Protein and cell wall polysaccharide carbonyl determination by a neutral pH 2,4-dinitrophenylhydrazine-based photometric assay

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ABSTRACT

A new 2,4-dinitrophenylhydrazine (DNPH)-based photometric assay is developed for the quantification of carbonyls in protein samples from any biological source by protein carbonyl-DNPH hydrazone formation at acidic pH in the presence of denaturing urea, and subsequent hydrazone solubilization in the presence of SDS and stabilization from acid hydrolysis at pH 7.0. At this neutral (ntr) pH, interfering unreacted DNPH is uncharged and its thus increased hydrophobicity permits its 100% effective removal from the solubilizate with ethyl acetate/hexane wash. The ntrDNPH assay is more reliable and sensitive than the standard (std) DNPH photometric assay because it eliminates its main limitations: (i) interfering unreacted DNPH (pKa 1.55) that is non-specifically bound to the TCA (pKa 0.7)-protein pellet is not effectively removed after wash with EtOH: ethyl acetate because it is positively charged, (ii) acid (TCA-induced) hydrolysis of the protein carbonyl-DNPH hydrazone, (iii) sample protein concentration re-determination, (iv) loss of sample acid (TCA)-soluble proteins, (v) DNA interference, and (vi) requires high protein quantity samples ($\geq$ 1 mg). Considering ntrDNPH assay's very low protein limit (1 µg), its cumulative and functional sensitivities are 2600- and 2000-fold higher than those of the stdDNPH assay, respectively. The present study elucidates the DNA interference mechanism on the stdDNPH assay, and also develops a standardized protocol for sample protein treatment and fractionation (into *cytoplasmic/aqueous*, *membrane/lipid-bound*, and *histone/DNA-bound* proteins; see Supplement section V) in order to ensure reproducible carbonyl determination on defined cell protein fractions, and to eliminate assay interference from protein samples containing (i) Cys sulfenic acid groups (via their neutralization with dithiothreitol), and (ii) DNA (via its removal by streptomycin sulfate precipitation). Lastly, the ntrDNPH assay determines carbonyl groups on cell wall polysaccharides, thus paving the way on studies to investigate cell walls acting as antioxidant defense in plants, fungi, bacteria and lichens.

1. Introduction

Protein oxidative modifications are used as markers of oxidative damage and cellular stress. They are measured by a wide variety of methods from simple global to the detection of individual oxidatively modified amino acid residues [1], with mass spectrometry being the most informative method [2].

Protein carbonylation is considered a well-established marker of global protein oxidative stress damage in organisms (blood, tissues, cells etc.), and the result of various oxidative reactions on amino acids involving reactive oxygen species (ROS) [3]. For instance,

carbonylation can occur on Ser, Lys, Arg, Thr and Pro by hypochlorous acid and via metal-catalyzed oxidation [2]. Protein structure determines the preferential sites of carbonylation. ROS (e.g. hydroxyl radicals) could directly create carbonyls on the amino acids Lys, Arg, Pro, and Thr [4–6]. Amino acids such as His, Cys and Lys are carbonylated indirectly by a mechanism that involves their covalent binding with reactive carbonyl species (RCS) such as 4-hydroxy-2-nonenal (4HNE) [7]. This mechanism is prominent on conserved Cys residues in cellular proteins collectively termed the redox/electrophile-responsive proteome, which are expressed to protect cells from oxidative damage [8]. RCS originate from non-enzymatic and enzymatic peroxidation of

lipids (especially arachidonic acid), which generates α,β-unsaturated aldehydes (e.g., 4HNE, acrolein, crotonaldehyde) and α,β-unsaturated ketones (e.g., cyclopentenone, prostaglandins) [8]. Another indirect mechanism, prominent on Lys, generates adducts of amino acid-secondary aldehydes by reaction with simple sugars (forming Amadory product), malondialdehyde, glyoxal and 4-hydroxynonenal (the latter two Lys-adducts being not reactive with hydrazines) [6]. It should be noted that carbohydrate groups of glycoproteins do not contribute to carbonyl level increase [9]. The most investigated carbonyl derivates are represented by the γ-glutamic semialdehyde, which is generated via the degradation of Arg and Lys-derived α-aminoadipic semialdehyde [10].

Carbonylated proteins are involved in various biological phenomena such as formation of advanced glycation end products [11], age-associated disorders, cytotoxicity, Parkinson's and Alzheimer's disease, cancer, chronic lung disease, renal failure, diabetes, sepsis, arthritis, skeletal muscle dysfunctions, chronic arterial occlusion and thalassemia major, acute pancreatitis etc [12–16]. Thus, the quantification of carbonylated proteins provides a new diagnostic (possibly presymptomatic) biomarker for oxidative damage-associated human diseases and biological dysfunctions [12].

Carbonylation has attracted great research and clinical attention because it is mostly irreversible and unrepairable, which makes it the most reliable accumulative biomarker of severe oxidative protein damage [12]. Moreover, protein carbonyl levels are appreciably greater than other oxidative modifications [5]. Although moderately carbonylated proteins are degraded by the proteasomal system [17], the heavily carbonylated ones tend to form high-molecular-weight aggregates that escape proteolytic degradation by the proteasome [18,19]. Such aggregates are accumulated in cells as damaged or unfolded proteins [20], can inhibit proteasome activity [19], amplify protein aggregation and cross-linking in non-dividing (post-mitotic) cells, and induce apoptosis [20,21]. On the other hand, limited degradation of carbonylated protein aggregates by proteasomes may be due to structural constrains that prevent their recognition by the catalytic sites within the cylinder of the proteasome complex [20]. Another reason is that proteasomes themselves could become the target of carbonylation (e.g. subunit S6 ATPase) and other oxidative modifications (e.g., glycoxidation, modification with lipid peroxidation products) [22], ending up (e.g. 26S proteasome) in decreased activity [23]. Other advantage for using carbonylated proteins as indicators of oxidative stress is their chemical stability, which allows easy detection even upon sample long storage [24].

There are numerous assays for protein carbonyl group detection and quantification [3,25–31] (some being available as commercial kits [5]). Over the past 30 years, protein carbonyls are quantified mainly by derivatization with 2,4-dinitrophenylhydrazine (DNPH) [32–34]. The DNPH-based assays evaluate quantitatively carbonyls in samples of total proteins via the standard photometric DNPH assay (thereafter referred as stdDNPH assay) or qualitatively after their fractionation by gel electrophoresis in bands or by HPLC as elution peaks. Carbonyl determination involves coupling of the photometric DNPH assay to protein fractionation by HPLC and 1/2-D gel electrophoresis [35], and isoelectric focusing and microsequencing [35]. Similar goals are served by the anti-DNP antibody-based immunocytochemical detection procedures such as ELISA (coupled with an HRP-conjugated secondary antibody) [36–38], Western immunoblotting [9,14,28,36,39–41], and other variations of immunohistochemical detection [35,42]. Anti-DNP antibody immunocytochemical detection has also been used to qualitatively evaluate the spatial distribution of carbonylated proteins in cells and tissues [42–45].

The strengths and weaknesses of the aforementioned DNPH-based assays have been extensively examined [3,6,46,47]. The immunohistochemical methods offer high sensitivity in the detection of the protein carbonyl-DNPH hydrazone [39,48]. However, they are qualitative, costly and time consuming (e.g., derivatization of cellular

protein carbonyls by DNPH requires overnight incubation [49]). Moreover, the ELISA and Western immunobloting techniques produce variable data possibly due to the non-specific binding of free DNPH to proteins and to DNA (interferes with the stdDNPH assay [34,50,51]), and to the inefective access of the antibody to all carbonyl-DNPH hydrazone sites in the protein. Limitations have been also attributed to problems arising from variations in sample preparation protocols, derivatization conditions, protein handling, self-preparation of standards and lack of internal controls [3,6]. There are also unidentified reproducibility problems in terms of major quantitative differences between the various DNPH assay versions [41].

1.1. The stdDNPH photometric assay: limitations

The photometric stdDNPH assay is the simplest method for the routine quantitative determination of total protein carbonyls. However, this assay has many limitations both at the procedural and non-procedural level, which justify the need for the development of a more reliable and sensitive version. To illustrate them, a brief outline of the principle of the stdDNPH assay follows: It is based on the reaction of DNPH (normally used at 10 mM in 2.5 M HCl) with the carbonyls of previously pelleted (via trichloroacetic acid, TCA) proteins at pH $\sim$ 0 for $\geq$ 1 h at RT, and the formation of a protein carbonyl-DNPH hydrazone. Given that free DNPH exhibits peak absorbance at 370 nm same as its protein carbonyl hydrazone, any unreacted DNPH that is non-specifically bound to the protein pellet is removed by two TCA washes, followed by three EtOH:ethyl acetate (EA) washes. Finally, the TCA/EtOH-EA-washed protein pellet is solubilized in urea or guanidine at acidic pH (2.3), and its carbonyl-DNPH hydrazone groups are quantified (per mg protein) by the DNPH absorption extinction coefficient 22,000 M^{-1} cm^{-1} at 370 nm [52].

The main procedural limitations of the stdDNPH assay are as follows:

1. DNPH reaction with the carbonyls of the TCA-pelleted proteins (applied upon by the stdDNPH assay) is expected to be less efficient than with proteins in solution. TCA-pelleted proteins actually exist in a 'molten globule-like' intermediate conformation state(s) [53], which may not allow equal accessibility of DNPH to all carbonyl groups. This limitation is an important factor of the high variability in the data obtained by the stdDNPH assay [32].
2. Any interfering unreacted DNPH that is non-specifically associated with the TCA(pKa 0.7)-pelleted protein is expected to be present as hydrazinium cation [54,55]. Being at a such highly polarized state, DNPH's complete solubilization in the EtOH:EA organic mixture used by the stdDNPH assay's wash procedure is greatly reduced and its removal from the pelleted protein is inefficient.
3. Another limitation factor in the stdDNPH assay is that the initial sample protein quantity is decreased by every protein pellet TCA wash. This is amplified by the fact that the recovery of proteins by TCA precipitation alone can be as low as 24% [56].
4. During the inefective protein TCA precipitation any acid soluble proteins in the sample are lost.
5. Limitations 3 and 4 require re-quantification of the protein content in the DNPH-treated sample.
6. The stdDNPH assay requires the use of very large sample protein quantities (1–10 mg ml^{-1}) to counteract the aforementioned procedural high protein loss.
7. The high protein sample requirement of the stdDNPH assay decreases its sensitivity substantially, making it inappropriate for samples with very low protein content.
8. Another unreliability in certain variations of the stdDNPH assay derives from the photometric estimation of the protein concentration in the sample blank (at 276–280 nm), by assuming an equal protein concentration in the DNPH-treated sample as well [32,33].
9. An important limitation of the stdDNPH assay is the hydrolysis-

prone hydrazone double bond (–C=N–) at acidic pH [57–59]; consequently, and that of the protein carbonyl-DNPH hydrazone [60,61]. This problem has been overlooked thus far although it is known that hydrazone bonds are generally stable within the pH range 5 to 9 [58]. Moreover, it has been shown that the protein carbonyl-DNPH hydrazone bond is stable at pH 9 but not at the acidic pH (~0) used by the stdDNPH assay [61]. At pH ~0, DNPH hydrazone hydrolyzes back to the carbonyl compound and free DNPH [59].

The stdDNPH assay is also prone to the following interfering factors:

1. DNA interfers with the stdDNPH assay [34,50,51], but the interference mechanism has not been elucidated [51].
2. Sulfenic acid, the product of ROS-mediated oxidation of the Cys -SH group [8], interfers with the stdDNPH assay [62].
3. The stdDNPH assay overestimates carbonyls in samples containing high amounts of heme proteins, such as cytochrome *c* and hemoglobin (Hb), due to their absorption near DNPH's 370 nm [28]. However, this problem can be addressed by appropriate protein sample blanks.
4. Non-reproducible data may derive from the application of different versions of the stdDNPH assay at non-standardized DNPH concentrations in the presence of guanidine hydrochloride (gndHCl) or urea used at various concentrations for reasons not justified. Indicatively, DNPH concentration variations range from 425 μM (in 6 M gndHCl) [63], 2.5 mM (in 4 M urea) [64], 5 mM [61,63,65], 10 mM [32–34,40,41,66–71] to 12.5 mM DNPH [50].
5. The lack of attention to experimental details on the correct application of the stdDNPH assay is another claimed source of interference [5,16].

1.2. A new DNPH-based photometric assay suitable for both protein and cell wall polysaccharide carbonyls

The present study introduces a new photometric assay based on an extensive modification of the stdDNPH assay. It is designed to eliminate all the aforementioned procedural limitations of the stdDNPH assay (100% efficient unreacted DNPH removal, protein carbonyl-DNPH hydrazone stability, and protein quantity as low as 1 μg). The present study elucidates also the mechanism of DNA interference affecting the stdDNPH assay, and shows that DNA does not interfere with the new assay. Moreover, the present study develops a comprehensive procedure for standardized protein sample preparation and fractionation (see Supplement Section V) in order to minimize any sample protein treatment related problems [3] in carbonyl content determination. This procedure will integrate interfering DNA removal via precipitation by streptomycin sulfate (SS), neutralization of any interfering Cys-sulfenic acid groups by dithiothreitol (DTT), and separation of proteins from interfering cytoplasmic or extracellular ketones and aldehydes by a near 100% effective protein precipitation method based on TCA and deoxycholate (DOC).

The present study will extend the new photometric assay for the determination of carbonyls on cell wall polysaccharides. By doing so, the new assay aims in paving the way on studies that will investigate whether cell walls may possess an antioxidant role besides its other known roles [72]. This is justified by the fact that cell wall polysaccharide constituents (such as cellulose) are known to oxidize to monocarbonyls, diketones and aldehydes (besides other oxidation forms) [73]. Moreover, carbonyls are known to form on cellulose pulp upon bleaching with the well-known ROS generators hypochloride and O_3 [74,75]. The methods currently available for the determination of carbonyl groups in cell wall polysaccharides (e.g., cellulose) are based on their condensation with various reagents (such as oxime formation by carbonyl reaction with hydroxylamine [76]), their oxidation or reduction (to carboxyls or hydroxyls, respectively) [76], their titration

with cyanide and its subsequent quantification by silver nitrate [77], and their determination via the copper number (with Cu^{2+} salts, which are then reduced and determined titrimetrically [78]). These methods provide only sum parameters and suffer from limited comparability, because they are either based on conversions with unknown mechanisms and they indirectly measure carbonyls (the copper number method), or they are non-reproducible (the oxime and cyanohydrin methods) [79]. A more carbonyl-specific method is based on the fluorescent carbazole-9-carboxylic acid [2-(2-aminooxy-ethoxy)-ethoxy]-amide (CCOA). As such, it requires CCOA release (via triflic acid) from the labeled cellulose, and determination of its concentration (and of any CCOA-derived products) by HPLC. Besides being quite cumbersome for testing many samples, this method requires samples in the mg range [80–83].

