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Assessment of in situ cellular glutathione labeling with naphthalene-2,3-dicarboxaldehyde using high-performance liquid chromatography

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Abstract

We have presently studied a dialdehydic reagent, i.e. naphthalene-2,3-dicarboxaldehyde (NDA), as a fluorogenic probe for the labeling of intracellular reduced glutathione (GSH), using a yeast strain *Candida albicans* as a cell model. Chemical reactivity of NDA with both amino and sulfhydryl groups of the GSH molecule leads to a highly selective detection. Moreover, fluorescence properties of the resulting adduct fit well with most of modern instruments adapted for in situ measurements, and equipped with an argon laser. After incubation of cells with 100 μM of NDA for 20 min, cells were harvested and corresponding lysates obtained after a freezing cycle, were suspended in 0.2 M borate buffer pH 9.2 and analysed with HPLC (column: Spherisorb ODS-2 (125 mm × 4.6 mm i.d.) 5 μm; mobile phase: methanol–0.01 M phosphate buffer pH 6.5 (20:80, v/v) at a flow rate of 0.8 mL min^{-1}; spectrofluorimetric detection: λ_{exc} = 430 nm and λ_{em} = 530 nm). The GSH-NDA adduct was identified in the yeast strain extracts using the reported HPLC technique and quantified versus a calibration curve of NDA derivatized with an excess of GSH (linearity range: 9–230 nM). The cell loading step of the free probe NDA and the extraction efficiency of the resulting NDA-GSH adduct were optimized.

Keywords: Reduced glutathione; Naphthalene-2,3-dicarboxaldehyde; Yeast strain; Cellular staining

1. Introduction

Oxidative stress is a well known process implied in many pathological diseases. It corresponds to an excess of reactive oxygen species (ROS) production, when cellular antioxidant defenses are overwhelmed, leading to free radical damages of cell components [1–3]. The tripeptide glutathione (γ-glutamylcysteinylglycine; GSH) is the main intracellular low molecular mass thiol in most living cells, from microorganisms to humans. Its reduced form is present at millimolar levels and it highly participates to the fight against ROS. As a matter of fact, GSH is implied into numerous antioxidant mechanisms, either as a strong reducing chemical compound or as the cosubstrate of enzymes involved into detoxification processes, such as glutathione-S-transferases (GSTs) and glutathione peroxidases. Intracellular GSH is kept at a constant level, especially by its de novo synthesis cycle [4]. Oxidative stress has more recently become an interesting research field in environnemental microbiology, especially as a mechanism occurring during disinfection with oxidants, e.g. peroxide and chlorine species [5,6].

Most of methods devoted to GSH measurement in cells and tissues rely upon separative techniques (i) HPLC with either direct electrochemical detection or coupled with a derivatization reaction, and (ii) capillary electrophoresis (CE) with either direct UV detection or derivatization reaction (for review, see refs. [7,8]). Those iterative techniques

