbonyl Western Blot principle). Three independent approaches have been developed, depending on when in the process the DNPH derivatization step is carried out.

It can be performed before isoelectrofocusing step [47]; right after isoelectrofocusing [48,49] or post-electrophoretically [50]. DNPH derivatization prior gel electrophoresis of proteins requires very low pH (1M HCl) and typically the excess of the reagent is removed by precipitation of proteins, which can lead to uncontrolled loss of proteins. At the same time the DNPH derivatization changes protein mobility and therefore it is not possible to compare the patterns of carbonylated and non-carbonylated proteins directly. For such experiments it is mandatory to prepare control samples by treating protein extracts in the same way as for DNPH labeling, but without DNPH. Post-electrophoretic or isoelectrophoretic staining overcomes those problems and allows direct comparison between labeled and non-labeled patterns, which facilitates the quantitation process and MS identification [51,52].

Carbonyl specific detection of proteins separated by polyacrylamide gel electrophoresis can also be achieved by labeling with fluorescent carbonyl-reactive probes, for example with fluorescent hydroxylamine [53], fluorescein-5-thiosemicarbazide [54], or fluorescent hydrazides (discussed in more details below). Also an approach based on biotin hydrazide derivatization followed by visualization with avidin fluorescein probes has been developed [55].

One of the major advantages of Carbonyl Western Blot approach, as mentioned above, is that the excess reagent does not interfere with analysis because it is effectively removed during SDS-PAGE. Gel-based protein separation prior to detection provides additional advantage — it minimizes signal detection originating from non-protein carbonyl derivatives, such as nucleic acids [19]. Differentially carbonylated proteins can be subsequently identified by mass spectrometry analysis (Figure 1). The limitation of Carbonyl Western Blot approach is that the extent of carbonylation of distinct protein bands is determined in relation to another sample (e.g., healthy versus diseased) and it is not possible to determine an absolute measure of carbonyl groups per protein. Therefore an absolute quantitative analysis has to be undertaken in combination with DNPH-ELISA approach. Another drawback of the method is extensive sample consumption. Ideally, each sample should be analyzed in three experiments, one being actual DNPH derivative, second being derivatization control, and third protein load control, detected with protein-specific stain such as Coomassie Blue. Such controls are necessary because they assure reliability of the data obtained from the actual Carbonyl Western Blots. One other issue is related to the detection system. Chemiluminescent approach although fast and straightforward is not as reproducible and linear as fluorescence detection, which so far has not been included into the standard Carbonyl Western Blot.

## DNPH dot blot

High specificity of the anti-DNPH antibodies has been explored for developing a dot blot (or slot blot) approaches for quantitation of protein carbonylation [40,56,57]. The newest modifications to the protocol have been introduced by Levine's group [56] and increase the sensitivity of the assay by at least an order of magnitude as compared to the Carbonyl Western Blot. In dot blot experiment protein samples (of various complexities) are derivatized with DNPH in the presence of dimethyl sulfoxide (DMSO) and directly spotted onto PVDF membrane. Unbound DNPH is removed by acidic washes prior to immunodetection, performed essentially like for the Carbonyl Western Blot. However, in dot blot presented by Levine's group [56] the secondary antibody was conjugated to infrared fluorophore allowing for fluorescence-based detection of carbonyl content. Direct spotting onto PVDF membrane rather than electrotransferring in-gel separated samples significantly reduces processing time and allows simultaneous analysis of multiple samples and/or replicates improving analysis throughput. Replacement of chemiluminescence (HRP-conjugated secondary antibody) with infrared fluorescent detection is a major advancement for quantitative analysis. It significantly reduces the amount of sample required for analysis (60 ng protein compared to 10–20  $\mu\text{g}$  typically used in Carbonyl Western Blot experiments). Additionally, application of infrared-conjugated secondary antibodies maximizes sensitivity, allowing as little as  $\sim 0.2$  pmol of carbonyl groups to be detected. The signal response is linear, reproducible, and stable over time, however, the exact dynamic range of detection is not known [56,57]. Interestingly, the authors report that presence of DNA does not affect measurements [56]. This is rather surprising considering that it is a known issue for techniques where polyacrylamide-based protein separation is not used [19]. The limitation of dot blot as compared to Carbonyl Western Blot is that it measures total carbonyl levels and cannot distinguish between differentially carbonylated individual proteins.

