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SHORT COMMUNICATION

HPLC study of the host–guest complexation between fluorescent glutathione derivatives and β-cyclodextrin

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Abstract RP-HPLC and the van't Hoff law were used to study the association in which β-cyclodextrin forms inclusion complexes with aminothiol–phthaldialdehyde derivatives prepared from either glutathione (GSH) or γ-glutamylcysteine (γ-glucys) and either naphthalene-2,3-dicarboxaldehyde (NDA) or *o*-phthaldialdehyde (OPA). Elution was carried out at pH 8.5, the derivatization pH which gave the highest fluorescence signal during batch experiments. The variation of the retention factor (*k*) was monitored as a function of column temperature (10–35 °C) and β-cyclodextrin concentration (0–5 mM) in the mobile phase. Apparent binding constants, enthalpy and entropy were calculated from van't Hoff plots for the complexation reaction. These data lay the groundwork for the improvement of high throughput GSH quantification methods using fluorimetry in biological and vegetal samples.

Keywords β-Cyclodextrin · Glutathione · HPLC · Inclusion complexes · van't Hoff

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Abbreviations

K_f	Apparent association constant
CD	Cyclodextrin
γ-glucys	γ-Glutamyl-L-cysteine
GSH	Glutathione
NDA	Naphthalene-2,3-dicarboxaldehyde
OPA	*o*-Phthaldialdehyde
k	Retention factor

Introduction

Reduced glutathione (γ-L-glutamyl-L-cysteinylglycine; GSH) is the principal non-protein thiol ubiquitous in animals, plants and even in microorganisms. It is the most prominent cellular antioxidant and the chief indicator of oxidative stress [1]. As an example, intracellular levels of GSH in human tissues normally range from 0.1 to 10 mM [1], concentrations that have made this compound difficult to measure until now.

Because of the key role and widespread presence of GSH in biochemical systems, reports of non-separative and separative detection and quantification methods for GSH have been published from several laboratories including ours [1–4]. Fluorimetry is currently the most popular technique for GSH detection. In this method, GSH reacts with a probe to form a fluorogenic adduct. Derivatizing agents such as aromatic dialdehydes, i.e. *o*-phthaldialdehyde (OPA) and naphthalene-2,3-dicarboxaldehyde (NDA) are widely used to label nucleophilic primary amino compounds, especially amino acids and peptides. GSH and its biosynthetic precursor, γ-glutamyl-L-cysteine (γ-glucys) have similar nucleophilic properties which permit their highly selective detection [2–6]. NDA and OPA are

inexpensive and the derivatization reaction to form fluorescent isoindolic adducts is quite rapid at ambient temperature. For this reason, aromatic dialdehydes such as NDA and OPA have been extensively used for thiol derivatization in HPLC [2, 3] and capillary electrophoresis [4] methods, and more recently in non-separative techniques such as microplate reading [5] and even bioimaging [6]. A simple and reliable quantification method for this important compound is reported below that will serve as a key biochemical tool. The sensitivity of these available quantification methods can potentially be increased by inclusion of the fluorescent derivatives into cyclodextrins.

Cyclodextrins (CD) are crystalline, water soluble, cyclic, non-reducing, oligosaccharides of six (α-CD), seven (β-CD) or eight (γ-CD) glucopyranose units linked α-(1,4). According to Connors [7] the CD cavity diameter is about 0.5 nm for α-CD, 0.7 nm for β-CD and 0.8 nm for γ-CD; according to Szejtli [8] it is about 0.6, 0.8 and 0.9 nm, respectively. The CDs are wreath-shaped truncated cones. Their cavities are hydrophobic, while their external surfaces are hydrophilic. Its conical shape gives CD host properties that allow selective incorporation of various types of guest substrates having particular size and polar characteristics [8–11]. The reactivities of CDs can be adjusted by chemical modification of the lateral hydroxyl groups [12]. These compounds are widely used to mimic natural enzymes [13, 14], and they are also used as selective agents in HPLC and capillary electrophoresis systems [15]. The inclusion of a fluorescent compound into CD may lower the limits of its measurement by protecting it against quenching [16], a finding that gives further impetus to our work. Several authors have noted the influence of CD on the fluorescence intensity of OPA and NDA adducts: Nakamura and Tamura [17] reported a signal improvement when β-CD was added to a reaction medium containing OPA and taurine; Bantan-Polak et al. [18] reported that the fluorescence of 15 amino acids–NDA adducts was enhanced by 70% with the addition of β-CD at a 10 mM final concentration; Wagner and McManus [19] observed a threefold increase in the fluorescence signal of the glycine–OPA adduct in the presence of 10 mM of hydroxypropyl-β-CD. To our knowledge, the interaction of aminothiol–dialdehyde adducts with CD have not been studied to date. HPLC has demonstrated its usefulness in studies of molecular interaction. The inclusion complexes with CD can be examined by HPLC in two different ways. First, the Hummel–Dreyer method [20] employs a mobile phase containing the guest molecule into which the solution of CD is injected. In the second method the guest compound is injected and the mobile phase contains various concentrations of CD [21]. Both methods permit the calculation of the apparent association constant (K_f) from the change in retention factor (k).

