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Original article

# Benchmarking of protein carbonylation analysis in *Caenorhabditis elegans*: specific considerations and general advice

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ABSTRACT

Oxidative stress has been extensively studied due to its correlation with cellular disorders and aging. In proteins, one biomarker of oxidative stress is the presence of carbonyl groups, such as aldehyde and ketone, in specific amino acid side chains such as lysine, proline, arginine and threonine, so-called protein carbonylation (PC). PC study is now a growing field in general and medical science since PC accumulation is associated with various pathologies and disorders.

At present, enzyme-linked immunosorbent assays (ELISA) seem to be the most robust method of quantifying the presence of carbonyl groups in proteins, despite having some recognised caveats. In parallel, gel-based approaches present cross-comparison difficulties, along with other technical problems. As generic PC analyses still suffer from poor homogeneity, leading to cross-data analysis difficulties and poor results overlap, the need for harmonisation in the field of carbonyl detection is now widely accepted. This study aims to highlight some of the technical challenges in proteomic gel-based multiplexing experiments when dealing with PC in difficult samples like those from *Caenorhabditis elegans*, from protein extraction to carbonyl detection.

We demonstrate that some critical technical parameters, such as labelling time, probe concentration, and total and carbonylated protein recovery rates, should be re-addressed in a sample-specific way. We also defined a procedure to cost-effectively adapt CyDye™-hydrazide-based protocols to specific samples, especially when the experimental interest is focused on studying differences between stimulating conditions with a maximised signal-to-noise ratio. Moreover, we have improved an already-existing powerful solubilisation buffer, making it potentially useful for hard-to-solubilise protein pellets. Lastly, the depicted methodology exemplifies a simple way of normalising carbonyl-related signal to total protein in SDS-PAGE multiplexing experiments. Within that scope, we also proposed a simple way to quantify carbonyl groups by on-gel spotting diluted dye-containing labelling buffer. Proof of the robustness of the procedure was also highlighted by the high linear correlation between the level of carbonyls and the ultraviolet exposure duration of whole worms ($R^2 = 0.993$).

Altogether, these results will help to standardise existing protocols in the growing field of proteomic carbonylation studies.



## 1. Introduction

It is well established today that oxidative stress is associated with cellular disorders and aging [1–6]. Oxidative stress is mainly a consequence of increased production of reactive oxygen species (ROS) which takes place when detoxification mechanisms are overflowed or impaired [7]. ROS are represented by highly reactive molecules such as hydrogen peroxide ($H_2O_2$), the hydroxyl radical ($\cdot OH$) and the superoxide anion ($O_2 \cdot^-$), which can be generated through leakage of the mitochondrial electron transport chain or by enzymatic activities, such as that of NADPH oxidase [8–11]. ROS can react in many ways with almost all cell components such as DNA, lipids, carbohydrates and proteins [7]. In turn, these modifications alter the structure and functions of the targeted molecules [1,12]. In proteins, one marker of oxidative stress is the presence of carbonyl groups, such as aldehyde and ketone, on specific amino acid side chains such as lysine, proline, arginine and threonine, so-called protein carbonylation (PC) [5]. PC is mainly

*Abbreviations:* PC, Protein carbonylation; UTC, the numbers that follow relate to individual component molarity or detergent percentage, urea-thiourea CHAPS-containing buffer; *C. elegans*, *Caeonorhabditis elegans*; DNPH, dinitrophenylhydrazine; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Hz, hydrazide

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considered as an irreversible post-translational modification, despite recent reports arguing for a decarbonylation process [1,13,14]. PC can be generated through different mechanisms including metal catalysed oxidation, lipid peroxidation adducts and glycoxidation. At present, the individual contribution of each oxidation mode in PC generation is still an open debate and probably relies upon the specific oxidation conditions of the cellular microenvironment [5,15].

Under normal non-accumulative conditions, PC is eliminated and maintained to sub-stoichiometric basal level through protein turnover. This process is carried out by the ATP/ubiquitin-independent 20S proteasome and the calpain family [16–19]. However, when oxidative stress increases, PC accumulates and augments protein hydrophobicity. This physico-chemical modification, along with protein misfolding, coordinates to generate aggregates that ultimately impair protein degradation processes and leads to different pathologies and disorders, such as Alzheimer's disease, chronic lung disease and atherosclerosis [2,3,12,20]. In view of this, PC study is now a growing field in general and medical science.

There are currently several detection techniques available to study oxidative stress in general and PC in particular. These assays are predominantly based on a specific reaction of carbonyl moieties with hydrazine, hydrazide and hydroxylamine compounds [6,21–24]. Today, most of them are conjugated with labelling molecules, such as fluorescent probes [25], trapping molecules, such as biotin [26]; or even with reporter ions, such as iTRAQH 114/117 (Isobaric Tags for Relative and Absolute Quantitation Hydrazide) [27], and range from traditional biochemical techniques to the latest mass spectrometry analyses. Each system has its advantages and disadvantages with regard to the main objectives of the analysis considered [6,23,24].

The pioneering work of Stadman and Levine has defined some parameters for the use of a dinitrophenylhydrazine (DNPH)-based methodology as well as general guidelines for dealing with PC [28–30]. Yan et al. have also summed up protocols and good laboratory practices focused on carbonylated protein handling [21]. Despite this, several PC analyses still suffer from poor homogeneity, leading to difficult cross-data analysis and poor results overlap [6,23,24,31–33].

At present, enzyme-linked immunosorbent assays (ELISA) seem to be the most robust method used across laboratories to quantify the presence of carbonyl groups in proteins, despite having some recognised caveats mainly related to “in-lab” protocol discrepancies [34]. In parallel, gel-based approaches do indeed suffer from cross-comparison difficulties, but they also provide additional information, such as the carbonyl distribution across protein pattern [34–36]. Moreover, gel-based approaches allow easy identification of protein band or spot selection for further characterisation, *e.g.* by mass spectrometry analysis [35,36]. Lastly, fluorescent multiplexing tools have greatly improved the reproducibility of gel-based experiments and facilitated data interpretation [37].

This study aims to highlight some of the technical challenges in proteomic gel-based multiplexing experiments when dealing with PC in the wider context of an oxidative stress mechanism study. To achieve this, we focused on the nematode *Caeonorhabditis elegans* (*C. elegans*), a commonly-used and well-known animal model [38–41]. Within that scope, benchmarking already-existing procedures to this experimental model appeared essential to obtain robust and reproducible results [6,23,24,34]. The first step focused on protein extraction parameters notably in terms of carbonyl status maintenance in *C. elegans*, a model organism that is difficult to extract [42]. Based on a protocol modified from Tamarit et al. which used fluorescent hydrazide probes, the second step was mainly dedicated specifically to setting up crucial PC labelling parameters, such as probe concentration and labelling time [25]. In a third step, special attention was paid to protein purification and buffer exchange protocols which are critical when studying PC, due to the need to remove excessive unreacted carbonyl labelling molecules, as well as carbonyl containing molecules other than proteins, without excessive and non-homogeneous loss of proteins in general and carbonylated proteins in particular, which are more hydrophobic and more prone to precipitation [6,12,23,24].