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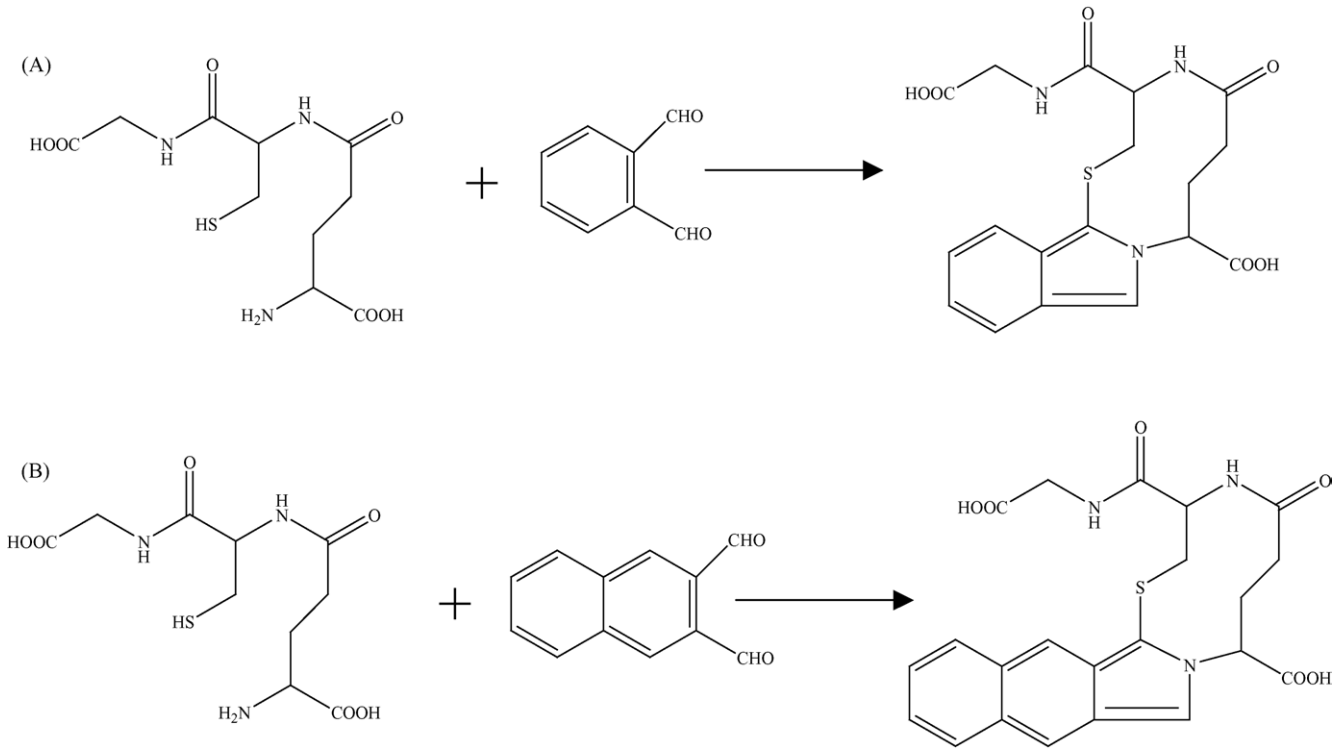

Fig. 1. Scheme of derivatization reaction of (A) OPA and (B) NDA with reduced glutathione (GSH).

implied time consuming sample treatment steps and careful storage conditions. Thus, there is a need for the development of non invasive techniques.

Cell GSH variations during oxidative stress processes have already been investigated in situ in different eucaryotic cell lines using fluorogenic reagents, mainly monochlorobimane [9–11] and 7-amino-4-chloromethylcoumarin [10,11], which conjugation with GSH is catalysed by cytosolic GSTs. The expression and/or activity of these enzymes vary between cell lines and during oxidative stress, thus, the corresponding fluorescence signal cannot be fully related to GSH levels. To counteract these drawbacks, probes only offering a chemical reactivity with GSH have been reported for GSH staining in cells: monobromobimane [12,13], *N*-[1-pyrene]maleimide [13], BODIPY® maleimides [14], and aromatic dialdehydic probes, i.e. *ortho*-phthaldialdehyde (OPA) [11,12] and naphthalene-2,3-dicarboxaldehyde (NDA) [11,15]. Monobromobimane and maleimide probes react with most of thiols (low molecular mass compounds and proteins), giving rise to a non selective fluorescent signal.

OPA and NDA have been widely used in HPLC techniques as derivatizing reagents of amino acids, and various endo- and exo-genous amino compounds, concomitantly with another nucleophilic reagent such as a thiol [16]; the resulting isoindolic adducts exhibit a high fluorescence yield. Otherwise, OPA alone has been used as a selective reagent for either pre- [17] or post-column [5,6,18,19] derivatization of GSH in HPLC, because it reacts with both amino and sulfhydryl groups of the GSH molecule (Fig. 1A); NDA presents a similar reactivity (Fig. 1B) and has been reported as a GSH derivatizing reagent in CE with laser induced fluorescence detection (LIF) [15,20,21]. OPA and NDA have already been tested for labeling intracellular GSH in mammalian cells [11,12,15], but data available in the literature concerning their real uptake by cells and their reactivity with GSH inside the cells are poorly informative. Moreover, at our knowledge, yeast staining with either OPA or NDA has not yet been investigated. Presently, NDA was preferred to OPA, because this latter yields adducts with an excitation wavelength in the UV range, thus, precluding use of the argon laser. As a matter of fact, the argon laser presents an emission line at 488 nm and it is provided with numerous instruments devoted to in situ measurement of cellular compounds labeled with fluorogenic probes, e.g. confocal microscopy and flow cytometry. The fluorescence properties of the NDA-GSH adduct are fully adapted to the argon laser, as already demonstrated in CE-LIF [15,20,21].

