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# Spectrofluorimetric determination of serotonin and 5-hydroxyindoleacetic acid in urine with different cyclodextrin media

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ABSTRACT

Alternative and sensitive spectrofluorimetric methods for the determination of hydroxyindoles, such as serotonin (**5HT**) and 5-hydroxyindoleacetic acid (**5HIA**), were developed on the basis of supramolecular interaction with cyclodextrin (**CD**) nanocavities (**βCD** and hydroxypropyl-**βCD**, **HPCD)** at different pH values. Both substrates and receptors have acidic protons, therefore the interactions produced in different systems were considered. The effects of neutral **CD** at pH 2.00 and 6.994, and of anionic **CD** at pH 13.00 on the specific acid-base species of the compounds at each pH were determined. In all the conditions studied, the fluorescence of the substrates in the presence of **CD** increased. The association constants ($K_A$, $mol^{-1}$ L) between the substrates and **CD** were determined (30–300) and interpreted. A zero-crossing first-derivative spectrofluorimetric method with and without **HPCD** was developed for the simultaneous determination of **5HT** and **5HIA**. The limits of detection ($\mathbf{L_D}$, ng $mL^{-1}$) for the best conditions were 0.37 for **5HT** and 0.50 for **5HIA** at pH 2.00 with **HPCD**. These $\mathbf{L_D}$ proved to be better than others reported. The applicability of the direct and derivative spectrofluorimetric methods to urine samples was demonstrated with good recoveries 92–110% and R.S.D. 1–10%.



## 1. Introduction

Hydroxyindoles are very important biological compounds as hormones, neurotransmitters or pharmaceutical products [1]. In this work we selected serotonin (5-hydroxytryptamine, **5HT**) (Fig. 1a) and 5-hydroxy-3-indolylacetic acid (**5HIA**) (Fig. 1b) as models of hydroxyindoles. **5HT** is an important neurotransmitter of the central nervous system involved in numerous physiological processes [2]; its principal metabolite is **5HIA**, commonly referred to as 5-hydroxyindoleacetic acid. The abnormal production of **5HT** and **5HIA** has been related to cancerous tumours [3]. These abnormalities have also been associated with mental disorders such as depression, bipolar behaviour, schizophrenia [4] and as mediators in the migraine syndrome [5]. Hence, these metabolites require sensitive analytical methods for their quantitative determination with low limits of detections ($\mathbf{L_D}$). The simultaneous determination of **5HT** and **5HIA** is considered vital as their altered level indicates a disturbance in human physiology.

Cyclodextrins (**CDs**) are cyclic oligosaccharides consisting of six (**αCD**), seven (**βCD**) or eight (**γCD**) units of α-D-glucose linked by α-(1,4) bonds. Among the derivatives of native **CDs**, hydroxypropyl-β-cyclodextrin (**HPCD**) has higher solubility in water than its homologue and some analytical advantages, including binding affinities and selectivity [6]. These macrocycles have a nanocavity (0.7 nm internal diameter **βCD**) which allows them to act as hosts and to form inclusion complexes with guest molecules in the solid state or in solution [7]. The complex formation produces, in many cases, changes in the physical and chemical properties of the substrates included [6,7]. The acid-base property of **CDs** ($pK_a$ = 12.2) [8] also provides either a neutral or an anionic receptor depending on the pH of the medium.

**5HT** and **5HIA** have two acidic protons, and different acid–base species with particular fluorescent characteristics are found at a given pH value. These compounds, like other indoles, show quenching of the fluorescence in acid and basic media [9].

Several analytical methods have been reported for the determination of the concentration of **5HT**. The oldest and common method used for blood solution at pH 4 is fluorimetry preceded by an extraction procedure [10]. Other methods differ in the extraction procedure and in the final pH (diluted HCl) for the fluorimetric measurement [11], the solvent (ethanol 30–35% in HCl 1.5 mol $L^{-1}$) [10], or the required derivatization reagent (ninhydrin [10] or o-phtaldehyde [10]). Cyclic voltammetry with glassy or fiber carbon electrodes modified with carbon nanotubes or gold nanoparticles has also been applied [12–15]. Other methods proposed include HPLC with fluorescent [16–18] or electrochemistry [19–22] detection, in some cases with pre-column [23–25] or post-column [26] fluorescent or chemiluminescent [27,28] derivatization. Radioim-

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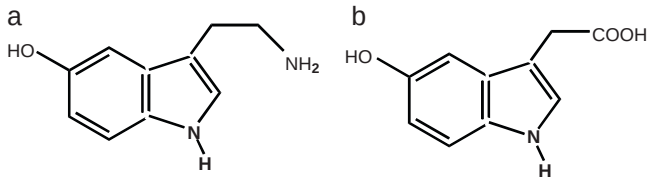



**Fig. 1.** Chemical structures: (a) **5HT** and (b) **5HIA**.

munoassay has also been reported for the chemical conversion of **5HT** in melatonin [29].

The determination of **5HIA** levels was first obtained by spectrophotometry, based on the absorbance of a violet chromophore formed with 1-nitroso-2-naphtol in acid media, a current reference method [30]. A variation of this method was also reported involving the formation of a derivative with 2-mercaptoethanol [31]. Both methods have been applied to urine samples previously extracted with ether and buffer pH 7. The Ehrlich reagent (p-dimethylaminobenzaldehyde in acidic media) has also been used; however, this is not a specific method of analysis [10]. Direct spectrofluorimetry in acid media or after derivatization with o-pthaldehyde has been applied to urine and human fluids previously eluted from chromatographic columns [32,33]. In addition, RIA [10], GC-TD after methylation with diazomethane [10], and HPLC with fluorescence [34–36] or electrochemistry [37] detection, have been reported.

These analytical methods have both advantages and disadvantages. The detection sensitivity of UV/vis method does not generally reach the lower level required for analysis of real sample (detection limit ($L_D$) ~ 12–30 $\mu g\,mL^{-1}$) [10,30,31]. Electroanalytical methods with conventional electrodes normally have low sensitivity, and the preparation of modified electrodes requires further time ($L_D$ ~ 1–10 $ng\,mL^{-1}$) [12–14]. Chromatography-based techniques involve costly equipment and solvents along with complex pre-treatment processes ($L_D$ ~ 1–500 $ng\,mL^{-1}$) [16–28,34–37]. Spectrofluorometry is a high sensitive method for the determination of these analytes ($L_D$ ~ 10 $ng\,mL^{-1}$) [10,32,33]. The development of novel supramolecular fluorescent methods attracts particular attention in analytical and organic studies intended to avoid time-consuming procedures and to control optimal medium conditions.

