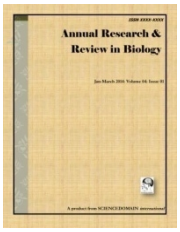

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Current Methods Used in the Protein Carbonyl Assay

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Authors' contributions

This work was carried out in collaboration between all authors. All authors were involved in data and information gathering, analysis, manuscript writing and critical reviewing. All authors read and approved the final manuscript.

Mini-review Article

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ABSTRACT

Some oxidative stress effects, such as lipid peroxidation or the DNA damage, have been extensively assessed and reliable biomarkers have been associated to characterize the degree of the damage, while the attack of reactive oxygen species on proteins and the formation of protein carbonyls were investigated only in the recent years. Taking into account that protein carbonyls may play an important role in the early diagnosis of pathologies associated with reactive oxygen species overproduction, a robust and reliable method to quantify the protein carbonyls in complex biological samples is also required.
This minireview investigates up-to-date methods used for the separation, identification and quantitative assay of protein carbonyls, a special attention being paid to the advantages and disadvantages of each technique.

Keywords: Protein carbonyls; oxidative stress; biomarkers; assays.

1. INTRODUCTION

Normal cellular metabolism, as well as some external factors (such as exposure to certain xenonobiotics) can generate reactive oxygen species (ROS). The most reactive ROS are

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free hydroxyl radical (HO•) and singlet oxygen ($^1O_2$). When the ROS generation exceeds the neutralizing capacity of the living organisms (the anitoxidant capacity), the oxidative stress is induced [1].

In other words, the oxidative stress represents the aggression produced at the molecular level by the imbalance between pro-oxidant and antioxidant agents, in favor of pro-oxidants, with severe functional consequences in all organs and tissues. An overproduction of ROS results in oxidative damages especially to proteins (the main target of ROS), as well as in lipids,or DNA. Glycation and oxidative stress are closely linked, and both phenomena are referred to as ‘‘glycoxidation’’. All steps of glycoxidation generate oxygen-free radical production, some of them being common with lipidic peroxidation pathways.

Glycation is one of the major causes of spontaneous damage to cellular and extracellular proteins in physiological systems; advanced glycation end products (AGEs) form when proteins interact with aldose sugars for an extended period of time (weeks, months). The initial glycation reaction is followed by a cascade of chemical reactions resulting in the formation of intermediate products (Schiff base, Amadori and Maillard products) and finally to a variety of derivatives named advanced glycation end products (AGEs). In hyperglycemic environments and in natural aging, AGEs are generated in increased concentrations; their levels can be evaluated in plasma due to the fact that they are fluorescent compounds.

Specific biomarkers of oxidative stress are currently investigated in order to evaluate the oxidative status of a biological system and/or its regenerative power. Generaly, malondialdehyde, 4-hydroxy-nonenal (known together as thiobarbituric acid reactive substances - TBARS), 2-propenal and F2-isoprostanes are investigated as markers of lipid peroxidation, while the measurement of protein thiols, as well as S-glutathionylated protein are assessed as markers of oxidative damage of proteins [2,3]. In most cases, the oxidative damage of the DNA has 8-hydroxy-2^l-deoxyguanosine (8-OHdG) as a marker [4,5].

The lipid peroxidation and the DNA damage have been extensively investigated, while oxidative attack on proteins was assessed later. At the same time the oxidative degradation of proteins plays an important role in the early diagnosis of pathologies associated with ROS overproduction [6].

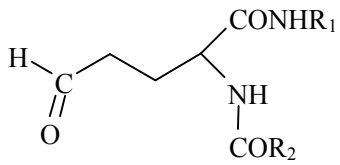
Oxidative modification of the protein structure may take a variety of forms, including the nitration of tyrosine residues, carbonylation, oxidation of methionine, or thiol groups, etc.