We have presently studied NDA as a probe for monitoring level of GSH in cultured cells of a yeast strain. We have developed and validated a reversed-phase HPLC method in order to identify and quantify the corresponding NDA-GSH adduct produced in cells loaded with the free probe. The method was used to assess whether NDA penetrates the cell and reacts with GSH, then to optimize the loading step of the probe. The monitoring of GSH variations was also preliminary investigated.

2. Experimental

2.1. Chemicals, reagents and standards

All chemicals and solvents were of analytical or HPLC reagent grade and were used without further purification. GSH and buthionine sulfoximine (BSO) were obtained from Sigma (Saint-Quentin Fallavier, France), OPA, NDA from Fluka (Saint-Quentin Fallavier, France), and γ-glutamylcysteine (γ-glucys) from Bachem (Voisins-le-Bretonneux, France). Phosphate buffer saline (PBS) solution contains 120 mM NaCl, 2.7 mM KCl, and 10 mM K_2HPO_4, and is adjusted to pH 7.0 with 1 M hydrochloric acid before sterilization. The stock solutions of OPA and NDA were prepared in ethanol at a concentration of 1 mg mL^{-1} (7.45 and 5.43 mM, respectively) and stored at −80 °C. Further dilutions were realized in 0.2 M borate buffer pH 9.2 for calibration curve (5 points) and in sterile PBS solution for loading cultured cells.

2.2. HPLC system and operating conditions

The HPLC system consisted of a low-pressure gradient solvent delivery pump (model PU 980, Jasco, Nantes, France), an autosampler equipped with a cooling sample device and a column oven (model AS-300, Thermo, Les Ulis, France), a spectrofluorimetric detector (model FP-920, Jasco), and a data processing software (model AZUR™ V3.0, Datalys, Saint-Martin d'Heres, France). The tray compartment containing sample vials was cooled at 4 °C. A guard column (8 mm × 4 mm i.d.) and an analytical column (125 mm × 4.6 mm i.d.) were packed with Spherisorb ODS-2 5 μm (Macherey-Nagel GmbH & Co, Düren, Germany) and used at a temperature of 40 °C. NDA-GSH and OPA-GSH adducts were eluted with methanol–0.01 M phosphate buffer pH 6.5 (20:80 and 5:95, v/v, respectively) at a flow rate of 0.8 mL min^{-1}.

Spectrofluorimetric detection of NDA-GSH and OPA-GSH adducts was operated at λ_{exc} = 430 nm and λ_{em} = 530 nm and at λ_{exc} = 340 nm and λ_{em} = 440 nm, respectively.

2.3. Pre-column derivatization of NDA

One hundred microliters of each standard NDA solution used for the calibration curve was mixed with 1 mL of 0.2 M borate buffer pH 9.2 and 100 μL of a freshly prepared GSH solution at 1 mg mL^{-1} (3.25 mM) in 0.01 M hydrochloric acid; the resulting solution was left at 20 ± 2 °C for 15 min, then immediately stored in the autosampler at 4 °C for a maximum delay period of 1 h before injection into the HPLC system. OPA standard solutions were treated in a similar way.