The importance of **5HT** and **5HIA** levels in daily clinical controls lies in simple cost and effective methods for the rapid monitoring of the two compounds. Therefore, the aim of this work is to determine the best conditions for the direct and simultaneous spectrofluorimetric determination of **5HT** and **5HIA** depending on the pH of the media, and the effect of cyclodextrins as receptors, simplifying the experimental requirements and avoiding derivatization reactions.

## 2. Experimental

### 2.1. Apparatus

UV–vis and spectrofluorimetric determinations were carried out on a Shimadzu UV-2101 PC and a Jasco FP-777 respectively. The pH was measured on an Orion model 720 (resolution 0.001; relative accuracy ± 0.002) at (25.0 ± 0.1) °C using a Ross combination pH electrode. The pH-meter was calibrated using standard buffers (pH 2.932; 4.955; 6.994 and 9.155) prepared according to the literature [38]. An ultrasonic bath (Testlab tb02) was used for the dissolution of the reagents. Data analysis was performed with Sigma Plot (Scientific Graph system) version 8.00 (Jandel Scientific) and Info Stat Statistical Package, version beta (Facultad de Ciencias Agropecuarias, Universidad Nacional de Córdoba, Argentina).

### 2.2. Reagents

The water was obtained using a Millipore apparatus. **5HT** and **5HIA** (99% purity, Sigma–Aldrich), **βCD** (Roquette), **HPCD** degree of substitution 5.5 (Cerestar) and glucose (Anedra) were used as received. Potassium *p*-sulfonatocalix[6]arene and *p*-sulfonato-decylethercalix[6]arene (**DC6S**) were synthesized and characterized according to the literature [39,40]. The buffers were prepared according to literature procedures [38] and buffer solution at pH 6.994 (0.020 $mol\,L^{-1}$ monopotassium dihydrogenphosphate, 0.030 $mol\,L^{-1}$ disodium hydrogenphosphate, and 0.020 $mol\,L^{-1}$ sodium chloride were used as reference solution. The basic (pH 13.00 is 0.1 $mol\,L^{-1}$ NaOH) and acid (pH 2.00 is 0.01 $mol\,L^{-1}$ HCl) solutions were prepared from concentrated solutions of NaOH (1 $mol\,L^{-1}$) or HCl (4 $mol\,L^{-1}$) respectively. All constituents of the buffers were commercial reagents of analytical grade. Methanol was HPLC grade (Sintorgan).

### 2.3. General procedure

A concentrated solution of substrate in water (2 mg/10 mL) was stored in the refrigerator (4 °C) for a maximum of 20 days. The stability of the stock solutions was periodically checked by spectrophotometry before preparing the appropriate dilutions for fluorimetric determinations. Water solutions were prepared by adding the stock solution of substrate to the reference buffer solution prepared as indicated above and diluting to the mark with water. The concentration of the buffer in the final solution was 95% of the original reference buffer solution. All solutions were covered with aluminium foil. For emission and excitation fluorescence spectra the photomultiplier gain was medium (acidic or neutral media) or high (basic media) with 10 nm emission and excitation bandwidths. The fluorescence emission spectra were taken with excitation wavelength ($\lambda^{ex}$) equal to the wavelength of maximum absorption. All the determinations were made at (25.0 ± 0.1) °C, and the temperature of the cell compartment was controlled with a Haake circulator. The solutions were not degassed. A solution of 2.40 $\mu mol\,L^{-1}$ of the substrates at pH 6.994 was used as reference for the fluorimetric measurements. The ionic strength ($\mu$) of all solutions was 0.124 $mol\,L^{-1}$ by adding NaCl when required.

For the determination of the overall association constant at neutral pH, two solutions of the same substrate concentration (one without receptor and the other with the maximum concentration of the receptor used) were mixed in the proper proportion, in order to vary the receptor concentration and minimize the changes in fluorescence resulting from changes in the substrate concentration [41]. The solutions were stabilized for 3 h before the measurement. However, in acid or basic media, individual solutions with the appropriate concentration of all the reagents (except the substrate) were prepared; then the substrate was added and the volume was completed with water. In these cases, the fluorescence of the final solution was measured immediately with good reproducibility due to spectral changes were observed over time at these pH values.

For the spectrofluorimetric determination, the total area below the fluorescence spectrum ($F$) (Eq. (1)) and the fluorescence intensity at a fixed emission wavelength ($F_\lambda$) (Eq. (2)) were measured:

$$F = B\sum \varepsilon_i \phi_i [i] \quad (1)$$

$$F_\lambda = B\sum \varepsilon_i \phi_i \gamma_i [i] \quad (2)$$

where $B$ is a constant which depends on the instrumental set-up, $\varepsilon_i$ is the molar absorptivity at the $\lambda^{ex}$, $\phi_i$ is the fluorescence quantum yield, $\gamma_i$ is the fraction of the total emission intensity at a given wavelength and $[i]$ indicates the concentration of each fluorescent

species *i*. In all cases the absorbance of the solution was <0.025, where Eqs. (1) and (2) are valid.

### 2.4. Procedure for analyzing binary mixtures by zero-crossing first-derivative spectrofluorimetry

The solutions were prepared at 95% of buffer pH 6.994 in a final volume of 10.00 mL, with 1.20 μmol $L^{-1}$ of the substrate acting as interference and 0.50–8.00 μmol $L^{-1}$ of the substrate to be determined. This pH value was selected since the values of the zero-crossing points obtained were better than those found at the other pH studied (2.00 and 13.00). In the solutions containing **HPCD**, the final concentration was 10 mmol $L^{-1}$ by adding 2.00 mL from a concentrated stock solution 0.05 mol $L^{-1}$ in buffer. The zero-order fluorimetric spectra were recorded in the range between 300.0 and 485.0 nm; the data interval was 0.5 nm; and the excitation wavelength was 277 nm. The selection of optimum instrumental parameters was done in order to achieve an adequate signal-to-noise ratio. The scanning speed was examined between 100.0–500.0 nm/min, and 200.0 nm/min was selected. The response time was varied between 0.5–4.0 s, and 1.0 s was chosen. The derivative spectra were obtained at different $\Delta\lambda$ between 3.0 and 10.0 nm. The $\Delta\lambda$ value preferred was 3.0 nm in buffer solutions with or without **HPCD**.