2. GENERATION OF PROTEIN CARBONYLS

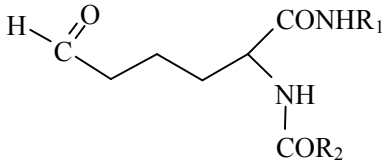
The carbonylation of protein represents the introduction of carbonyl groups (aldehyde or ketone) in the protein structure, through several mechanisms: by direct oxidation of the residues of lysine, arginine, proline and threonine residues from the protein chain, by interaction with lipid peroxidation products with aldehyde groups (such as 4-hydroxy-2-nonenal, malondialdehyde, 2-propenal), or by the interaction with the compounds with the carbonyl groups resulting from the degradation of the lipid or glycoxidation [7]. All of these molecular changes occur under oxidative stress conditions.

Compared with other oxidative changes (such as cysteine disulfide bond formation), the carbonylation process is irreversible, therefore the final compounds are stable and can be quantified [8]. There is a pattern of carbonylation, meaning that only certain proteins can undergo this process and protein structure determines the preferential sites of carbonylation.

The most investigated carbonyl derivates are represented by gamma-glutamic semialdehyde (GGS) generated from the degradation of arginine residue and α-aminoadipic semialdehyde (AAS) derived from lysine Fig. 1 [9].



gamma-glutamic semialdehyde (GGS)



α-aminoadipic semialdehyde (AAS)

Fig. 1. The structures of the most investigated carbonyl derivates

3. THE RELEVANCE OF PROTEIN CARBONYLS IN NONPHYSIOLOGICAL PROCESSES

A number of studies [1,7,10] have shown that the generation of protein carbonyl groups is associated with normal cellular phenomena like apoptosis, and cell differentiation and is dependent on age, species and habits (eg. smoking) or severe conditions' exposure (as starvation or stress). According to Dalle-Donne et al [11], the physiological levels of protein carbonyls in the plasma range within 0.5 to 4.0nmol/mg proteins, in the serum between 0.1 to 1.0 nmol/mg proteins, and in human brain cortex vary between 1.5 to 6.4nmol/mg proteins.

The formation and accumulation of protein carbonyls is increased in various human diseases such as Parkinson's and Alzheimer's disease, amyotrophic lateral sclerosis, cataractogenesis, cystic fibrosis, diabetes and cardiovascular disease, rheumatoid arthritis, etc [1,10,12,13].

In diabetes, oxidative stress is secondary to persistent hyperglycemia. The generated ROS lead to structural changes of proteins (including carbonylation process) and of the biomolecules (lipids, DNA, carbohydrates), enzyme inhibition and impairment of immune status. Different studies investigated the protein carbonyls in plasma of patients with diabetes [10] or the vitreous humor from patients with diabetic retinopathy [14].

Recently, Nystrom [7] suggested that the carbonylation process is associated with the physiological and not to the chronological age of the organism and the carbonylation may be one of the causes of aging and cell senescence; therefore it can be used as the marker of these processes. Jha and Rizvi, [15] proposed the quantification of protein carbonyls in the erythrocyte membrane as a biomarker of aging.

The formation of protein carbonyls was studied both in human cell culture (human fibroblasts, hepatocytes) or animal cells (myocytes or neurons) as well as in biological samples from patients with various diseases: in plasma and lymphocyte cells taken from the hippocampus and the inferior parietal lobe of patients with Alzheimer's disease [16], in plasma of patients with diabetes mellitus [17], in bronchoalveolar lavage from patients with severe sepsis [18] or from patients with acute respiratory distress syndrome [19], in synovial fluid from patients with rheumatoid arthritis [20].

The assessment of protein carbonyls offers some advantages because it is a marker that occurs in the early stages of pathology and remains in circulation for a long time, compared to other biomarkers of oxidative stress (as malondialdehyde or 4-hydroxy-2-nonenal or glutathione). Their chemical stability (for 3 months at - 80^0C during storage) in different types of biological samples Table1 and its clinical accessibility makes protein carbonyls suitable for routine laboratory measurement [21].