## Fluorophores with carbonyl reactive groups

Properties of chemical probes suitable for detection of protein bound carbonyls have been reviewed recently [58]. A large group of such probes carries fluorophore moiety, which enables detection and quantitation of carbonyls using fluorescent scanner. In an experiment using fluorophores with carbonyl reactive groups protein samples are derivatized with carbonyl reactive hydrazide-labels under denaturing conditions. Generated Schiff base is then stabilized by reduction with sodium cyanoborohydride and proteins are precipitated with TCA, to remove unbound tag. Protein pellets after extensive washes are subjected to gel-based separation (either 1D or 2D). Protein-bound carbonyls are detected directly in-gel using fluorescent scanner. For each sample replicate gel is prepared and stained for total protein content using complementary fluorescent

dye. The two gels are then overlaid and changes in carbonylation levels are corrected by changes in protein abundance levels [53,54,59,60]. Fluorescent hydrazides possess strong advantages over both Carbonyl Western Blot and DNPH dot blot. They provide enhanced selectivity in carbonyl labeling as compared to DNPH, known for its cross-reactivity with sulfenic acids [19]. Despite additional reduction and protein precipitation steps sample processing time is reduced by electrotransfer and lengthy immunodetection. Fluorescence detection is advantageous for its signal stability and sensitivity, increasing depth of the analysis [60,61].

Several different hydrazides have been used to detect carbonyls, for example, fluorescein-5-thiosemicarbazide [44,54,62], Alexa 488 Fluorescent Hydroxylamine [53], Cy5 and Cy3 hydrazide [60,61] or BodipyFL hydrazide [60]. Each of the dyes has some specific advantages. In particular, use of CyDyes allow for simultaneous analysis of two carbonylated samples in the Difference Gel Electrophoresis (DIGE) format [60,61]. Despite their numerous advantages, limitations of fluorescent hydrazides exist. For example, requirement of special reagents and equipment, in particular for CyDye based multiplex analysis, fluorescent laser-based scanner with narrow band pass filters is necessary for accurate detection and to prevent overlap from one fluorescent channel to the other. Another issue of CyDye hydrazides is that they shift derivatized proteins from their original spot position making it difficult to overlap with corresponding spots from total protein stain. Importantly, dynamic range of detection with fluorescent hydrazides does not differ from the one provided by chemiluminescent 2DE DNHP approach [60]. This, however, might be improved in the future, when infrared fluorophore-coupled hydrazides become available.

## GC and HPLC detection of carbonyls

Several analytical methods including gas chromatography (GC), high performance liquid chromatography (HPLC), and liquid chromatography coupled to tandem mass spectrometry (LC-MSMS) have been applied in order to either gain more accurate quantitative information about protein carbonylation and also to gain further insight about the site of carbonylation. These will be briefly reviewed in the following section.

In order to overcome the shortcomings in the spectrophotometric assay such as removal of excess reagent and low solubility of the protein pellets in guanidine a new approach involving gel filtration using HPLC had been proposed [63]. DNPH derivatization is performed in 6M guanidine, pH 2.5 or in 6% SDS, followed by injection onto an HPLC equipped with a gel filtration column. Guanidine at such high concentration is very viscous and generates high back pressure, which is why HPLC is preferred to FPLC to perform separation. Most HPLC system cannot tolerate strong acids and some proteins are not solubilized in acid, which is an argument for performing

derivatization in guanidine. However, such high concentration of guanidine leads to crystallization and corrosion of the HPLC affecting the pump, seals, and injector. In contrast, the SDS derivatization is straightforward and does not lead to such drawbacks. Derivatization in SDS is performed by preparing the sample in a minimum 6% SDS using DNPH in TFA (10%). In all cases the column used is a gel filtration column at a 2 ml/min flow rate and pre-filtration or pre-column is necessary in order to avoid clogging of the gel filtration column. Detection of the hydrazine is at 370 nm and monitoring protein at 276 nm with elution time of less than 10 min. However, this is still a rather imprecise and relatively inaccurate method (Table V).