### 2.5. Extraction and analysis of biological samples

Urine samples of a healthy adult collected and stored at pH 2.00 as recommended in the literature [42], and extracted as described below, were used for the method of standard addition (MOSA) in direct and derivative apparent recovery analysis. The drugs and diets that could produce false positive or false negative results were restricted to 3 or 4 days prior to and during collection, as reported in the literature [42].

The efficiency of the analyte extractions from spiked samples of acid urine was evaluated. The conventional method [10] consisting in one extraction with ether and a re-extraction with aqueous buffer at pH 7.00 showed good recoveries for **5HIA**, ionized at this pH; however, no good results were found with **5HT**. Several extraction procedures were tested in order to optimize this process. Thus, the best results were achieved when the acidic urine sample was adjusted to pH 10.00, extracted with butanol and re-extracted with buffer pH 6.994. This extraction procedure yielded recoveries (50–70%) similar to those informed in the literature [3].

In the apparent recovery analysis, a final volume of 0.200 mL of the buffer urine extract was placed in a 10.00 mL volumetric flask in the buffer or with the appropriate **HPCD** concentration in the buffer. Four different amounts of the respective hydroxyindole compound were added in order to give concentrations between 0.5 and 8.0 μmol $L^{-1}$. The same procedure was performed in the presence of the interference for the derivative experiments. In all cases, the fluorescence signal of the corresponding blank was 10% lower than that obtained for the smallest spiked concentration, subtracted from the overall signal. All the determinations were done in triplicate.

## 3. Results and discussion

### 3.1. Effect of pH and supramolecular media

UV/vis spectra of the substrates (not shown) were not affected by pH changes from 2.00 to 9.00. The first deprotonation for each substrate, the carboxylic acid ($pK_a \sim 5$) for **5HIA** or the amino group ($pK_a \sim 9$) for **5HT** are not found in the UV/vis spectrum since these groups are not conjugated with the indole chromophore. The molar absorptivity at the maximum (276.0 and 278.0 nm) of the reference solution at pH 6.994 was $(53.1 \pm 0.5) \times 10^2$ $cm^{-1}$ $mol^{-1}$ L and $(53.7 \pm 0.4) \times 10^2$ $cm^{-1}$ $mol^{-1}$ L for **5HT** and **5HIA**, respectively. A second band at 323.00 nm, according to the phenol deprotonation, was observed in both cases in basic media (pH 13). Therefore, a titration curve of absorbance as a function of the pH (6.00–13.00) allowed determining the $pK_a$ values of $(11.12 \pm 0.01)$ for **5HT** and $(11.16 \pm 0.01)$ for **5HIA**. The presence of **CDs** produces only small shifts (1 nm) in the maxima of absorbance in all the pH range studied.

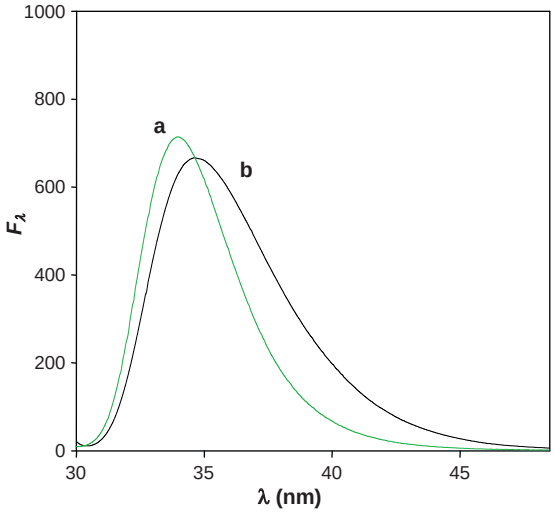



**Fig. 2.** Fluorescence emission spectra of reference solutions 2.4 μmol $L^{-1}$ at pH 6.994 and 25.0 °C: (a) **5HT** and (b) **5HIA**.

The fluorescence behaviour shown for **5HT** is similar in all the pH range 2.00–7.00 with emission wavelength ($\lambda^{max}$ = 339.5 nm) (Fig. 2) since the acid is present. At pH 13.00 ($\lambda^{max}$ = 354.0 nm) a decrease in fluorescence of 97% was observed. This was interpreted as the effect of the ionization of the ammonium ($pK_a \cong pK_a^{tryptamine} = 9.59$ [43]) and the phenol groups, without the possibility of distinguishing them. In addition, the alkaline quenching for the indole group is operating at this pH [9].

In the case of **5HIA**, the 80% decrease in fluorescence, observed from pH 6.994 ($\lambda^{max}$ = 346.0 nm) (Fig. 2) to pH 2.00 ($\lambda^{max}$ = 338.0 nm), indicated the protonation of the carboxylate species (Fig. 3). The corresponding fluorescence titration curve gave a $pK_a$ value $(4.74 \pm 0.01)$ similar to that of indole-3-acetic acid $(4.48 \pm 0.02)$ [44]. From pH 6.994 to 10.00 the fluorescence was con-

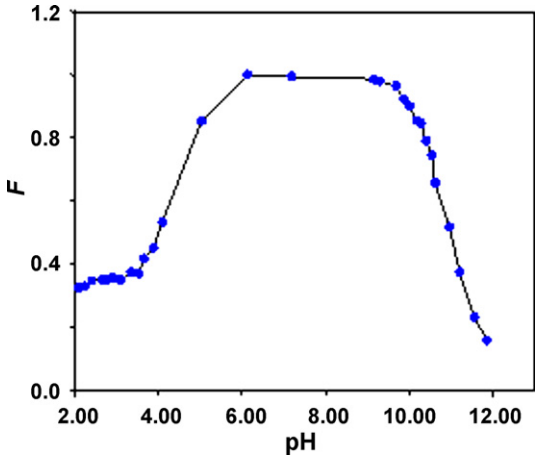



**Fig. 3.** Profile of fluorescence versus pH for solutions 2.4 μmol $L^{-1}$ of **5HIA** at 25.0 °C.

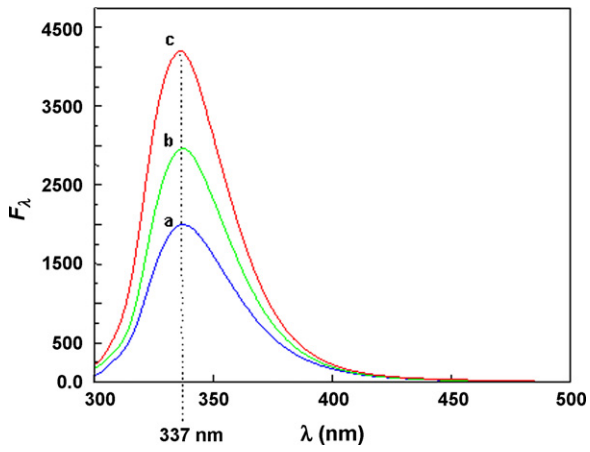



**Fig. 4.** Fluorescence emission spectra of solutions 2.4 μmol $L^{-1}$ of **5HIA** at pH 2.00 and 25.0 °C: (a) buffer solution, (b) with 10 mmol $L^{-1}$ of **βCD**, and (c) with 10 mmol $L^{-1}$ of **HPCD**.

stant, and then diminished due to the deprotonation of the phenol and to the alkaline quenching ($\lambda^{max}$ = 379.0 nm at pH 13).

