# Combined proteomics and structural analyes of hair keratins as a reliable read-out of hair shafts damage/protection

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## Abstract

*Introduction***:** Hair damage induced by stress factors, such as UV irradiation, fine dust pollution, heat-styling tools could tradionnaly be assessed with spectroscopic and microscopic imaging or hair physical characteristics tests (shine, strength, suppleness, etc. ). However, these approches are not suitable to evaluate hair damage at daily equivalent stress doses precluding the evaluation of the efficacy of novel products or active compounds in conditions that are closed to consumer's uses.

*Methods:* Specific *ex-vivo* experimental models of daily above mentionned stress, reproducing closed to real life exposures, have been developped using hair tresses and combined to proteomics approaches for the quantification of carbonylated (oxidized) proteins and structural analyses of cuticle and cortex. The absolute quantifcation of hair protein oxidation (carbonylation) has been achieved by differential in gel electrophoresis, while the vizualisation and semi-quantification on both cuticle and cortex oxidative damage has been assessed by *in situ* detection.

*Results:* A significant dose-dependent increase in protein oxidation (carbonylation), related to both both keratins, and keratins associated proteins, was observed upon exposures of hair to stress factors, starting from daily stress exposure equivalent doses. However, protein carbonylation was prevented in the presence of anti-oxidant compounds and, interestingly, the application of hair care product treatment on damaged hair tresses resulted in decrease in protein oxidation.

*Discussion and Conclusion:* Taking together these results lead to a new generations of high-sensitivity and wide-range tools for the assessment of hair protection and repair from daily equivalent stress-induced damages.

**Keywords:** Hair, Oxidative damage, Exposome

## Introduction

Hair fibers physico-chemical properties are the direct result of their structural elements arrangement, most significantly proteins which constitute almost 95% of hair fiber weight (1,2). However, while hair grows and while the fiber ages over time, both the internal structure of the cortex as well as the integrity of the external cuticle may become damaged due to cumulative environmental effects, such as exposition to daily solar ultraviolet light or urban pollutants (3); as well as some weathering or chemical products used for decoloring-coloring, permanent waving, chemical relaxing (lanthionization) (4). Since hair fibers are not metabolically active, they do not have any active protection/reparation mechanisms for counteracting oxidative damage. It is their intrinsic structure which is in charge of its preservation against external insults. The maintenance of hair fiber structural intactness is essential for consumers' perception of healthy and shiny hair.

Accumulation of damaged macromolecules, including oxidatively damaged (carbonylated) proteins, is a hallmark of oxidative stress and cellular and organism aging (5). This accumulation has been viewed as the combined result of increased production of reactive oxygen species (ROS) and other toxic compounds coming from both cellular metabolism and external factors (UV irradiation, cigarette smoke) as well as the failure of protein maintenance systems (i.e. degradation and repair) with age. Protein carbonylation is particularly detrimental as the resulting damages can render carbonylated proteins inactive and lead to cellular functional abnormalities (6) Protein carbonylation is induced either directly by ROS or indirectly by reactions with secondary products of oxidative stress such as reactive aldehydes as 4-hydroxy-2-nonenal (4-HNE) and malondialdehyde (MDA). These aldehydes are produced by the peroxidation of polyunsaturated fatty acids of membrane lipids and can react with protein to form covalent Michael adducts with the side chains of cysteine, histidine and lysine residues (5). Proteins can also be carbonylated through the reaction of arginine and lysine side chains with reducing sugars or reactive dicarbonyl compounds such as glyoxal and methylglyoxal, based on the Maillard reaction (7). Formation of these lipid peroxidation and glycation adducts are found in many tissues and believed to contribute to aging and variety of age-associated diseases.

Although "anti-pollution" and "anti-oxidant" claims for hair products are more and more represented in the market, the anti-oxidant protection of hair proteins is not yet a read-out of choice for efficacy testing, due to the absence of sensible and reliable tools for analysis. In this study, we will show that since hair cannot be restored after deterioration, the protection of hair proteins from carbonylation by preventing external factors from affecting the hair could be an efficient approach for hair protection against urban stress.