At the same time, protein carbonyls level fulfill the four conditions of the ideal biomarker of oxidative stress: (1) accurately indicates the level of the oxidative damage, (2) it is an early indicator of the pathological process (3) it gives significant information of the pharmacologic response to a therapeutic intervention and (4) it evaluates the efficacy of antioxidants.

Table 1. Biological samples used for quantification of protein carbonyls

Sample	Reference
Serum, plasma, blood	[7,10,17]
Lymphocytes	[16]
Erythrocytes	[15]
Synovial fluid	[20]
Vitreous humor	[14]
Bronchoalveolar lavage (BAL) fluid	[18,19]
Cerebrospinal fluid	[22]
Subretinal fluid	[23]
Postmortem brain tissue	[24]
Skeletal muscle	[25]
Cultured human colon cells	[26]

4. CURRENT STATUS OF THE ANALYTICAL METHODS

In the last decade, various analytical methods for the assessment of protein carbonyls were developed and validated: spectrophotometric assays [16, 24,27,28], high-performance liquid chromatography with diode-array or fluorescence detectors [29,30], enzyme-linked immunosorbent assays (ELISA) [31,32], one- or two-dimensional electrophoresis and Western Blot immunoassays [28] or capillary electrophoresis with laser induced fluorescence detectors [20]. Detailed protocols and techniques can also be found in literature [3,11]

4.1 Spectrophotometric Assays

The standard method for assessing protein carbonyls in various biological materials (plasma, cellular extracts, erythrocytes, or isolated proteins) involves the derivatisation Fig. 2 of the carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH) in order to obtain the 2,4-dinitrophenylhydrazones (DNP) [28]. This stable product (yellow) has a absorption maximum at 370 nm and can be quantified spectrophotometrically but can also be detected using immunoassays as ELISA or Western blotting techniques [31,32,33].

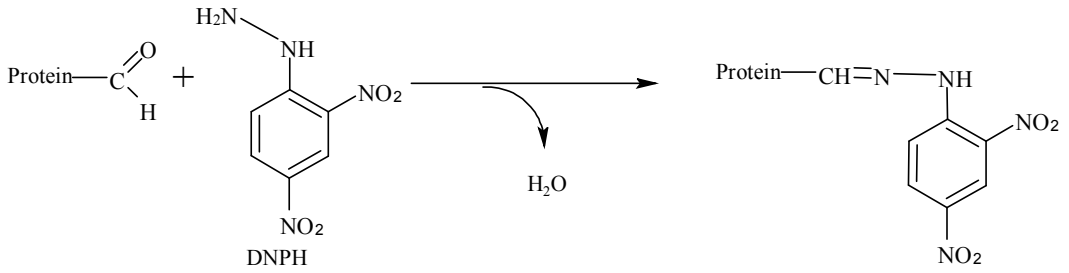


Fig. 2. Derivatisation of carbonyl group with DNPH

In order to express the protein carbonyl content (nmol/mg) total protein, the UV determination of total protein (at 280nm) is required. The major disadvantages of this method are: (1) the interference of other compounds with chromophores that also absorb at 360-380 nm (such as heme-containing proteins or retinoids); (2) usually requires milligram amounts of proteins (3) the need to remove the nucleic acids that also react with DNPH and might lead to overestimation of the real concentration of protein carbonyls; (4) the UV absorption of hydrazone also interfering with the determination of total protein; (5) it does not provide any information on the identity of the molecular nature of the protein carbonyls. Another major disadvantage of the method is related to protein loss during washing steps.

Several improvements were performed mainly on the techniques to remove the additional chromophores (by successive extraction steps with acetone/water) and to remove the traces of free DNPH (by washing with trichloroacetic acid/ethanol and extraction with ethyl acetate) [27]. These steps might lead to reducing the sensitivity of the method.