2.4. Cell culture, probe loading and extraction conditions

Yeast cells (*Candida albicans* VW 32 strain) were obtained from a preculture and were grown in Sabouraud medium, as previously described [22], for 6 h at 25 °C under stirring at 300 rpm, to obtain an absorbance measured at 620 nm versus the cell free culture medium, between 0.6 and 0.8. In such conditions, cells are in their exponential growth phase. After incubation, 20 mL of cell suspensions was centrifuged at 10,000 × *g* for 5 min at 4 °C. Cell pellets were washed twice by resuspending in 10 mL of PBS solution and centrifuging as above. The final pellets were resuspended in 5 mL of PBS solution containing 20, 100 and 500 μM of NDA and the resulting suspensions were stirred at 300 rpm, at 20 ± 2 °C, for 10, 20 and 30 min, in darkness. For experiments in presence of BSO, cells were exposed at 1 and 5 mM BSO concentrations in the culture medium, for 4 h. Then, the cell suspensions were centrifuged at 10,000 × *g* for 5 min at 4 °C and the resulting cells pellets were washed twice by resuspending in 5 mL of PBS solution and centrifuging as above.

The final cell pellets were freezed at −80 °C for 12 h, then quickly thawed and resuspended in 1 mL of 0.2 M borate buffer pH 9.2. Some experiments were realized using an ultrasonic probe (model Bandelin Sonopuls, Berlin, Germany) dipped in cell suspensions kept in ice at a power of 15 W for 5 min. The obtained suspensions were then centrifuged at 10,000 × *g* for 5 min at 4 °C, and the resulting supernatants were kept at 4 °C till HPLC analysis, for a maximum delay period of 1 h. When the concentration was upper than the highest limit of the linearity range, samples were diluted in borate buffer. The protein concentration was determined in the corresponding pellets by the Lowry method [23], using bovine serum albumin as standard for calibration curve.

3. Results and discussion

3.1. Optimization and validation of the HPLC method

The fluorescence of the yeast cells loaded with NDA was observed using confocal microscopy in a qualitative way (*data not shown*). The main scope for the development of the presently reported HPLC technique is to verify the real uptake of the probe NDA by cells and to estimate its reactivity level with GSH inside the cells. Thus, it was necessary to settle a method which can identify the resulting NDA-GSH adduct and quantify it. For this purpose, a classical isocratic reversed-phase HPLC technique was used and quantification was operated using NDA standard solutions derivatized with an excess of GSH. A GSH/NDA molar concentration ratio of ca. 120 was used at the highest limit of the linearity range. First, the optimum reaction time in borate buffer to obtain the maximum adduct formation at room temperature (presently 20 ± 2 °C) was examined (Fig. 2A): a maximum was reached after 5 min and a plateau was observed for a reaction time up to 30 min; a reaction time of 15 min was retained in further experiments. Next, the stability of the NDA-GSH adduct formed in the conditions defined above, was studied at 4 °C

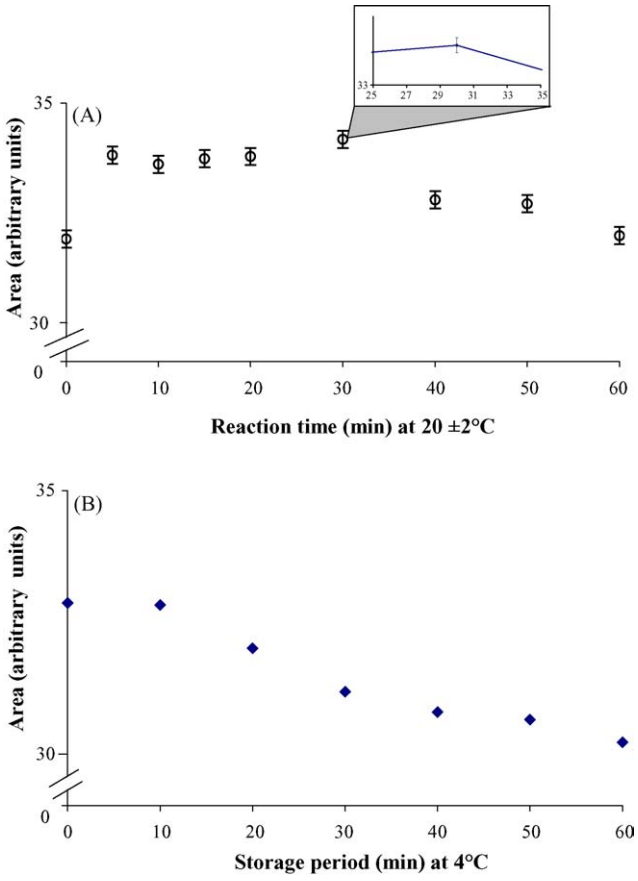

Fig. 2. Formation of the NDA-GSH adduct as a function of (A) reaction time at 20 ± 2 °C and (B) its stability study at 4 °C; concentration of NDA tested: 8.5 ng mL^{-1} (45 nM). Values are the mean of three independent experiments ± standard deviation.