**Material & Methods**

*Hair samples Preparation*. Hair tresses (Light Brown hair from Caucasian donors) used in this study were free from chemical treatment and significant physical damage. The hair strands were washed for 1hour with 0.1% of Triton X-100, rinsed with water and dried naturally in ambient conditions. The hairs were stocked in dark environment at room temperature until analysis.

*UV irradiation*. Hair strands were gently cut into small pieces (1cm) and incubated with agitation into water or anti-oxidant treatment for one hour. Water was absorbed on paper towel, and dry hair strands were disposed into small petri dish. UV-A irradiation (365nm) was performed using Oxiproteomics irradiation chamber at 3 doses (Dose 1: 1.8kJ, Dose 2:3.2kJ, Dose 3: 5.6kJ).

*Exposure to Urban Pollution*. Oxiproteomics internal urban pollution stress is a combination of particulate matter (particulate matter HAP from European Reference Material CZ100) deposition and UV exposure. Hair strands were gently cut into small pieces (1cm) and incubated with agitation into water or anti-oxidant treatment for one hour and into a particulate matter solution for 15min. UV exposure was simulated using Oxiproteomics UV-A (365nm) irradiation chamber (5.6 kJ).

*Carbonylated proteins analy*sis. The proteins were extracted from 10 mg of hair samples by shaking into an extraction buffer containing chaotropic agents, detergents and reductants. The extracts were clarified by centrifugation. The concentration of proteins was determined with the Bradford method using calibrated BSA as standard.. Carbonylated proteins were labeling using functionalized fluorescence probes specific for carbonyls groups and proteins were resolved by high-resolution electrophoresis (8). Total proteins were post-stained with SyproRubyTM protein gel stain. Image acquisition for carbonylated and total proteins was performed using the Ettan® DIGE imager (GE Healthcare). Image processing and densitometric analysis of protein bands was performed using ImageJ. Statistical analyses were accomplished using GraphPad Software (La Jolla, California, USA). Carbonylated protein signal was normalized by total protein signal for each sample in order to obtain a Carbonyl Score.

*In situ* detection of carbonylated proteins. After stress exposure, carbonylated proteins were labeled in situ on hair strands with a specific fluorescent probe. Hair images were collected by Epi-Fluorescence Microscopy (LEICA DMi8 – 63X) and were elaborated by ImageJ software.
Identical conditions of acquisition time, exposure, focus and resolution were performed for all experimental groups.

*Anti-oxidant treatment*. Hair strands were gently cut into small pieces (1cm) and were incubated with agitation one hour into a solution of 0.5% and 2% of N-acetyl-cysteine.

## Results & Discussion

### UV-A irradiation induces protein carbonylation in a dose-dependent manner

At a molecular level, hair damage is usually determined by evaluating hair proteins total levels. Protein degradation is often quantified via tryptophan decomposition, which only study the modification of one amino acid, or Bradford assay whose results are influenced by melanin content. Both methods present low sensitivity and selectivity.

Protein oxidation in hair has been previously studied only in sulfur containing amino-acids (methionine and cysteine) by labeling their SH group with probes such as N-(9-acridinyl)maleimide and N-(7-dimethyl-amino-4-methylcoumarinyl) maleimide. However, during oxidation the SH group and the S-S bond are changed to cysteic acid (SO3H), which results into hair damage. As cysteic acid does not react with the above-mentioned dyes, it is assumed that the damage level estimation, obtained by these approaches using the SH group as a biomarker for hair damage, does not accurately reflect the reality. In this study, we have developed proteomics approaches for hair damage evaluation by detecting and quantifying carbonylated proteins in hair tresses using differential in gel electrophoresis.