## 2. Results and discussion

In this work, batches of a thousand *C. elegans* worms were synchronized as much as possible in order to always work with the same stage and avoid differences in protein content or nature due to their short lifecycle [43]. Carbonyl moieties were introduced into *C. elegans* proteins by ultraviolet (UV) exposure of whole worms or already-extracted clear lysates. The first methodology was used to refine the extraction parameters and the second was to set up carbonyl labelling. The fact that no chemicals are introduced into the experimental system is one of the major advantages of UV stimulation, facilitating data interpretation due to limited experimental bias (see below). To ensure complete bacteria removal (external and internal ), specific washing steps were designed to last minimum 1 h at 20 °C before extraction or sample freezing [42].

### 2.1. Extraction parameters

Extraction parameters were carefully investigated with regard to the potentially applicable lysis buffers and associated disruptive methods. For *C. elegans*, a sufficiently powerful technique had to be used to allow worm cuticle breakdown and a substantial protein recovery rate, without modifying their carbonyl group status [24,44,45].

In this study, two main extraction methods (Potter or grinder) and two main buffers (Radioimmunoprecipitation Assay lysis buffer (RIPA) or the urea-thiourea-3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS)-containing buffers) were tested. As no major changes were observed between all the tested conditions, neither in protein or carbonylated protein pattern and yield, a modified version of the RIPA extraction buffer (see the purification parameters section for full explanation) combined with the grinder apparatus (commonly used for *C. elegans* [44]) was selected for all subsequent experiments.

However, 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris)-based buffers like RIPA (containing primary amines) could potentially react with aldehydes before carbonyl labelling and compete in the reaction with hydrazine-based compounds [23]. Here, a comparison was made between two versions of the RIPA buffers, where Tris was replaced by an equivalent molarity of 2-bis(2-hydroxyethyl)amino-2-(hydroxymethyl)-1,3-propanediol (Bis-Tris) compound which does not contain primary amines. Fig. 1 shows the limited impact of this change on unstimulated samples, while no statistical difference was observed for UV-stimulated worms under our experimental conditions. For this reason, Tris-generated results were considered valid within the scope of this analysis.

In order to investigate potential carbonyl group introduction in protein via grinder extraction, several experiments were carried out using increasing 1,4-dithiothreitol (DTT) concentrations *e.g.* before extraction, after extraction and during labelling. DTT is used as a reductant in many carbonylation-associated publications and some papers recommend its use in the lysis buffer up to 50–100 mM [24]. However, thiol reductants at these molarity ranges have also been shown to introduce additional carbonyl groups in proteins via Fenton reaction after iron reduction, and should

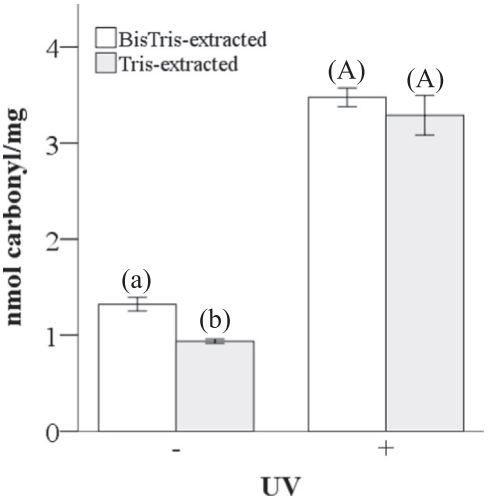


**Fig. 1.** Effect of primary amines-containing buffers on carbonyl detection. After 3 × 10 min UV exposure, 3000 worms were extracted for each replicate in 30 mM Tris- or 30 mM Bis-Tris-containing buffer. Then, 15 μg of protein extract was labelled and purified as described in the Section 5. At the end of the process, 8 μg of protein was loaded on top of a 4–15% SDS-PAGE gradient gel, separated and scanned on a Typhoon system at the selected wavelengths. Results are expressed as a mean ± SD (nmol of carbonyl per mg of total protein clear lysate extract), n=3. UV− and UV+ groups were considered as individual statistical groups (small/capital lettering). Within each group, treatments with dissimilar letters are statistically different (Student's t-test; $p < 0.01$).

therefore be considered with caution [46]. Moreover, some published works also argue for the impact of DTT on dye labelling efficiency [47], although to our knowledge no study has tested the influence of DTT on endogenous carbonyl status.

Firstly, the influence of DTT on carbonyl labelling efficiency was tested on the same protein extract with DTT spiking in the labelling buffer before addition of the dye (Fig. 2, panel 1). This figure shows a decrease in carbonyl detection associated with an increase in DTT concentration, highlighting a partial impairment of the labelling procedure under these conditions or a decrease in the non-specific Hz-related signal, in accordance with Chaudhuri et al. [47]. Secondly, the impact of DTT during the lysis process was investigated on three separate batches of worms from the same initial pool (Fig. 2, panel 2). This figure shows a decrease in the carbonyl signal along with an increased DTT concentration to the same extent as that observed in Fig. 2, panel 1. Trends are similar before and after extraction, strongly suggesting that the selected extraction protocol introduces little or no carbonyl groups in proteins. Thirdly, the effect of DTT on carbonyl groups has been addressed by overnight incubation at 4 °C of clear protein lysates with in-sample spiking of 100 mM DTT. A comparison with control samples showed a similar level of carbonyls (Fig. 2, panel 3), demonstrating that DTT does not reduce carbonyl groups. Moreover, UV-stimulated samples harboured a small, but statistically significant difference with or without DTT. This slight increase in PC with DTT is in accordance with the hypothesis of protein oxidation via Fenton reaction, as suggested by Luo et al. (Fig. 2, panel 3) [46].

Altogether, these results strongly suggest that no or a limited amount of carbonyl groups are introduced during the extraction stage. Therefore, a minimal amount of 2 mM DTT was kept for all subsequent experiments. It should be noted that different temporal stimulations of worms and divergent timing due to experimental requirements could explain batch-to-batch variations as regards the estimated nmol of carbonyl per mg of total protein extract.

## 3. Protein carbonyl labelling parameters

### 3.1. DNPH labelling: a former method with some issues

As a first step, samples were treated with DNPH labelling molecule according to Millipore's OxyBlot kit method. However, many technical problems were observed with this method. For example, despite the high sodium dodecyl sulfate (SDS) load, *C. elegans* protein samples precipitated when mixed with the DNPH-containing solution, probably due to the highly acidic pH of the derivatisation mixture which contains strong acid. Moreover, a general absence of carbonyl-related signal for protein migrating under the 30 kDa limit was observed, despite a corresponding normal uncarbonylated protein pattern (Fig. 3). However, it could be expected that a physico-chemical reaction, like carbonylation, especially generated by UV exposure of a clear protein lysate (Fig. 3), should produce a homogeneously distributed protein profile. Various published data using polyclonal serum from a rabbit source have shown similar results, while newer techniques, like hydrazide-based carbonyl detection, have generated a distributed signal that corresponds with the total protein pattern (see below) [47,48]. In addition to this, a major loss of the carbonyl-related signal was observed after TCA precipitation which precluded buffer exchanges and prevented multiplexing experiments or cleaning steps on the sample (Fig. 3, panel 1).