(Fig. 2B): over an 1 h storage period, no significant signal decrease was observed.

Validation of the HPLC technique was realized concerning the following points:

(i) Selectivity was first tested versus the reagent: no peak was observed for the reagent blank, containing NDA but no GSH. Next, selectivity was studied with regard to different amino-acids (cysteine, histidine, histamine, arginine) and a peptide, i.e. γ-glutamylcysteine (γ-glucys). All these compounds can potentially react with NDA, as previously mentioned [15]. The experimental approach consisted in NDA derivatization with the different compounds cited above, instead of GSH, and at the same concentration than GSH. No signal was observed for all the compounds tested but for γ-glucys, which is the biosynthetic precursor of GSH in the cytosolic compartment and exists at very low levels in most of eukaryotic cells. We observed for the corresponding derivative a baseline resolution with the peak of NDA-GSH adduct (the relative retention time (RRT) versus NDA-GSH adduct was equal to 0.81).

(ii) Linearity was observed between 1.7 and 42 ng mL^{-1} (9–230 nM): the equation of the regression line was $y = (5.160 \pm 0.003)x - (0.66 \pm 0.52)$ ($r^2 = 0.9999$; $n = 3$), where y is the area of the peak corresponding to the NDA-GSH adduct and x is the NDA concentration expressed in ng mL^{-1}.

(iii) Limits of detection (LoD) and quantification (LoQ) were calculated using European Pharmacopeia definition [24]: resulting values obtained for LoD and LoQ were 0.7 ng mL^{-1} (4 nM) with a signal-to-noise ratio (S/N) of 3, and 1.7 ng mL^{-1} (9 nM) with S/N = 10, respectively.

(iv) Repeatability: coefficients of variation ($n = 5$) were calculated for three concentrations in the linearity range (42, 8.3 and 1.7 ng mL^{-1}, this latter corresponding to the LoQ): values were 1.84, 2.08 and 0.86%, respectively.

A typical chromatogram corresponding to a NDA standard solution derivatized with GSH is shown in Fig. 3A.

3.2. Optimization of cell extraction

We observed that the NDA-GSH adduct was unstable both in a perchloric acid solution, and in the mobile phase used in the present HPLC system, which preclude their use for the extraction from cells. Thus, we tested both PBS solution pH 7 and borate buffer pH 9.2, for the extraction step. Two physical processes were also compared for cell lysis: operating a freezing cycle or using an ultrasonic probe. According to data shown in Fig. 4, combining a cell freezing cycle and borate buffer gives rise to the highest efficiency of extraction of NDA-GSH adduct. Moreover, full selectivity was obtained versus endogenous compounds, as demonstrated by the chromatograms of cell extracts loaded or not with NDA (Fig. 3B and C). In cell extracts, several unidentified peaks including a major one eluting later than the NDA-GSH (RRT = 1.1 versus NDA-GSH adduct) were observed. NDA-GSH was clearly identified and quantified using a calibration curve realized with NDA standard solutions derivatized with an excess of GSH. No NDA- γ-glucys adduct was detected in the cell extracts. At last, the supernatant obtained after cell loading with NDA was analyzed: no NDA-GSH adduct peak was observed, which demonstrates that neither release of NDA-GSH adduct from cells nor extracellular GSH reaction with NDA occur during that step.