2. Materials and methods

2.1. Reagents

Acetic acid glacial (Sigma, cat. no. 537020)
Acetate, sodium salt (Sigma, cat. no. S5889)
Acetone (AC; Merck, cat. no. 01-6300117)
Adenosine 5′-monophosphate (AMP; Sigma, cat no. A1752)
Ammonium iron (II) sulfate hexahydrate $(NH_4)_2Fe(SO_4)_2·6H_2O$; Sigma, cat. no. 215406)
Butylated hydroxyanisol (BHA; Sigma-Aldrich, cat. no. B1253)
Bovine serum albumin (BSA; Sigma, cat. no. A9418)
Carboxymethyl cellulose sodium salt (CMCellu; Sigma cat. no. 21904)
Chloroform ($CHCl_3$; Merck, cat. no. 1.02445)
Coommasie Briliant Blue G-250 (CBB G-250; Serva, cat. no. C.I. 42655)
Cytidine 5′-monophosphate (CMP; Sigma, cat. no. C1006)
Deoxycholic acid, sodium salt (DOC; Sigma-Aldrich, cat. no. D6750)
2,4-Dinitrophenylhydrazine (DNPH; Sigma, cat. no. D198501)
DNA type III from salmon testes (Sigma, cat. no D1626)
Dithiothreitol, DL- (DTT; Sigma cat. no. D0632)
Ethanol, absolute (EtOH; Merck cat. no. 159010)
Ethyl acetate (EA; Sigma cat no. 270989)
Ethylenediaminetetraacetic acid, disodium (EDTA; Merck, cat. no. 324503)
Glucose, monohydrate (Sigma, cat no. 49159)
Guanidine-HCl (gndHCl; Sigma, cat. no. G4505)
Guanosine 5′-monophosphate (GMP; Sigma, cat. no. G8377)
Hexane (Merck, cat. no. 104374)
H_2SO_4, concentrated (96% or 18.11 M; Sigma-EMD MILLIPORE, cat. no. 1.00714)
Hydrochloric acid (HCl, ≥ 37% w/w; Fluka, cat. no. 84415)
Hydrogen peroxide (H_2O_2; 30% w/w, Merck, cat. no. 107209)
Hypochlorite, sodium salt (12–13% w/w or 1.67 M active chlorine; CL Chemicals, cat. no. CL02.1438)
Lysozyme from chicken egg white (Sigma, cat no. L6876)
Methanol (MetOH; 100%) for HPLC (Sigma-Aldrich, cat. no. 34860)
Pepsin from porcine gastric mucosa (Sigma, cat no. P6887)
Phenol (≥ 99.0%, Sigma cat. no. P4161)
Sodium borohydride ($NaBH_4$; Sigma, cat. no. 213462)
Sodium chloride (NaCl; Sigma, cat. no. 433209)
Sodium dodecyl sulfate (SDS; Bio-Rad, cat. no. 1610302)
Sodium hydroxide (NaOH; Merck, cat. no. 567530)
Sodium (di-) phosphate ($Na_2HPO_4·2H_2O$; Merck, cat. no. 106580)
Sodium (tri-) phosphate dodecahydrate ($Na_3PO_4·12H_2O$; Merck, cat. no. 106578)
Streptomycin sulfate (SS; Sigma cat. no. S9137)
Thymidine 5′-monophosphate (TMP; Sigma, cat. no. T7004)
Trichloroacetic acid (TCA; Merck, cat. no. 1.00807.0250)
Tris-base (MP Biomedicals, cat. no. 02103133)

Urea (Sigma-Aldrich, cat. no. U1250)
All other reagents used were of the highest purity

2.2. Instrumentation

Balance (Kern, 770/65/6J)
Bench top centrifuge (Hermle, model Z206A)
Centrifugal vacuum concentrator
Glass Pasteur pipettes (i.d. 0.5 cm, 22 cm length, by Hirschmann Laborgeräte GmbH & Co, Germany)
Microcentrifuge clear tubes, 1.5 and 2 ml (VWR, cat. no. 89000-028)
Micropipettes (adjustable volume) 2.5 µl, 10 µl, 20 µl, 100 µl, 200 µl, 1 ml, and tips (Eppendorf Research)
Microcuvette for absorbance measurements (12.5 × 12.5 × 45 mm external dimensions, 4 mm internal window and 9 mm bottom, 1.16 ml, quartz; Starna 9/B/9/Q/10)
Mechanical homogenizer Ultra-Turrax T25 (by IKA Labortecnik) homogenizer fitted with an (8 mm diam) S25N-8G disperser
Microcuvette for fluorescence measurements (45 × 4 mm, 0.5 ml, quartz; Starna SOG/Q), and associated adapter (Starna FCA4)
pH meter (Metrohm, 827 pH lab)
Sonicator (UP-50H, Dr Hielscher GmbH) equipped with a MS2 micro-tip (2 mm diameter)
Speedvac apparatus for vacuum drying
Spectrofluorometer (Shimadzu, model RF-1501)
Spectrophotometer (Hitachi, model UV–VIS U-1800)
Dialysis membrane (Spectra/Por 4-Dialysis Membrane Tubing MWCO 12–14,000, Spectrum Laboratories Inc)

3. Method procedure

The new DNPH-based assay involves the formation of a protein carbonyl-DNPH hydrazone under acidic conditions, followed by a 100% effective removal of unreacted DNPH via aqueous extraction with organic solvents from the DNPH-treated proteins while being solubilized at hydrazone stabilizing neutral (ntr) pH in the presence of the protein denaturing reagent sodium dodecyl sulfate (SDS). The ntrDNPH assay's protocol strategy contrasts with the ineffective removal of unreacted DNPH by solvent wash of the hydrazone destabilizing TCA-acidified protein pellet followed by the stdDNPH assay. The following ntrDNPH assay procedure has been developed after extensive investigation of its various parameters in comparison to the stdDNPH assay (presented in the Supplement):

3.1. Protocol of the ntrDNPH assay

The optimal conditions for the ntrDNPH assay established in Supplement Sections II to V are the following: Assay reaction takes place in minimum 0.24 ml, at final 1 mM DNPH (in 0.67 M HCl and 3.33 M urea) for a minimum incubation period 30 min at RT in the dark. Removal of free DNPH is achieved at a hydrazone-stabilizing neutral pH (7.0) by 3x wash with 0.5 ml EA:hexane (5:2 v-v) after extention of the assay reaction mixture (0.24 ml) to a final 0.5 ml DNPH-wash solution. This 0.5-ml extention originates from the 0.24 ml-assay reaction mixture brought to pH 7.0 by a master mix buffer with its components at final 0.32 M Tris-base, 0.32 M acetic acid and 15 mM SDS. Protein sample is prepared for the ntrDNPH assay as in Supplement Section V, which establishes standardized fractionation and solubilization conditions for the main protein fractions contained in any biological sample. The optimal ntrDNPH assay procedure is as follows:

3.1.1. Reagent setup

- **50 mM NaOH - 4 M Urea:** Dissolve 2.4 g urea in 8.18 ml 61 mM

NaOH.
- **50 mM NaOH - 8 M Urea:** Dissolve 4.8 g urea in 6.36 ml 78.6 mM NaOH at 37 °C.
- **250 mM SDS:** Dissolve 72 mg SDS in 0.93 ml ddH$_2$O (enough for ~ 33 samples).
- **0.8/0.8 M Tris-base/acetate buffer, pH 7.0:** Dissolve 2.9 g Tris-base and 1.38 ml glacial acetic acid in final 30 ml ddH$_2$O, and adjust to pH 7.0 by dropwise addition of 10 M NaOH.
- **M HCl:** Mix 18 ml concentrated HCl with 36 ml ddH$_2$O.
- **6 mM DNPH - 4 M HCl:** Prepare an initial DNPH solution by dissolving ~ 25 mg DNPH in 10 ml 4 M HCl at 25 °C (by continuous stirring). Then, determine the concentration of this initial DNPH solution (cleared by centrifugation if needed) by measuring the absorbance of various dilutions in 0.8/0.8 M Tris-base/acetate buffer (pH 7.0), and using the extinction coefficient of DNPH at 370 nm 22,000 M^{-1} cm^{-1}. Subsequently, adjust the concentration of the initial DNPH solution to 6 mM by appropriate dilution with 4 M HCl.
- **M NaOH:** Dissolve 2 g NaOH in ddH$_2$O to final 10 ml.
- Master mix solution (pH ~ 10): Mix 1 ml 5 M NaOH with 6.67 ml 0.8/0.8 M Tris-base/acetate buffer, pH 7.0 (enough for ~33 samples).

IMPORTANT NOTES: 1. During the ntrDNPH assay procedure, 0.23 ml of the master mix solution is mixed (for each protein sample) with 0.24 ml of the assay reaction mixture, and the resulting (unreacted DNPH-wash) solution should have pH ≤ 7.0 ± 0.3 (due to variations in the reagents in use). The resulting wash solution pH can be set to ~7.0 by slightly decreasing the volume (1 ml) of the 5 M NaOH component of the master mix solution. 2. Accuracy of the ntrDNPH assay during its application on a series of samples is ensured as long as the following precautions are met: (i) all samples are treated with a common master mix solution, (ii) the master mix solution is prepared fresh and used at constant RT (due to the known temperature-dependent pH variation of Tris buffers), and (iii) the assay reagents are of the highest purity.

EA:hexane (5:2 v-v): Mix 40 ml EA with 16 ml hexane (enough for ~37 samples).

3.1.2. Procedure (timing 60 min)

1. In each of two 1.5 ml-microcentrifuge tubes (one for the sample, S, and one for the sample blank, SB), add 200 µl of sample protein solubilizate (for solubilization options see **step 5** in sample protein fractionation described in Supplement Section V). Sample protein solubilizate (and its dilutions) can be made in 50 mM NaOH - 4 M Urea (see Supplement Section V, **step 5.1**, *Procedure 1*). For higher accuracy, determine protein carbonyls on at least three protein solubilizate dilutions. Alternatively, sample protein solubilizate (and its dilutions) can be made in 50 mM NaOH (see also Supplement Section V, **step 5.1**, *Procedure 2*), and, in this case, S and SB can be prepared each from a 100-µl protein sample (dissolved in 50 mM NaOH), mixed with 100 µl 50 mM NaOH - 8 M Urea. For sample protein solubilizates of very low protein/carbonyl content and made in 50 mM NaOH, the maximum volume of such samples for the ntrDNPH assay is 200 µl, supplemented with 48 mg urea (to final ~ 4 M). For biofluids with low protein/carbonyl content (e.g. aqueous humor; blood serum or plasma have high protein content) that are going to be tested for carbonyls directly (i.e. without prior fractionation of their proteins by the fractionation procedures described in Supplement Section V), the maximum sample protein solubilizate volume that could be used by the ntrDNPH assay is 154 µl (sample volumes < 154 µl can be also tested by adjustment to 154 µl with H$_2$O). The 154 µl volume is subsequently brought to final 200 µl by mixing with 48 mg urea and 10 µl 1 M NaOH. The resulting protein sample solution will be 50 mM NaOH and 4 M urea.

*IMPORTANT NOTES: 1. If the maximum sample protein solubilizate volume of 200 μl is used, and produces a net absorbance value (at 360 nm; see **step 5**) below the net carbonyl-DNPH absorbance detection limit ~0.015 set for the ntrDNPH assay (using the UV–visible spectrophotometer in the present study or others of same capability), such protein solubilizate samples could be increased in protein content by preparation from higher weights of the starting biological sample. 2. For protein solubilizates of limited quantity, their SB can be prepared only for the lowest tested dilution, and its absorbance value (at 360 nm; see **step 5**) can be proportionally extended to the other tested higher dilutions.*

In two microcentrifuge tubes (one for the reagent blank, RB, and the other for the reagent used for zeroing the spectrophotometer, and designated RZ in the *NOTE 2* of Supplement Section III) add 200 μl 50 mM NaOH - 4 M Urea.

IMPORTANT NOTE: For each protein solubilizate sample separate S and SB tubes are prepared, while tubes RB and RZ can be common for all samples.

2. To complete the assay reaction mixtures, add 40 μl 6 mM DNPH - 4 M HCl to each of the S and the RB tube (resulting in final 1 mM DNPH), and 40 μl 4 M HCl to the SB and RZ tubes. Incubate for 30 min at RT in the dark.

*IMPORTANT NOTE: Do not skip the 30 min-incubation step for the SB tube; otherwise, its absorbance reading at 360 nm (see **step 5**) will be overestimated (by ~7 fold) possibly because urea may not have exerted its denaturing effect on the protein in the tube.*

3. Bring all the resulting 0.24 ml-mixtures (in the tubes S, SB, RB and RZ) to pH 7.0 by mixing each with 0.23 ml of the Master mix solution (see *IMPORTANT NOTE 1*). Finally, add each tube with 0.03 ml 250 mM SDS, and mix gently to avoid bubble formation (which, however, can be eliminated by centrifugation).

*IMPORTANT NOTES: 1. If the color of the resulting S and RB mixtures (i.e. those containing the DNPH reagent) turns brown their pH will be likely above 7 (towards alkaline). In that case, the pH of the Master mix stock solution should be rechecked. 2. Any DNA initially present in mix with the sample protein solubilizate and subsequently in the S and the SB tube (if present it will show up as precipitate in these tubes due to acidification by HCl of their protein content in **step 2**) will not be re-solubilized after addition of the Master mix solution in **step 3**. This has been shown in the subsequent Part **C** that which investigates DNA interference.*

4. The resulting clear S, SD, RB, and RZ wash solutions (0.5 ml) are extracted 3x with 0.5 ml EA:hexane (5:2 v-v); S and RB EA:hexane-extraction will remove unreacted DNPH and also any reagents' impurities, while SB and RZ extraction will remove the latter. Specifically, the 0.5-ml-wash solutions are mixed (by vigorous vortexing for at least 30 s) with 0.5 ml EA:hexane (5:2 v-v), followed by centrifugation at 16,000*g* for 3 min at RT. The upper organic phase (EA:hexane, containing most of the free DNPH and the reagents impurities) is discarded first, and the 0.5 ml EA:hexane wash procedure is repeated 2x on the aqueous bottom phase (see *IMPORTANT NOTE 1*). Finally, the 3x-washed 0.5 ml clear aqueous bottom phase (corresponding to the initial S, SD, RB, and RZ wash solutions) is collected (after discarding the upper EA:hexane phase), and treated as in the following **step 5**.