Lastly, a non-specific carbonyl-related signal on underivatised *C. elegans* samples and other technical problems were observed, such as poor transfer reliability or migration defects probably generated by the high load of salts in the sample (Fig. 3, panel (1), lane 1 and 2).

### 3.2. Fluorescent hydrazide-based technology: a promising tool

In order to circumvent the above-depicted issues, we decided to take advantage of the hydrazide-based technology where carbonyl group labelling is done in less acidic conditions, ranging from pH 5 to 7. Moreover, the associated CyDye™ technology (CyDye™-hydrazide or CyDye™-Hz) easily enables multiplexing experiments analysing carbonyl moieties (Cy5™-Hz in our case) and total protein using molecules targeting NH2 from lysines (Cy2™-NHS ester, Cy2™ different from Cy5™). Furthermore, direct in-gel reading of the signals can be achieved as these fluorophores, *e.g.* Cy2, Cy5, are linked to proteins via a reactive part, *e.g.* Hz, NHS ester, which precludes the need for blotting procedures and limits experimental variation [31]. Altogether, these modifications should reduce experimental workflow variations. Fig. 4 illustrates carbonylation patterns obtained by this approach. The carbonylated protein pattern generated by this hydrazide-based method is clearly different to the one obtained with the traditional DNPH-based assay and is similar to other results from the literature [47]. The full range of protein molecular weights is now covered (Fig. 4) and, whereas the total protein pattern is similar between control and stimulated samples (Fig. 4), the carbonylated protein pattern is different. This is more in line with a non-enzymatically induced chemical modification, contrary to DNPH-acquired results.

Fig. 4 shows that the carbonyl-related signal is more diffuse than the total one, which harbours well-defined bands. This could correlate with the small mass shift introduced by the carbonyl tag. As positive control samples were generated by high non-physiological doses of UV irradiation, some proteins may harbour several carbonyl groups, which could probably explain the smear shape of the one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (1D-SDS-PAGE) migration lane. This result is in agreement with other data from literature [47].

The impact of pH values on carbonyl labelling efficiency was

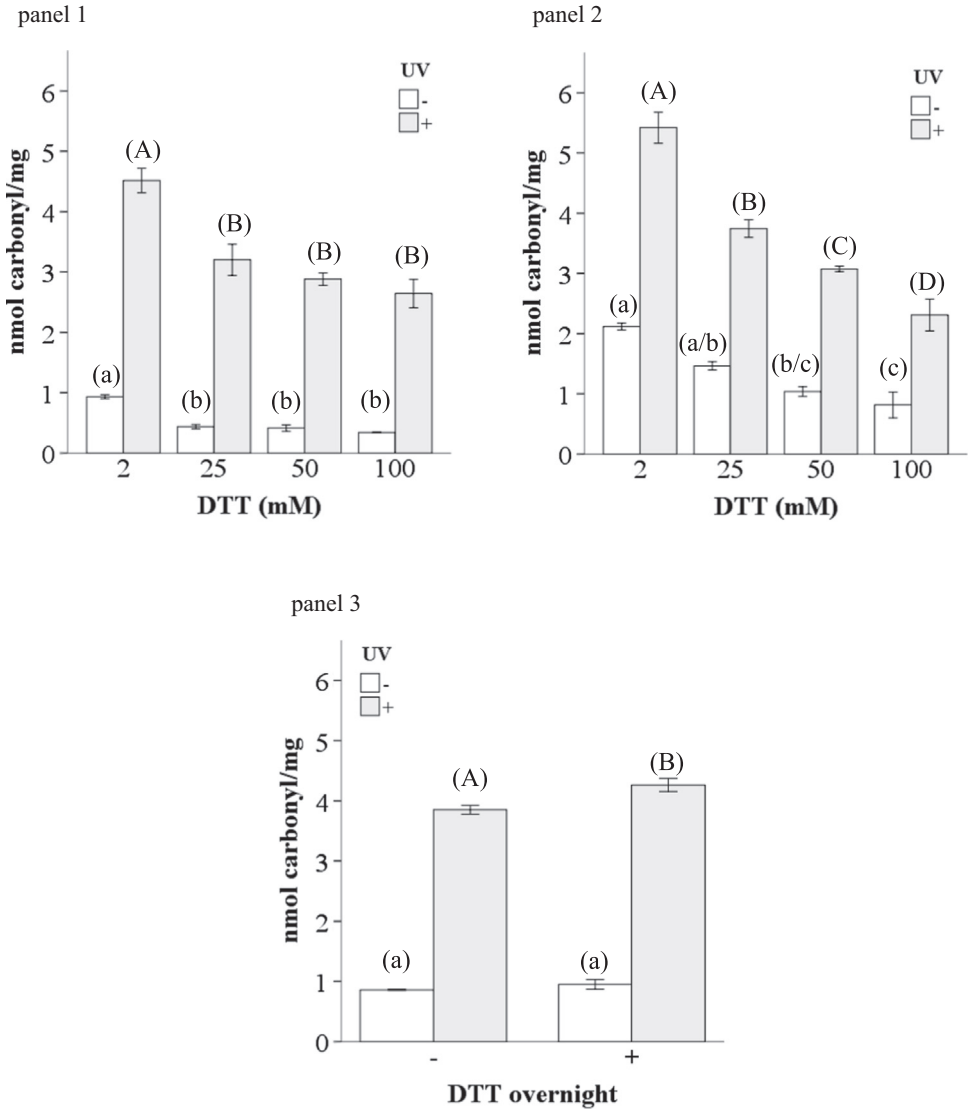


**Fig. 2.** Effect of DTT concentration on carbonyl detection. Panel (1), effect of DTT on carbonyl labelling; panel (2), effect of DTT on carbonyl during extraction; panel (3), effect of DTT on already-extracted carbonyl. 3000 worms were extracted in a modified version of the RIPA buffer and UV stimulated (3 × 10 min). Then, except for the experimental tested parameter the protocol is described under the Section 5: 15 μg of protein extract was labelled and purified for each individual sample. At the end of the process, 8 μg of protein was loaded on top of a 4–15% SDS-PAGE gradient gel, separated and scanned on a Typhoon system at the selected wavelengths. Results are expressed as a mean ± SD (nmol of carbonyl per mg of total protein clear lysate extract), $n=3$. UV− and UV+ groups were considered as individual statistical groups (small/capital lettering). Within each group, treatments with dissimilar letters are statistically different (Student's *t*-test; $p<0.05$).