Recently we noted that the fluorescence signal of GSH–NDA adducts can be significantly enhanced by the addition of β-CD to the derivatization mixture [5].

This paper reports host parameters of β-CD for the inclusion of the aminothiol aromatic dialdehyde adducts shown in Fig. 1: GSH–NDA (a), GSH–OPA (b), γ-glucys–NDA (c), and γ-glucys–OPA (d). Our strategy utilizes an RP-HPLC system developed in our laboratory for the isolation of these adducts. Variations in k as a function of β-CD concentration were measured at various temperatures (10–35 °C). The apparent K_f values were calculated by Fujimura's method [21]. The apparent enthalpy, entropy and Gibbs free energy for association were also calculated. This parametric information will permit the development of high throughput non-separative and separative quantification methods for GSH and γ-glucys in various complex biological samples.

Experimental

Chemicals and reagents

All chemicals and solvents were of analytical or HPLC grade and used without further purification. GSH was obtained from Sigma (Lyon, France) and γ-glucys from Bachem (Weil am Rhein, Germany). OPA and NDA were purchased from Fluka (Lyon, France). β-CD was obtained from Acros (Geel, Belgium). GSH and γ-glucys stock solutions were prepared at 3.4 mM in 0.1 M HCl added with 2 mM EDTA, and stored at −20 °C for up to one month. Working standard solutions were prepared daily from each stock solution by diluting in 0.1 M HCl added with 2 mM EDTA.

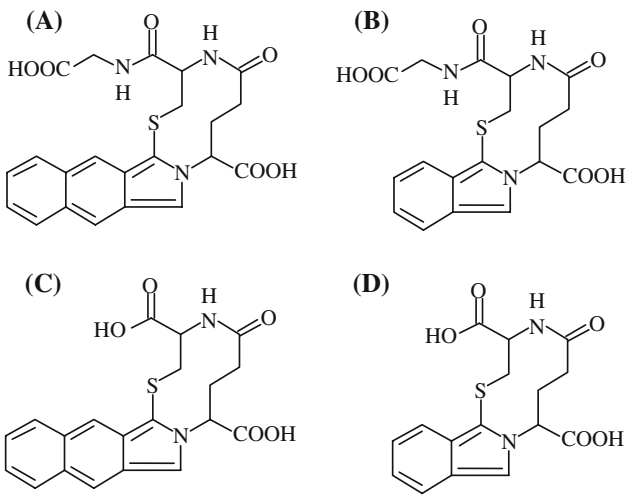

Fig. 1 Chemical structures of the aminothiol–aromaticdialdehydes adducts studied: GSH–NDA (**a**); GSH–OPA (**b**); γ-glucys–NDA (**c**); γ-glucys–OPA (**d**)

Derivatization procedure

A 1 mg mL^{-1} OPA or NDA solution was prepared in ethanol and stored at −20 °C for up to one month without loss of activity. The derivatization procedure was performed as follows: to 160 µL of 0.2 M borate buffer pH 9.2 was added 20 µL of OPA solution or NDA solution and 20 µL of 3.4 µM of GSH or γ-glucys standard solutions. After shaking for 10 min in the dark at 20 ± 2 °C, the reaction medium was cooled at 4 °C and 20 µL samples were injected into the HPLC system.