Reverse phase RP-HPLC has been successfully implemented to determine released protein carbonyls such as formaldehydes, acetone, isobutyraldehyde, glyoxylic acid released from oxidized amino acid such as alanine, valine, leucine, aspartic acid [64]. This is performed using a 5 -  $\mu$ m C18 column and the following settings: a flow rate

of 1 ml/min applying a gradient of solvent A (10% methanol in acetonitrile) and B (10% methanol in acetate buffer). The detection is performed using UV detection of hydrazine and quantified using authentic standards. A variation of that approach was also developed, where protein sample is hydrolyzed prior derivatization and analyzed by HPLC equipped with the same reverse phase column and similar solvent, quantifying DNPH-derivatized amino acids by absorbance at 370 nm [65]. Identification of derivatized amino acid was performed by simultaneous detection using a MS detector scanning in the positive mode between  $m/z$  50-600 and single ion monitoring (SIM mode for  $m/z$  209 and 298, respectively, for Trp, and Met+ His). These methods have been so far used sporadically meaning that the limit of detection and the sensitivity are not documented. In addition, they often require the preparation of "homemade" standards for identification and quantitation and their full implementation may represent several challenges.

Table V. Summary of the methods used for detection of protein carbonyls.

Method	Sensitivity	Linearity	Advantages	Pitfalls	Starting protein amount
Spectrophotometry	0.1 nmol/mg	At least 20 nmol/mg	Independent of antibody enhanced signal. Simple and fast.	Precipitation with TCA denatures protein and resulting pellet is difficult to wash free of excess DNPH and solubilize for spectrophotometry.	1 mg
Carbonyl Western Blot <sup>a</sup>	Non-quantitative	10 fold range	Provides information about proteins from a complex sample	Only relative quantitation is possible. Derivatization affects protein pI.	20 $\mu$ g
Dot blot	0.19 $\pm$ 0.04 pmol	n/a	High throughput, very sensitive		60 ng
ELISA	0.1 nmol/mg	8 nmol/mg	High throughput. Very sensitive. Highly reproducible within batches.	Standardization varies between available kits and individual laboratories. No correlation with results from spectrophotometric method [58]	1 $\mu$ g
GC-MS	0.1 pmol	1000 fold	Sensitive also for non-purified sample when using SIM	Hydrolysis of sample necessary. No commercially available markers, need to be synthesized and purified.	10–200 $\mu$ g
LC-Fluorescence or MS	4 and 10 fmol	At least 1 nmol/mg	Sensitive also for non-purified sample when using MS (SIM)	Derivatization necessary. No commercially available markers, need to be synthesized and purified.	mg
2 DE	Non quantitative	1000 fold range	Combined with mass spectrometry can identify oxidized proteins in complex mixtures.	Only relative quantitation is possible. Derivatization before electrophoresis affects protein pI.	50 $\mu$ g
MS	(atto-molar)		Allows identification of oxidized proteins and oxidation sites in proteins. Relative and absolute quantitation is possible.	Very complex method; requires specialized equipment; selective enrichment of oxidized proteins/peptides is necessary.	mg

<sup>a</sup>The determination of protein carbonyls by Carbonyl Western Blot is usually relative between test and control. Occasionally, standard commercially oxidized protein may be incorporated. Linearity of western blotting is affected by antibody concentration and time of development with chemiluminescent reagent. The linear range is generally considered to be 10-fold when comparing a faint band to a dense band. Beyond this, the signal becomes saturated and signal does not increase with increasing amount of antigen.