IMPORTANT NOTES: 1. After every wash with EA:hexane draw as much as possible of the upper organic phase, even draw a small volume (~ 25 μl) of the bottom aqueous phase (~0.5 ml). However, you will need to re-measure the final volume of the 3x washed bottom aqueous phase, as to be able to accurately measure the contained moles of protein carbonyls (and express them as, e.g.

*nmoles per mg protein). 2. If DNA is present in the sample protein solubilizate, it will be distributed, during the EA:hexane-wash steps, in the bottom of the microcentrifuge tube (after centrifugation) as an invisible precipitate. Therefore, care should be taken as not to touch the bottom of the tube while drawing the bottom aqueous phase in the S and SD tubes for the absorbance measurements performed in **step 5**. 3. If the protein content in the S (and SB) tube exceeds the ntrDNPH assay's protein limit (1.5 mg in 0.24 ml assay volume, as established with BSA_{red}), its excess will be visible in the aqueous/EA-hexane interphase (after the 3rd EA:hexane wash) either as a white or clear gel-like protein zone interphase. However, the undissolved protein may not appear as a zone in the interface, and the bottom aqueous phase could still produce $netA_S$ (see **step 5**) readings, which are not proportional to the (three) different sample (and SB) dilutions fold suggested to be used in **step 1**; $netA_S$ readings may end up being even negative. The reason being, the insoluble protein may exist within the bottom aqueous phase as a suspension of very small clear protein particles, which are invisible to the eye. The best way to solve this problem is to use higher dilutions of the sample protein solubilizate. However, the disproportional reaction mixtures in the S and SD tubes can still be salvaged (for their $netA_S$ to be re-determined) as follows: Isolate the S and SB tube aqueous phase (~0.5 ml) and dilute it by mixing (via vortexing) with 0.25 or 0.5 ml of a mixture made e.g. of 0.25 ml 0.8/0.8 M Tris-base/acetate buffer (pH 7.0), 0.22 ml ddH$_2$O and 0.03 ml 250 mM SDS; complete mixing may require a brief sonication (possible carbonyl formation by the hydroxyl radicals generated by the sonication will not cause any interference problems because free DNPH has been already removed). 4. The S, SD, RB, and RZ washed solutions (totaling 0.5 ml) will require the use of quartz cuvettes since the absorbance reading is in the UV range, and preferably microcuvetts (such as the 0.58 ml cuvette Starna 18/B/9 used in the present study) since they allow the use of small and concentrated protein samples. In general, the ntrDNPH assay reaction and DNPH wash solution volumes can be adjusted appropriately to fit to the maximum accepted volume by the cuvette in use.*

5. Finally, measure the absorbance of the S, SB and RB (EA:hexane-washed) aqueous phases at 360 nm (designated A_S, A_{SB} and A_{RB}, respectively), having previously zeroed the spectrophotometer with the RZ (also EA:hexane-washed) aqueous fraction. Before placing the aqueous phase in the cuvette to measure its absorbance at 360 nm (see *Note* in Supplement Section IV), make sure to remove any minute air bubbles in it by centrifugation at 16,000*g* for 2 min at RT because they will increase the absorbance readings. Then, determine the net absorbance of the S phase by the equation:

$netA_S = A_S - (A_{SB} + A_{RB})$

Then, convert the $netA_S$ value (determined for and being proportional to at least three sample dilutions) to nmoles carbonyls (in the actual volume of the washed S aqueous phase; S volume is determined as in the *IMPORTANT NOTE 1* of **step 4**) by the DNPH molar extinction coefficient 22,000 M^{-1} cm^{-1}, and express protein carbonyl content per mg of the tested protein.

NOTE: Absorbance readings A_S, A_{SB}, and A_{RB} are stable for at least 12 and 24 hrs in the dark at RT and 4 °C, respectively.

*IMPORTANT NOTE: The difference of factors A_S and $(A_{SB} + A_{RB})$ in the equation for $netA_S$ is calculated for different sample dilutions (at least three, as suggested in **step 1**), and each of the resulting netAs values should be proportional to the corresponding dilution factor. If this does not hold (especially for samples with very low carbonyl content), the protocol should be applied on lower dilution samples (i.e. having higher protein content). However, the low protein carbonyl content A_S, A_{SB}, and A_{RB} values can be possibly savaged by plotting separately the value for the difference ($A_S - A_{RB}$) and the value for A_{SB} against their*

corresponding dilutions as to fit to a straight line that crosses Y (absorbance values) and X (sample dilution values) axes at the zero value. If this holds true, the resulting slope values for the (As - A_{RB}) and the A_{SB} straight-line curves are determined, and netA_S will be equal to the slope value of the (As - A_{RB}) curve minus the slope value of the A_{SB} curve.

3.1.3. Statistical treatment of raw data and statistical precision of the ntrDNPH assay

The assay was evaluated on human serum carbonyls, the values of which (Table 4) are expressed as mean (of 10 male and 10 female subjects of middle age) ± standard deviation (SD) after checking (with the statistical package SPSS Inc, 2001, release 11.0.0, USA) for equality of error variances between values of males and females (Levene's test) with two-way ANOVA analysis of variance (to identify significant differences between values), and with the parametric post-hoc multiple comparison Bonferroni test (p < 0.05). Measurements on certain protein fractions carbonyls from other biological sources (Tables 3, 4) were also analyzed with the SPSS software and expressed as mean of at least five independent experiments ± SD.

The ntrDNPH assay is also analyzed statistically for *precision* (i) during a single analytical run (*within-run, within-day precision* or *repeatability*), and (ii) with time (*between-run,* or *between day repeatability,* also termed *intermediate precision*). The minimum statistical variation of the protein carbonyls quantified by the ntrDNPH assay was determined by analyzing at least three successive dilutions of human serum samples the same day of blood collection, and calculating their mean value. The within-day % coefficient variation is calculated as SDx100/mean, and the variance of intermediate precision (σ^2_{total}) is defined as the sum of between day variance ($\sigma^2_{between}$) - associated with the day-to-day variation - and the variance of repeatability (σ^2_{within}).

3.2. Evaluation of the ntrDNPH assay against the stdDNPH assay

The stdDNPH assay removes unreacted DNPH from the assay reaction mixture by three EtOH:EA (1:1 v/v) wash steps, which follow the two TCA protein precipitation steps [32]. The present experiment tests the efficiency of the EtOH:EA wash steps of the stdDNPH assay versus those of the ntrDNPH assay by the following procedures: (i) the three EtOH:EA wash steps of the stdDNPH assay are extended to six and compared with an equal number of the EA:hexane (5:2 v/v) wash steps used by the ntrDNPH assay; (ii) the protein pellet from the 3rd wash step of the stdDNPH assay is dissolved in the assay reagent mixture used by the ntrDNPH assay (i.e., 0.24 ml 0.67 M HCl and 3.33 M urea, excluding the DNPH reagent), and is comparatively treated by the DNPH-wash procedure established for the ntrDNPH assay. The ntrDNPH assay is performed on BSA$_{ox}$ (200 μg) in a reaction mixture (0.24 ml) containing 1 mM DNPH, 0.67 M HCl and 3.33 M urea, and for 30 min incubation at RT. The stdDNPH assay is also performed on 200 μg BSA$_{ox}$ in a reaction mixture (0.5 ml) containing 10 mM DNPH and 2.5 M HCl, for 60 min incubation (Fig. 1). This experiment also establishes the sensitivity of the ntrDNPH assay against the stdDNPH assay (Table 1).

3.2.1. Reagent setup
For the stdDNPH assay:

- **10 mM DNPH - 2.5 M HCl:** As in Supplement Section II.
- **10, 100% TCA:** As in Supplement Section II.
- **EtOH:EA (1:1 v-v):** As in Supplement Section II.
- **5 M Urea, pH 2.3:** As in Supplement Section II.
- **1 mg ml^{-1} BSA$_{ox}$:** As in Supplement Section II.

For the ntrDNPH assay:

- **6.6 M Urea:** Dissolve 7.92 g urea in ddH$_2$O to final 20 ml.
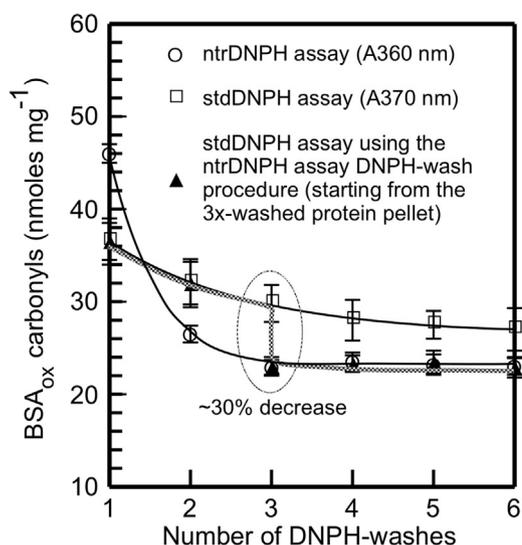- **6 mM DNPH - 4 M HCl:** As in 3.1.1 Reagent setup

Fig. 1. Comparative evaluation of the ntrDNPH and stdDNPH assay on the effective removal of unreacted DNPH. BSA$_{ox}$ was used in measuring the protein carbonyls by comparing the effectiveness in cleaning the BSA$_{ox}$-DNPH hydrazine from the interfering unreacted DNPH by the wash procedures of each assay. The stdDNPH assay's three wash steps (with EtOH:EA 1:1 v/v, following the two TCA protein precipitation steps) were extended to six steps, and were compared with an equal number of the wash steps (with EA:hexane 5:2 v-v) employed by the ntrDNPH assay. Additionally, the BSA$_{ox}$ pellet from the third wash step of the stdDNPH assay was treated by the ntrDNPH assay wash procedure (using four EA:hexane wash steps) as illustrated by the grey curve. Note that the BSA$_{ox}$ carbonyl concentration (determined by the stdDNPH assay after the 3rd wash step) drops by 30% (marked in oval) down to the value obtained by the ntrDNPH assay (after 3 wash times) after been treated by this assay's DNPH-wash procedure (meaning that this assay overestimates protein carbonyls by at least 30%). Error bars designate SD.

Table 1
Sensitivity of the ntrDNPH vs the stdDNPH assay.

Assays Sensitivities	ntrDNPH [stdDNPH]	ntrDNPH/stdDNPH sensitivity ratio
A. Carbonyl detection limit (in pmoles)	341[a] [443][a]	1.3 (= 443/341)
B. Minimum protein (in μg)	≤ 1 [2,000]	2,000 (= 2,000/1)
C. Minimum protein (in μg) per 1 nmole carbonyl at its detection limit[c]	2.93 (= 1/0.341) [4,545] (= 2,000/0.443)	1,541 (= 4,515/2.93)
Cumulative sensitivity limit (= AxB)	341 [886,000]	~ 2,600
Functional sensitivity limit (= AxC)	1,000 [2,013,435]	~ 2,000

[a] Values are based on a minimum netA$_{360/370nm}$ = 0.015 measured in the (0.5 ml) DNPH-wash solution, which correspond to 341 pmoles for both assays because of the same DNPH extinction coefficient 22,000 M^{-1} cm^{-1} at 360 and 370 nm (used by the ntrDNPH and the stdDNPH assay, respectively). Numbers in brackets are for the stdDNPH assay, the carbonyl detection limit (341 increased by 30% = 443 pmoles) of which is increased by minimum 30% due to 30% overestimation by the non-specific binding of DNPH in the stdDNPH in comparison to the ntrDNPH assay (Fig. 1). bMinimum sample protein quantities used in this table for the ntrDNPH and stdDNPH assays are 1 and 2,000 μg, respectively, with that stated for the stdDNPH assay reported elsewhere [32,33].

- **Master mix solution:** As in 3.1.1 Reagent setup
- **250 mM SDS stock:** As in 3.1.1 Reagent setup
- **EA:hexane (5:2 v-v):** As in 3.1.1 Reagent setup
- **2.5 mg ml^{-1} BSA$_{ox}$:** Dilute 100 μl 32 mg ml^{-1} BSA$_{ox}$ stock

(Supplement *Section I*) with 1.18 ml ddH$_2$O.

3.2.1.1. Procedure for the stdDNPH assay.

In separate microcentrifuge tubes precipitate seven samples of BSA$_{ox}$ (200 µg) with 10% TCA (by mixing 200 µl 1 mg ml^{-1} BSA$_{ox}$ with 22 µl 100% TCA) after 10 min-ice-water bath incubation, and centrifugation at 16,000g for 5 min at 4 °C. Dissolve each of the seven protein pellets in 0.5 ml 10 mM DNPH - 2.5 M HCl and incubate for 1 h in the dark at RT. Then, to each add 55 µl 100% TCA, incubate for 15 min in an ice-water bath and centrifuge at 16,000g for 5 min at 4 °C. Wash the seven pellets with 0.5 ml 10% TCA by vortexing and centrifugation at 16,000g for 5 min at 4 °C. Discard the supernatant and wash all seven protein pellets with 0.5 ml EtOH:EA (1:1 v-v) by vortexing, followed by centrifugation at 16,000g for 3 min at RT, and save one pellet for urea-solubilization and absorbance measurement. The same EtOH:EA wash step is repeated for a 2nd time in the remaining six 1x-washed pellets and one of them (the 2x-washed) is saved as previously. Then, the EtOH:EA wash step is repeated for a 3nd time in the remaining 5 1st-time-washed pellets and two of them (the 3x-washed) are saved as previously, one of them to be treated with the procedure for the ntrDNPH assay. The procedure is repeated with the remaining pellets until completion of the treatment of the remaining 4x-, 5x- and 6x-washed pellets. Then, all six variously EtOH:EA-washed pellets are solubilized in 0.5 ml 5 M urea, pH 2.3, and the net absorbance at 370 nm of each pellet solubilizate is read against a common reagent blank (made of 5 M urea, pH 2.3), and against wash-number-corresponding sample blanks (prepared with 2.5 M HCl in place of the 10 mM DNPH - 2.5 M HCl solution), and converted to nmoles BSA$_{ox}$ carbonyls mg^{-1} protein (after determination of the protein concentration in samples and corresponding sample blanks, and using the DNPH extinction coefficient 22,000 M^{-1} cm^{-1}).