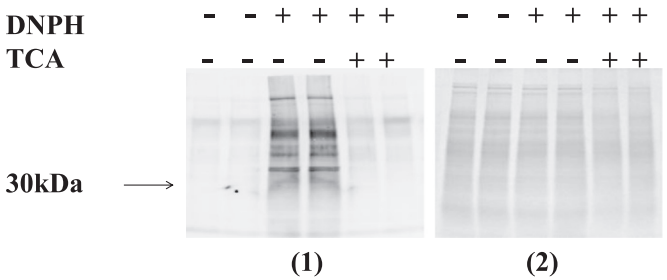


**Fig. 3.** Example of DNPH tagging experiment obtained after UV exposure of *C. elegans* clear lysates. Panel (1) corresponds to a Cy5™ antibody DNPH labelling scan, carbonyl related. Panel (2) corresponds to a Coomassie staining scan, total protein-related. 3000 worms were extracted in a modified version of the RIPA buffer, UV stimulated (3 × 10 min) and 15 μg of protein extract was labelled according to the OxyBlot Protein Oxidation Detection Kit instructions. At the end of the process, 8 μg of protein was independently loaded on top of two 4–15% SDS-PAGE gradient gels, separated and scanned on a Typhoon system at the selected wavelengths. One gel was used for carbonyl detection, the second for total protein estimation. Results are presented for two out of six similar replicates.

tested. Four conditions were assessed, from pH 5 to 8 as per the manufacturer's instructions. The potency of CyDye™-Hz to label carbonyl groups between pH 5 and 7 was first confirmed and associated with a clear reduction in the fluorescent signal when the pH value reached 8 (data not shown). Lastly, an intermediate labelling condition of pH 6 was selected.

The amount of dye needed to provide the best signal-to-noise ratio with the aforementioned settings was also investigated. In general, concentrations in terms of mM of dye are used when labelling μM of total protein extract [23,25]. This reaction is shown to be relatively inefficient in organic media [28,49], but also non-specific, yielding by-products [28,49,50]. For this reason, we decided to test the labelling response on UV-stimulated *C. elegans* over a large range of dye concentrations. In order to cover the full range of useful concentrations, the commonly-used dye amount was serially diluted by a factor of ten, until obtaining an equimolar amount of dye and protein extract. Fig. 5 shows an increase in Hz signal related to the engaged dye amount. Comparison of the UV+ carbonyl ratio between 0.025 and 0.25 mM Cy5™-Hz shows a

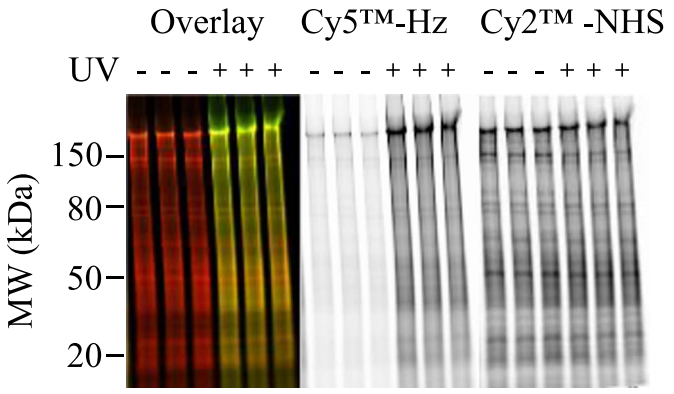


**Fig. 4.** Example of in-gel carbonyl or total protein-related signal obtained after UV stimulation of whole worms. Overlay corresponds to a superposition of both channels' signals; Cy5™-Hz corresponds to carbonyl-related signal; Cy2™-NHS corresponds to total protein-related signal. After 3 × 10 min UV exposure, 3000 worms were extracted for each replicate in a modified version of the RIPA buffer. Then, except for the experimental tested parameter the protocol is described under the Section 5: 15 μg of protein extract was labelled and purified for each individual sample. At the end of the process, 8 μg of protein was loaded on top of a 4–15% SDS-PAGE gradient gel, separated and scanned on a Typhoon system at the selected wavelengths.

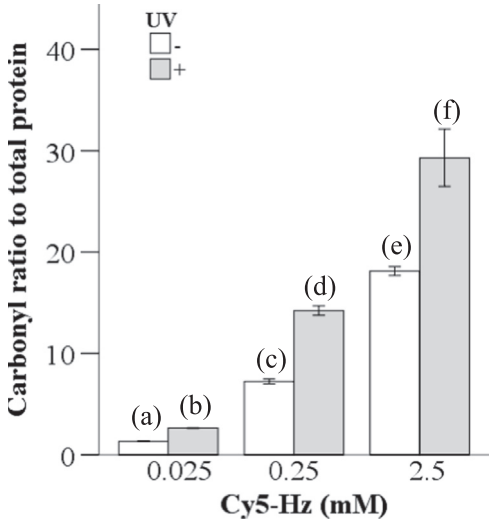


**Fig. 5.** Impact of Cydye™-Hz concentration on carbonyl detection. 3000 worms were extracted in a modified version of the RIPA buffer and UV stimulated (3 × 10 min). Then, except for the experimental tested parameter the protocol is described under the Section 5: 15 μg of protein extract was labelled and purified for each individual sample. At the end of the process, 8 μg of protein was loaded on top of a 4–15% SDS-PAGE gradient gel, separated and scanned on a Typhoon system at the selected wavelengths. Results are expressed as a mean ± SD (ratio of Hz-related signal normalised with the corresponding Coomassie signal, arbitrary units), $n=3$. UV− and UV+ groups were considered as individual statistical groups (small/capital lettering). Within each group, treatments with dissimilar letters are statistically different (Two-way ANOVA; $p<0.001$).

signal magnification of 6, whereas it decreases to 2 between 0.25 and 2.5 mM Cy5™-Hz. The ratio of 6 (instead of the expected 10) suggests that a portion of the dye does not react with carbonyl moieties during the labelling process. The ratio decrease to 2 seems to indicate a plateau phase tendency around 2.5 mM. The result is similar for the control (UV- samples) between 0.025 and 0.25 mM Cy5™-Hz, but not between 0.25 and 2.5 mM Cy5™-Hz where the carbonyl ratio is around 3. At 2.5 mM Cy5™-Hz, under our condition, UV- seems to increase, suggesting a higher rate of by-product formation that modifies the UV+/UV− ratio. As the best signal-noise ratio is required, the concentration of 0.25 mM Cy5™-Hz was selected for the subsequent experiments. This procedure reduces the required amount of CyDye™-Hz, decreasing the cost previously associated with this technique tenfold [23,25]. However, if the objective is absolute quantitation of the carbonyl content, other labelling conditions could be used, but non-specific signal should be assessed for the method's accuracy. It can be noted that experiments carried out here with 2.5 mM CyDye™-Hz produced a carbonyl content signal less than two times higher than with 0.25 mM, included in a variation range recently described in the literature [24,33,34].