Another method which has recently received some attention is derivatization using p-aminobenzaldehyde (ABA) of the oxidation products of lysine, arginine and proline. Indeed metal-catalyzed oxidation of lysine has been shown to lead to deamination and formation of  $\alpha$ -amino adipic acid semialdehydes (AAS) while oxidation of proline and arginine lead to the formation of gamma-glutamic semialdehydes (GGS) [66]. The semialdehydes react with the primary amino group to form a Schiff base, which is subsequently reduced using cyanoborohydride ( $\text{NaCNBH}_3$ ). Adducts are stable and the method has been optimized in terms of derivatizing reagents concentration and reaction time [67]. It was reported that 25 mM ABA and 25 mM  $\text{NaCNBH}_3$  and a reaction time of 90 min gave the best results for derivatization of biological sample. The quantitation limit using this method is 10 fmol for AAS and 4 fmol for GGS at a signal to noise ratio of 10. The amount reported in biological samples range from 20 to 300 pmol/mg protein for AAS and lower values for GGS ranging from 3 to 60 pmol/mg protein. AAS and GGS were also shown for BSA to represent 23% of the total carbonyl groups when comparing with the DNPH derivatization methods. This method has been further developed [68] using tissue sample and using a mass spectrometric analysis. A quadrupole ion trap mass spectrometer equipped with electrospray ionization interface mass spectrometer with post-LC separation was used, which allowed identification of the molecular ions for AAS-ABBA and GGS-ABA with respective  $m/z$  at 267 and 253. Quantitation using SIM has been performed using homemade standards. The advantage of this method is that the preparation of AAS and GGS standards is easily performed with  $N\alpha$ -acetyl-L-lysine and  $N\alpha$ -acetyl-L-ornithine using lysyl oxidase from the egg shell membrane. Briefly, standards are prepared using egg shell membrane (10 g) which is incubated with individual compounds (10 mM) in phosphate buffer pH 9 at 37°C for 24 h, and after adjustment of the pH to 6 the aldehydes are aminated with ABA. The difficulty result in the purification of the obtained AAS-ABA and GGS-ABA compounds which has been reported to be performed using gel filtration followed by thin layer chromatography (TLC) and preparative HPLC. Nevertheless, this method has been receiving some attention but has only been tested with tissues and plasma and has not been fully validated, for limit of detection, minimum amount of protein required, or robustness.

Amici et al. and Requena et al. were the first to demonstrate that  $\alpha$ -amino adipic acid semialdehydes and  $\alpha$ -glutamic semialdehydes are the two main oxidation products of metal catalyzed oxidation of proteins and used GC-MS with isotopic dilution to demonstrate it [66,69]. They reduced the semialdehydes to their corresponding alcohols, 5-hydroxy-2-aminovaleric acid (HAVA) and 6-hydroxy-2-aminocaproic acid (HACA) and after acid hydrolysis of the protein, methylation of the alcohol to their trifluoroacetyl-derivatives was performed. Samples were injected onto a GC equipped with a mass spectrometer and detected using SIM with  $m/z$  280, 285, 294, and 298 corresponding to HAVA, d5-HAVA, HACA, and

d4-HACA, respectively. Both HAVA and HACA as well as their deuterated derivatives are not commercially available but the precursors glutamic acid and lysine and their deuterated counterparts can be synthesized in the laboratory. The coefficient of variation for HAVA was reported to be between 5% and 8% and for HACA ranged from 5% to 13% depending on the amount of protein material used, the number of repeats was  $n = 8$  or  $n = 9$ . The amount detected ranged from 300 mmol/mol glutamyl synthase to 3 mmol/mol lysozyme. A previous study using GC-MS reported that HAVA could be detected at a level ranging from 1 to 5  $\mu\text{mol}/\text{ng}$  protein in liver samples [70].

These analytical methods can be used to identify and quantify carbonylated protein, however, they have not been standardized and are not yet widely used. The lack of available standards and the lack of systematic quantitation make them difficult to implement. However, these are promising and especially AAS and GGS which have received a lot of attention since they seem to give more precise, and accurate measurement of protein carbonylation when compared to the classical spectrophotometric DNPH methods.