HPLC system and operating conditions

The HPLC system consisted of a Merck L6200 pump system, a Merck L4000 UV–vis detector (Darmstadt, Germany) and a Rheodyne manual injector fitted with a 20 µL loop. Temperature regulation of the column was ensured by placing it inside a straight double walled condenser. Water from a constant temperature bath was pumped through the jacket of this condenser, and a thermocouple was placed alongside the column to monitor the actual separation temperature. The temperatures tested were: 10, 15, 20, 25, 30 and 35 °C. The HPLC separation procedure was adopted from the optimized derivatization conditions of GSH by NDA [5]. Methanol mixed with aqueous 0.05 M phosphate and adjusted to a final pH of 8.5 was used as the mobile phase at a flow rate of 0.8 mL min^{-1}. The aqueous phase was 5% (v/v) for the OPA derivatives and 20% for the NDA derivatives. The column was an HPLC cartridge (250 × 4.6 mm i.d.) packed with endcapped Purospher STAR RP-18 (5 µm). This material can tolerate alkaline mobile phases. Following injection of each sample, the adducts were immediately eluted with buffer mixed with β-CD at concentrations of 0, 1, 2, 3, 4, or 5 mM. These concentrations produced wide variations of retention factor (k) values for each temperature used. Spectrophotometric detection was tuned at 330 nm for aminothiol–OPA adducts and at 458 nm for aminothiol–NDA adducts Acquisition and integration of chromatograms was carried out with Azur® software (Datalys, Saint Martin d'Heres, France).

Microplate assay

Samples were prepared as previously described [14]. Briefly, 160 µL of 0.2 M borate buffer pH 9.2 containing variable amounts of β-CD was pipetted into each of a 96-well black microplate, then 20 µL of 3.25 µM GSH or γ-glucys solution and 20 µL NDA or OPA stock solution were successively added. The final concentration of β-CD added to the borate buffer in the well varied from 0 to 6 mM. The microplates were swirled for 10 min at 500 rpm in the dark at 20 °C. The microplates were then analyzed by a Victor[3] (Perkin-Elmer, Courtaboeuf, France) microplate fluorescence reader. Excitation wavelengths were: 355 ± 40 nm for OPA adducts and 485 ± 14 nm for NDA adducts; emission wavelengths were: 460 ± 25 nm for OPA adducts and 535 ± 25 nm for NDA adducts. Two blank samples were used: (i) 180 µL of borate buffer without β-CD and 20 µL of NDA or OPA stock solution and (ii) 180 µL of borate buffer with different concentrations of β-CD and 20 µL of NDA or OPA stock solution. Each sample was measured in triplicate.

Results and discussion

Results of the HPLC study

Figure 1 shows the chemical structures of the four aminothiol–aromaticdialdehyde adducts (a, b, c, d) studied. This series probes structural effects on complexation with β-CD, of the additional glycine residue (compare γ-glucys to GSH), and of an additional aromatic ring (compare OPA to NDA). Typical chromatograms are shown in Fig. 2. An HPLC-system back-pressure of close to 10^7 Pa was needed for the retention times obtained. Baseline resolution ($R > 1.2$) was achieved between the peaks corresponding to the GSH–NDA and γ-glucys–NDA adducts with a β-CD concentration up to 2 mM and a temperature higher than 30 °C. At higher β-CD and lower temperatures, these adducts co-eluted. Media containing only one adduct were then injected. Baseline resolution was also obtained between the peaks of the two aminothiol–OPA adducts, using the above eluting conditions. The measured k values vary from 1.86 to 22.16 for the aminothiol–NDA adducts and from 2.54 to 11.46 for the aminothiol–OPA adducts. Classical linear relationships between k and the β-CD concentration in the mobile phase were then obtained for each temperature tested.

K_f for complexation of adducts a–d with β-CD

We report complexation constants (K_f) between these adducts and β-CD. Because samples of the adduct–β-CD complexes prepared before injection were not retained in the HPLC system, we were able to apply the method of Fujimara et al. [21] which assumes that complexation by β-CD increases adduct solubility in the mobile phase thereby decreasing retention time and the retention factor (k).