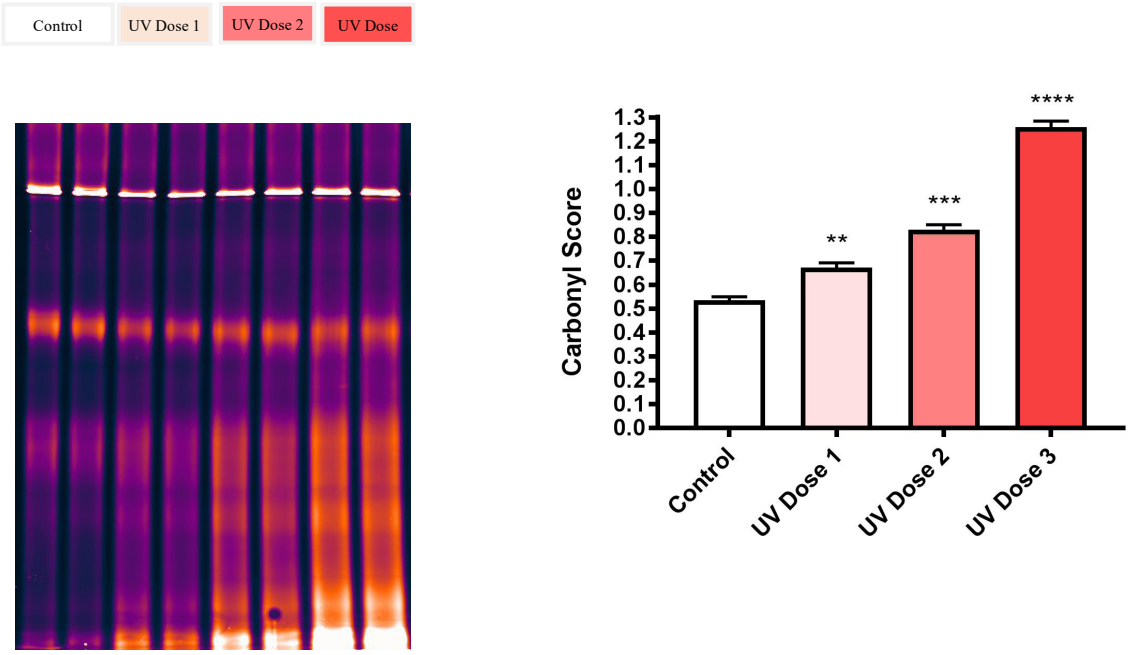


**Figure 1. Protein carbonylation upon UV-irradiation of hair shafts:** *Left panel*: Carbonylated proteins on each sample without normalization by total protein signal represented as a continuous intensity histogram. The maximum intensity levels are in white and lower levels in deep purple/black. *Right panel*: Quantification of carbonylated proteins by experimental group after normalization by total protein signal, bars represents the mean of the three replicates ± SD. Statistical analysis (One way ANOVA): *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

UV irradiation is known to inflict irreversible oxidative damages on hairs (9). We used this model to validate the use of protein carbonylation as hair damage biomarker. As depicted in Figure 1, both keratins, and keratins associated proteins were carbonylated upon UV irradiation. The level of hair carbonylation increases significantly in a dose-dependent manner upon UV irradiation making it a quantitative readout for hair damage.

**Innovative urban pollution ex vivo models for hair care efficacy testing**

In this study, we have developed an innovative and reliable urban pollution model, which corresponds to a combination of UV irradiation and particulate matter on hair tresses.
The application of this innovative urban pollution model shows an increase of hair protein carbonylation compare to the control (Figure 2). This model inflicts irreversible damages to hair strands and could be used as pollution simulation for novel product efficacy testing assessment.