### Mass spectrometry for identification and quantitation of oxidative protein modifications

Mass spectrometry can be used to analyze any protein modification without *a priori* assumptions of what type of modification it is. Based on the mass shift between the genome deduced protein sequence and peptide masses experimentally observed it is possible to identify any protein modification (reviewed in [71]). However, this approach is tedious and not applicable to high throughput studies of complex protein mixtures due to the lack of appropriate database search algorithms capable of coping with such data [71]. The majority of proteomics and mass spectrometry based strategies are focusing on a particular group or type of protein modifications. This is mainly achieved via a specific enrichment and/or chemical derivatization methods that are targeting a certain class of modifications (reviewed in [71]). Approaches targeting oxidized proteins are discussed in the subsequent section.

Protein mass spectrometry (MS) is an analytical tool that is used to determine the masses of proteins or peptides and allows elucidating their chemical structures and composition. MS is an ideal tool for studying protein modifications because covalent addition or loss of a chemical moiety from an amino acid leads to an increase or decrease in the molecular mass of that residue. For example, oxidation of a methionine residue (131 Da) increases its mass to 147 Da by the addition of single oxygen atom (16 Da). Through the observation of a discrete mass increment or decrement of intact protein or peptide it is possible to assign a respective modification. Additionally, the tandem mass spectrometry allows the site-specific assignment of modifications at the resolution of individual amino acids in proteins [72–74].

Modified proteins exist in cells and tissues at very low levels. Therefore analytical strategies very often require modification-specific detection and enrichment techniques combined with electrophoretic and microfluidic separations and advanced mass spectrometry. Analysis of oxidized proteins is exceptionally challenging because there are many different types of modifications of proteins that are induced by ROS (for a comprehensive inventory of oxidative modifications to proteins please see [21]). Those modifications can be introduced in different amino acids and can co-exist in oxidized proteins together making the analysis even more challenging. Due to the different properties of the different oxidative modifications to proteins several dedicated approaches specific for particular type of modification have been developed and are briefly summarized in the following section.

Mass spectrometry based analysis of oxidized proteins and peptides is highly specific, because as mentioned above, each oxidation modification leads to a characteristic increase or decrease in the molecular mass of that residue. This rule, however, has few exceptions, for example, oxidation of proline to glutamic semialdehyde or hydroxyproline, which represent both the same mass shift of 16 Da. Still using modification specific tags, for example, biotin hydrazide, it is possible to distinguish between those two. Glutamic semialdehyde contains a carbonyl residue, which is reactive toward a hydrazine group, whereas hydroxyproline does not.

Unlike “bottom up” experiments that rely on sample proteolysis prior to mass spectrometric detection, top-down experiments detect and identify intact proteins. This type of experiments tend to provide higher individual protein information, including full characterization of each protein form present and its modifications [75]. However top-down proteomics is a relatively young field compared to bottom-up proteomics, and currently suffers from several limitations [76].

### Quantitation of peptides and proteins by mass spectrometry

Sensitivity of modern mass spectrometry instruments for the detection of peptides is at sub-femtomole levels [77]. Studies have shown that either with shotgun proteomics experiments [78] or with targeted proteomics assays [79] it is possible to detect proteins that exist in less than 100 copies per cell. However, although MS has been mainly used to identify proteins or their PTMs, it can also be used to determine their abundances.

The most common strategy is relative quantitation, which measures changes in the abundance of proteins and their PTMs between two or more samples. Such strategies predominantly use stable isotopes ( $^2\text{H}$ ,  $^{13}\text{C}$ ,  $^{15}\text{N}$  and  $^{18}\text{O}$ ) for sample labeling. Incorporation of isotopes has an effect on mass but little effect on the physiochemical properties of proteins/peptide. This means that identical peptides from differentially labeled samples of different origins can be distinguished by mass in a single MS analysis. The ratio

of their peak intensities corresponds to the relative abundance ratio of the peptides (and proteins) present in the original samples. Stable isotopes can be introduced as metabolic labels during protein synthesis using SILAC (Stable Isotope Labeling by Amino acids in cell Culture) approach [80,81] or by various chemical labeling approaches, for example, trypsin-catalyzed  $^{18}\text{O}$  labeling [82] or dimethyl labeling [83,84]. An additional chemical labeling strategy known collectively as isobaric labeling, that is, Isobaric Tag for Relative and Absolute Quantitation (iTRAQ) and Tandem Mass Tag (TMT) is also commonly used. In this case, samples representing different biological conditions are digested with trypsin, derivatized with respective labels, pooled together in an equimolar ratio and analyzed by MS. The different tags are isobaric in terms of the precursor ion (unlike SILAC and other methods mentioned above), however, upon fragmentation a reporter ion species is released. The intensities of these reporter ions, present in the low  $m/z$  range, are relative to the abundance of the precursor peptide to which it was attached.