4.2 High-performance Liquid Chromatography (HPLC)

The interferences of unmodified proteins in the carbonyl proteins assays based on formation of the 2,4-dinitrophenylhydrazone were resolved by Agarwal et al. [29] by developing a high-performance liquid chromatography (HLPC) method with diode-array detector (at 370nm) after an initial separation of the protein fractions by gel-filtration. The separation of compounds is based on their molecular weight. Using this additional step, the method can be applied to tissue homogenates or cellular extracts. However, some disadvantages of this method can be noted: (1) the extreme pH value used for derivatisation with DNPH (2M HCl); (2) the problems related to solubilisation of the proteins; (3) co-elution of proteins with closer molecular weights and (4) lack of information on the structural identity of the protein carbonyls.

Akagawa et al. [30] developed and validated another HPLC method with fluorescence detection for the assessment of gamma-glutamic semialdehyde (GGS) and α-aminoadipic semialdehyde (AAS). The technique involves a reductive amination step with sodium cyanoborohydride and the coupling with the fluorescent reagent (p-aminobenzoic acid) followed by HPLC analysis, using 50 mM sodium acetate buffer (pH 5.4) as mobile phase. The highly fluorescent derivatives were detected with excitation and emission wavelengths set at 283 and 350nm, respectively.

Bollineni et al. [34] tried to identify the protein carbonylation sites using a complex strategy that implies the DNPH derivatisation, followed by liquid chromatography (LC) separation and

analysis by MS matrix- assisted laser-desorption/ionization with DNPH as the reactive matrix. The mass list generated for each LC fraction, representing mostly DNP-modified peptides, is analysed in the subsequent nano reversed-phase chromatography, coupled to an electrospray ionization mass spectrometer.

4.3 Gas Chromatography (GC)

Requena et al. [35] developed a complicated procedure for the assessment of glutamic and aminoadipic semialdehydes based on their reduction with sodium borohydride to 5-hydroxy-2-aminovaleric acid and 6-hydroxy-2-aminocaproic acid, respectively. This step was followed by derivatization to their volatile N,O-trifluoroacetyl methyl esters, which were further analyzed by GC and assessed using a mass spectrometry detector. The ions with m/z=280, 285, 294, and 298 have been used to quantify the protein carbonyls. The method requires internal standards and is time-consuming and multiple-stage procedure.

4.4 Fluorescent Methods

Ahn et al. [36] developed a method for monitoring the presence of carbonyl groups based on the derivatisation with fluorescein isothiocyanate. The fluorescently labeled proteins (thiosemicarbazones or hydrazones) are further separated on lithium dodecyl sulfate-polyacrylamide gels and fluorometrically detected. The drawback of the assay is related to the instability of fluorescently labeled proteins at room temperature.

A derivatizing method based on reductive amination of glutamic and aminoadipic semialdehydes with fluoresceinamine and sodium cyanoborohydride has been reported by Daneshvar et al. [37]. Nevertheless, the resulting fluoresceinamine derivatives are unfortunately degraded to nonfluorescent decarboxylated derivatives by acid hydrolysis.

Yoo et al. [38] developed a two-dimensional electrophoresis method (2-DE) using two-step labeling: first the biotinylation with biotin-hydrazide and then the staining with avidin-fluorescein isothiocyanate. The method is sensitive (limit of detection almost 0.64 pmol of carbonyl proteins) but requires long analysis and is not useful for small amounts of samples, such as those obtained from needle biopsies or single cells.

Recently, Tamarit et al [39] developed a method based on derivatization with fluorescent Bodipy-hydrazide followed by 2D-gel electrophoresis. The method also implies derivatization with combination of Cy3 and Cy5 hydrazides, this derivatization allowing multiplexing analyses in a single two-dimensional gel. The procedure presents multiple advantages, especially a great accuracy and reproductibility. The properties of Bodipy-hydrazide such as low molecular weight and no net charge, are esential for the perfect match between the Bodipy-derivatized spot and that of the protein stain. The Bodipy/Flamingo signal ratio (B/F) can be calculated for every spot in the gel, allowing a comparison of the degree of carbonylation at different protein spots.