3.2.1.2. Procedure for the ntrDNPH assay.

In separate microcentrifuge tubes mix six portions of 80 µl (200 µg) 2.5 mg ml^{-1} BSA$_{ox}$, each with 40 µl 6 mM DNPH - 4 M HCl and 120 µl 6.6 M urea. The resulting six 0.24 ml assay reaction mixtures (to be subsequently EA:hexane washed for 1, 2, 3, 4, 5 and 6 wash steps) are incubate for 30 min in the dark at RT. Additionally, the 3x-washed protein pellet prepared in the preceding procedure for the stdDNPH assay is solubilized in 120 µl 6.6 M urea, 80 µl ddH$_2$O, and 40 µl 4 M HCl (final volume 0.24 ml). Then, to this and the resulting six 0.24-ml reaction mixtures add 230 µl Master mix solution and 30 µl 250 mM SDS, ensuring that the pH in the resulting six DNPH-wash solutions (each 0.5 ml) should be ~ 7.0. Excluding the 3x-washed protein solubilizate wash solution (which will be washed four times with EA:hexane (5:2 v-v), the other six wash solutions (where the unreacted DNPH is ~ 1 mM, and has an initial absorbance ~ 22 at 370 nm) are mixed with 0.5 ml EA:hexane (5:2 v-v) by vortexing followed by centrifugation at 16,000g for 3 min at RT, the bottom aqueous layers are collected (one time-washed), and one of them is saved for absorbance reading at 360 nm. The same wash step is repeated for a 2nd time in the remaining five (1x-washed) solutions, and one of them (the 2x-washed one) is saved for absorbance measurement. The procedure is repeated with the remaining wash solutions until completion of the preparation of the remaining 3x-, 4x-, 5x and 6x-washed solutions. Then, the net absorbance at 360 nm of each of the six variably washed solutions is read against its wash-number-corresponding reagent blank (with ddH$_2$O in place of 2.5 mg ml^{-1} BSA$_{ox}$) and its wash-number-corresponding sample blank, using DNPH extinction coefficient for conversion of their net absorbance at 360 nm to nmoles carbonyls mg^{-1} BSA$_{ox}$.

3.3. DNA interference on DNPH and mechanism

It has been also reported that DNA interferes with the stdDNPH assay [34,50,51], but the mechanism has not been uncovered. The present experiment will use the ntrDNPH assay to investigate whether DNPH can react with the nucleoside mono phosphates (NMP' s) TMP,
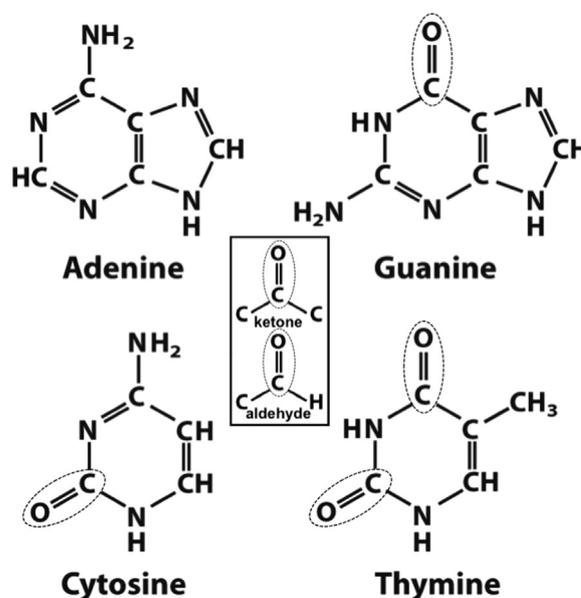

Fig. 2. DNA carbonyl groups shown (in ovals) present in thymine, guanine and cytosine (adenine lacks carbonyls). Carbonyl groups of aldehydes and ketones (known to react with DNPH) are also shown (in the orthogonal insert).

GMP, and CMP (since thymine, guanine, and cytosine contain 2, 1 and 1 carbonyl groups, respectively), using AMP as control since its adenine base does not contain carbonyl groups (Fig. 2). Moreover, the ntrDNPH assay will be applied on pure DNA to determine the nature and degree of its interference. It will also be investigated whether this interference can be eliminated by the ntrDNPH assay in protein samples containing DNA (omitting its removal by SS-precipitation; for DNA SS-precipitation see Supplement Section V). Finally, the degree of interference of DNA on the stdDNPH assay will be determined in comparison with the ntrDNPH assay.

3.3.1. Reagent setup

- **NMP's stock solutions:** These were prepared in 0.5 ml 10 mM phosphate buffer, pH 7.0, and their concentrations (20, 5.4, 4.7 and 6.6 mM for TMP, AMP, GMP and CMP, respectively) were determined by their respective absorption extinction coefficients (8.56, 15.06, 12.18 and 7.1 mM^{-1} cm^{-1}) at 260 nm [84].

- **1.85 mg (4.281 µmoles carbonyls) ml^{-1} DNA stock solution:** Dissolve 3.7 mg DNA (from salmon testes) in 2 ml 10 mM phosphate buffer, pH 7.0 (by gentle inversion e.g. in a 2-ml microcentrifuge tube), and let stand overnight at 4 °C for complete solubilization. The theoretical concentration of this solution in carbonyl groups is determined as follows: DNA is composed of the deoxynucleoside monophosphates (dNMP's) dAMP, dCMP, dGMP, and dTMP, having molecular weights (MW) 331.2, 307.2, 347.2, 322.2 (average MW 327 per dNMP) and containing 0, 1, 1, 2 carbonyl groups (in their respective bases; Fig. 2), respectively. With DNA dNMP's being in the pairs AT and GC, each mole dNMP pair contains 2 moles carbonyl groups. Moreover, the AT/GC ratio in any DNA (58.8%/ 41.2% in salmon sperm DNA) is insignificant for carbonyl group content determination because the average MW for dAMP and dTMP in the pair AT (326.7) and that for dCMP and dGMP in the pair CG (327.2) differ only ± 0.1% from the average MW (327) of the dNMP's. Thus, the 1.85 mg ml^{-1} DNA stock solution contains an average 5.657 µmoles carbonyl groups ml^{-1} (or 3058 nmoles carbonyl groups per mg DNA).

- **8 M urea in 100 mM NaOH stock solution:** Dissolve 4.8 g urea in 6.36 ml 157 mM NaOH.

- Remaining reagents of the ntrDNPH assay: As in 3.1.1 Reagent setup

Table 2

Interference of nucleoside monophosphate (NMP) and DNA carbonyl groups on the ntrDNPH and stdDNPH assays.

Assays	Interference from direct reaction of DNPH with the NMP carbonyls[a]	Interference from DNPH's non-specific binding to DNA[a] Tested DNA in mg, and in corresponding |nmoles carbonyls|		DNPH non-specific binding on DNA (0.2 mg) as fold-decrease over the stdDNPH assay
		0.1 mg |306|	0.2 mg |612|	
ntrDNPH	(0[b] or 0%)[a]	(0 or 0%)	(0 or 0%)[a,da,d]	292
		[0 or 0%][e]	[0.39 or 0.06%][e]	(= 17.5%/0.06%)
stdDNPH	NP[b]	(50.8 or 16.6%)	(105 or 17.2%)[a,da,d]	1 (control baseline)
		[53.9 or 17.6%][e]	[109 or 17.8%][e]	(= 17.5%/17.5%)

[a] DNA % interference is defined as the number of nmoles DNA carbonyl groups (shown by the 1st number in parentheses) that are detected out of theoretical 100 nmoles contained in the tested quantity of DNA. It is expressed as %, and is shown by the 2nd number in parentheses.

[b] TMP, CMP, GMP and AMP were each tested at 11.6 nmoles, or at corresponding 23.2, 11.6, 11.6 and 0 nmoles carbonyl groups. Carbonyl zero values for all NMP's tested are derived from testing their stated carbonyl values, which are well above (35–70-fold) the sensitivity limit of the employed ntrDNPH assay (Table 1). NA stands for "Not Possible" for the stdDNPGH assay. cConversion of DNA mg to nmoles DNA carbonyls is based on the correspondence of 3.06 nmoles carbonyls per µg DNA (for its derivation see Part C). The data obtained by the stated assays are normalized to the tested DNA quantities, 0.1 and 0.2 mg.

[d] Zero values in parentheses are derived from the absorbance values of the ntrDNPH assay's solubilization solution due to the presence of solubilized DNA (after the initial solubilization of its pellet in NaOH). The obtained zero carbonyl values may be explained by the observation that the ntrDNPH assay's solubilization solution does not solubilize the DNA that precipitates during the application of the assay (see *Part C*). Therefore, this result can be due to either the non-solubilization of DNA, or to the overrun of the sensitivity limit of the assay to detect the DNPH reagent that may have been non-specifically bound on the minor quantity of DNA that could have been solubilized. DNA insolubility in the solubilization solution of the ntrDNPH assay is the main reason that this assay escapes DNA interference (even at its statistically insignificant degree determined at 0.06%) when testing carbonyls in protein samples that may be contaminated with DNA. Interference of DNA is non-specific because the carbonyl groups in TMP, CMP, and GMP do not react with DNPH (nor DNPH reacts with the non-carbonyl group-containing control AMP). In contrast, the solubilization solution used by the stdDNPH assay solubilizes completely the DNA that is also precipitated during the application of this assay. The quite high interference of DNPH non-specifically bound to DNA for the stdDNPH assay (average 17.5%, and 290-fold higher than the DNA non-specific binding of DNPH by the ntrDNPH assay) can be only eliminated, for use by the stdDNPH assay, by removing DNA from samples via SS-precipitation (see Supplement Section V).

[e] Values in brackets are derived from the absorbance values of the ntrDNPH assay's solubilization solution, measured after initially subjecting the insoluble DNA precipitate to NaOH solubilization (as also described in the preceding *Note 'd'*), and then mixing the resulting solubilizate with the corresponding assay solubilization solutions. The DNA pellet alkaline pre-solubilization procedure was employed in order to determine the degree (0.06%) of the non-specific interference of DNA on the ntrDNPH assay. Interference of DNA quantities carrying at least less than 306 nmoles carbonyls is not detected by the ntrDNPH assay due to its sensitivity limit. On the other hand, the aforementioned alkaline pre-solubilization of precipitated DNA when applied on DNA treated with the stdDNPH assay gave a DNA interference degree, which coincided with that (17.5%) derived by the direct solubilization of precipitated DNA in the solubilization solution of this assay (see preceding *Note 'd'*).

- **8.2 mM DNPH in 4 M HCl:** By adjustment of the preparation shown in Supplement Section II.
- **5 M Urea, pH 2.3:** as in Supplement Section II.
- **Remaining reagents of the stdDNPH assay:** As in 3.2 Evaluation of the ntrDNPH assay against the the stdDNPH assay

3.3.1.1. Procedure for the NMP carbonyl groups. The same procedure of the ntrDNPH assay described in the preceding *Part B* is followed on the tested NMP's, with exception the preparation of the sample (S) and the sample blank (SB). S and SB are made (in 1.5 ml-microcentrifuge tubes) each as a 0.2 ml solution composed of 0.1 ml 0.116 mM for each NMP (made in 10 mM phosphate buffer, pH 7, by dilution of the NMP's stocks with ddH$_2$O) and 0.1 ml 8 M urea in 100 mM NaOH (final 4 M urea and 50 mM NaOH). The final concentration of TMP, CMP, GMP and AMP is the same and 58 µM (or 11.6 nmoles in 0.2 ml), and respectively in carbonyl groups 23.2 (=2×11.6), 11.6, 11.6 and 0.0 nmoles (Table 2). In two microcentrifuge tubes (one for preparing RB and the other for RZ), 0.1 ml ddH$_2$O is mixed with 0.1 ml 100 mM NaOH - 8 M urea. Then, the **steps 2** to **4** described in *Part A* are followed, and finally the absorbance of the S (A$_S$), SB (A$_{SB}$) and RB (A$_{RB}$) aqueous phases is measured at 360 nm against the absorbance of the RZ aqueous phase. The net absorbance (netA$_S$ = A$_S$ - A$_{SB}$ - A$_{RB}$) of the washed S aqueous phase is zero, and corresponds to 0 moles carbonyl groups (Table 2). This is verified from the fact that the zero netA$_S$ results in the presence of the initially tested 11.6 nmoles NMP's in the washed S and SB aqueous fractions. This is confirmed by measuring their quantity (at 260 nm, against the RZ absorbance, and using the corresponding extinction coefficients), which was found to be near equal to their initially tested 11.6 nmoles. Therefore, no reaction takes place between DNPH and the carbonyl groups of the NMP's.

3.3.1.2. Procedure for the DNA carbonyl groups by the ntrDNPH assay. Following the same above procedure for ntrDNPH, S and SB are prepared in microcentrifuge tubes by mixing 0.1 ml of various DNA quantities (up to 200 µg by dilution of the 1.85 mg ml^{-1} DNA stock with 10 mM phosphate buffer, pH 7.0) with 0.1 ml 100 mM NaOH - 8 M urea. For RB and RZ preparation, in a 3rd and a 4th microcentrifuge tube 0.1 ml ddH$_2$O is mixed with 0.1 ml 100 mM NaOH - 8 M urea. Then **step 2** described in *Part A* is followed (consisting of the addition of 40 µl 6 mM DNPH - 4 M HCl to the S and the RB tube, and of 40 µl 4 M HCl to the SB and the RZ tube, followed by incubation for 30 min at RT in the dark), where DNA in the S and the SB tube precipitates (due to neutralization of the phosphate deoxyribose backbone by HCl). Thereafter, the following comparative sub-procedures were tested on the resulting ntrDNPH assay reaction mixtures

1. *Sub-procedure 1.* The assay reaction mixtures follow **steps 3** to **5** described in *Part A*, at the end of which DNA (in the S and SB tubes) remains as precipitated pellet in the pH 7.0-buffered DNPH-wash solution (see DNA pellet further treatment in the following *Sub-procedure 2*). That is, the Master mix solution added in the **step 3** to bring the assay reaction mixtures to pH 7.0 does not solubilize the DNA precipitate (in the S and SB tubes; only 3.5% is dissolved as determined by its absorbance at 260 nm described below), and the net zero absorbance of S at 360 nm shows that no reaction of DNPH with DNA is detected (Table 2) even with the ∼ 3.5% soluble DNA (at netA$_S$ = 0.015, the detection limit of the ntrDNPH assay). The concentration of the slightly soluble fraction from the initially tested DNA is determined (in the SB tube) by comparison with 0.1 ml-solutions of known DNA concentration (made with e.g. 100 µg DNA, dissolved in 0.1 ml 10 mM phosphate buffer, pH 7.0), mixed with 0.1 ml 100 mM NaOH - 8 M urea, 0.23 ml Master mix solution, 30 µl

250 mM SDS, and 40 μl 4 M HCl (added last to avoid DNA precipitation), followed by a 3x-wash with 0.5 ml AE:hexane (5:2 v-v) and centrifugation at 16,000g for 3 min at RT. Therefore, insolubility of the DNA pellet in the DNPH-wash solution accounts for the non-interference of the ntrDNPH assay on protein samples containing DNA. An additional important factor that eliminates the non-specific binding of DNPH to DNA (and verified by the following *Sub-procedures 2, 3*) is the 100% effective (at pH 7) wash of DNA from DNPH; this is relevant to protein samples contaminated with DNA, where minor quantities of DNA may be co-solubilized with the proteins by the Master mix solution.