Due to the sub-stoichiometric nature of oxidative modifications and the consequent need for enrichment it is likely that rather large amounts of starting material (pre enrichment) will be used. This has an impact on the choice of labeling strategy. One could label pre-enrichment but for some labels (iTRAQ, e.g.) this could be prohibitively expensive. There is also the option of labeling post-enrichment, however, this will introduce significant technical error into the workflow as enrichment procedures are often not highly reproducible. This problem is similarly inherited with label free approaches where sample preparation must be extremely reproducible to achieve significant results. All of these strategies may be used in a data dependent analysis of protein oxidation. That means that no particular protein or peptide species is targeted for analysis, but a global overview is obtained. However, some may also be used in conjugation with data independent analysis or targeted analysis.

Multiple reactions monitoring (MRM) now more commonly referred to as single or selected reaction monitoring (SRM) is such a targeted approach. In this technique, specific peptides of interest are selected according to their  $m/z$  and subjected to fragmentation. The resulting fragment ions confirm the identity of the precursor and their intensity is proportional to its abundance. This technique is often described as “western blotting in the mass spectrometer”. Although it currently outperforms blotting in terms of throughput allowing for simultaneous quantitation of up to 100 proteins in one LC MS-SRM experiment [85]. This technique has the potential to exceed ELISA levels of sensitivity with further improvements in instrument sensitivity (reviewed in [86]). Typically, in SRM experiments synthetic isotope labeled peptide equivalents are used as internal standards to enable relative or absolute quantitation. However, the majority of oxidative modifications are not available through commercial sources of synthetic peptides. Nevertheless, SRM has the potential to be a powerful technique for monitoring

oxidative modifications if combined with a labeling strategy, such as SILAC.

Although we have mentioned the limitations of label free approaches in a workflow where PTM enrichment is involved, it still may be useful where protein abundance changes as well as PTM level changes are to be monitored. Both levels of information are important, as a distinction needs to be made between PTMs, which are altered in level due to a real PTM abundance change and those, which are apparent only due to changes in protein abundance. Among such label free approaches, various methods of spectral counting are the most commonly applied [86]. Their general principle is that protein abundance is directly reflected by the number of peptide-to-spectrum matches (PSMs). In other words, more MSMS spectra will be dedicated to identification of peptides from a high abundance protein compared to one of low abundance. The Exponentially Modified Protein Abundance Index (emPAI) index is a well-known estimate of protein abundance provided with every MSMS-based database search using the Mascot search engine [87].

Thanks to the advances in the mass spectrometers and MS-based platforms absolute quantitation of the protein samples is now also feasible. Here again several possibilities exist to determine exact quantities of analyzed samples. This is often achieved by spiking known amounts of heavy-labeled standards into the sample prior to LC-MSMS analysis and subsequently comparing the intensities of such standards and analyte. Examples of this approach are AQUA [88] and QconCAT [89], which utilize isotope labeled peptides.

Due to the wide variety of mass spectrometry based approaches for the detection and quantitation of oxidized proteins and the variety of instrumental and experimental setups it is very difficult to obtain inter-laboratory standardization. Several international initiatives have been undertaken during the last few years. Perhaps the most potent is The Human Proteome Organization (HUPO) Proteomics Standard Initiative (PSI). Its major focus is standardization in proteomics to facilitate data validation, accessibility, and experimental transparency within and outside of the proteomics field [90]. The three major pillars of HUPO PSI are publication guidelines—Minimum Information About a Proteomics Experiment (MIAPE), data handling, including file formats, storage and transfer, and consistency in terminology and language.