Lastly, the impact of labelling time was tested by performing a kinetic curve based on various literature data. This investigation was conducted because several papers argued for side reactions when hydrazine or hydrazine-like compounds are used for prolonged incubation periods [15,23,29,47,50,51]. In a first trial, four different times, ranging from 15 min to 2 h were assessed, corresponding to the most extreme labelling conditions found in the literature [22,23,33,52]. Induction of carbonyls between control and stimulated samples decreased with the incubation time demonstrating, as described in Stadman and Levine's original paper with DNPH, that a short incubation period must be used when performing this kind of assay [28,29]. In order to fully investigate this parameter, a second trial using shortened times was conducted. Fig. 6 shows that 15 min' incubation at 25 °C under 500 rpm gave the best labelling condition by maximising the signal ratio between UV+ and UV− conditions. At 30 min, the UV+ signal is still increasing, but slightly less than the UV− signal, thus suggesting potential by-product formation. This kind of over-labelling will result in an overestimation of the control's carbonyl content. It should be noted that the number of carbonyl groups quantified here per milligram of protein is in accordance with *in vitro*-generated results from Augustyniak et al. [34].

### 3.3. Purification parameters

As total protein labelling with CyDye™-NHS requires buffer exchanges, a purification procedure appeared mandatory. Various protocols can be found in the literature, each with their own advantages and disadvantages, but most use 10% (v/v) trichloroacetic

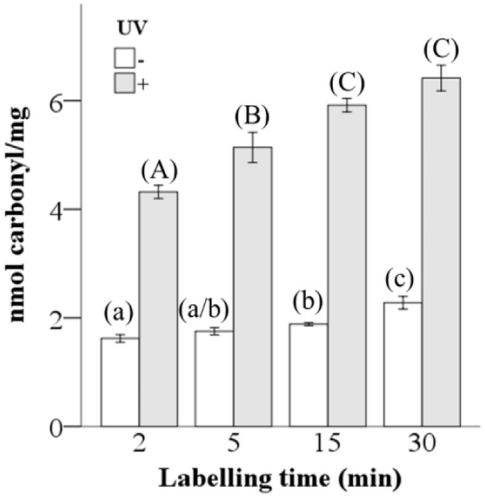


**Fig. 6.** Impact of carbonyl labelling time on carbonyl detection. 3000 worms were extracted in a modified version of the RIPA buffer and UV stimulated (3 × 10 min). Then, except for the experimental tested parameter the protocol is described under the Section 5: 15 μg of protein extract was labelled and purified for each individual sample. At the end of the process, 8 μg of protein was loaded on top of a 4–15% SDS-PAGE gradient gel, separated and scanned on a Typhoon system at the selected wavelengths. Results are expressed as a mean ± SD (nmol of carbonyl per mg of total protein clear lysate extract), $n=3$. UV− and UV+ groups were considered as individual statistical groups (small/capital lettering). Within each group, treatments with dissimilar letters are statistically different (One-way ANOVA; $p<0.05$).

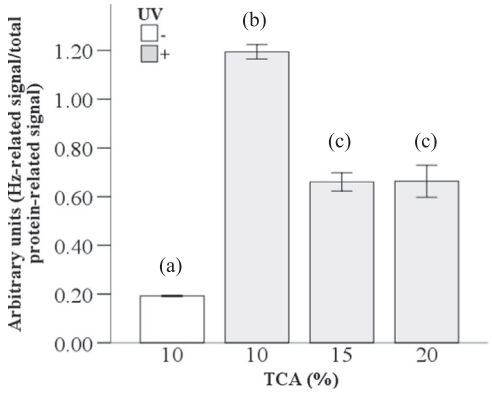


**Fig. 7.** Impact of final TCA concentration on carbonyl detection. 3000 worms were extracted in a modified version of the RIPA buffer and UV stimulated (3 × 10 min). Then, except for the experimental tested parameter the protocol is described under the Section 5: 15 μg of protein extract was labelled and purified for each individual sample. At the end of the process, 8 μg of protein was loaded on top of a 4–15% SDS-PAGE gradient gel, separated and scanned on a Typhoon system at the selected wavelengths. Results are expressed as a mean ± SD (ratio between carbonyl Hz-related signal and NHS-total protein-related signal, arbitrary units), $n=3$. UV− and UV+ groups were considered as one statistical group, treatments with dissimilar letters are statistically different (One-way ANOVA; $p<0.001$).

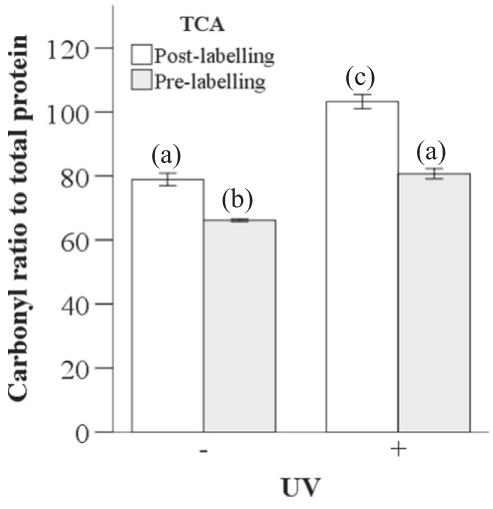


**Fig. 8.** Impact of TCA precipitation steps on carbonyl detection. 3000 worms were extracted in a modified version of the RIPA buffer and UV stimulated (3 × 10 min). Then, except for the experimental tested parameter the protocol is described under the Section 5: 15 μg of protein extract was labelled and purified for each individual sample. At the end of the process, 8 μg of protein was loaded on top of a 4–15% SDS-PAGE gradient gel, separated and scanned on a Typhoon system at the selected wavelengths. Results are expressed as a mean ± SD (ratio of Hz-related signal normalised with the corresponding Coomassie signal, arbitrary units), $n=3$. UV− and UV+ groups were considered as one statistical group, treatments with dissimilar letters are statistically different (One-way ANOVA; $p<0.001$).

acid (TCA) precipitation [6,23,24,31,32]. The purification procedure can be divided into three main steps (precipitation, washing and pellet solubilisation). Each was assessed in different ways to determine the best combination to use in PC study. Lastly the recovery of the whole procedure was assessed.

### 3.4. Precipitation step

Since measurement of the post-translational modification studied here can potentially be influenced during processing, the impact of a clean-up procedure was assessed. The clean-up has two main advantages, first it removes contaminants such as DNA and lipids that could interfere with the assay and secondly, it produces a better spot resolution in 2D-SDS-PAGE [22]. In a first step, TCA precipitation conditions were discussed, then, a comparison of carbonyl induction was performed on identical sample pools subjected to pre- and/or post-derivatisation purification steps.

Rajalingam et al. showed that 10–15% (v/v) TCA gives the best protein precipitation rate [53]. To our knowledge, no data are yet available on carbonylated proteins. Three reasonable TCA concentrations (10%, 15% and 20% (v/v) final) were therefore tested on UV-stimulated samples to ensure proper visualisation of the effect, while keeping the final volume constant to avoid any bias related to protein concentration. As shown in Fig. 7, 10% (v/v) TCA seems to be the best condition to recover a maximum amount of carbonylated proteins. Indeed, 15% (v/v) and 20% (v/v) TCA yielded 85% and 80% of the 10% (v/v) TCA total protein-related signal, whereas these TCA compositions yielded 53.5% and 55% loss in the carbonyl-related signal, respectively (data not shown). Then, as shown in Fig. 7 after correction to total protein amount, the carbonyl-related signal dropped to 60% of the one observed with 10% (v/v) TCA when 15% (v/v) and 20% (v/v) TCA are used.