If the stoichiometry of the adduct:β-cyclodextrin complex is 1:1, the complexation constant K_f is given by the expression in Eq. 1 where $[ACD]_m$ is the concentration of complex in the mobile phase, $[A]_m$ is the concentration of adducts in the mobile phase, and $[CD]_m$ is the concentration of β-CD in the mobile phase. The expressions (2) and (2′) give the retention factors where $n_{(A)s}$ is the quantity of

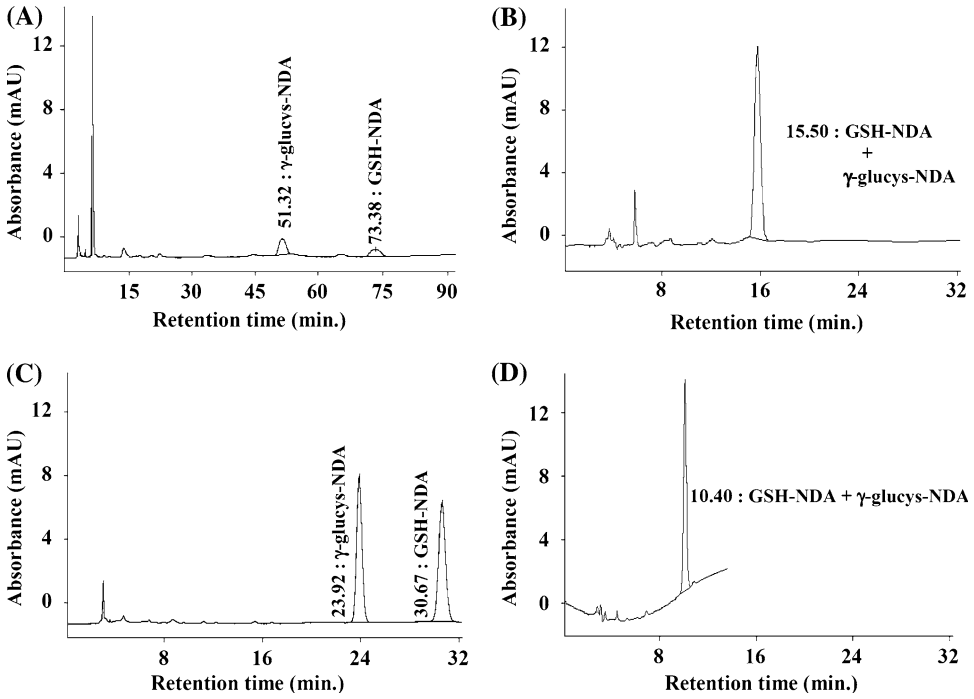

Fig. 2 Typical chromatograms obtained for aminothiol–NDA adducts without β-CD in mobile phase at 10 °C (**a**); with 5 mM β-CD in mobile phase at 10 °C (**b**); without β-CD in mobile phase at 35 °C (**c**); with 5 mM β-CD in mobile phase at 35 °C (**d**)

the adduct in the stationary phase, $n_{(ACD)m}$ is the amount of the complex in mobile phase, $n_{(A)m}$ is the adduct amount in the mobile phase, k is the retention factor for each adduct and k_0 is the retention factor in the absence of cyclodextrin. Since retention factors are used, the void volume of the HPLC system, including the column, need to be known. The minor disturbance method [22] was used to determine the void volume of the HPLC system. This was found to be 2.6 ± 0.15 mL (the average ± 1 standard deviation over all 288 injections used in the study).

After mathematical manipulation from (1), (2) and (2′), the expression (3) is the affinity relationship between $1/k$ and $[CD]_m$.

$$K_f = \frac{[ACD]_m}{[A]_m \cdot [CD]_m} \quad (1)$$

$$k = \frac{n_{(A)s}}{n_{(A)m} + n_{(ACD)m}} \quad (2)$$

$$k_0 = \frac{n_{(A)s}}{n_{(A)m}} \quad (2')$$

$$\frac{1}{k} = K_f \frac{[CD]_m}{k_0} + \frac{1}{k_0} \quad (3)$$

For the NDA adducts (a) and (c), plots of $1/k$ vs. $[CD]_m$ gave straight lines with $r > 0.99$ for each temperature tested (10, 15, 20, 25, 30 and 35 °C). An example is presented in Fig. 3a. This indicates that the stoichiometry of the complexation between the NDA adducts and β-cyclodextrin is 1:1. K_f values corresponding to each temperature are reported in Table 1. These results provide a basis for the associative character between aminothiol–NDA adducts and β-cyclodextrin; this in turn will give theoretical reinforcement to a quantitative method for γ-glucys and GSH quantification using spectrofluorimetry without a separative step [14].

The aminothiol–OPA adducts b and d, on the other hand, did not give clear linear relationships ($r > 0.97$) for Eq. 3 over the concentration and temperature ranges studied. This suggests that complexes other than 1:1 are being formed. Equations 4 and 5 apply to the 1:2 adduct:CD case, where K_f becomes $K_{f1:2}$. In fact, plots of $1/k$ vs. $[CD]_m^2$ for the aminothiol–OPA adducts gave straight lines with $r > 0.99$ at each temperature tested. An example is presented at Fig. 3b.