3.3. Optimization of cell loading

The staining of cellular GSH with NDA, in the yeast strain presently used as a cellular model, was optimized as a function of the probe concentration (20, 100 and 500 μM), and the incubation time of cells with the probe (10, 20 and 30 min). Data shown in Fig. 5 indicate that 100 μM and 20 min are optimum NDA concentration and loading period, respectively,

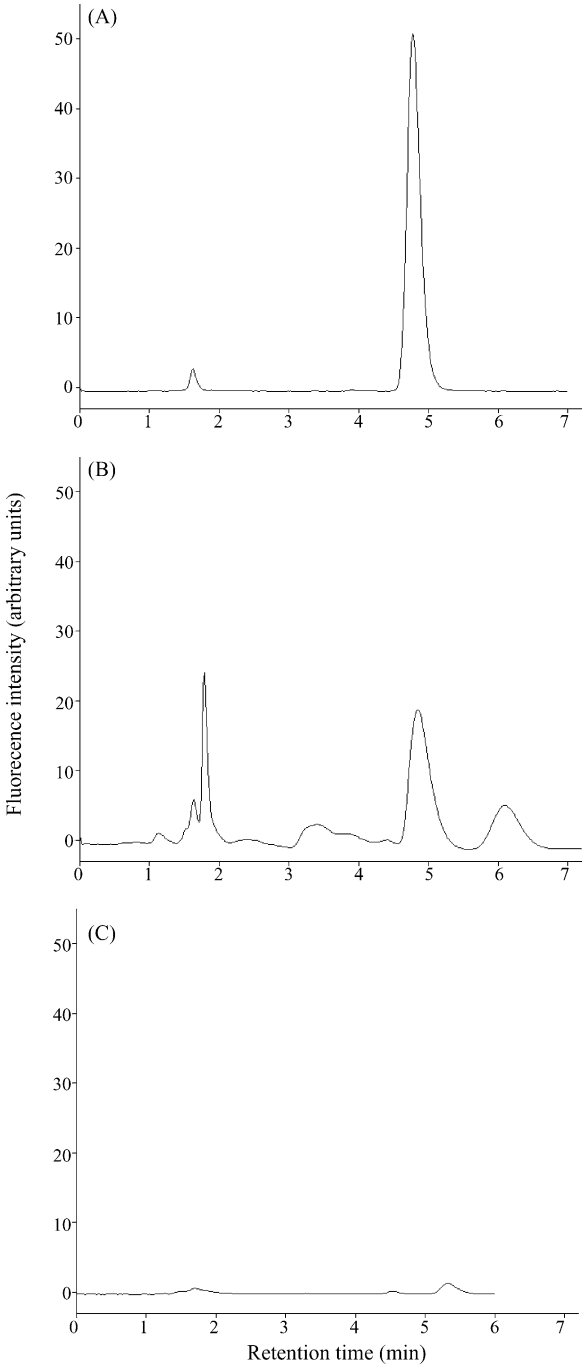

Fig. 3. Typical chromatograms corresponding to (A) a NDA standard solution at a concentration of 230 nM derivatized with GSH; (B) cells incubated with NDA (100 μM for 20 min) and extracted with borate buffer pH 9.2 after a freezing cycle; and (C) cell lysate without NDA loading. For chromatographic conditions, see Section 2.

when considering the maximum NDA-GSH adduct concentrations (3.15 ± 0.29 nmol mg^{-1} protein; $n = 3$) measured by HPLC. These parameters have probably to be optimized for any other prokaryotic or eukaryotic cell types studied and the presently described HPLC method should be a very helpful analytical tool for that purpose.

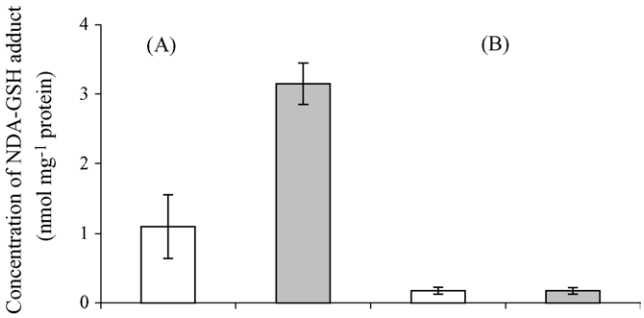

Fig. 4. Variation of cellular NDA-GSH concentration as a function of extractive conditions: cells incubated with NDA (100 μM for 20 min) are treated by (A) freezing cycle, (B) ultrasonic probe, and suspended in PBS pH 7.0 (□) or in borate buffer pH 9.2 (■). Values are the mean of three independent experiments ± standard deviation.