Next, the optimal way to obtain 10% (v/v) final TCA in the samples was investigated by adding 100% (with a minimum added volume), 50% (v/v) or 20% TCA (v/v) (by doubling the volume of the former sample), respectively (data not shown). 50% (v/v) TCA gives the highest carbonyl recovery, associated with the minimal inter-sample variation, whereas the commonly-used 20% (v/v) TCA solution results in a decrease in the carbonylated protein recovery rate, probably due to excessive sample dilution [15,23,25,47]. On the other hand, the addition of 100% TCA showed a higher variability than the 50% (v/v) solution given the tiny volumes added and the solution's viscosity.

A higher recovery rate from TCA precipitation is observed for proteins in general and for carbonylated proteins in particular when low to mild chaotropic lysis buffers are used. This was highlighted by the use of the commercially-available bovine serum albumin-dinitrophenyl-linked purified protein (BSA-DNP), which was diluted in modified RIPA or UTC924 buffers. 25–50% of the BSA-DNP signal was retained in the supernatant when UTC buffer was used, whereas 10–20% was retained when RIPA was used (data not shown). This confirms Rajalingam's results, showing that TCA is less potent to precipitate protein in a disordered state, provided here by the high urea and thiourea loads [53]. For these reasons, we chose a modified version of the RIPA buffer for extractions.

Lastly, Fig. 8 shows that the pre-labelling TCA procedure is associated with a slight significant decrease in carbonyl content between both control and stimulated samples, probably due to the need to use two successive TCA precipitations. In this respect, we decided to align workflow parameters with TCA precipitation after carbonyl labelling, in order to maximise the signal-to-noise ratio and minimise the number of steps in the workflow and their associated variations. General induction was maintained even when TCA precipitation was performed before carbonyl labelling, suggesting that TCA does not introduce a major source of carbonyl moieties in proteins.

### 3.5. Washing step

We next focused on the washing steps, where two main solvents are used when performing TCA precipitation. Acetone is the most common and is generally included in commercially available clean-up kits. However, acetone is poorly compatible with SDS-containing samples since it produces almost insoluble pellets (Vertommen, D., personal communication). For these reasons, ethyl acetate/ethanol (50% v/v) is sometimes preferred and pellets were effectively easily re-suspended in that way [23].

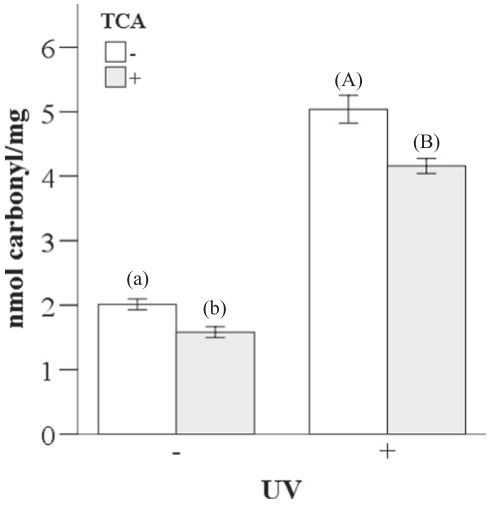


**Fig. 9.** Technique-associated carbonyl recovery rate. After 3 × 10 min UV exposure, 3000 worms were extracted for each replicate in a modified version of the RIPA buffer. Then, except for the experimental tested parameter the protocol is described under the Section 5: 15 μg of protein extract was labelled and purified for each individual sample. At the end of the process, 8 μg of protein was loaded on top of a 4–15% SDS-PAGE gradient gel, separated and scanned on a Typhoon system at the selected wavelengths. Results are expressed as a mean ± SD (nmol of carbonyl per mg of total protein clear lysate extract), $n=3$. UV− and UV+ groups were considered as individual statistical groups (small/capital lettering). Within each group, treatments with dissimilar letters are statistically different (Student's *t*-test; $p<0.001$).

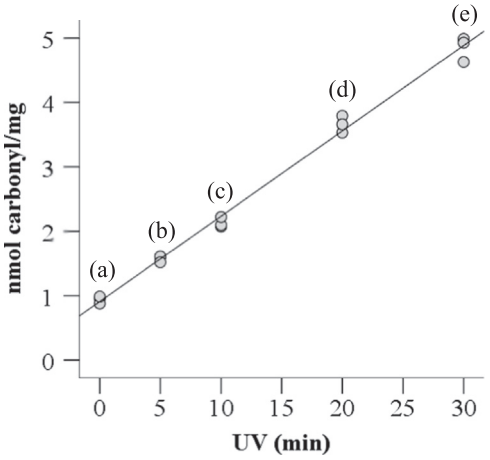


**Fig. 10.** Carbonyl dose-response curve for pre-extraction UV stimulation. After UV stimulation (dose-response, see graph for details), 3000 worms were extracted for each replicate in a modified version of the RIPA buffer. 15 μg of protein extract was labelled and purified for each individual sample. At the end of the process, 8 μg of protein was loaded on top of a 4–15% SDS-PAGE gradient gel, separated and scanned on a Typhoon system at the selected wavelengths. Results are expressed as a mean ± SD (nmol of carbonyl per mg of total protein clear lysate extract), $n=3$. Treatments with dissimilar letters are statistically different (ANOVA; $p<0.01$). Line represents simple linear regression: ($F_{1,14}=1966.3$, $p<0.01$, carbonyl concentration $= 0.909+0.132\times$ UV, $r^2=0.993$).

### 3.6. Solubilisation step and recovery of the procedure

This critical parameter was investigated by using various solubilisation buffers (6 M guanidine, 0.5 M NaOH, Laemmli loading buffer, UTC924, UTC-(ASB14)-9231) along with different incubation times (1, 2, 4 and 16 h), mixing conditions (800, 1200 and 1600 rpm) and temperatures (25 and 30 °C). These buffers were selected from the literature or patents associated with PC study [23,25,28,32]. Only an increase in pH and the use of new detergents (like sulfobetaine ASB-14), also used for membrane protein solubilisation, were successful within the scope of this analysis [54]. Ultimately, a hybrid solubilisation buffer composed of 9 M urea, 2 M thiourea, 3% (w/v) CHAPS, 1% (w/v) ASB-14 and 20 mM Tris with a pH of 9.5 was selected and used at 30 °C with a 1600 rpm agitation rate. As shown in Fig. 9, after correction to total protein amount, the carbonylated protein recovery rate reached 80–85%. This corresponded to 80–85% of total protein recovery associated with a recovery rate close to 100% for the carbonyl groups (data not shown).