The prediction of a 1:2 OPA adduct: β-CD complex can be visualized with molecular models, in which the adduct is "sandwiched" between two β-CD molecules. The OPA adducts presents relatively plane shape, the aromatic moieties could interact with the hydrophobic cavities of two β-CD units without inclusion. On the contrary, we can conclude that the naphthalene moiety of the adduct is inside the cavity of a single β-CD. For both OPA and NDA adducts the two carboxyl groups are converted into carboxylates at pH 8.5. The presently proposed models respect this characteristic by presenting no inclusion of the peptidic moiety in the hydrophobic cavity of β-CD. These results are consistent with a molecular modeling program with energy minimized structures using ChemOffice software MOPAC® method (Fig. 4a, b).

$$K_{f1:2} = \frac{[ACD]_m}{[A]_m \cdot [CD]_m^2} \quad (4)$$

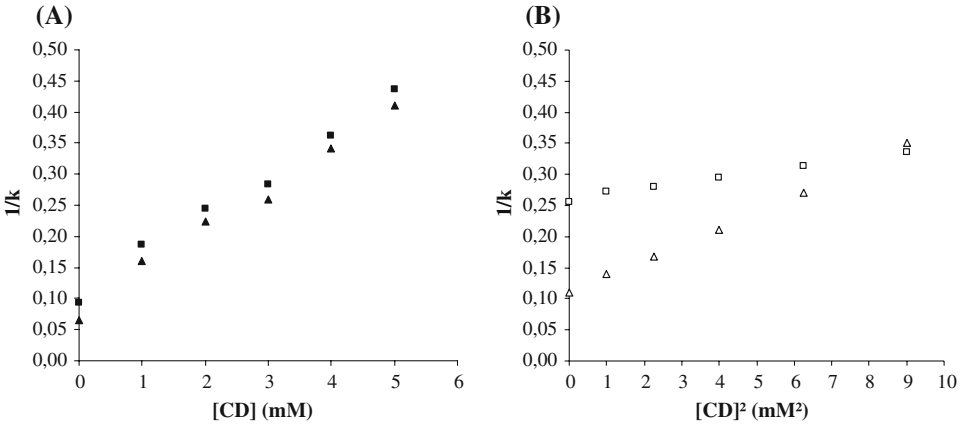

Fig. 3 **a** Plots of $1/k$ versus $[CD]_m$ for elution of GSH–NDA (▲); γ-glucys–NDA (■) adducts at $T = 20$ °C. **b** Plots of $1/k$ versus $[CD]_m^2$ for elution of GSH–OPA (△) or γ-glucys–OPA (□) adducts at $T = 20$ °C

Table 1 K_f values for complexation between aminothiol–aromaticdialdehyde adducts and β-CD

T (°C)	GSH–NDA $K_{f1:1}$	GSH–OPA $K_{f1:2}$	γ-Glucys–NDA $K_{f1:1}$	γ-Glucys–OPA $K_{f1:2}$
10	1308	931	875	129
15	1191	1662	775	323
20	934	2394	663	316
25	818	3356	582	426
30	699	4499	538	465
35	535	5977	430	560

$$\frac{1}{k} = K_{f1:2}\frac{[CD]_m^2}{k_0} + \frac{1}{k_0} \quad (5)$$

One of the main goals of this work was to evaluate the influence of complexation of adducts with β-CD on their fluorescence intensities. This influence was tested with a microplate fluorescence reader. Figure 5a plots fluorescence intensity of the derivation mixes containing NDA vs. β-CD concentration. A linear relationship ($r > 0.99$) between fluorescence intensity and β-CD concentration is observed until β-CD is raised to 3 mM. Another linear relationship has been reported between fluorescence intensity and aminothiol–NDA adducts concentration in the mix [5]. These measurements confirm a 1:1 stoichiometry of the complexation between the NDA adducts and β-CD. Furthermore fluorescence intensity of aminothiol–NDA adducts doubles when 3 mM of β-CD are added to the derivatized mixture, and without change of the emission wavelength (inset in Fig. 5a).