2. *Sub-procedure 2*. To quantify the DNPH moles possibly bound on the washed DNA precipitate (thus, to quantify their possible interference on the ntrDNPH assay), the DNA precipitate in the bottom of the S and SB tubes, prepared in *Sub-procedure 1*, is separated from the corresponding liquid phase, which is then saved (totaling ∼0.5 ml). The DNA precipitate is then solubilized in 30 μl 5 M NaOH, which is mixed with the saved ∼ 0.5 ml of the corresponding liquid phase, and with 30 μl 4 M HCl (to adjust pH at 7.0), and centrifuged at 16,000g for 5 min at RT. Then, the absorbance of the resulting S and SB supernatants (∼ 0.56 ml each) is measured at 260 nm (to verify the presence of the initial quantities of DNA tested), and at 360 nm (to derive the netA$_S$ value and express it in moles carbonyls as shown in **step 5** in *Part A*; see data in Table 2). If the DNPH possibly non-specifically bound to DNA has been efficiently washed from the DNA pellet by the EA:hexane solvent during *sub-procedure 1*, then the carbonyl group value derived from the *Sub-procedure 2* is expected to be the same with that derived from the following *Sub-procedure 3*, where the EA:hexane-wash of non-specifically bound DNPH is performed after solubilization of the pelleted DNA.

3. *Sub-procedure 3*. The assay reaction mixtures in the S and SB tubes that contain the tested DNA as precipitate, are first centrifuged at 16,000g for 5 min at RT and the corresponding supernatants are saved. The resulting DNA pellets are first dissolved in the 30 μl 5 M NaOH and then mixed with 0.2 ml 0.8/0.8 M Tris-base/acetate buffer (pH 7.0, the other component of the Master mix solution), with the saved corresponding S and SB supernatants (∼ 0.24 ml), and finally with 30 μl 250 mM SDS. Then, the **steps 4** and **5** in *Part A* are followed, and the DNA concentration in the DNPH-washed S and SD is determined in comparison with control solutions of known quantities of DNA as in *Sub-procedure 1* (data are shown in Table 2).

3.3.1.3. Procedure for the DNA carbonyl groups by the stdDNPH assay. DNA sample, S, is prepared in microcentrifuge tubes in 0.1 ml solutions (containing various DNA quantities up to 200 μg by dilution of the 1.85 mg ml^{-1} DNA stock with ddH$_2$O), and is precipitated with 11 μl 100% TCA (final 10%) by incubation for 10 min in an ice-water bath and centrifugation at 16,000g for 5 min at 4 °C. Verification of DNA precipitation is performed by measuring the absorbance of the TCA supernatant at 260 nm against 10% TCA (∼ 98% of the tested DNA initial quantities is precipitated). The resulting S DNA pellet is mixed with 0.5 ml 8.2 mM DNPH in 4 M HCl (using 0.5 ml 4 M HCl as RB) and incubated for 1 h at RT in the dark. Then, the S DNA suspension (and the RB) is brought to 10% TCA by addition of 55 μl 100% TCA, incubated for 10 min in an ice-water bath, and centrifuged at 16,000g for 5 min at 4 °C. The resulting S DNA pellet is treated as in *Part B* (subsection "*Procedure for the stdDNPH assay*"), where it is washed with 1 ml 10% ice-cold TCA (by vortexing, followed by centrifugation at 16,000g for 5 min at RT), and the resulting DNA pellet is 3x-washed with 0.5 ml EtOH:EA (1:1 v-v), followed by centrifugation at 16,000g for 5 min at RT. Thereafter, the following alternative sub-procedures were applied on the resulting TCA-EtOH:EA-washed S DNA pellet (data are shown in Table 2):

1. *Sub-procedure 1*. The washed S DNA pellet is dissolved in 20 μl 1 M

NaOH, to which 0.5 ml 5 M urea (pH 2.3) is added, and its absorbance is measured at 370 and 260 nm (against 0.5 ml 5 M urea, pH 2.3, mixed with 20 μl 1 M NaOH). Having established for this procedure that DNPH absorbs both at 370 and 260 nm with a ratio A$_{370\,nm}$/A$_{260\,nm}$ = 1.22 (in 5 M urea, pH 2.3) while DNA absorbs at 260 but not at 370 nm, washed S DNA absorbance at 260 nm will be overestimated with DNPH also present. However, the overestimated absorption of DNA at 260 nm (designated A) due to the absorption of DNPH at 370 nm (designated B) and 260 nm, can be corrected by use of the determined DNPH A$_{370\,nm}$/A$_{260\,nm}$ ratio in the correction the formula: corrected-A$_{DNA260nm}$ = A - (B/1.22). This corrected absorbance value can be then converted to the quantity of DNA using control solutions of known DNA quantities made in 5 M urea, pH 2.3. Thus, SB is not needed to determine the concentration of carbonyl groups/μg DNA in the S, given that the DNA recovery is not the same in S and SB during the stdDNPH assay.

2. *Sub-procedure 2*. The washed S DNA pellet is dissolved directly in 0.5 ml 5 M urea, pH 2.3, and (after centrifugation) and its absorbance is measured at 370 and 260 nm (against 0.5 ml 5 M Urea, pH 2.3), with the latter absorbance corrected as in *Sub-procedure 1*. The complete solubilization of the TCA-EtOH:EA-washed S DNA pellet in 0.5 ml 5 M urea, pH 2.3, was verified by comparison to control solutions of known DNA quantities made (from stock DNA) in 5 M urea, pH 2.3. DNA solubilization in the Urea solvent of the stdDNPH assay is a crucial factor that will contribute to the interference on the assay by protein samples containing DNA. An additional important interfering factor (evaluated by both *sub-procedures*) is the ineffective wash at acidic pH of the non-specifically bound DNPH to proteins.

3.4. Cell wall polysaccharide carbonyl content determination by the ntrDNPH assay

The ntrDNPH assay was cross-tested on the following CMCellu-based control solutions: CMCellu artificially carbonylated by NaClO (designated as CMCellu$_{NaClO-carbnylated}$), CMCellu$_{NaClO-carbnylated}$ decarbonylated by NaBH$_4$ (CMCellu$_{NaBH4-decarbonylated}$), CMCellu treated as for CMCellu$_{NaClO-carbnylated}$ but with the omission of the NaClO treatment step (CMCellu$_{NaClO\ treatment\ omitted}$), using CMCellu as overall control solution.

3.4.1. Reagent setup

- **0.2 M acetate buffer, pH 4.8**: Dissolve 0.25 gr sodium acetate in 12 ml ddH$_2$O, adjust pH to 4.8 dropwise with glacial acetic acid, and then adjust to final 15 ml with ddH$_2$O.
- **5% Phenol solution**: Dissolve 5 g in 100 ml ddH$_2$O and store frozen.
- **Hypochlorite (NaClO)-carbonylated carboxymethyl cellulose (CMCellu$_{NaClO-carbnylated}$) stock solution:** Since carbonyls generated on cellulose pulp by ozone and hypochlorite bleaching [74,75], the present study tested the ntrDNPH assay on carbonyls which were artificially generated on CMCellu by an extensive modification of the NaClO-based method described elsewhere [75]. Specifically, 15 mg CMCellu is dissolved in 3 ml 0.2 M acetate buffer, pH 4.8 in a glass tube, and to the resulting 0.5% (w/v) CMCellu solution 3.34 mM active ClO$^-$ (or 5% of the weight of CMCellu) is added, followed by incubation for 1 h at 75 °C. A control solution with same quantity of CMCellu is prepared and processed by omission of the NaClO step (designated CMCellu$_{NaClO\ treatment\ omitted}$), in order to test if the thermal and the following procedure steps could have artificially generated carbonyls. Then, CMCellu$_{NaClO-carbnylated}$ in the resulting 3 ml of the NaClO-treated CMCellu solution is isolated by the sequential treatment of this solution in 0.4 ml fractions as follows: Starting with the 1st 0.4 ml fraction, this is transferred to a 2-ml microcentrifuge tube and the contained CMCellu$_{NaClO-carbnylated}$ is

precipitated with 1.2 ml EtOH by vortexing, and to the pellet (collected at 18,500g for 3 min at RT) is added the 2nd 0.4 ml NaClO-treated CMCellu fraction, and the procedure is repeated until all 0.4-ml fractions of the initial 3 ml NaClO-treated CMCellu solution are similarly processed. The combined CMCellu$_{NaClO-carbnylated}$ wet pellets are washed once with 1 ml ice-cold acetone (by vortexing while being fractured into small pieces by the narrow angled tip of a metal spatula), followed by centrifugation at 18,500g for 3 min at RT and vacuum-dried. The resulting combined dried pellet of CMCellu$_{NaClO-carbnylated}$ (and also the CMCellu$_{NaClO treatment omitted}$) is weighted (~17 mg) and solubilized in minimum 4.25 ml ddH$_2$O (final 4 mg ml^{-1}; complete solubilization may require a few sec sonication) for expressing its carbonyls per mg CMCellu$_{NaClO-carbnylated}$. The CMCellu$_{NaClO-carbnylated}$ (and also the CMCellu$_{NaClO treatment omitted}$) stock solution is also quantified in glucose equivalents (by a glucose standard curve shown in a subsequent sub-section) for expressing control polysaccharide carbonyls per mg glucose equivalents.

- **NaBH$_4$-decarbonylated CMCellu (CMCellu$_{NaBH4-decarbonylated}$) stock solution:** For preparing a CMCellu$_{NaBH4-decarbonylated}$ stock solution a modification of a procedure described elsewhere [36] is applied: Specifically, in a 2-ml microcentrifuge tube 2.5 mg CMCellu$_{NaClO-carbnylated}$ (from the dry pellet obtained during preparation of the CMCellu$_{NaClO-carbnylated}$ stock solution) is dissolved in 1.5 ml ddH$_2$O (by vortexing), to which 2.5 mg NaBH$_4$ (final 44 mM) is added and dissolved by vortexing. The 1.5-ml mixture is then incubated for 30 min at RT and the generated H$_2$-gas bubbles are eliminated by centrifugation at 16,000g for 3 min at RT. Excess NaBH$_4$ is neutralized by dropwise addition of 10 M HCl while keeping pH 7.0. Then, CMCellu$_{NaBH4-decarbonylated}$ in the 1.5 ml NaBH$_4$-treated CMCellu$_{NaClO-carbnylated}$ is isolated by sequential treatment in 0.4 ml fractions as described in the sub-section entitled "Hypochlorite (NaClO)-carbonylated carboxymethyl cellulose (CMCellu$_{NaClO-carbnylated}$) stock solution". The resulting combined dried pellet CMCellu$_{NaBH4-decarbonylated}$ is weighted (~ 3 mg) and solubilized in minimum 0.625 ml ddH$_2$O (final 4 mg ml^{-1}) for expressing its carbonyls per mg CMCellu$_{decarbo}$, and the CMCellu$_{NaBH4-decarbonylated}$ solution is also measured in glucose equivalents (by the glucose standard curve shown below) for expressing its carbonyls per mg glucose equivalents.

3.4.1.1. Glucose standard curve vs mg glucose and CMCellu. A modification of the phenol-based glucose determination procedure [85] is followed: Stock glucose solution (0.18 mg ml^{-1}) is serially diluted (10, 6.66, 5, 4, 3.33, 2.86, 2.5, 2.22 and 2 fold), and 0.2 ml of the resulting dilutions (containing 20, 30, 40, 50, 60, 70, 80, 90 and 100 nmoles glucose, respectively) are mixed in 1.5-ml-microcentrifuge tubes 1st with 0.2 ml 5% phenol stock and 2nd with 1 ml concentrated H$_2$SO$_4$. A reagent blank (RB) is also made with 0.2 ml ddH$_2$O in place of the glucose sample. Sample blanks (SB) are also prepared by mixing the same 0.2 ml dilutions with 0.2 ml ddH$_2$O (instead of 5% phenol) and with 1 ml concentrated H$_2$SO$_4$. The tubes are allowed to stand for 10 min at RT (with their caps open due to high heat development), and then are mixed (by inversion after capping) and placed for 20 min at RT in the dark. Then, the net absorbance (netA$_S$) of each sample (A$_S$) is measured against reagent and sample blank absorbance (A$_{RB}$ and A$_{SB}$, respectively) at 490 nm (netA$_S$ = A$_S$ - A$_{SB}$ - A$_{RB}$), and plotted vs mg glucose (contained in the 1.4 ml assay volume). A standard curve is also made vs known mg CMCellu (from a 1.67 mg ml^{-1} CMCellu stock solution). The resulting slope was one half the slope of the standard curve in mg glucose, and this was also confirmed with the CMCellu$_{NaClO-carbnylated}$ and CMCellu$_{NaBH4-decarbonylated}$ stock solutions. The concentration of the CMCellu$_{NaClO-carbnylated}$ and CMCellu$_{NaBH4-decarbonylated}$ stock solutions used is 4 mg ml^{-1} in polysaccharide or 2 mg ml^{-1} in glucose equivalents.

3.4.1.2. Procedure of the ntrDNPH assay on CMCellu$_{NaClO-carbnylated}$ and corresponding controls. The following CMCellu-based control stock solutions were tested by the ntrDNPH assay: The stock solution CMCellu$_{NaClO-carbnylated}$ and the control solutions of CMCellu, and CMCellu$_{NaBH4-decarbonylated}$, and CMCellu$_{NaClO treatment omitted}$. Samples of 0.2 ml each, containing 0.4–0.8 mg polysaccharide (corresponding to 0.2–0.4 mg glucose equivalents, respectively) are mixed with 1 µl 10 M NaOH (final 50 mM NaOH), with urea been omitted from the samples - one of the differences with the protocol for protein carbonyls. Then, the procedure follows the ntrDNPH assay protocol steps described in *Part A*, with an additional difference the incubation of the sample with the DNPH reagent for minimum 1 h at RT (compared to the 30 min for protein carbonyls). For the aforementioned polysaccharide samples, there was no need for using sample blank (SB) and reagent zero (RZ). Instead, the spectrophotometer was zeroed with ddH$_2$O. The carbonyl values of the tested control polysaccharide samples, expressed both per mg polysaccharide and glucose equivalents are shown in Table 5. It should be noted that the netA$_S$ of the tested DNPH-polysaccharide product was constant after storage for 1 day at 4 °C. Moreover, the sample maximum concentration used by the ntrDNPH assay is 4 mg polysaccharide ml^{-1} (for assay maximum sample volume 0.2 ml, this would correspond to a maximum 0.8 mg polysaccharide). It was attempted to also test the above CMCellu-based polysaccharides with the stdDNPH assay (as in *Part B*). However, they are not precipitated with TCA (to remove non-specifically bound DNPH), nor are they readily dissolved in the assay's solubilization buffer (5 M urea, pH 2.3).