In order to determine the effect of the neutral **CD** on the fluorescence of each acid–base species of the substrates (**$S^i$**), pH 2.00 and 6.994 were selected for **5HIA** (**S** or **$S^{-1}$**), and only pH 6.994 for **5HT** (**S**). The effect of the ionized cyclodextrin on the dibasic species of the substrates (**$S^{-2}$**) was observed at pH 13.00. The different equilibriums are shown in the following Scheme, where the upper, middle and lower parts represent the association constant between the diprotonated substrate (**S**) and the neutral **CD,** the mono-protonated substrate (**$S^{-1}$**) and the neutral **CD**, and the di-basic substrate (**$S^{-2}$**) and the ionized **CD** (**$CD^-$**), respectively.

For both substrates, an increase in the fluorescence intensity (14–321%) and small shifts of their maxima (1–4 nm, red shift for **5HT** and blue shift for **5HIA**, at pH 2.00–7.00; blue shift for **5HT** and red shift for **5HIA** at pH 13) were observed with **βCD** and **HPCD** (Table 1) (Fig. 4 is representative). This behaviour was not exhibited in the presence of α-CD, γ-CD or glucose (weight equivalent to 10 mmol $L^{-1}$ **βCD**) confirming that there is some specific interaction with **βCD** and **HPCD**. These changes evidence the inclusion complex formation [45] as observed in other indole derivatives [44,46].

The association constants for one-to-one (1:1) stoichiometry ($K_A$) and the fluorescent quantum yield ratios between free and complex substrate ($\phi_{SiCD}/\phi_{Si}$) can be fitted from non-linear analysis of *F* at different receptor concentrations (Eq. 3) at pH 2.00, 6.994 and 13.00 (Table 2) (Fig. 5 is representative).

$$\frac{F}{F_0} = \frac{\{1 + (\phi_{SiCD}/\phi_{Si})\,K_A\,[CD]\}}{\{1 + K_A\,[CD]\}} \quad (3)$$

In order to determine the $K_A$ values for the species **$S^{-2}$** ($pK_a \sim 11.10$) with neutral CD, some considerations were taken into account. In the case of **5HT** the experiments were carried out at pH 12.50 where only **$S^{-2}$** was present and could interact with neutral and ionized **CD** (see Scheme 1). From Eq. (1), expressed in terms of **$S^{-2}$**, **$S^{-2}$CD** and **$S^{-2}CD^-$** in the presence of **CD** and the mass balance for **$S^{-2}$**, it was possible to obtain $K_A^{S\text{-}2CD}$ and $(\phi_S{}^{-2}{}_{CD}/\phi_S{}^{-2})$ (Table 2) from the term *A* of Eq. (4). The terms *A* and *B* were defined by Eqs. (5) and (6), where $X^i$ represents the molar fraction of the species "i" of **CD**. The values required in Eq. (6) of $K_A^{S\text{-}2CD^-}$ and $(\phi_S{}^{-2}{}_{CD^-}/\phi_S{}^{-2})$ were independently determined at pH 13.00.

$$\frac{F}{F_0} = \{1 + A + B\} \quad (4)$$

**Table 2**
Values of $K_A$ determined and $(\phi_S{}^i{}_{CD}/\phi_S{}^i)$ for **5HT** and **5HIA** in different media.[a]

| $S^i$:CD | $K_A$[a] (10 mol$^{-1}$ L) | $(\phi_S{}^i{}_{CD}/\phi_S{}^i)$ |
|---|---|---|
| **5HT:βCD**[b] | 17 ± 4 | 1.19 ± 0.02 |
| **5HT:HPCD**[b] | 3.4 ± 0.2 | 2.01 ± 0.03 |
| **$5HT^{-2}$:βCD**[c] | 30 ± 20 | 2.2 ± 0.5 |
| **$5HT^{-2}$:HPCD**[c] | 10 ± 7 | 10 ± 5 |
| **$5HT^{-2}$:βCD**[d] | 6.8 ± 0.5 | 4.6 ± 0.1 |
| **$5HT^{-2}$:HPCD**[d] | 16 ± 5 | 4.9 ± 0.5 |
| **5HIA:βCD**[e] | 5.6 ± 0.9 | 1.9 ± 0.4 |
| **5HIA:HPCD**[e] | 14.8 ± 0.8 | 3.2 ± 0.2 |
| **$5HIA^{-1}$:βCD**[a] | 7.3 ± 0.5 | 1.20 ± 0.05 |
| **$5HIA^{-1}$:HPCD**[a] | 14 ± 4 | 1.18 ± 0.04 |
| **$5HIA^{-2}$:βCD**[f] | 3 ± 1 | 2.2 ± 0.5 |
| **$5HIA^{-2}$:HPCD**[f] | 7 ± 5 | 1.3 ± 0.1 |
| **$5HIA^{-2}$:βCD**$^-$ | 7 ± 3 | 1.8 ± 0.2 |
| **$5HIA^{-2}$:HPCD**[d] | 7 ± 4 | 4.1 ± 0.9 |

[a] At $\mu$ = 0.124 mol $L^{-1}$ and 25.0 °C.
[b] pH 6.994.
[c] pH 12.50.
[d] pH 13.00.
[e] pH 2.00.
[f] pH 11.20.