3.4. Comparison of cell loading by OPA and NDA

Cells were loaded with OPA in the same conditions than those previously optimized for NDA, i.e. a 100 μM concentration and an incubation period of 20 min. Next, OPA-GSH adduct was extracted by applying a cell freezing cycle and resuspending in borate buffer and the resulting cell extracts were analyzed in the same HPLC conditions than for NDA-GSH adduct, except for the mobile phase composition with a lower methanol content, i.e. 5% (v/v) instead of 20% (v/v). Typical chromatograms are shown in Fig. 6.

Linearity was verified in the concentration range 1.7–42 ng mL^{-1} (12.5–311 nM): the equation of the regres-

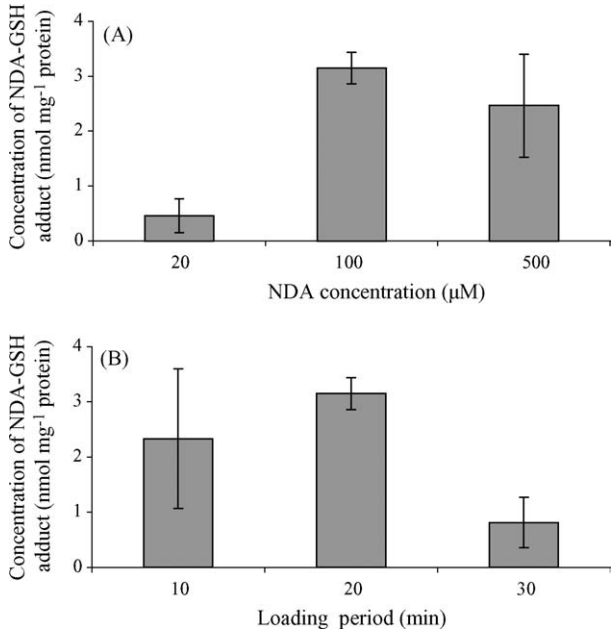

Fig. 5. Optimisation of staining conditions of cells as a function of (A) NDA concentration (cells are incubated with NDA (20, 100 and 500 μM) for 20 min) and (B) loading period (cells are incubated with NDA (100 μM) for 10, 20 and 30 min). Values are the mean of three independent experiments ± standard deviation.