3.5. Calculations and expected results

3.5.1. NtrDNPH assay optimal parameters vs the stdDNPH assay

The optimum parameters of the ntrDNPH assay were calibrated with control proteins (BSA, lysozyme, pepsin) that were artificially carbonylated, and also used as non-carbonylated controls by elimination of any background carbonyl groups (via NaBH$_4$ reduction; see Supplement Section I). These parameters established a shorter assay reaction time (30 min) and a much more efficient wash of the interfering unreacted DNPH in comparison to the stdDNPH assay (Suppl. Fig. 1). The low HCl concentration (0.67 M) used in the reaction of DNPH with proteins by the ntrDNPH assay allows its easy adjustment to pH 7.0 for the following key modifications in comparison to the stdDNPH assay: (i) The 100% efficient removal of the unreacted DNPH, (ii) the stabilization of the protein carbonyl-DNPH hydrazone (by preventing its hydrolysis at acidic pH [58,61]), and (iii) keeping it soluble by SDS during the EA:hexane DNPH-wash (Suppl. Figs. 2, 3). These conditions also permit the use of sample protein quantities as low as 1 µg (and up to 1.5 mg in an assay reaction volume of 0.24 ml). Hexane in mixture with EA lowers the polarity of EA (due to hexane's higher hydrophobicity), and (together with SDS and urea in the DNPH wash solution) prevents protein precipitation during the wash of unreacted DNPH and also enhances the partition of the latter to the EA:hexane phase. DNPH partition is further strengthened via decrease of its water solubility by the inclusion of acetate in the DNPH wash solution [86]. Such protocol strategy skips the removal of unreacted DNPH via protein TCA-precipitation of the stdDNPH assay.

The optimum pH range for attaining stable absorbance for the protein carbonyl-DNPH hydrazone (after washing unreacted DNPH) is 2.3 to ~7.0 (irrespective of urea's presence/absence; Suppl. Fig. 3A). Extending this pH range to ~0 under the assumption that the protein carbonyl-DNPH hydrazone extinction coefficient (22,000 M^{-1} cm^{-1} at 370 nm) does not change, is not a valid proposition because the present study found that at pH 0 the maximum absorbance (at 370 nm) of the hydrazone is 2-fold lower than at pH ≥ 2.3 (data not shown). Therefore, methods that quantify photometrically the protein carbonyl-DNPH hydrazone at pH ~0 (e.g., at 2 M HCl [67,87]) are expected to greatly underestimate its value. Above pH 7.0, the protein carbonyl-DNPH hydrazone extinction coefficient (as also of free DNPH) decreases

rapidly (Suppl. Fig. 3A), followed by a progressive visible change of its yellow to dark brown color, and this may be due to an alkaline pH-induced ionization in the N-N bond [59]. The color shift of free DNPH due to the formation of an ionizing intermediate above pH 7.0 is suggested by the rapid absorbance increase exhibited by the reagent blank also above pH 7.0. This may be due to the generation of an ionic form of DNPH above this pH, which is insoluble in the EA:hexane solvent. Therefore, the optimum pH for measuring protein carbonyl-DNPH hydrazones is 7.0 but not less than pH 5 (as hydrazone bonds are generally stable at the pH range 5–9 [58]), and for the additional reason that at this pH range the reagent blank maintains its lowest absorbance value (Suppl. Fig. 3A). It should be noted that the presence of SDS in the DNPH-wash solution shifts the absorbance maximum of free DNPH to 350 nm and that of the protein (BSA_{ox}) carbonyl-DNPH hydrazone to 360 nm, but without changing their maximum absorbance value (Suppl. Fig. 3B). Therefore, DNPH absorbance extinction coefficient ($22,000\,M^{-1}\,cm^{-1}$) applies both to the protein carbonyl-DNPH hydrazone at 360 and 370 nm (in the presence and absence of SDS, respectively), and to free DNPH at 350 and 360 nm (in the presence and absence of SDS, respectively) for the pH range 2.3–7.0.

Unreacted DNPH wash procedure of the ntrDNPH assay is considerably more effective ($\geq 99.8\%$) than that of the stdDNPH assay. As shown in Fig. 1, the 3x-wash with EtOH:EA (1:1 v-v) used by the stdDNPH assay is much less effective than that of the ntrDNPH assay even when increased to 6x. Moreover, the stdDNPH assay was found to require at least four EtOH:EA wash steps for optimal (but not complete) removal of free DNPH (Fig. 1). The unreliability of the stdDNPH assay may be attributed to the following factors: (i) loss of sample protein during each wash, (ii) hydrolysis of the protein carbonyl-DNPH hydrazone by the acidic pH still existing inside the protein pellet (e.g., due to trapped TCA, and to protonated amino acid side chains), and (iii) ineffective removal of the non-specifically bound free DNPH from the ('molten globule-like' [53]) protein pellet. Factor (iii) is supported by the fact that when the protein pellet obtained by the 3rd EtOH:EA wash of the stdDNPH assay was subsequently washed once with EA:hexane (used by the ntrDNPH wash procedure), there was a ~ 30% decrease of its carbonyl value (enclosed by the oval shape in Fig. 1). This observation translates to a minimum 30% higher washing efficiency and greater reliability for the ntrDNPH vs the stdDNPH assay. The ~ 100% efficiency of the ntrDNPH assay in removing unreacted DNPH may be due to the following reasons: Wash of unreacted DNPH by EA:hexane is performed with the protein in solution, at protein denaturing conditions (by urea and SDS), and at pH 7.0. Here, DNPH is uncharged and more easily soluble in organic solvents than when positively charged (as $DNP-N_2H_5$ [54,55]) at the near zero pH used by the stdDNPH assay. This together with the hydrogen bonding potential of the $DNP-N_2H_5$ nitro groups (acting as proton acceptors in hydrogen bonding [88]) will promote DNPH non-specific binding to proteins, resulting in at least 30% overestimation of protein carbonyls by the stdDNPH assay. In contrast, non-specific binding of DNPH to proteins is not favored by the DNPH-wash solution of the ntrDNPH assay, because its neutral pH eliminates the positive charge of DNPH and makes it readily soluble in the EA:hexane solvent, while its components urea and SDS minimize DNPH's hydrogen bonding and hydrophobic interactions, respectively. Moreover, urea's presence in the assay's 1st step (where DNPH reacts with protein carbonyls), and also in the subsequent steps partially unfolds proteins by expansion of their hydrophobic core in combination with their solvation by water [89].

Overestimation by the stdDNPH assay of protein carbonyls by ~30% is considered minimum as it could vary depending on the experimenter's experience. Another factor contributing to the high statistical variability of the stdDNPH assay is the observation by the present study that the high concentration of DNPH (10 mM) used by this assay decreases the efficiency of its TCA-protein precipitation step by 50% (data not shown). Consequently, this results in high protein loss, and explains the need for the stdDNPH assay to use samples of high

protein concentration ($1–2\,mg\,ml^{-1}$). All these factors undermine the use of the DNPH extinction coefficient ($22,000\,M^{-1}\,cm^{-1}$) as sensitivity limit of the stdDNPH assay. In light of these considerations and in terms of minimum assay's protein quantity used, the cumulative and functional sensitivity of the ntrDNPH assay is 2600- and 2000-fold higher, respectively, compared to those of the stdDNPH assay (Table 1).

The ntrDNPH assay is also advantageous to another DNPH-based assay, which measures the protein carbonyl-DNPH hydrazone after solubilization in a medium at alkaline pH (in 1 M NaOH) without removing unreacted DNPH via TCA-protein precipitation [90]. Alkaline solubilization of protein carbonyl-DNPH hydrazones by this method (thereafter designated alkDNPH assay) is claimed to decrease the interference from the absorbance of unreacted DNPH at 370 nm by shifting the maximum absorbance wavelength of the hydrazone from 370 to 450 nm, thus allowing its direct quantification without prior DNPH washing. However, the alkDNPH assay has the following limitations:

(i) It uses the extinction coefficient $22,000\,M^{-1}\,cm^{-1}$ of the stdDNPDH assay as equal to the molar absorptivity at 450 nm and at pH 10 of the carbonyl-DNPH hydrazone formed by any oxidized protein by showing non-significant difference based only on the absorbance peak of BSA_{ox} carbonyl-DNPH hydrazone. However, this claim overlooks the fact that the extinction coefficients of the maximum absorption peaks of various hydrazones show (at alkaline pH) significant variation both in their maximum wavelengths (they vary from 428 to 508 nm) and absorption values (they differ up to ~ 50%) [60]. These facts render both the absorbance peak at 450 nm and the extinction coefficient $22,000\,M^{-1}\,cm^{-1}$ used by the alkDNPH assay unreliable for the accurate estimation of the average molar absorbance value among the various types of hydrazone carbonyls that may exist in an unknown protein sample. This argument applies even to the DNPH-hydrazone of BSA_{ox} used for the calibration of the alkDNPH assay, as suggested by the non-symmetrical shape of its absorption spectrum peak at 450 nm at alkaline pH [90]. Such peak shape indicates a sum peak absorbance of the individual absorbance peaks of the various hydrazone carbonyls formed within the same protein. This is supported by the symmetrical absorbance peak of the BSA_{ox} carbonyl-DNPH hydrazone at 370 nm shown in the same study [90]. This symmetry suggests derivation from a single peak, which is supported by the fact that the maximum absorbance of the various hydrazones formed on BSA_{ox} (at neutral pH) mostly peak at ~ 370 nm [60].

(ii) Performs the reaction of protein carbonyls with DNPH at similar conditions to those of the stdDNPH assay (10 mM in $0.5\,M\,H_3PO_4$, at pH ~0), but it uses 10 min incubation time instead of the minimum 60 min established for the stdDNPH assay and also verified in the present study (Suppl. Fig. 1).

(iii) Attempts to overcome the instability of the DNPH-hydrazones by controlling the time they are kept solublized in the alkalized (by NaOH) reaction medium at exactly 10 min before photometric reading. However, this is not practically feasible when testing many samples (as also cautioned by the same study).

(iv) Assumes that proteins are completely solubilized at the pH 14 of the employed solubilization medium, although it is known that alkaline solubilization of proteins is optimal within the pH interval 10.5–11.5, beyond which solubilization it decreases sharply [91].

(v) It does not eliminate completely the absorbance interference of free DNPH, given that the study admits that there is small interference from the absorption of DNPH [90]. However, DNPH interference was not quantified at the employed quite high concentration (10 mM) of this reagent.

3.5.2. DNA interference

It was assessed by the ntrDNPH assay both via the direct reaction of DNPH with the carbonyl groups of NMP's (Fig. 2) and with pure DNA.

NMP's were not tested with the stdDNPH assay because its TCA-precipitation step with proteins is not applicable to NMP's. In contrast, the ntrDNPH assay takes advantage of the fact that during the effective removal of unreacted DNPH by EA:hexane the NMP's in solution (at pH 7.0) do not partition into the EA:hexane phase. It was found that no direct reaction of DNPH with the NMP's takes place (Table 2). Additionally, the ntrDNPH assay was tested against the stdDNPH assay for DNA interference. It was found that pure DNA (up to 0.2 mg) produced a 17.5% interference (on average) with the stdDNPH assay. Therefore, the DNA interference on the stdDNPH assay previously reported [34,50,51] is non-specific, and can be attributed to the ineffective wash of unreacted DNPH shown by the present study. In contrast, the DNPH-washing procedure of the ntrDNPH assay produces a negligible interference (0.06%), verifying again that the free DNPH wash procedure is ~ 100% effective (Table 2; data in parentheses). The main reasons for the DNA non-interference on the ntrDNPH assay are two-fold (see notes in Table 2 for more detailed explanation): Firstly, DNA is insoluble in the corresponding protein solubilization solution used by the ntrDNPH assay. Thus, no removal of DNA from samples (by SS precipitation; see Supplement Section V) is required when using the ntrDNPH assay for protein carbonyl content quantification. Secondly, the wash procedure of the ntrDNPH assay for unreacted DNPH non-specifically bound to DNA is much more effective than the one employed by the stdDNPH assay. Indeed, the effectiveness by the ntrDNPH assay in removing DNPH from its associated DNA is ~290-fold higher than that by the stdDNPH assay (Table 2; data in brackets).

3.5.3. Applicability of the ntrDNPH assay on oxidized proteins and statistical precision

These were enhanced by the development of a new standardized procedure (outlined in Suppl. Fig. 4) for sample protein treatment with the following considerations in mind: A. To avoid possible carbonyl overestimation resulting from ROS-generated Cys sulfenic acid groups (eliminated via neutralization by DTT in place of the previously used ß-mercaptoethanol [62]), and from the presence of DNA (removed by SS precipitation). As previously explained, DNA does not interfer with the ntrDNPH assay. Nonetheless, the standardized procedure may be followed by any other protein carbonyl assay not only prone to DNA interference (all DNPH-based assays), but also to other known interferences and variability and reproducibility factors that are usually involved in protein carbonyl determination [3]. B. To extend the applicability of the assay (and any other protein carbonyl assay) to the **cytoplasmic/aqueous**, **membrane/lipid-bound**, and *chromosomal* protein fractions (see Supplement Section V) of any biological source.

The ntrDNPH assay was tested on various animal and plant protein sources as indicative examples for quantifying the carbonyl content of cytoplasmic protein fractions (Table 3), and also to test its detection limit for small size protein fractions such as histones. The ntrDNPH assay was also compared against the stdDNPH assay on human blood serum protein carbonyl content. Moreover, indicative comparisons were made human blood serum/plasma protein carbonyls determined

Table 3
Evaluation of the ntrDNPH assay for its applicability to indicative sample protein fractions.

Samples	cytoplasmic / histone[a] (nmoles mg^{-1} protein)
Cauliflower (*B. oleracea*)	9.9 / ND
Lettuce (L. *sativa*)	25.5 / ND
Rat brain stem	0.58 / ND
Rat intestine	0.72 / ND

[a] Values for cytoplasmic and histone protein fractions are shown by the numbers left and right to the separating slash, respectively. Values are averages from at least 5 independent measurements (SD, not shown, is < 10% of the average). ND for the carbonyl content of histones designates Not Detected (possibly because it falls below the assay's sensitivity limit).