Figure 5b shows the effect of added β-CD on the intensity of the aminothiol–OPA adduct fluorescence when β-CD is added to the derivatization mix. No linear relationship is observed between fluorescence intensity and β-CD concentration but one is observed between fluorescence intensity of GSH adducts and the square of the β-CD concentration until β-CD is raised to 6 mM ($r > 0.99$). This is further evidence that the complexation of aminothiol–OPA adducts with β-CD is a 1:2 ratio.

On another hand, microplate results presented on Fig. 5a permits the calculation 1:1 association constants between NDA adducts and β-CD using the Benesi Hildebrand method [23]. For NDA adducts $K_{1:1}$ are calculated using Eq. 6 where ΔF is the fluorescence intensity in the microplate mix in presence of β-CD divided by background, ΔF_c is the part of the fluorescence intensity generated by the included adduct and [CD] is the β-CD concentration in the microplate well. For both GSH–NDA and γ-glucys–NDA adducts, Eq. 6 presents straight lines ($R > 0.99$), their equation permits the determination of 1:1 association constants. For GSH–NDA the calculated association constant with β-CD is 1,264 and for γ-glucys–NDA the calculated association constant with β-CD is 869 at room temperature around 20 °C. Theses results are comparables to the ones obtained by HPLC method.

$$\frac{1}{\Delta F} = \frac{1}{\Delta F_c \cdot K_{1:1} \cdot [CD]} + \frac{1}{\Delta F_c} \quad (6)$$

Thermodynamic constants

From a thermodynamic point of view, K_f shows two different behaviors: the complexation of β-CD with aminothiol–OPA adducts is promoted by temperature. The opposite effect is observed with aminothiol–NDA adducts (Table 1). These observations confirm that the aromatic part of the aminothiol–aromaticdialdehyde adducts is a key feature of the complexation process. A thermodynamic study of the data adds to our understanding of the findings above. The van't Hoff law (7) concerns the effects of a change in temperature on equilibrium and yields ΔH_f and ΔS_f for complexation. These parameters will give further insight on the complexes between the aminothiol adducts and β-CD.

$$\ln K_f = -\frac{\Delta G_f}{RT} = -\frac{\Delta H_f}{RT} + \frac{\Delta S_f}{R} \quad (7)$$