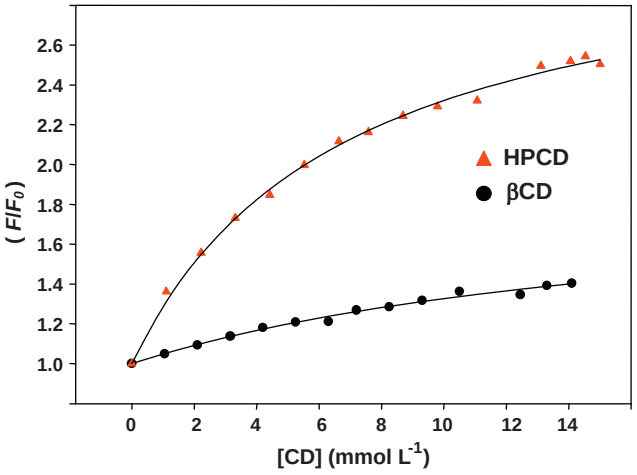
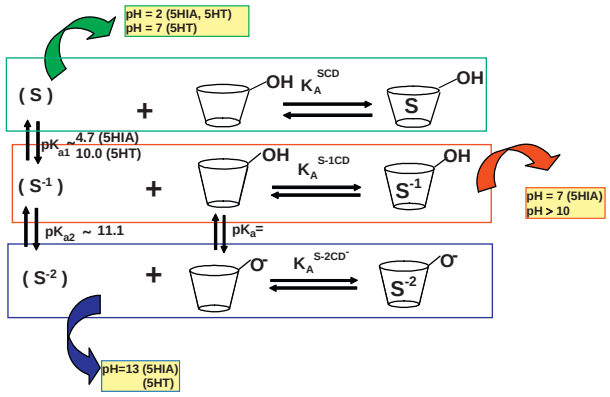



**Fig. 5.** Relative fluorescence of solutions 2.4 μmol $L^{-1}$ of **5HIA** as a function of **CD** concentrations at pH 6.994 and 25.0 °C: (●) **βCD**, (▲) **HPCD**.

**Table 1**
Fluorescence characteristics of **5HT** and **5HIA** in different media.[a]

| S | $F/F_0$ ($\lambda^{max}$, nm) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | pH 2.00[b] | | | pH 6.994[c] | | | pH 13.00[d] | | |
| | Buffer | βCD | HPCD | Buffer | βCD | HPCD | Buffer | βCD | HPCD |
| **5HT** | 1.00 (339.0) | 1.15 (340.0) | 1.16 (340.0) | 1.00 (338.0) | 1.14 (340.0) | 1.28 (340.0) | 1.00 (379.5) | 2.50 (378.0) | 3.21 (378.5) |
| **5HIA** | 1.00 (337.0) | 1.60 (336.5) | 2.13 (336.0) | 1.00 (346.5) | 1.20 (343.5) | 1.18 (343.5) | 1.00 (379.0) | 1.24 (383.0) | 1.25 (383.0) |

[a] Substrate concentration 2.4 μmol $L^{-1}$, $\mu$ = 0.124 mol $L^{-1}$ and 25.0 °C in buffer or with 10 mmol $L^{-1}$ of **βCD** or **HPCD** as indicated.
[b] HCl 0.01 mol $L^{-1}$.
[c] Buffer prepared as indicated in Section 2.
[d] NaOH 0.1 mol $L^{-1}$.




**Scheme 1.** Equilibriums of hydroxyindoles in the presence of **CD** at different pH values as indicated.

$$A = \left[\left(\left(\frac{\phi_{S^{-2}CD}}{\phi_{S^{-2}}}\right) - 1\right)\left(\frac{K_A^{S^{-2}CD} X^{CD}\,[CD_0]}{1 + K_A^{S^{-2}CD} X^{CD}\,[CD_0]}\right)\right] \quad (5)$$

$$B = \left[\left(\left(\frac{\phi_{S^{-2}CD^-}}{\phi_{S^{-2}}}\right) - 1\right)\left(\frac{K_A^{S^{-2}CD^-} X^{CD^-}\,[CD_0]}{1 + K_A^{S^{-2}CD^-} X^{CD^-}\,[CD_0]}\right)\right] \quad (6)$$

For **5IHA** at pH 12.50 the term *A* does not change significantly with the increase in **CD** concentration. Therefore, in this case, in order to obtain $K_A^{S\text{-}2CD}$, the experiments were carried out at pH 11.10 where two species of substrate (**S**$^{-1}$ and **S**$^{-2}$) were present in both free and complex forms with only neutral **CD**. According to Eq. (1) expressed in terms of **S**$^{-1}$, **S**$^{-1}$**CD**, **S**$^{-2}$ and **S**$^{-2}$**CD** in the presence of **CD** and the mass balance for each **S**$^{i}$, the complete expression obtained has the same form as that found in Eq. (4). The co-existence of two independent equilibriums for **S**$^{-1}$ and **S**$^{-2}$ was considered in order to express the molar fractions $X^{Si}$ and $X^{SiCD}$ (Eq. (7)), since the **CD** concentration ranged from $5 \times 10^2$ to $5 \times 10^3$ times higher than that of the total S concentration. Terms *A* and *B* are defined by Eqs. (7) and (8), where $f^{Si}$ is the fluorescent fraction at this pH for each species **S**$^{i}$ in the absence of **CD**. Term *A* can be calculated from the values obtained at pH 6.994 for $S^{-1}$; from term *B*, it was possible to determine the values of $K_A^{S\text{-}2CD}$ and $(\phi_S{}^{-2}{}_{CD}/\phi_S{}^{-2})$ (Table 2).

$$X^{S^iCD} = \left[\frac{\left(K_A^{S^iCD}\,[CD]\right)}{\left(1 + K_A^{S^iCD}\,[CD]\right)}\right] \quad (7)$$

$$A = \left\{f^{S^{-1}} X^{S^{-1}CD}\left[\left(\frac{\phi_{S^{-1}CD}}{\phi_{S^{-1}}}\right) - 1\right]\right\} \quad (8)$$

$$B = \left\{f^{S^{-2}} X^{S^{-2}CD}\left[\left(\frac{\phi_{S^{-2}CD}}{\phi_{S^{-2}}}\right) - 1\right]\right\} \quad (9)$$

In all cases, the data $(F/F_0)$ plotted according to a linearized equation [45,47] (not shown) also gave the same values of $K_A$ within the experimental error, confirming the (1:1) relationship between the substrate (**S**$^{i}$) and the receptor (**CD**) [48]. The values of $K_A$ determined for **5HT** and **5HIA** (Table 2) range from 30 to 300 $mol^{-1}$ L mainly indicating aromatic inclusion as in the case of other aromatic compounds [7] and related indoles [9]. **HPCD** showed a better interaction with **5HIA** than **βCD** ($K_A^{SiHPCD} > K_A^{Si\beta CD}$), the former being higher with the neutral (**S**) and the carboxylate-phenol substrate (**S**$^{-1}$) than with the carboxylate-phenolate substrate (**S**$^{-2}$) and without being influenced by the ionization of the receptor (**HPCD** or **HPCD**$^{-}$). $K_A^{Si\beta CD}$ values were practically the same for all the species **S**$^{i}$ of the substrate. **5HT** showed the best interaction with neutral **βCD** for the ammonium substrate (**S**) and the amine-phenolate substrate (**S**$^{-2}$) ($K_A^{SiHPCD} < K_A^{Si\beta CD}$), but with the ionized **HPCD**$^{-}$ for **S**$^{-2}$ ($K_A^{SiHPCD-} > K_A^{Si\beta CD-}$) at pH 13.