Table 4
Human blood protein carbonyls.

Serum carbonyls[a]

	ntrDNPH assay	stdDNPH assay[a]
Males	1.09 ± 0.07[abc]	1.45 ± 0.07[ac]
Females	1.17 ± 0.09[abc]	1.53 ± 0.13[ac]

Serum/plasma (unspecified gender) indicative carbonyl values by other DNPH-based assays

photometric DNPH assays	ELISA assay
0.50 ± 0.15 (plasma) [69]	0.04 (serum[d]) [97]
1.2 ± 0.1 (plasma) [68]	
3.7 (serum) [67]	
0.5 (plasma) [41]	

[a] Values (in nmoles mg^{-1}) are averages (± SD) from 10 males and 10 females in middle age. Values by the stdDNPH assay performed in the present study.

[b] Comparison of the new assays for the same and between sex; there is no statistical difference for values statistically significant at p < 0.05.

[c] Comparison between males and females for the three assays; there is a ~ 30% statistical difference for the stdDNPH assay for values statistically significant at p < 0.05.

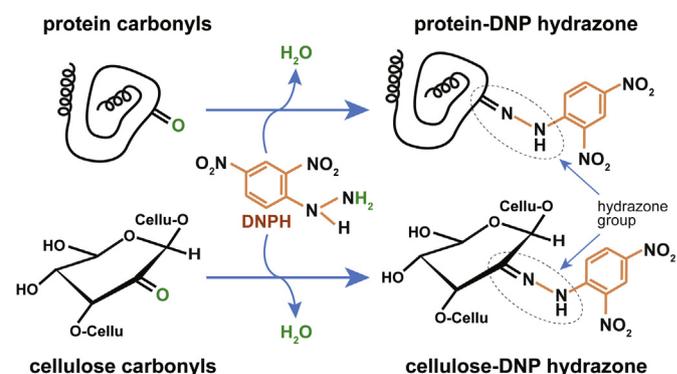[d] Serum was sampled before performing coronary heart surgery on the subject.

Fig. 3. Mechanism of the reaction of DNPH with protein and cell wall polysaccharide (cellulose) carbonyls upon application of the ntrDNPH assay.

Table 5
Determination of cell wall polysaccharide carbonyls by the ntrDNPH assay: Measurement of CMCellu$_{NaClO-carbnylated}$ and controls.

Samples	nmoles carbonyls mg^{-1} glucose equivalents	nmoles carbonyls mg^{-1} polysaccharide
NaClO-carbonylated CMCellu polysaccharide		
CMCellu$_{NaClO-carbnylated}$	8.0 ± 0.2	4 ± 0.1
CMCellu olysaccharide controls		
CMCellu$_{NaBH4-decarbonylated}$	0.0	0.0
CMCellu	2.2 ± 0.2	1.1 ± 0.1
CMCellu$_{NaClO\ treatment\ omitted}$	2.4 ± 0.2	1.2 ± 0.1

by indicative studies with other DNPH-based assays (Table 4). The ntrDNPH assay produced protein carbonyl values for human serum, which were ~ 30% lower those determined by the stdDNPH assay. However, greater variation in the carbonyl values determined by other DNPH-based assays on human blood serum/plasma is observed in comparison to those obtained by the ntrDNPH assay, which can be attributed to variability factors already mentioned in the *Introduction* section. Most notable is the ~28-fold lower serum carbonyl value obtained by the ELISA assay, which could be attributed to certain interfering factors stated elsewhere [41], combined with its high degree of variability [6].

The ntrDNPH assay was also analyzed for precision, that is, the evaluation of the closeness of individual measures of protein carbonyl

Table 6
Key advantages of the ntrDNPH assay over the stdDNPH assay.

The protein carbonyl-DNPH hydrazone is stable (for at least 2 days) due to its quantification at neutral pH
Assay high reproducibility and no DNA interference
Short assay reaction incubation time (30 min vs 1–2 hrs for the stdDNPH assay)
Protein re-quantification is not required after DNPH treatment for correspondence to the measured carbonyls in the initial protein sample quantity
Assay sensitivity is not limited by sample protein concentration (can use low as 1 µg), and its functional sensitivity is ~ 2000-fold higher than that of the ntrDNPH assay
No ineffective protein TCA precipitation steps are involved. This preserves acid soluble proteins in samples
Highly accurate assay (vs > 30% overestimation by the stdDNPH assay)
Quantifies cell wall polysaccharide carbonyls (plus aldehydic and ketone groups), but not the stdDNPH assay

when the assay is applied repeatedly to multiple aliquots of a single homogeneous volume of a biological sample and at different days. The *within-day* % coefficient variation for the ntrDNPH assay is < 4.0% and the variance of *intermediate precision* (or *between day repeatability*) is < 4.7%.

3.5.4. Application of the ntrDNPH assay on cell wall polysaccharide carbonyls

DNPH is expected to form the same kind of hydrazones on cell wall polysaccharides as with proteins (Fig. 3). The assay was applied on CMCellu polysaccharide samples artificially carbonylated by NaClO and verified against NaBH$_4$-decarbonylated and other relevant controls (Table 5). The ntrDNPH assay was adjusted for application to cell wall polysaccharides after the following minor modifications in its main procedure: Urea is omitted from the reaction of the polysaccharide sample with DNPH, and the reaction incubation time is extended from 30 to 60 min. The stdDNPH assay is not applicable to polysaccharide samples because they cannot be precipitated by TCA. It should be noted that the absorbance of the DNPH-CMCellu product formed by the ntrDNPH assay is as stable as that with proteins, and that the assay can use samples with polysaccharide concentration as high as 4 mg ml^{-1}. It was also found that the native carbonyl content of the CMCellu polysaccharide determined by the ntrDNPH assay (~ 1 µmole g^{-1}) is in agreement with the average concentration of carbonyls in plant polysaccharides (e.g. in cellulose it is in the range of few µmoles g^{-1} [79]). Since DNPH reacts with ketones [92] and aldehydes [62,93], the ntrDNPH assay is expected not to discriminate them from carbonyls as all theses groups are formed in oxidized polysaccharides [73]. It could possibly react with the polyaldehyde products of periodate-oxidized cellulose [94] and glycogen [95,96]. The ability of the ntrDNPH assay to quantify the oxidation of cell wall polysaccharides could pave the way on oxidative stress studies in plants, fungi, bacteria and lichens. These could uncover a new role for cell wall polysaccharides, that of an antioxidant wall barrier.

Concluding, the main advantages of the ntrDNPH assay over the stdDNPH assay are outlined in Table 6.

3.5.5. Caveats

Interference from reagents possibly present in various sample treatments can be eliminated by sample dilution and/or by testing the appropriate reagent blank. For example, DTT used in the standardized sample protein treatment (in Supplement Section V) does not interfer when present in the ntrDNPH assay reaction mixture at 25 mM.

Acknowledgments

Declarations of interest

None

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.redox.2018.04.010.

References

[1] E. Shacter, Quantification and significance of protein oxidation in biological samples, Drug Metab. Rev. 32 (2000) 307–326.
[2] I. Verrastro, S. Pasha, K.T. Jensen, A.R. Pitt, C.M. Spickett, Mass Spectrometry-based methods for identifying oxidized proteins in disease: advances and challenges, Biomolecules 5 (2015) 378–411.
[3] D. Weber, M.J. Davies, T. Grune, Determination of protein carbonyls in plasma, cell extracts, tissue homogenates, isolated proteins: focus on sample preparation and derivatization conditions, Redox Biol. 5 (2015) 367–380.
[4] D.A. Butterfield, E.R. Stadtman, Protein Oxidation Processes in Aging Brain, Elsevier, 1998.
[5] E.R. Stadtman, R.L. Levine, Free radical-mediated oxidation of free amino acids and amino acid residues in proteins, Amino Acids 25 (2003) 207–218.
[6] E. Augustyniak, A. Adam, K. Wojdyla, A. Rogowska-Wrzesinska, R. Willetts, A. Korkmaz, M. Atalay, D. Weber, T. Grune, C. Borsa, D. Gradinaru, R. Chand Bollineni, M. Fedorova, H.R. Griffiths, Validation of protein carbonyl measurement: a multi-centre study, Redox Biol. 4 (2015) 149–157.
[7] P.A. Grimsrud, H. Xie, T.J. Griffin, D.A. Bernlohr, Oxidative stress and covalent modification of protein with bioactive aldehydes, J. Biol. Chem. 283 (2008) 21837–21841.
[8] K. Doyle, F.A. Fitzpatrick, Redox signaling, alkylation (carbonylation) of conserved cysteines inactivates class I histone deacetylases 1, 2, and 3 and antagonizes their transcriptional repressor function, J. Biol. Chem. 285 (2010) 17417–17424.
[9] E. Shacter, J.A. Williams, M. Lim, R.L. Levine, Differential susceptibility of plasma proteins to oxidative modification: examination by western blot immunoassay, Free Radic. Biol. Med. 17 (1994) 429–437.
[10] J.R. Requena, R.L. Levine, E.R. Stadtman, Recent advances in the analysis of oxidized proteins, Amino Acids 25 (2003) 221–226.
[11] J. Liggins, A.J. Furth, Role of protein-bound carbonyl groups in the formation of advanced glycation endproducts, Biochim. Biophys. Acta 1361 (1997) 123–130.
[12] I. Dalle-Donne, D. Giustarini, R. Colombo, R. Rossi, A. Milzani, Protein carbonylation in human diseases, Trends Mol. Med. 9 (2003) 169–176.
[13] T. Nyström, Role of oxidative carbonylation in protein quality control and senescence, EMBO J. 24 (2005) 1311–1317.
[14] Y.J. Suzuki, M. Carini, D.A. Butterfield, Protein carbonylation, Antioxid. Redox Signal. 12 (2010) 323–325.
[15] I. Rahman, A.A. van Schadewijk, A.J. Crowther, P.S. Hiemstra, J. Stolk, W. MacNee, W.I. De Boer, 4-Hydroxy-2-nonenal, a specific lipid peroxidation product, is elevated in lungs of patients with chronic obstructive pulmonary disease, Am. J. Respir. Crit. Care Med. 166 (2002) 490–495.
[16] R.L. Levine, Carbonyl modified proteins in cellular regulation, aging, and disease, Free Rad. Biol. Med. 32 (2002) 790–796.
[17] T. Grune, K. Merker, G. Sandig, K.J. Davies, Selective degradation of oxidatively modified protein substrates by the proteasome, Biochem. Biophys. Res. Commun. 305 (2003) 709–718.
[18] D.A. Bota, K.J. Davies, Lon protease preferentially degrades oxidized mitochondrial aconitase by an ATP-stimulated mechanism, Nat. Cell Biol. 4 (2002) 674–680.
[19] I. Dalle-Donne, G. Aldini, M. Carini, R. Colombo, R. Rossi, A. Milzani, Protein carbonylation, cellular dysfunction, and disease progression, J. Cell Mol. Med. 10 (2006) 389–406.
[20] T. Grune, T. Jung, K. Merker, K.J. Davies, Decreased proteolysis caused by protein aggregates, inclusion bodies, plaques, lipofuscin, ceroid, and 'aggresomes' during oxidative stress, aging, and disease, Int. J. Biochem. Cell Biol. 36 (2004) 2519–2530.
[21] S.R. Powell, P. Wang, A. Divald, S. Teichberg, V. Haridas, T.W. McCloskey, K.J. Davies, H. Katzeff, Aggregates of oxidized proteins (lipofuscin) induce apoptosis through proteasome inhibition and dysregulation of proapoptotic proteins, Free Radic. Biol. Med. 38 (2005) 1093–1101.
[22] T. Jung, A. Höhn, T. Grune, The proteasome and the degradation of oxidized proteins: Part III—redox regulation of the proteasomal system, Redox Biol. 2 (2014) 388–394.
[23] T. Ishii, T. Sakurai, H. Usami, K. Uchida, Oxidative modification of proteasome: identification of an oxidation-sensitive subunit in 26 S proteasome, Biochemistry 44 (2005) 13893–13901.
[24] I. Dalle-Donne, R. Rossi, D. Giustarini, A. Milzani, R. Colombo, Protein carbonyl groups as biomarkers of oxidative stress, Clin. Chim. Acta 329 (2003) 23–38.