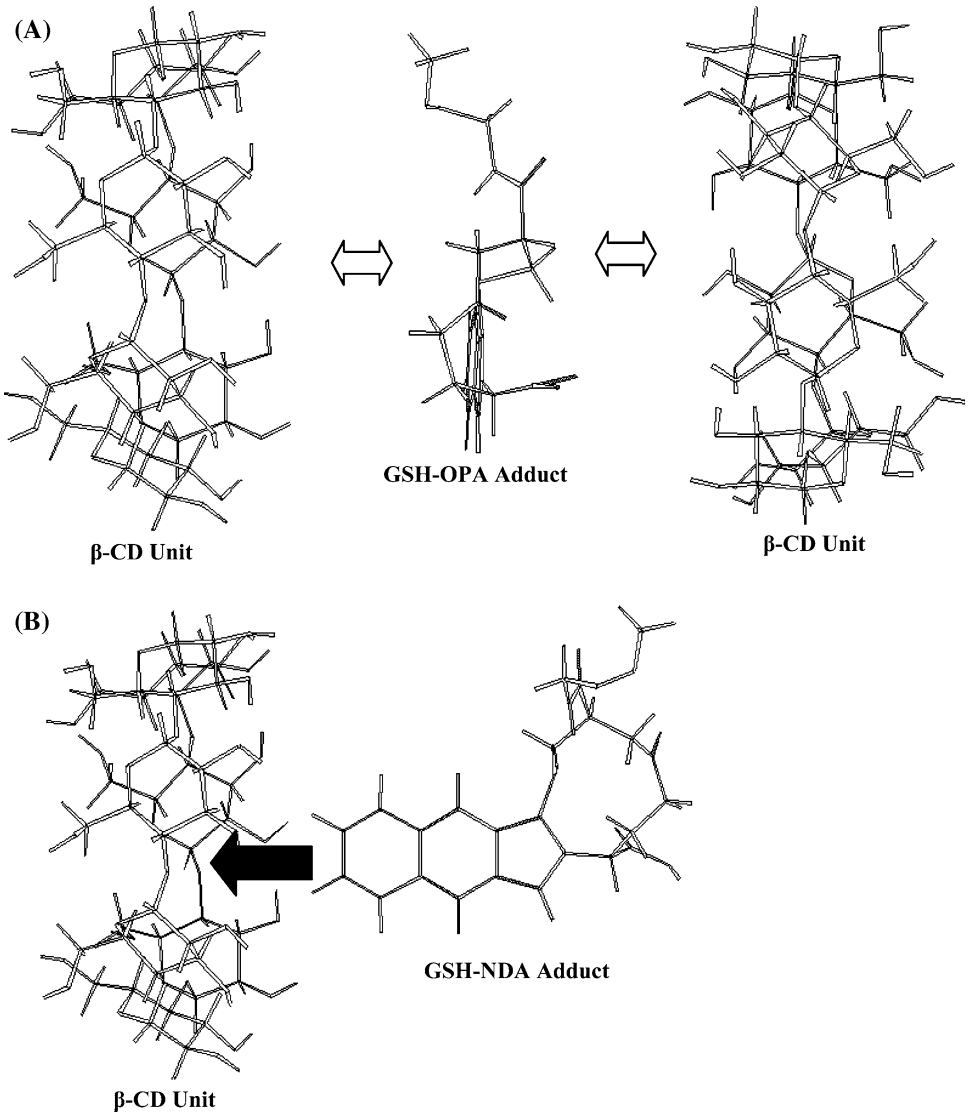

Fig. 4 **a** Hypothesis for the geometry of 1:2 GSH–OPA adduct:β-CD complex. White arrows symbolize interactions between hydrophobic cyclodextrin cavity and GSHOPA adduct. **b** Hypothesis for the geometry of 1:1 GSH–NDA adduct:β-CD complex. Black arrow symbolizes inclusion of GSH–NDA adduct in hydrophobic cyclodextrin cavity

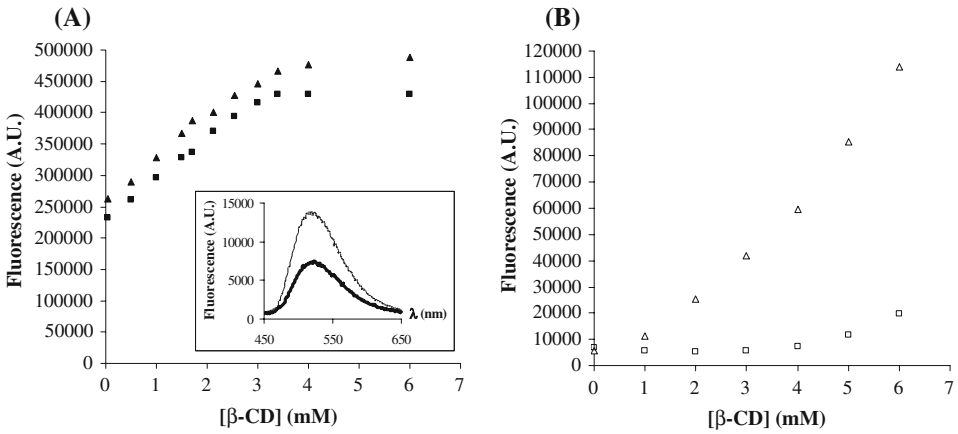

Fig. 5 Variations of the fluorescence intensity of aminothiol–aromatic dialdehyde adducts at concentrations of 3.4 μM as a function of β-CD concentration for **a** GSH–NDA (▲) and γ-glucys–NDA (■) adducts; and for **b** GSH–OPA (△) and γ-glucys–OPA (□) adducts. Inset: fluorescence emission spectra of GSH–NDA adduct without β-cyclodextrin (▬) and with 1 mM β-cyclodextrin (−)

First, it is necessary to determine if the chromatographic behaviors of the four adducts are similar. K_t is the transfer constant from mobile to stationary phase; Eq. 8 links K_t with the retention factor k and ϕ the phase ratio of the column.

$$K_t = \frac{[A]_{stat}}{[A]_{mob}} = \frac{k}{\phi} \Rightarrow \ln k = \ln K_t + \ln \phi = -\frac{\Delta H_t}{RT} + \frac{\Delta S_t}{R} + \ln \phi \quad (8)$$