In the case of **5HIA**, the observed $K_A^{SiHPCD} > K_A^{Si\beta CD}$ for (**S**) and (**S**$^{-1}$) and the lack of influence by the ionization of the receptor ($K_A^{S\text{-}2CD} \approx K_A^{S\text{-}2CD-}$), correlate very well with those found for 3-Indole acetic acid [9,44]. These results could be interpreted as the absence of interaction between the lateral carboxylic chain in C-3 of the substrate outside the cavity of the cyclodextrin, with a better aromatic interaction between the indolic compounds and the more hydrophobic **HPCD**. Furthermore, the aromatic inclusion is more influenced by the repulsion between the included negative deprotonated hydroxyl group in position 5 of the substrate (**S**$^{-2}$) and the hydrophobic cavity of the neutral or the negatively charged receptor ($K_A^{S\text{-}1HPCD} > K_A^{S\text{-}2HPCD} \approx K_A^{S\text{-}2HPCD-}$). The results observed with **5HT** could indicate a major influence of the amino lateral chain on C-3 of the indole, since the neutral amino-phenolate substrate (**S**$^{-2}$) interacts better with both cyclodextrins than the protonated amino-phenol substrate (**S**) ($K_A^{S\text{-}2CD} > K_A^{SCD}$). Also, the better interactions with neutral **βCD** than with **HPCD** for the charged compounds **S** and **S**$^{-2}$ ($K_A^{SiHPCD} < K_A^{Si\beta CD}$) are indicative of the less hydrophobic cavity of **βCD**, these results contrast with those found for 5-methoxytryptamine due to the less polar characteristic of the methoxy group [9]. In general, the higher values of $K_A$ obtained for **5HT** than for **5HIA** ($K_A^{5HTCD} > K_A^{5HIACD}$) could probably due to a deeper inclusion of the aromatic moiety in **5HT** (ethylamine group in C-3) than in **5HIA** (methylcarboxylic group in C-3). This interpretation is in accord with the mode of inclusion proposed for related indoles from fluorescence quenching studies in acid and basic media and from Induced circular dichroism [9], where mainly the C-2 and the –NH group of the indole are included into the cavity of **CD**. In all cases the interaction with **CD** indicates the more protected environment that the receptor offers for the excited state of the substrate since the values of $(\phi_S{}^{i}{}_{CD}/\phi_S{}^{i})$ range from 1.20 to 10 with possible analytical applications.

The preceding interpretations of the observed changes in guest fluorescence produced by **CD** were based mainly from the evidence of inclusion complex formation, hydrophobic interactions and literature data, but they provide limited structural information on the geometry and mode of inclusion. Direct evidence in solution can be obtained from NMR studies, which can show some specific interactions between specific parts of the guest and host, and thus direct evidence of particular mode of inclusion [7,45]. Accordingly, the $^1$H NMR (Bruker 400 MHz) experiments in $D_2O$ for solutions of **CD** and (**S**+**CD**) were performed. The chemical shift differences ($\Delta\delta$, ppm) between the complex and free inner protons (3 and 5) of **CD** were −0.045 and −0.06 for the complex with **5HIA** and smaller with **5HT** (−0.03 and −0.04). These experiments were performed with the best ratio between the cyclodextrin and the substrate concentration that produce the higher concentration of complex according to the small $K_A$ values and the substrate solubility. Although these $\Delta\delta$ values are small, the changes observed indicate the effect of the aromatic inclusion in the **CD** cavity, as previously reported for related compounds [9]. These $^1$H NMR results correlate with the fluorescence study.

In order to evaluate the specificity of the supramolecular interaction, the influence of *p*-sulfonatocalix[6]arene (**C6S**) and *p*-sulfonato-decylethercalix[6]arene (**DC6S**) as receptors was also investigated by fluorescence. To avoid the absorption of the receptor, the experiments with **C6S** were done at pH 13.00 with an excitation wavelength of 323.0 nm. With **DC6S** the pH was 6.994 with an excitation wavelength of 280.0 nm since the absorption of the maximum concentration of the receptor used was five times lower than that of the absorbance of the substrates. In all cases, no changes in fluorescence were observed, this behaviour no suggest some type of supramolecular interaction between calixarenes and

**Table 3**
Analytical parameters for **5HT** and **5HIA** by direct spectrofluorimetry in different media.[a]

| S | pH | Parameter | Media | | |
|---|---|---|---|---|---|
| | | | Buffer | βCD | HPβCD |
| **5HT**[e] | 2.00 | $\boldsymbol{m}$ ($10^5$ mol$^{-1}$ L)[b] | 4.05 ± 0.02 | 4.37 ± 0.01 | 4.78 ± 0.07 |
| | | $\mathbf{s_B}$ ($10^{-3}$)[c] | 0.42 | 0.18 | 0.34 |
| | | $\mathbf{L_D}$ (ng/mL)[d] | 0.550 ± 0.003 | 2.100 ± 0.005 | 0.370 ± 0.005 |
| | 6.997 | $\boldsymbol{m}$ ($10^5$ mol$^{-1}$ L)[b] | 4.11 ± 0.04 | 4.73 ± 0.07 | 4.98 ± 0.05 |
| | | $\mathbf{s_B}$ ($10^{-3}$)[c] | 0.45 | 0.48 | 0.50 |
| | | $\mathbf{L_D}$ (ng/mL)[d] | 0.580 ± 0.009 | 0.54 ± 0.01 | 0.530 ± 0.005 |
| | 13.00 | $\boldsymbol{m}$ ($10^5$ mol$^{-1}$ L)[b] | 6.1 ± 0.2 | 24.3 ± 0.5 | 31.3 ± 0.3 |
| | | $\mathbf{s_B}$ ($10^{-3}$)[c] | 42 | 370 | 50 |
| | | $\mathbf{L_D}$ (ng/mL)[d] | 37 ± 1 | 88 ± 2 | 9.0 ± 0.1 |
| **5HIA**[f] | 2.00 | $\boldsymbol{m}$ ($10^5$ mol$^{-1}$ L)[b] | 4.24 ± 0.01 | 5.98 ± 0.01 | 8.36 ± 0.02 |
| | | $\mathbf{s_B}$ ($10^{-3}$)[c] | 0.60 | 0.65 | 0.75 |
| | | $\mathbf{L_D}$ (ng/mL)[d] | 0.800 ± 0.03 | 0.600 ± 0.001 | 0.500 ± 0.001 |
| | 6.997 | $\boldsymbol{m}$ ($10^5$ mol$^{-1}$ L)[b] | 3.63 ± 0.07 | 4.05 ± 0.04 | 4.25 ± 0.06 |
| | | $\mathbf{s_B}$ ($10^{-3}$)[c] | 0.58 | 0.58 | 0.56 |
| | | $\mathbf{L_D}$ (ng/mL)[d] | 0.92 ± 0.02 | 0.820 ± 0.008 | 0.75 ± 0.01 |
| | 13.00 | $\boldsymbol{m}$ ($10^5$ mol$^{-1}$ L)[b] | 4.3 ± 0.1 | 6.2 ± 0.2 | 5.3 ± 0.1 |
| | | $\mathbf{s_B}$ ($10^{-3}$)[c] | 20 | 130 | 21 |
| | | $\mathbf{L_D}$ (ng/mL)[d] | 27.0 ± 0.6 | 114 ± 4 | 23.0 ± 0.4 |