4.5 Radioactive Labelling

A radioactive labeling procedure used for the quantification of protein carbonyls was developed by Lenz et al. [19] and involves the reduction of the carbonyl groups with sodium tritiated borohydride (^{3}H) in solution or prior to gel electrophoresis, the radioactivity of the obtained compound been then detected by standard radioactive counting. This procedure

has two disadvantages: (1) low selectivity due to the nonspecific incorporation of tritium into existing Schiff bases that can lead to high backgrounds and (2) the need to take safety precautions to radioactive detection of biomolecules. Therefore, the method is applicable only on purified samples.

Using the same principle of reduction with tritiated sodium borohydride, Yan and Sohal [40] developed a gel electrophoretic method, where the tritiated proteins were separated on SDS-PAGE and then quantified by liquid scintillation. This method has the advantage that unreacted tritiated sodium borohydride does not need be removed.

4.6 Immunoassays

The quantification of protein carbonyls is mainly done using immunobloting assays, especially using the commercial kits developed in the last years.

Robinson et al. [26] developed a technique where the protein samples were slot-blotted onto a polyvinylidene difluoride membrane, which sequentially suffered DNPH derivatisation, staining with anti-dinitrophenyl (DNP) antibody followed by interaction with peroxidase-conjugated second antibody. The limit of detection of the method corresponds to 60 pmol/mg protein, meaning at least 1700 times lower that the DNPH spectrophotometric assay and 500 lower than that for HPLC assay. It is important to underline that the method requires only small amounts of sample and does not imply DNA by precipitation with streptomycin sulfate in order to eliminate the DNA interference in the determination.

Another sensitive immunological procedure described by Bautista et al. [41] is based on the labelling of protein carbonyls with digoxigenin-hydrazide followed by dot blotting detection with anti-digoxigenin antibodies conjugated to alkaline phosphatase. This is a non-radioactive technique, with a low limit of detection (1.26 picomole of protein carbonyls on microblot), aplicable not only on isolated proteins but also on homogenates from different tissues.

Winterbourne and Buss [32] developed a sensitive enzyme-linked immunosorbent assay (ELISA) based on formation of 2,4-dinitrophenylhydrazone (through DNPH derivatization), followed by nonspecific adsorption of the hydrazone to the ELISA plate, staining with biotinylated anti-dinitrophenyl(DNP) antibody and interaction with steptavidin-linked horseradish peroxidase. Free DNPH and other non-protein constituents interferences la 450 nm are minimal, therefore the accuracy and sensitivity is higher compared with spectrophotometric assays. The ELISA test requires small amounts of protein (micrograms), therefore is applicable in cases where there is a limited amount of sample (e.g. tracheal aspirates from newborn babies). Currently, there are commercial ELISA kits on the market widely used for the quantification of protein carbonyls in biological fluids (serum, plasma, bronchoalveolar lavage fluid, cerebrospinal fluid), in cell extracts or other soluble protein samples [22]. The main disadvantage of the assay is related to DNA and small molecule interferences that underestimate the total protein carbonyls content. Unfortunately, like other methods, ELISA does not provide any information on the molecular nature of protein carbonyls and on the extent of carbonylation of a particular protein in a complex mixture.

Recently, Wehr and Levine [42] developed an immunochemical dot blot method for quantitation of protein carbonylation in purified proteins or homogenates. The method uses of dimethyl sulfoxide as the solvent, because it dissolves 2,4-dinitrophenylhydrazine, extracts proteins from tissues and keeps them soluble and wets PVDF membranes. The

method requires only 60 ng protein for one analysis and is having the detection limit is less than 0.2pmol, this indicating a greater sensitivity compared with Western blot assay.

4.7 Capillary Electrophoresis

Currently capillary electrophoresis is an extremely versatile method, widely used in the separation and determination of different proteins. Unfortunately, only a few methods were applied on protein carbonyls.