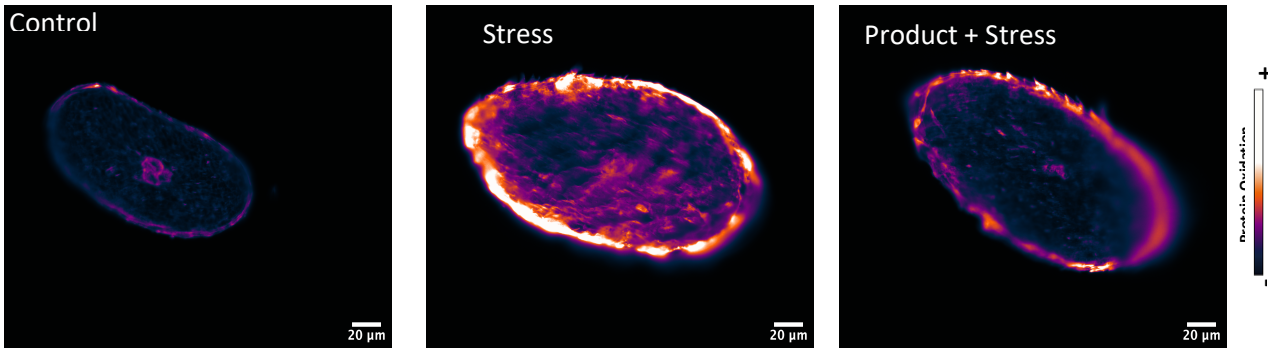


***Figure 2. Structural visualization of proein carbonylation on* hair shafts cross-sections.** The fluorescence emission signal was detected at specific wavelengths (excitation and emission). Increased carbonylation levels are evidenced in both cortex and cuticle regions upon stress (urban pollution; particulate matters PM and UV-A irradiation). The presence of an anti-oxidant product, applied before stress exposure, protected both cortex and cuticle from the stress-induced increase of carbonylation.

**Protein oxidation induced by urban pollutants is prevented by anti-oxidants**

The prevention of irreversible molecular damages such as protein carbonylation can preserve hair superficial aspect and physical properties. By applying the well-known anti-oxidant N-acetyl-cystein on hair strands previous to stress we could prevent the occurrence of protein carbonylation upon UV and Urban pollution stress both in the cuticle and the cortex (Figure 3). Thus, the accumulation of carbonylated proteins can be avoided or reduced by cosmetics approaches.

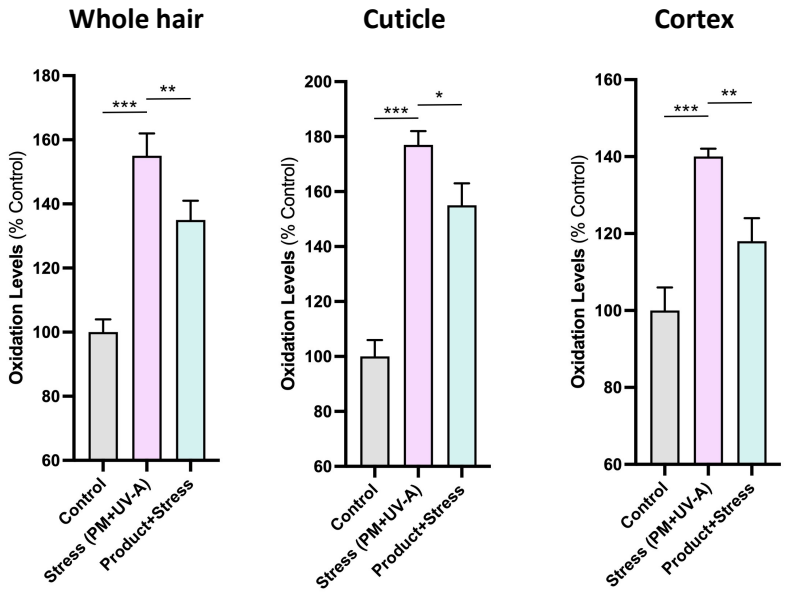


Figure 3. Quantification of carbonylation levels on hair cross-sections (whole hair; cuticle; cortex). The fluorescence emission signal intensity was integrated on the surface of evaluation. A significant increase in carbonylation levels is observed upon stress on both cortex and cuticle. The presence of an anti-oxidant product shown a significant protection against stress-induced increase of carbonylation levels. Statistics : ANOVA and Dunnett's post-hoc test multi-comparisons (vs. stress; *$p<0.05$, **$p<0.01$; ***$p<0.001$).

## Conclusions

Taken together, our results prove that ex vivo models coupled to reliable tools of analysis represent a valuable tool for hair care products and ingredients evaluation. The availability of sensible tools of analysis gives the possibility to test the protection from daily equivalent stress-induced damages.

**Conflict of Interest Statement**. NONE.

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