[a] At μ = 0.124 mol L$^{-1}$ and 25.0 °C in buffer or in buffer with 10 mmol L$^{-1}$ of **βCD** or **HPCD** as indicated.
[b] Slope of a calibration graph. The errors are those calculated by the fitting program.
[c] Standard deviation of 25 blanks.
[d] Error calculated by error propagation.
[e] $\lambda^{ex}$ = 276.0 nm.
[f] $\lambda^{ex}$ = 278.0 nm.

the substrates. No further evidence for some interaction between **C6S** and the substrates was obtained in NMR experiments.

Even though **C6S** and **CD** have a hydrophobic nanocavity with similar dimensions (internal diameter ~0.7 nm) and both hosts are soluble in water, their features, structural properties and nature of their driving interactions for complex formation differ significantly [7,39,40]. The results with **C6S** and **DC6S** indicate the specificity of the interaction between the substrates and **CD**.

### 3.2. Analytical parameters

Table 3 shows the analytical parameters, such as calibration sensitivity ($\boldsymbol{m}$), standard deviation of blank signal ($\mathbf{s_B}$), limit of quantification ($\mathbf{L_Q}$) and limit of detection ($\mathbf{L_D}$) calculated according to IUPAC definition (3.29 $\mathbf{s_B}/\boldsymbol{m}$) [49] for **5HT** and **5HIA** in the concentration interval 0.5–5.0 μmol L$^{-1}$, in the presence of 10 mmol L$^{-1}$ **βCD** or **HPCD** at pH 2.00, 6.994 and 13.00 at 25.0 °C. The precision of the method was determined by analysing 10 replicate samples of 2.4 μmol L$^{-1}$ of **5HT** or **5HIA**; in all cases the relative error was not higher than 5%.

All plots of the $y$-residuals in regression [50] obtained from ($y$–$\hat{y}$) values (which represent the differences between the experimental $y$-values and the fitted $y$-value) versus $\hat{y}$ show a normal distribution with variance independent of $\hat{y}$. The assessment of the fitted regression model was checked by $F$ test of linear model, which evaluates the null hypothesis $H_0$: $x$ and $y$ are not linearly related, on the basis of ANOVA principles [50]. In all cases, the value of the ratio obtained between mean square regression and mean square for residuals exceeds the critical value of $F$ for the corresponding degree of freedom (1 and at least 8) in each case at a 95% confidence level. Therefore we conclude that the fitted linear model seems to be statistically valid.

The best values of $\boldsymbol{m}$ were obtained employing **βCD** and **HPCD** as hosts, the higher being with the latter. The best $\mathbf{L_D}$ determined for **5HT** (0.370 ng mL$^{-1}$) and **5HIA** (0.500 ng mL$^{-1}$) were in the presence of **HPCD** at pH 2.00, mainly due to the increase of the $\boldsymbol{m}$ value with respect to buffer, and also because the $\mathbf{s_B}$ determined in the presence of **CD** were lower than in basic media.

The values of $\mathbf{L_D}$ reported here are lower than those determined by direct fluorescence or with derivative reactions (10 ng mL$^{-1}$) for **5HT** [10,11] and **5HIA** [32,33]. They are also several orders lower than those of the spectrophotometric reference Method for **5HIA** (12–30 μg mL$^{-1}$) [10,30,31].

### 3.3. Simultaneous determination by zero-crossing first-derivative spectrofluorimetric method

As shown in Fig. 2, the fluorescence spectra of **5HT** and **5HIA** are highly overlapped, a characteristic found in all the conditions studied (Table 1). In order to determinate simultaneously both analytes on the basis of previous results with simple indoles [44], the first-order derivative fluorimetric spectra were analyzed. These experiments indicate that the best changes are produced at pH 6.994 and with **HPCD**. The zero-crossing point (nm) was located at 340.0 for **5HT** and 347.0 for **5HT** in buffer, and 339.5 for **5HT** and 345.0 with **HPCD** (10 mmol L$^{-1}$) respectively. At the zero-crossing point of the interference (**5HIA** or **5HT**), it was possible to obtain good calibration plots for the signals corresponding to the first derivative of the other components of the mixture (**5HT** or **5HIA** respectively) in concentrations between 0.5 and 8.0 μmol L$^{-1}$ with 1.2 μmol L$^{-1}$ of the interference. The $\boldsymbol{m}$ and $\mathbf{L_D}$ values obtained

**Table 4**
Analytical Parameters for **5HT** and **5HIA** by zero-crossing first-derivative spectrofluorimetry in different media.[a]

| S (interference)[b] | $m$ ($10^5$, mol$^{-1}$ L) ($L_D$, ng mL$^{-1}$) | |
|---|---|---|
| | Buffer | HPCD |
| 5HT (–) | 3.84 ± 0.09 | 5.71 ± 0.06 |
| | (19.40) | (14.20) |
| 5HT (5HIA) | 4.07 ± 0.04 | 6.1 ± 0.1 |
| 5HIA (–) | 3.97 ± 0.05 | 4.09 ± 0.02 |
| | (17.40) | (18.50) |
| 5HIA (5HT) | 4.21 ± 0.04 | 4.11 ± 0.07 |

[a] At μ = 0.124 mol L$^{-1}$, 25.0 °C and in buffer pH 6.994 with or without 10 mmol L$^{-1}$ of **HPCD** as indicated.
[b] In the presence and absence of 1.2 μmol L$^{-1}$ of the interference as specified.