In summary, the range of quantitative tools available nowadays in proteomics is extremely broad. All of them having their advantages and limitations (reviewed [86, 91–93]). In order to take full advantage of the available technology the sample type, experimental aims, and the instrumentation at hand should be carefully considered.

### Identification and quantification of carbonylated proteins by mass spectrometry

Several proteomic and mass spectrometry-based strategies have been developed to enrich for and analyze carbonylated

proteins (reviewed in [94] and [21]). The majority of them rely on the reactivity of carbonyl group toward hydrazines and hydrazides [95]. The probes used include biotin hydrazide [96], Girard's P reagent [97], Solid Phase Hydrazide [98], iTRAQH [99], and APR [100]. The oxidation-dependent, carbonyl-specific, Element-Coded Affinity Mass Tag, O-ECAT [101] approach utilizes affinity methods to isolate labeled proteins. The most successful approach based on biotin hydrazide and avidin affinity contributed to the identification of several carbonylated proteins and peptides in yeast [96,102], rat [103,104], and human plasma [105,106], reviewed in [21]. However, due to the strong binding between biotin and avidin this approach can only be used for protein isolation. It is not applicable for modified peptides due to a very low recovery from avidin resins [94]. The limit of detection for biotin hydrazide method with FITC avidin detection after gel separation was estimated to 10 ng [94]. Methods for specific isolation of carbonylated proteins have been successfully combined with mass spectrometry based quantitation methods. Stable isotope coding allowed comparison of the degree of oxidation of a particular site between two or more samples. This has been achieved by using isotopomers of DNPH [107], Girard-P reagent [108], O-ECAT [101], Hydrazide-functionalized, Isotope-Coded Affinity Tag, HICAT [109], iTRAQ [106,110,111], iTRAQH [99] isotope-labeled Phenyl Isocyanate, PIC reagent [112], and targeted<sup>18</sup>O-labeling [113]. Most recently MRM based, label-free approach has been used to quantify relative expression of carbonylated peptides in human plasma samples [106].

In addition to classical hydrazide-based derivatives hydroxylamine-containing reagents were also successfully adopted from nucleic acid research for selective labeling of protein carbonyls [114]. O-(biotinylcarbonylmethyl) hydroxylamine (aldehyde reactive probe, ARP) has been recently tested for labeling efficiency and MSMS fragmentation behavior [100]. When used in optimal (acidic) conditions ARP outperformed DNPH and biotin hydrazide in labeling of both aldehyde and ketone-containing peptides. Additional advantage of ARP over biotin hydrazide is that it does not require stabilizing reduction after carbonyl labeling [100]. Concerning might be CID and ETD fragmentation patterns complicated by neutral losses [100]. However, given an excellent labeling efficiency this should not prevent from widespread usage of the probe in the analysis of carbonylated proteins.

Few attempts to use DNPH as MALDI (Matrix Assisted Laser Desorption Ionization) matrix to facilitate detection of carbonylated peptides have also been described in literature. These methods utilize the specific UV absorption properties of DNPH (370 nm) which are similar to wavelength of the Nd:YAG-laser typically used in MALDI MS analysis. Initially applied to identification of formylglycine containing peptides [115] and HNE modified peptides [116,117] it has been recently further adapted for global analysis of carbonylated proteins [118,119]. Complete analysis consists of four principal components.

Initially, carbonylated proteins are digested with trypsin and carbonyl-containing peptides derivatized with DNPH. Peptide mixtures containing both carbonylated and non-modified species are fractionated using hydrophilic interaction chromatography (HILIC). Each HILIC fraction is then analyzed by DNPH-LDI-MS. Retrieved m/z ratios of carbonylated peptides are converted to corresponding multiply charged forms and included in classical nano-reverse phase-nano-electrospray tandem mass spectrometry analysis to identify sequence of modified peptides. Although laborious, the strategy allows identification of *in vivo* generated carbonyls [119]. This method was applied for mapping protein carbonylation in HeLa cells under mild oxidative stress, identifying 210 carbonylated protein targets with total of 643 carbonylation sites [118]. Despite its potential in high throughput analysis of carbonylated proteomes the method currently suffers from lack of quantitation necessary in comparative redox proteomics.