### 3.7. Application of the full process, quantitation and latest considerations

Lastly, a simple way to quantify carbonyl groups in proteins has been established by making serial dilutions of the dye-containing labelling solution spotted on a 20–30-min air-dried gel and scanned along with experimental gels. Confirmation of the excitation and emission wavelengths from free- or protein-associated Cy5™-Hz dye was first achieved through the acquisition of a spectrum scan performed on a TECAN fluorimeter system. Maximal excitation and emission wavelengths for both were 638 nm and 670 nm, respectively. These values correspond to literature data and are similar to those obtained with the TYPHOON system used for the gel-scanning part of this analysis (excitation 635 nm; emission $>665$ nm). The Typhoon-associated counts from the calibration curve (6 points from 0 to 20 pmoles of Cy5™-Hz dye) were then expressed as a function of the corresponding amount of Cy5™-Hz.

As shown in Figs. 1, 2, 6, 9 and 11 of this article, the estimated level of protein carbonylation in control samples is between 1 and 2 nmol of carbonyl per mg of protein. This is in accordance with previous studies based on DNPH labelling of normal BSA (2.6–5 nmol of carbonyl per mg of protein) and other tests performed on total protein extracts [24,33,34].

An example of the robustness of the method described in this paper is provided in Fig. 10 where a high linear correlation between the carbonyl level and UV exposure duration of whole worms was observed ($R^2=0.993$).

Although the cell type is different, this result can be discussed together with that obtained by Augustyniak et al., using less energetic UV, and showing in some cases a decrease in the carbonyl-related signal between 5 and 15 min of UV exposure of rat protein liver clear lysates, despite the use of protease inhibitors [34]. According to Augustyniak et al., this decrease after high doses of UV was due to aggregation or precipitation of carbonylated proteins that were then lost. In our case, whole worms were exposed, and optimized protocol steps enable carbonylated protein signal follow-up even after a huge dose of UV.

Finally, this method fits with one aim of generating a protocol that allows a single processed sample to be used in either 1D- or in 2D-SDS-PAGE without major modification as final sample is in a buffer suitable for such analysis [54]. With this method, samples can be directly loaded on top of an IEF strip; and pH adjustment with Bis-Tris buffer (150 mM, pH 6.8, volume/volume) associated with the addition of loading buffer produces well-defined bands in 1D-SDS-PAGE experiments (Fig. 4).

## 4. Conclusions

This work highlighted several questionable protocol-related aspects of published data, including carbonyl labelling dye concentration, the associated labelling time, and TCA precipitation parameters. Within that scope, these parameters should be re-addressed in a sample-specific manner. We also defined a procedure to adapt CyDye™-hydrazide-based protocols to specific samples in a cost-effective way. We, furthermore, improved an

already-existing powerful solubilisation buffer, making it potentially useful for hard-to-solubilise protein pellets. The methodology described exemplifies a simple way to normalise carbonyl-related signal to total protein in 1D- or 2D-SDS-PAGE multiplexing experiments, associated with minor sample modification with regard to the considered technique, which will limit experimental variations. We also propose a simple way to quantify carbonyl groups via on-gel spotting of diluted dye-containing labelling buffer. Lastly, the method's robustness was confirmed by a high linear correlation between the carbonyl level and the UV exposure duration of whole worms. Altogether, these results will help to standardise existing protocols in the growing field of proteomic carbonylation studies.

## 5. Materials and methods

### 5.1. Reagents

All chemicals were purchased from Sigma (St Louis), except for OxiBlot and the BCA kit which were from Thermo Scientific (Waltham), the Cy5-goat anti-rabbit secondary antibody which was from Life Technologies (Carlsbad), the TGX pre-cast gels and 10X TG, 10X TGS buffers which were from BioRad (Hercules), and the hydrazide-related dye products which were from Interchim (Montuçon).

#### 5.1.1. C. elegans-related techniques

*5.1.1.1. Strain, culturing and synchronisation.* The *C. elegans* wild-type strain (Bristol N2) was purchased from the Caenorhabditis Genetics Center (MN, USA). The nematode stock was kept in the dark at 20 °C in nematode growth medium (NGM) agar seeded with *Escherichia coli* OP50 strain, under 70% relative humidity, according to the WormBook general guidelines [43]. Batch generation of *C. elegans* was done in a sterile modified S-BASE medium, under 130 rpm agitation, at 18 °C in the dark. *Ad libitum* growing conditions were ensured by the regular addition of 40-times concentrated bacterial suspension [43]. The worms used in the experiments were first synchronised by a bleaching procedure as per the WormBook guidelines [43].

*5.1.1.2. Washing steps.* The liquid cultures were supplemented with Triton X-100 to a final concentration of 0.01% (v/v) to prevent the worms' adsorption on plastic devices (Boag, P., personal communication). Worms were pelleted at 600 g for 2 min at 22 °C. An additional resting period of 5 min was applied to ensure maximum worm recovery. Worm pellets were then dispersed in a modified M9 medium (5 g/L NaCl, 25 mM $KPO_4$ buffer, 1 mM $MgSO_4$). These washing steps were repeated three times to ensure maximum bacteria removal. In order to remove any remaining bacteria from the digestive tract, the worms were left to stand for 3 × 10 min at room temperature with continuous agitation to limit hypoxic exposure. The worms were pelleted between washes, as described above, before M9 medium renewal. The related worm pellets were then pooled and counted under a stereomicroscope (ZEISS SteREO Discovery V20, Oberkochen), before being dispensed into 2-ml screw-cap tubes at 3000 worms per vial, snap frozen in liquid nitrogen and stored at −80 °C.

*5.1.1.3. Protein extraction parameters.* After rapid thawing, the *C. elegans* samples were centrifuged (2 min, 600 g, 4 °C), the small remaining volume of washing medium was removed to achieve optimum equivalent volumes. 200 µl of 0.5-mm diameter zirconium beads and an equal amount of lysis buffer (30 mM Tris–HCl pH 7.4, 150 mM NaCl, 1.0% (v/v) Igepal CA-630 (NP-40), 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulfate (SDS), 2% (v/v) glycerol, 2 mM 1,4-dithiothreitol (DTT), 1 µg/ml leupeptin, 1 µg/mlaprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM ethylenediaminetetraacetic acid (EDTA)) were added on top of worm pellets and incubated for 15 min on ice. The worms were then disrupted by three 6800-rpm cycles in the Precellys grinder system (Bertin Technologies, Montigny-le-Bretonneux). Resting cycles of 15 min and one hour were introduced between runs and at the end of the process, respectively. Clear lysates were obtained after insoluble materials were removed by 4 °C centrifugation at 13,500*g* for 15 min. Samples were aliquoted and snap frozen in liquid nitrogen. The protein concentration was assessed with the BCA kit (Thermo Scientific) according to the manufacturer's instructions. Before use, the samples were adjusted to 1 $mg/ml^{-1}$ with lysis buffer to ensure buffer homogeneity.