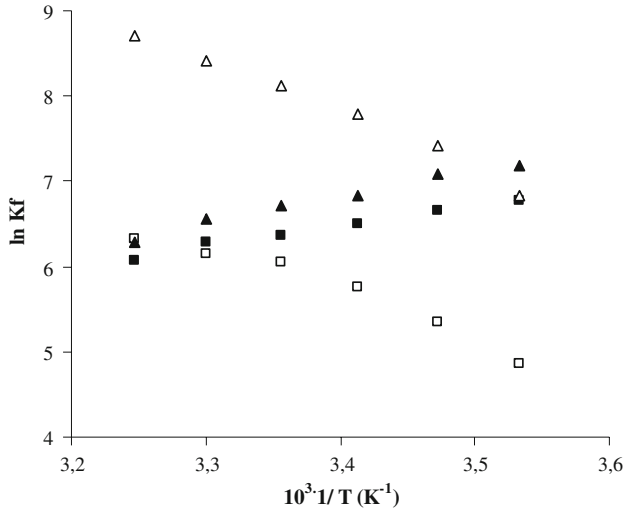

Fig. 6 van't Hoff plots generated using K_f values for β-cyclodextrin complexes of GSH–NDA–β-cyclodextrin (▲), γ-glucys–NDA–β-cyclodextrin (■), GSH–OPA–(β-cyclodextrin)$_2$ (△), γ-glucys–OPA–(β-cyclodextrin)$_2$ (□)

The ϕ value of the column used during this study is 0.88 as calculated from by the manufacturer's data. The representation of ln(k) as function of T^{-1} shows straight lines for each β-CD concentration and each adduct. In the β-CD concentration range, ΔH_t and ΔS_t values calculated for the four adducts are very close. For example, when the β-CD concentration is 1 mM, ΔH_t is -13 kJ mol^{-1} for GSH–NDA adduct and -15 kJ mol^{-1} for γ-glucys–NDA adduct and ΔS_t is -28 J K^{-1} mol^{-1} for GSH–NDA and -35 J K^{-1} mol^{-1} for γ-glucys–NDA. The nature of the adduct does not influence the chromatographic process.

Figure 6 presents the van't Hoff plots obtained for each of the four 1:1 stoichiometry aminothiol–aromaticdialdehydes and β-CD complexes, and for the two possible 1:2 stoichiometry aminothiol–OPA and β-CD complexes. Values of ΔH_f and ΔS_f for the complexation process are extracted from the slope and the intercept of the van't Hoff plot lines and are listed in Table 2. For the temperature range tested (10–35 °C), ΔG_f is negative for all the complexation studied as expected. It is important to note that an complexation of aminothiol–NDA adducts and β-CD give negative values for ΔH_f and ΔS_f, while complexation of aminothiol–OPA adducts and β-CD give positive values. These results suggest that the complex formation between GSH–NDA adducts and β-CD is largely driven by enthalpy ($\Delta H_f = -25.6$ kJ mol^{-1}). The slight entropy change ($\Delta S_f = -31$ J K^{-1} mol^{-1}) can be attributed to van der Waals interactions and solvent disordering or displacement of water molecules from the cavity during guest molecule inclusion [24]. Thus the process of complexation for NDA adducts are enthalpy driven whereas the process of complexation for OPA adducts are entropy driven. Only the inclusions of NDA adducts are effective with the extra aromatic moiety as the guest within the β-CD cavity.

Table 2 Enthalpy, entropy and Gibbs free energy for complexation between aromatic dialdehyde adducts of aminothiols and β-CD

	GSH–NDA	GSH–OPA	γ-glucys–NDA	γ-glucys–OPA
ΔH_f (kJ mol^{-1})	−25.6	52.4	−19.7	36.6
ΔS_f (J K^{-1} mol^{-1})	−31.0	242.8	−13.0	172.2
ΔG_f (kJ mol^{-1}) at 283 K	−16.8	−16.3	−16.1	−12.2
ΔG_f (kJ mol^{-1}) at 308 K	−16.0	−22.3	−15.7	−16.4

Conclusion

The present work points out notable differences in the complexes formed between aminothiol–NDA and β-CD compared to complexes formed between aminothiol–OPA and β-CD. Following our earlier conclusion [5, 6] that NDA adducts are superior for their inclusion properties and greater spectrometric sensitivity, we supply additional advantages for the NDA over OPA for analysis of GSH in problematical matrices such as biological samples. This work shows how thermodynamic considerations can typically be used to improve quantifications. The data reported reveals details of the association between the aminothiol–NDA adducts and β-CD. Details of the inclusion processes discussed here will be used to improve detection techniques for aminothiols in biological matrices with a possible application to imaging techniques.

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