### Novel carbonyl-reactive isobaric labels for quantitative analysis of protein-bound carbonyls

Isobaric labels are powerful tools in quantitative proteomics. Commonly used amine-reactive derivatives are successfully applied in expression proteomics as well as in quantitation of post-translational modifications, including protein carbonylation [110,120]. There, quantitation of protein carbonyl content is effected indirectly, since different tags are used for carbonyl labeling (biotin hydrazide) and general peptide labeling for quantitation (iTRAQ) complicating derivatization and enrichment schemes. Introduction of iTRAQ hydrazide (iTRAQH) overcomes these issues [99]. This dual-functionality tag was generated by simple, one step conversion of amine-reactive NHS ester to hydrazide moiety in presence of excess hydrazine [99]. iTRAQH seems superior to currently available carbonyl-derivatization reagents providing simultaneous identification and quantitation of carbonylated peptides. Additionally, isobaric nature of the tags allows multiplex analysis of up to 8 samples, increasing analysis throughput and quantitative precision as compared to isotopically labeled carbonyl-reactive derivatives [99]. Limitation is lack of specific enrichment which hampers detection of sub-stoichiometric quantities of carbonylated proteins especially from cell and tissue lysates.

An alternative to iTRAQH are carbonyl-reactive Tandem Mass Tag reagents. Equipped in aminoxy group for carbonyl labeling, they allow simultaneous quantitation of up to 6 samples [121]. Additional advantage is that labeled proteins/peptides may be immune-purified and/or immune-detected using anti-TMT antibody [122]. Interestingly these potent reagents have so far only been exploited in the field of glycomics and their application to protein carbonyl analysis is yet to be revealed.

### Conclusions

As indicated throughout the entire article, one of the greatest problems in analysis of oxidized proteins is preservation of the real situation and avoidance of artifactual changes that may occur during sample collection, preparation, and analysis. All experimental steps may interfere with a final result leading to either over- or underestimation of the amount of oxidized proteins. Factors (besides those directly linked to methodology and instrument) that influence the experimental outcome include the type of the sample, buffer composition, purity of chemicals, pH, temperature, atmospheric oxygen, light, time, number of steps, stabilizers, presence of other oxidized molecules, removal of excess reagents, and/or interfering substances, storage conditions, and enrichment procedures. Each method and experimental approach described above has its strengths and weaknesses (summarized in Table V). Due to their specificities we can make only few general recommendations:

- Measure as quickly as possible after sampling
- Reduce the number of experimental steps to minimum necessary
- Perform derivatization as soon as possible
- Use primary chemicals from a verified supplier
- Prepare fresh working solutions
- Optimize and standardize the entire procedure
- Introduce control samples and control steps to exclude background and interfering signals

The biochemistry and metabolism of ROS/free radical-modified proteins have been gaining increasing attention in the last two decades, imposing a requirement for unified measurement procedures and traceability to reliable standard(s). Besides defining primary standard(s) for the oxidized proteins, equally important is the networking of laboratories and *in vitro* diagnostic test manufacturers to participate in a ring trial aiming to test the applicability of standards for different methods and purposes (in respect to samples, species, disorders, or other variables). Inter-laboratory testing is expected to provide information on relative strengths and limitations of different methods and possibly, the assessment of complementation between methods. A ring trial may be useful to participants to assess their own expertise level. Proteomics research studies have demonstrated that the major challenges are associated with detection and accurate quantitation of minor proteins in complex media (such as physiological mixtures), and detection of isoforms, homologous and truncated proteins [123].

The ring testing would also assess the allowable error of a measurement. Finally, an agreement is required whether it is preferable to avoid false negative or false positive results, that is, to define the uncertainty of a method standardized using consensus-accepted primary standard. On the other side, the implementation of a common primary standard in the *in vivo* diagnostics test manufacture would harmonize analytical performances of

commercial assays and reduce producer to producer and lot to lot variability. Compatible numerical results from different laboratories and assays would, hopefully, lead to unification of decision-making criteria.

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### Declaration of interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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