Feng et al. [25] detected and quantified protein carbonyls by capillary sieving electrophoresis with laser induced fluorescence detection (CSE-LIF). The method used the labeling of carbonyl group with Alexa 488 hydrazide and the labeling of proteins with 3-(2-furoyl) quinoline-2-carboxaldehyde. The method is capable of detecting femtomole (fmol) amounts of mitochondrial carbonyl proteins of striated muscle with molecular weights ranging from 26 to 30 kDa.

The advantages of this technique are well defined: increased sensitivity, accuracy, small volume of sample used (microL), quantitative and qualitative characterization of protein carbonyls.

4.8 Other Methods

Different separation techniques of DNPH-derivatised proteins, prior to the interaction with anti-DNP antibodies are mentioned in the literature, like one-dimensional gel electrophoresis [43] or two-dimensional electrophoresis [33]. The interference of free DNPH is minimal, because it does not react with anti-DNP-antibodies, therefore its removal is not necessary. These techniques, especially two-dimensional electrophoresis associated with Western blot immunoassay are largely used for separation and characterization of protein carbonyls based on their relative molecular masses.

Alternatively, the DNPH derivatisation of protein carbonyls can be done after separation via gel-electrophoresis and blotting to polyvinylidene difluoride (PVDF) membrane, this procedure resolving the problems raised by alteration of the isoelectric points of proteins caused by hydrazone formation [44].

Moreover, several commercial kits are currently on the market. Even if the commercial kits were widely used, the quality of the results, especially the reproducibility is questionable [45]. Recent studies point out that measured values of protein carbonyls may be artificially increased by nucleic acid contamination, exposure to thiols that may stimulate Fenton reaction or through DNPH reaction with sulfenic acids [46].

Recently, even mass spectrometry-based techniques were used as proteomic tools to monitor the extent of protein carbonylation and to identify the targeted proteins [47].

Madian and Regnier [48] reported the first proteomic-based identification and characterization of oxidized and carbonylated proteins in human plasma. The method used implies following steps: isolation from the plasma with avidin affinity chromatography, then biotinylation of carbonyl groups with biotin hydrazide followed by sodium cyanoborohydride reduction of the resulting Schiff's bases. Avidin selected proteins were digested with trypsin, and the peptide fragments were separated by C18 reversed phase chromatography and identified and characterized by both electrospray ionization and matrix assisted laser

desorption ionization mass spectrometry. Using this technique, sixty-five high, medium, and low abundance proteins were identified and fifteen carbonylation sites carried on 7 proteins were detected.

If not just the identification of the carbonylated sites but also the extent of protein carbonylation are required, different new techniques as stable isotope coded affinity tagging (ICAT) or isobaric Tag for Relative and Absolute Quantification (ITRAQ) are available [49,50]. With the iTRAQ method it was possible to identify over 200 carbonylated proteins from rat skeletal muscle mitochondrial protein isolates [49].

Another recent technique developed by Hollins et al. [51] uses oxalyldihydrazide as a crosslinker for enrichment of carbonylated proteins within a microfluidic chip. Based on this approach, the chip is capable of capturing 7.5 µg of carbonylated protein after 100 min of continuous loading, suggesting the usefulness for low abundance protein samples.

5. CONCLUSION

Protein carbonyls constitute an important biomarker used in the early diagnosis of pathologies associated with an overproduction of reactive oxygen species; therefore robust and reliable methods to quantify the protein carbonyls in complex biological samples are required.

Comprehensive evaluation of the current state-of-the-art concerning protein carbonyl evaluation revealed that considerable progress has been made in the development of new methods and techniques and on their applicability on different biological samples. Still, one of the greatest problems linked to the oxidative stress and proteins meaning the elucidation of the molecular identity of protein carbonyls and investigation of which amino acid residues suffered the carbonilation process is still a challenge.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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