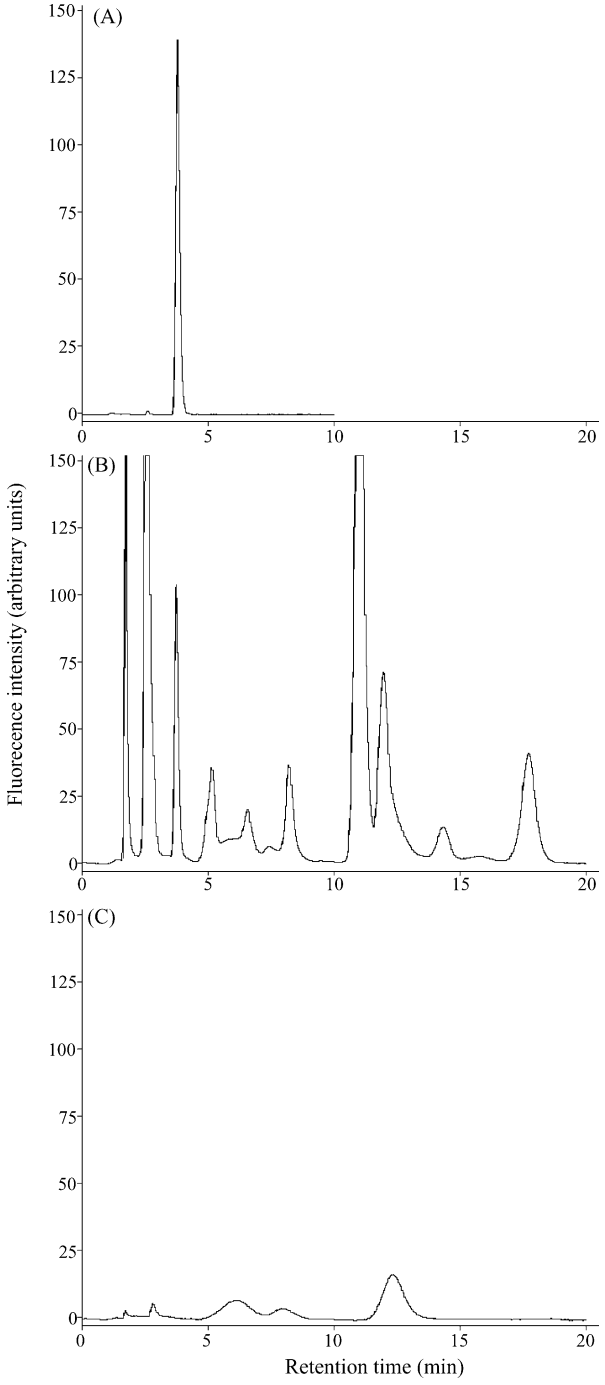

Fig. 6. Typical chromatograms corresponding to (A) a OPA standard solution at a concentration of 311 nM derivatized with GSH; (B) cells incubated with OPA (100 μM for 20 min) and extracted with borate buffer pH 9.2 after a freezing cycle; and (C) cell lysate without OPA loading. For chromatographic conditions, see Section 2.

sion line was $y = 35.76x + 13.11$ ($r^2 = 0.9999$), where y is the area of the peak corresponding to the OPA-GSH adduct and x the OPA concentration expressed in ng mL^{-1}). An OPA-GSH adduct concentration of 1.72 ± 0.06 nmol mg^{-1} protein ($n = 3$) was found in cell extracts, which appears 1.8-fold less than the NDA-GSH adduct concentration (see Section 3.4 and Fig. 5). NDA is more lipophilic than OPA, thus, it could penetrate the cell with a better yield than OPA (calculated partition coefficient $\log k_{ow}$ of OPA and NDA are equal to 2.13 and 3.42, respectively). However, other mechanisms can act with a different intensity, such as the reactivity of OPA and NDA with the amino or sulfhydryl groups of cell wall components. Moreover, many other peaks, corresponding to unidentified cytosolic components which react with OPA, appear on chromatograms (Fig. 6B and C). Thus, OPA seems less selective than NDA.

3.5. Monitoring cellular GSH variations

The usefulness of NDA as a fluorogenic probe able to monitor variations of GSH concentration in the yeast strain, was tested by varying the cellular GSH level with BSO. This compound is a selective inhibitor of γ-glutamylcysteinyl ligase (E.C. 6.3.2.2), which is a cytosolic key enzyme in the biosynthesis of GSH. Cells were treated or not with BSO added to the culture medium at two concentrations: 1 and 5 mM, for 4 h. A decrease of the NDA-GSH adduct concentration in cells was observed in presence of BSO: 1.31 ± 0.07 and 0.41 ± 0.01 nmol mg^{-1} protein ($n = 3$) for 1 and 5 mM of BSO, respectively (the NDA-GSH adduct concentration without BSO was 3.15 ± 0.29 nmol mg^{-1} protein). These values are opposite to previous data which conclude in no sensitivity of NDA for detection of cellular GSH variations [11]. However, our data were obtained in experimental conditions (yeast cells loaded with 100 μM of NDA) different than those previously used (mammalian cells loaded with 650 μM of NDA).

4. Conclusion

The presently described HPLC method has permitted to identify the NDA-GSH adduct formed in yeast cells after their loading with the free probe NDA. Moreover, the optimization of the probe loading step by cells was possible by quantifying the extracted NDA-GSH adduct. This approach could be applied to other cell lines than the presently used yeast strain. In conclusion, NDA seems a promising probe for intracellular GSH staining coupled with direct in situ methods, e.g. flow cytometry and confocal microscopy, as most of those equipments include an argon laser with an emission line at 488 nm, well fitted to the excitation of the NDA-GSH adduct.

At last, this is the first report of the measurement of the aromatic dialdehydes OPA and NDA using GSH as a derivatizing reagent in a HPLC system.

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