*5.1.1.4. Positive control generation.* Already-extracted clear lysates or whole worms (see captions for details) were exposed for the indicated time periods to UV at 254 nm, 99 $J/cm^2$ (Bio-Link Crosslinker) in a glass beaker or a Petri dish.

*5.1.1.5. Carbonyl and minimal lysine labelling*

*5.1.1.5.1. DNPH labelling.* 15 µg of protein clear lysates was labelled according to the manufacturer's instructions. After 1D-SDS-PAGE migration (see below), proteins were transferred on a polyvinylidene difluoride membrane in the Mini-PROTEAN tank (1 × Tris-Glycine buffer, BioRad, 1 h transfer at 100 V). Membranes were blocked with phosphate buffer saline solution (PBS) supplemented with 0.1% Tween-20% and 0.5% (w/v) bovine serum albumin (BSA) for 30 min at room temperature. Membranes were then incubated with the primary antibody from rabbit source directed against DNPH moiety for two hours at room temperature in PBS-Tween 0.1% (v/v) −0.5% BSA (w/v) (dilution: 1/150), before being washed three times with PBS-Tween 0.1% (v/v). The Cy5-goat anti-rabbit secondary antibody (A10523) was incubated for one hour at room temperature (dilution 1/6.600) in PBS-Tween 0.1% (v/v) −0.5% BSA (w/v). After three washes in PBS-Tween 0.1% (v/v), the membranes were washed two more times in PBS to remove detergent only, which is incompatible with the subsequent fluorescent scanning steps.

*5.1.1.5.2. Hydrazide-based labelling.* 15 µg of protein clear lysates was labelled in each individual condition. Carbonyl labelling dye (Cy5™-Hz MW 569.61 g/mol (FP-IO2490, Interchim) was diluted in dimethyl sulfoxide to obtain stock solutions at a final concentration of 50 mM. Cy5™-Hz was then diluted one hundred times in carbonyl labelling buffer (0.1 M sodium acetate, 1 mM EDTA, 1% (w/v) SDS, pH 5.1, other specified). It should be noted that a pH of 5.1 resulted in a final pH of 6 according to our extraction buffer. Samples and Cy5™-Hz-containing solution were mixed volume/volume and incubated in the dark at 25 °C under 500 rpm agitation for 20 min. The reaction was stopped by adding 8.25 µl of 2 M Tris and 1.35 µl of 0.2 M $NaBH_4$ with 10 min' incubation under the same conditions. Samples were then precipitated on ice for 20 min with the final 10% (v/v) TCA by adding 50% (v/v) TCA stock solution, before being centrifuged (4 °C) for 5 min at 13,500*g*. Pellets were washed three times with 200 µl of ice-cold ethyl acetate/ethanol (v/v) with a 5-minute resting period (on ice, in the dark) between two-minute centrifugation runs (4 °C, 13,500*g*). Afterwards, the pellets were air-dried under a chemical hood for 5 min, before being recovered in 6 µl of UTC9231 (9 M urea, 2 M thiourea, 3% (w/v) CHAPS, 1% (w/v) ASB-14, 20 mM Tris, pH 9.5) under agitation (1600 rpm) at 30 °C in the dark for two hours. The sample's pH was then adjusted to 8.6 with the addition of 6 µl of 50 mM Tris-HCl pH 8.5, before Cy2™-NHS ester minimal lysine labelling. 5 nmol of Cye2™-NHS dye (FP-LV2330**,** Interchim) was reconstituted in 7.5 µl of ultra-pure

anhydrous N,N-dimethylformamide (DMF). This stock solution was then diluted 6 times in DMF and 1 μl was added to the 12-μl samples. The labelling reaction was then incubated for 30 min on ice in the dark, before being stopped by the addition of 1 μl of 10 mM lysine and a further 10 min of incubation. These samples could then be run in 1D- or 2D-SDS-PAGE experiments. The pH of the 1D-dedicated samples needed to be adjusted to 6.8 by adding 3 μl of 150 mM Bis-Tris-HCl pH 6.8 and 5 μl of 5 × Laemli loading buffer (150 mM Tris, 100 mM DTT, 30% (v/v) glycerol, 2% (w/v) SDS, trace amounts of bromophenol blue, pH 6.8) prior to 1D-SDS-PAGE migration.

*5.1.1.5.3. Gel migration, image acquisition and quantitation.* 1D-SDS-PAGE was run in a Mini-PROTEAN Tetra Cell system (1658001, BioRad) with Mini-PROTEAN TGX pre-cast gels (4–20% 15 wells (4561096), 4–15% 15 wells (4561086), 4–20% 10 wells (4561094), 4–15% 10 wells (4561084), BioRad) in 1 × Tris-glycine-SDS (TGS) buffers at 100 V for 15 min and 150 V until loss of the migration front. The gels and membranes were scanned in fluorescence on a TYPHOON FLA 9500 imager (GE Healthcare) at a resolution of 100 μm with the appropriate wavelengths, filters and photo-multiplier tube settings. Images were analysed according to the manufacturer's instructions with ImageQuant TotalLab (IQTL, GE Healthcare) software.

The CyDye™-Hz-related signal was quantified with background subtraction provided by the analysis of an empty lane, specific to each experiment.

Quantitation was performed by on-gel spotting of Cy5™-Hz dye. To do this, 25-mn air-dried SDS-PAGE gel was placed below the experimental gels on the Typhoon system. The dye-Hz-containing solution (Hz labelling buffer) was diluted to 20 and 15 μM before being serially diluted two fold. 1 μl of each standard, namely 0, 2.5, 5, 7.5, 10, 15 and 20 pmol is spotted in triplicate on the dried gel. Typhoon-associated counts from this calibration curve were then expressed as a function of the corresponding Cy5™-Hz picomoles.

## 6. Statistical analysis

Results are expressed as a mean of three individual experiments ($n=3$) unless otherwise indicated, with a corresponding standard deviation (SD). Normality (Shapiro-Wilk test) and homogeneity of data variance (Levene's test) were tested before all statistical analyses. Effects of considered treatments (see in-text captions) were assessed using non-parametric Kruskal-Wallis tests when these two assumptions were not found, followed by a post-hoc Nemenyi test. When assumptions were met, the effects of the considered treatments (see in-text captions) were assessed using one- or two-way ANOVAs followed by a post-hoc pairwise comparison Tukey's test or Student's *t*-test. Simple linear regressions were run to explore the relationship between the CyDye™-Hz spiked amount and Typhoon-associated counts, or between UV exposure time and carbonyl content. All statistical analyses were carried out using the IBM SPSS software package, version 19 (StatSoft Inc., Tulsa, OK, USA), with α set at 0.05.

## Authors' contribution

Sébastien Pyr dit Ruys contributed to the experiment's conception and design, data acquisition, analysis and interpretation, and the article's drafting.

Sandrine Frelon contributed to the experiment's conception and design, data analysis and interpretation, and the article's drafting.

J.-M. Bonzom contributed to the statistical analysis of the data and the associated figures.

## Conflict of interest

The authors declare no competing financial interest.

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