**Table 5**
Average apparent recoveries ($R_A^A$) of **5HT** and **5HIA** in urine samples.[a]

| S | pH | $R_A^A$ (R.S.D.) Buffer | HPCD |
|---|---|---|---|
| **5HT** | 2.00 | 102 (2)[b] | 90[b] (1)[b,e] |
| | | 102 (2)[c] | 103 (3)[c] |
| | 6.997 | 101 (3)[b] | 93 (1)[b,e] |
| | | 99 (2)[c] | 102 (2)[c] |
| | 6.994[d] | 106 (2)[b,e] | 85 (8)[b,e] |
| | | 98 (2)[c] | 92 (8)[c] |
| | 13.00 | 70 (5)[b,e] | 54 (6)[b,e] |
| | | 99 (4)[c] | 102 (4)[c] |
| **5HIA** | 2.00 | 99 (5)[b] | 86 (3)[b,e] |
| | | 102 (4)[c] | 101 (3)[c] |
| | 6.994 | 115 (2)[b,e] | 101 (3)[b] |
| | | 101 (2)[c] | 100 (1)[c] |
| | 6.994[d] | 96 (2)[b,e] | 107 (10)[b] |
| | | 102 (3)[c] | 110 (10)[c] |
| | 13.00 | 97 (3)[b] | 93 (3)[b,e] |
| | | 104 (3)[c] | 99 (3)[c] |

[a] $R_A^A$ for the direct spectrofluorimetric method (or indicated).
[b] Values calculated with calibration parameters in the absence of matrix.
[c] Values calculated with calibration parameters in the presence of matrix.
[d] $R_A^A$ for the first-derivative spectrofluorimetric method in the presence of interference.
[e] The statistical test *t* indicates a matrix effect.

(Table 4) show better sensitivity (50%) for **5HT** in **HPCD** than in buffer and practically no change is noticed for **5HIA**, with a 27% lower $\mathbf{L_D}$ in the first case and with no improvement in the second one. Although the $\mathbf{L_D}$ determined by derivative spectrofluorimetry are higher than those obtained by the direct method, these values are in the same order as those reported by HPLC with fluorimetric detection [16–18].

### 3.4. Applications

Since **5HT** and **5HIA** concentrations in biological fluids are important markers for various diseases and ageing-related physiological conditions, it is considered necessary to detect these biomolecules in real matrices. However, whenever a compound is traceable in urine, urine sampling is always preferred to blood sample due to its non-invasive nature. Hence, the methods developed were applied for quantification of these molecules in urine samples.

Following the method described under Section 2.5, apparent recovery experiments were performed in urine samples spiked at five concentration levels between 0.5 and 8.00 μmol $L^{-1}$ of the analyte for the direct (pH 2.00, 6.994, 13.00) and in the presence of the interference (1.2 mol $L^{-1}$) for the derivative (pH 6.994) spectrofluorimetric method respectively, with or without **HPCD**, in triplicate in each case. Due to the complexity of the urine matrix, the MOSA was applied.

The average apparent recoveries ($R_A^A$) for the triplicates at five levels of fortification in each condition calculated with ***m*** in the absence of matrix reveal, in some cases, positive and negative effects (Table 5). The calibration parameters determined in the matrix provide satisfactory $R_A^A$ and a normalization of the proportional error (Table 5). The latter is indicated from the lower statistical test *t* calculated by Eq. (10) compared to the tabulated ($t_{(n-1),}\ \alpha = 0.05 = 3.18$).

$$t = \frac{(\%R_A^A - 100)\sqrt{n}}{s} \tag{10}$$

The comparison of the results obtained for the simultaneous determination of **5HT** and **5HIA** with the derivative method in the presence of **HPCD** and the HPLC-fluorescence detection method reported in the literature [25] was also performed in urine samples fortified at two levels of concentration (1.00 and 3.50 μmol $L^{-1}$) with equal concentration of each analyte. The values of the statistical test *t* calculated as Eq. (11) for the means obtained for both methods ($A_1$ and $A_2$) were lower than those tabulated ($t_{(nA+nB-2)} = 4.30$) at a 95% confidence level, indicating that both methods yield the same results.

$$t = \left[\frac{(\%R_{A1}^A - \%R_{A2}^A)}{s_g\sqrt{((1/n_1)+(1/n_2))}}\right] \tag{11}$$

## 4. Conclusions

Host-guest complexes with **βCD** and **HPCD** offer an alternative, efficient and direct spectrofluorimetric method for the determination of **5HT** and **5HIA** which depends on the pH of the medium, since the specific interactions between the acid–base species of the substrate and the receptor are different. In view of the spectral changes observed in the presence of **HPCD** (shifts in $\lambda^{em}$ and fluorescence enhancement), a zero-crossing first-derivative method for the simultaneous determination of **5HT** and **5HIA** was developed.

The $\mathbf{L_D}$ reported here for the direct (0.3–0.5 ng $mL^{-1}$) and derivative spectrofluorimetric methods (20 ng $mL^{-1}$) are better than, or in the same order as, the spectrophotometric [10,30,31], fluorimetric [10,32] and HPLC with fluorescence detection methods [16–18,23–26,34–36] informed in the literature. Some recent developments in electrochemical ($\mathbf{L_D}$ = 6 ng $mL^{-1}$) [51] and HPLC methods ($\mathbf{L_D}$ = 0.5–20 ng $mL^{-1}$) [52,53] report similar or higher values.

In addition, these $\mathbf{L_D}$ assess the quantification (quantification limits, $\mathbf{L_Q} = \mathbf{L_D} \times 10/3.29$) [49] of hydroxyindole levels excreted in urine, related to abnormalities or illnesses (>25 mg/day) as well as to normal levels found in healthy adults (<9 mg/day) [10]. These values are equivalent to 150 ng $mL^{-1}$ and 45 ng $mL^{-1}$ in the amount of diluted urine extract (0.2 mL of urine extract in 10 mL of final volume) used in the apparent recovery experiments (Section 2.5). The present spectrofluorimetric methods in the presence of **CD** as nanosensor, a natural and commercially available macrocyclic receptor, avoid potentially harmful experimental conditions, derivatization reactions, and the use of hazardous solvents by simplifying the experimental requirements.

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