[25] E. Shacter, Protein oxidative damage, Methods Enzymol. 319 (2000) 428–436.
[26] A.G. Madian, A.D. Myracle, N. Diaz-Maldonado, N.S. Rochelle, E.M. Janle, F.E. Regnier, Differential carbonylation of proteins as a function of *in vivo* oxidative stress, J. Proteome Res. 10 (2011) 3959–3972.
[27] I.M. Moller, A. Rogowska-Wrzesinska, R.S. Rao, Protein carbonylation and metal-catalyzed protein oxidation in a cellular perspective, J. Proteom. 74 (2011) 2228–2242.
[28] L.-J. Yan, M.J. Forster, Chemical probes for analysis of carbonylated proteins: a review, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 879 (17–18) (2011) 1308–1315.
[29] M.A. Baraibar, R. Ladouce, B. Friguet, Proteomic quantification and identification of carbonylated proteins upon oxidative stress and during cellular aging, J. Proteom. 92 (2013) 63–70.
[30] E. Cabiscol, J. Tamarit, J. Ros, Protein carbonylation: proteomics, specificity and relevance to aging, Mass Spectrom. Rev. 33 (2014) 21–48.
[31] M. Fedorova, R.C. Bollineni, R. Hoffmann, Protein carbonylation as a major hall-mark of oxidative damage: update of analytical strategies, Mass Spectrom. Rev. 33 (2014) 79–97.
[32] Z.A. Reznick, L. Packer, Oxidative damage to proteins: spectrophotometric method for carbonyl assay, in: N.J. Abelson, I.M. Simon (Eds.), Methods in Enzymology, Academic Press, New York, 1994, pp. 357–363.
[33] R.L. Levine, J.A. Williams, E.R. Stadtman, E. Shacter, Carbonyl assays for de-termination of oxidatively modified proteins, Methods Enzymol. 233 (1994) 346–357.
[34] R.L. Levine, D. Garland, N.C. Oliver, A. Amici, I. Climent, G.-A. Lenz, W.-B. Ahn, S. Shaltiel, R.E. Stadtman, Determination of carbonyl content in oxidatively mod-ified proteins, Methods Enzymol. 186 (1990) 464–479.
[35] L.J. Yan, W.C. Orr, R.S. Sohal, Identification of oxidized proteins based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, immunochemical detection, isoelectric focusing, and microsequencing, Anal. Biochem. 263 (1998) 67–71.
[36] H. Buss, P.T. Chan, B.K. Sluis, M.N. Domigan, C.C. Winterbourn, Protein carbonyl measurement by a sensitive ELISA method, Free Radic. Biol. Med. 23 (1997) 361–366.
[37] H. Buss, C.C. Winterbourn, Oxidative stress biomarkers and antioxidant protocols: protein carbonyl measurement by ELISA, in: D. Armstrong (Ed.), Methods in Molecular Biology, Humana Press Inc., New Jersey, 2002, pp. 123–128.
[38] D.H. Alamdari, E. Kostidou, K. Paletas, M. Sarigianni, A.G. Konstas, A. Karapiperidou, G. Koliakos, High sensitivity enzyme-linked immunosorbent assay (ELISA) method for measuring protein carbonyl in samples with low amounts of protein, Free Radic. Biol. Med. 39 (2005) 1362–1367.
[39] Y.J. Lee, E. Shacter, Role of carbohydrates in oxidative modification of fibrinogen and other plasma proteins, Arch. Biochem. Biophys. 321 (1995) 175–181.
[40] I. Dalle-Donne, R. Rossi, D. Giustarini, R. Colombo, A. Milzani, Actin S-glutathio-nylation: evidence against a thiol-disulfide exchange mechanism, Free Radic. Biol. Med. 35 (2003) 1185–1193.
[41] J.G. Mohanty, S. Bhamidipaty, M.K. Evans, J.M. Rifkind, A fluorimetric semi-mi-croplate format assay of protein carbonyls in blood plasma, Anal. Biochem. 400 (2010) 289–294.
[42] M.A. Smith, L.M. Sayre, V.E. Anderson, P.L. Harris, M.F. Beal, N. Kowall, G. Perry, Cytochemical demonstration of oxidative damage in Alzheimer Disease by im-munochemical enhancement of the carbonyl reaction with 2,4-dinitrophenylhy-drazine, J. Histochem. Cytochem. 46 (1998) 731–735.
[43] T. Jung, M. Engels, B. Kaiser, D. Poppek, T. Grune, Intracellular distribution of oxidized proteins and proteasome in HT22 cells during oxidative stress, Free Radic. Biol. Med. 40 (2006) 1303–1312.
[44] T. Jung, M. Engels, L.O. Klotz, K.D. Kröncke, T. Grune, Nitrotyrosine and protein carbonyls are equally distributed in HT22 cells after nitrosative stress, Free Radic. Biol. Med. 42 (2007) 773–786.
[45] T. Jung, A. Höhn, B. Catalgol, T. Grune, Age-related differences in oxidative pro-tein-damage in young and senescent fibroblasts, Arch. Biochem. Biophys. 483 (2009) 127–135.
[46] N.C. Purdel, D. Margina, M. Ilie, Current methods used in the protein carbonyl assay, Annu. Res. Rev. Biol. 4 (2014) 2015–2026.
[47] A. Rogowska-Wrzesinska, O. Nedić, C.P. Baron, H.R. Griffiths, Analysis of protein oxidation – pitfalls and promise in commonly used methods, Free Radic. Res. 48 (2014) 1145–1162.
[48] R.L. Levine, N. Wehr, J.A. Williams, E.R. Stadtman, E. Shacter, Determination of carbonyl groups in oxidized proteins, in: S.M. Keyse (Ed.), Stress Response: Methods and Protocols, Humana Press, 2000, pp. 15–24.
[49] V. Vemula, Z. Ni, M. Fedorova, Fluorescence labeling of carbonylated lipids and proteins in cells using coumarin-hydrazide, Redox Biol. 5 (2015) 195–204.
[50] G. Cao, R.G. Cutler, Protein oxidation and aging. I. Difficulties in measuring re-active protein carbonyls in tissues using 2,4-dinitrophenylhydrazine, Arch. Biochem. Biophys. 320 (1995) 106–114.
[51] S. Luo, N.B. Wehr, Protein carbonylation: avoiding pitfalls in the 2,4-dini-trophenylhydrazine assay, Redox Rep. 14 (2009) 159–166.
[52] G.D. Johnson, Correlation of color and constitution. I. 2,4-dinitrophenylhydrazones, J. Am. Chem. Soc. 75 (1953) 2720–2723.
[53] D. Rajalingam, C. Loftis, J.J. Xu, T.K. Kumar, Trichloroacetic acid-induced protein precipitation involves the reversible association of a stable partially structured in-termediate, Protein Sci. 18 (2009) 980–993.
[54] P. Bernheim, A. Dobos, A.M.M. Doherty, N. Haine, G. Stedman, Kinetics and me-chanism of the reaction of nitrous acid with 2,4-dinitrophenylhydrazine, J. Chem. Soc. Perkin Trans. 2 (1996) 275–280.
[55] R. Kruszynski, Intermolecular interactions in 2,4-dinitrophenylhydrazine hydro-chloride hydrate: x-ray structural and quantum mechanical study, Cent. Eur. J.

Chem. 6 (2008) 542–548.
[56] E. Fic, S. Kedracka-Krok, U. Jankowska, A. Pirog, M. Dziedzicka-Wasylewska, Comparison of protein precipitation methods for various rat brain structures prior to proteomic analysis, Electrophoresis 31 (2010) 3573–3579.
[57] P.D. Senter, D.L. Meyer, Monoclonal antibody drug conjugates for cancer therapy, in: V. Stella, R. Borchardt, M. Hageman, R. Oliyai, H. Maag, J. Tilley (Eds.), Prodrugs: Challenges and Rewards, Springer-Verlag, New York, 2007, p. 510.
[58] J. Kalia, R.T. Raines, Hydrolytic stability of hydrazones and oximes, Angew. Chem. Int. Ed. Engl. 47 (2008) 7523–7526.
[59] S. Uchiyama, Y. Inaba, N. Kunugita, Derivatization of carbonyl compounds with 2,4-dinitrophenylhydrazine and their subsequent determination by high-perfor-mance liquid chromatography, J. Chromatogr. B 879 (2011) 1282–1289.
[60] L.A. Jones, J.C. Holmes, R.B. Seligman, Spectrophotometric studies of some 2,4-dinitrophenylhydrazones, Anal. Chem. 28 (1956) 191–198.
[61] R. Fields, H.B. Dixon, Micro method for determination of reactive carbonyl groups in proteins and peptides, using 2,4-dinitrophenylhydrazine, Biochem. J. 121 (1971) 587–589.
[62] I. Dalle-Donne, M. Carini, M. Orioli, G. Vistoli, L. Regazzoni, G. Colombo, R. Rossi, A. Milzani, G. Aldini, Protein carbonylation: 2,4-dinitrophenylhydrazine reacts with both aldehydes/ketones and sulfenic acids, Free Radic. Biol. Med. 46 (2009) 1411–1419.
[63] R.L. Levine, Oxidative modification of glutamine synthetase. I. inactivation is due to loss of one histidine residue, J. Biol. Chem. 258 (1983) 11823–11827.
[64] P.C. Burcham, Modified protein carbonyl assay detects oxidised membrane pro-teins: a new tool for assessing drug- and chemically-induced oxidative cell injury, J. Pharmacol. Toxicol. Methods 56 (2007) 18–22.
[65] M.E. Murphy, J.P. Kehrer, Oxidation state of tissue thiol groups and content of protein carbonyl groups in chickens with inherited muscular dystrophy, Biochem. J. 260 (1989) 359–364.
[66] C.N. Oliver, B.W. Ahn, E.J. Moerman, S. Goldstein, E.R. Stadtman, Age-related changes in oxidized proteins, J. Biol. Chem. 262 (1987) 5488–5491.
[67] N. De Maria, A. Colantoni, S. Fagiuoli, G.J. Liu, B.K. Rogers, F. Farinati, D.H. Van Thiel, R.A. Floyd, Association between reactive oxygen species and disease activity in chronic hepatitis C, Free Radic. Biol. Med. 21 (1996) 291–295.
[68] Y. Jung, K. Chay, D. Song, S. Yang, M. Lee, B. Ahn, Protein carbonyl formation in blood plasma by cephalosporins, Arch. Biochem. Biophys. 345 (1997) 311–317.
[69] J. Mimić-Oka, T. Simić, M. Plješa, N. Stupar, S. Turković, Oxidative modifications of plasma proteins in different stages of chronic renal failure, Facta Univ.: Med. Biol. 8 (2001) 1–5.
[70] A.R. Chaudhuri, E.M. de Waal, A. Pierce, H. Van Remmen, W.F. Ward, A. Richardson, Detection of protein carbonyls in aging liver tissue: a fluorescence-based proteomic approach, Mech. Ageing Dev. 127 (2006) 849–861.
[71] T.J. Caperna, A.E. Shannon, L.A. Blomberg, W.M. Garrett, T.G. Ramsay, Identification of protein carbonyls in serum of the fetal and neonatal pig, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 156 (2010) 189–196.
[72] K.H. Caffall, D. Mohnen, The structure, function, and biosynthesis of plant cell wall pectic polysaccharides, Carbohydr. Res. 344 (2009) (1879-900).
[73] J. Łojewska, P. Miśkowieca, T. Łojewsk, L.M. Proniewicz, Cellulose oxidative and hydrolytic degradation: In situ FTIR approach, Polym. Degrad. Stab. 88 (2005) 512–520.
[74] F. Pouyet, C. Chirat, A. Potthast, D. Lachenal, Formation of carbonyl groups on cellulose during ozone treatment of pulp: consequences for pulp bleaching, Carbohydr. Polym. 109 (2014) 85–91.
[75] J. Perrin, F. Pouyet, C. Chirat, D. Lachenal, Formation of carbonyl and carboxyl groups on cellulosic pulps: effect on alkali resistance and ageing, BioResources 9 (4) (2014) 7299–7310.
[76] H. Sihtola, L. Neimo, R. Sumiala, Classification of carbonyl groups in cellulose on the basis of their reaction rates at oximation, J. Polym. Sci.: Part C 2 (1963) 289–310.
[77] M. Lewin, J.A. Epstein, Functional groups and degradation of cotton oxidized by hypochlorite, J. Polym. Sci. 58 (166) (1962) 1023–1037.
[78] Tappi Test Methods 1989-1999, T430 Tappi Press, Atlanta, 1998.
[79] J. Röhrling, A. Potthast, T. Rosenau, H. Sixta, P. Kosma, Determination of carbonyl functions in cellulosic substrates, Lenzing. Ber. 81 (2002) 89–97.
[80] J. Röhrling, A. Potthast, T. Rosenau, T. Lange, G. Ebner, H. Sixta, P. Kosma, A novel method for the determination of carbonyl groups in cellulosics by fluorescence la-beling. 2. Validting and applications, Biomacromolecules 3 (5) (2002) 969–975.
[81] J. Röhrling, A. Potthast, T. Rosenau, T. Lange, G. Ebner, H. Sixta, P. Kosma, A novel method for the determination of carbonyl groups in cellulosics by fluorescence la-beling. 1. Method development, Biomacromolecules 3 (5) (2002) 959–968.
[82] A. Potthast, J. Röhrling, T. Rosenau, A. Borgards, H. Sixta, P. Kosma, A novel method for the determination of carbonyl groups in cellulosics by fluorescence la-beling. 3. monitoring oxidative processes, Biomacromolecules 4 (3) (2003) 743–749.
[83] M. Kostic, A. Potthast, T. Rosenau, P. Kosma, H. Sixta, A novelapproach to de-termination of carbonyl groups in DMAc/LiCl-insoluble pulps by fluorescence la-beling, Cellulose 13 (2006) 429–435.
[84] M.J. Cavaluzzi, P.N. Borer, U.V. Revised, extinction coefficients for nucleoside-5′-monophosphates and unpaired DNA and RNA, Nucleic Acids Res. 32 (2004) e13.
[85] M. DuBois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Colorimetric method for determination of sugars and related substances, Anal. Chem. 28 (1956) 350–356.
[86] M. Kahlweit, R. Strey, P. Firman, D. Haase, J. Jen, R. Schomäcker, General patterns of the phase behavior of mixtures of H_2O, nonpolar solvents, amphiphiles, and electrolytes. 1, Langmuir 4 (1988) 499–511.
[87] L.H. Stockwin, M.A. Bumke, S.X. Yu, S.P. Webb, J.R. Collins, M.G. Hollingshead,

D.L. Newton, Proteomic analysis identifies oxidative stress induction by adaphostin, Clin. Cancer Res. 13 (2007) 3667–3681.

[88] W.F. Baitinger, P. v.R. Schleyer, T.S.S.R. Murty, L. Robinson, Nitro groups as proton acceptors in hydrogen bonding, Tetrahedron 20 (1964) 1635–1647.

[89] B.J. Bennion, V. Daggett, The molecular basis for the chemical denaturation of proteins by urea, Proc. Nat. Acad. Sci. USA 100 (2003) 5143–5147.

[90] C.S. Mesquita, R. Oliveira, F. Bento, D. Geraldo, J.V. Rodrigues, J.C. Marcos, Simplified 2,4-dinitrophenylhydrazine spectrophotometric assay for quantification of carbonyls in oxidized proteins, Anal. Biochem. 458 (2014) 69–71.

[91] Y. Hrynets, D.A. Omana, Y. Xu, M. Betti, Comparative study on the effect of acid- and alkaline-aided extractions on mechanically separated turkey meat (MSTM): chemical characteristics of recovered proteins, Process Biochem. 46 (2011) 335–343.

[92] G.E. Anthon, D.M. Barrett, Modified method for the determination of pyruvic acid with dinitrophenylhydrazine in the assessment of onion pungency, J. Sci. Food Agric. 83 (2003) 1210–1213.

[93] O.L. Brady, G.V. Elsmie, The use of 2:4-dinitrophenylhydrazine as a reagent for aldehydes and ketones, Analyst 51 (599) (1926) 77–78.

[94] U.J. Kim, S. Kuga, M. Wada, T. Okano, T. Kondo, Periodate oxidation of crystalline cellulose, Biomacromolecules 1 (3) (2000) 488–492.

[95] M. Abdel-Akher, F. Smith, Oxidation of glycogen with periodic acid, J. Am. Chem. Soc. 81 (7) (1959) 1718–1721.

[96] M. Bertoldo, G. Zampano, L. Suffner, E. Liberatic, F. Ciardelli, Oxidation of glycogen "molecular nanoparticles" by periodate, Polym. Chem. 4 (2013) 653–661.

[97] U. Pantke, T. Volk, M. Schmutzler, W.J. Kox, N. Sitte, G. T, Oxidized proteins as a marker of oxidative stress during coronary heart surgery, Free Radic. Biol. Med. 27 (1999) 1080–1086.