Fluorimetric Determination of \( \text{p}-\text{Hydroxybenzoic Acid} \) in Beer as \( \alpha \)-Cyclodextrin Inclusion Complex

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LUMINESCENCE

Fluorimetric Determination of p-Hydroxybenzoic Acid in Beer as α-Cyclodextrin Inclusion Complex

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Abstract: The inclusion complex of p-hydroxybenzoic acid and α- and β-cyclodextrin has been studied by fluorescence spectroscopy. To describe quantitatively complex formation between α-cyclodextrin (α-CD) and p-hydroxybenzoic acid, an association constant of 967 ± 14 M⁻¹ at 21°C was obtained. The inclusion complex has been used to determine p-hydroxybenzoic acid in the range 0.15–1.00 mg L⁻¹ (RSD 4.5%, n = 8). Application of the method to determination of p-hydroxybenzoic acid in beer samples gave an endogenous content of 1.25 mg L⁻¹.

Keywords: α-cyclodextrin, fluorimetry, inclusion, p-hydroxybenzoic acid

INTRODUCTION

p-Hydroxybenzoic acid (PHB) occurs naturally in various fruits that suffer changes during ripening and storage. It is used in pharmaceutical preparations and to obtain esters, which are very useful as food preservatives. Liquid chromatography with ultraviolet and electrochemical detection is the most commonly used technique to detect phenolic acids.
Fluorimetric Determination of \( p \)-Hydroxybenzoic Acid

[Escarpa and González 2001; Naczk and Shahidi 2004; Ayaz et al. 2005]. However, under optimum conditions of separation and detection of phenolic compounds, it is not possible to detect PHB.

Alternatively, PHB can be determined by a technique that offers selectivity and low detection limits. In phosphorimetry it has been found that the signal is proportional to PHB amounts, above 140 ng, with a detection limit of 20 ng [Wandruska and Hurtubise 1977]. In spite of the low detection limit, this technique is poorly used compared with fluorimetry. Unfortunately, hydrobenzoic acid derivatives are not fluorescent, but their photophysical properties can be altered by inclusion in the cyclodextrin cavity. Because of the organizing ability of cyclodextrin media, luminescent phenomena are enhanced, the molecules in the internal cavity are isolated from the surrounding environment, and their excited states are shielded from extinction processes.

Cyclodextrin (CD) complexations show selectivity depending on the relative size of the cavity and guest molecules [Saenger 1980; Szretli 1982; Dodziuk 2006], with the lower \( \alpha \)-CDs displaying higher selectivities. The determination of a variety of organic compounds has been developed by exploiting the \( \beta \)-CDs [Pola et al. 2000; Kjoniksen et al. 2005; Dupuy et al. 2005; Pinto et al. 2005], whereas only a few analytical applications of \( \alpha \)-CD have been described [García, Carnero, and Heredia 1994; Sanchez et al. 1994].

This work discusses the inclusion of PHB in \( \alpha \)-CD, permitting fluorimetric determination with a low detection limit.

EXPERIMENTAL

Reagents and Standards

\( p \)-Hydroxybenzoic acid (PHB) was obtained from Sigma (St Louis, USA), and \( \alpha \)-CD and \( \beta \)-CD were kindly given by Amaizo (USA). A stock solution of PHB (1000 \( \mu \)g L\(^{-1} \)) was prepared in water. \( \alpha \)-Cyclodextrin was purified by recrystallization once from boiling water, and 10\(^{-2} \) M aqueous solutions were prepared. All solvents were of analytical grade from Merck (Darmstadt, Germany). Deionized water previously purified through a Millipore 60 system (Bedford, MA, USA) was employed for all aqueous solutions.

Instrumentation

Emission measurements were made with a LS-50 Perkin-Elmer luminescence spectrometer (Beaconsfield, UK). Information was sent
via the RS232C interface of the fluorescence instrument to an external computer. Instrumental parameters were controlled by Fluorescence Data Manager (FLDM) software.

**Procedures**

**General Procedures**

In a 10 mL standard flask was placed an aliquot (≤1 mL) of PHB solution to give a final concentration between 0.1 and 1 mg mL⁻¹, 2 mL of pH 3.6 acetate buffer was added to make up the volume with 10⁻² M of α-CD solution. The samples were sonicated for 20 min and the relative fluorescence intensity was measured at λ₀ exc = 263 nm and λ₀ em = 333 nm against a blank. The relative fluorescence intensity was converted into units of concentration by applying a regression equation or calibration graph.

**Extraction Procedure**

Beer samples were degassed by agitation and moderate heating (between 30–35°C) and the pH was adjusted to 2 with 1 M HCl. From this acidified sample, 25 mL was taken and extracted with 2 × 25 mL portions of n-hexane. The resulting two phases were separated, and the aqueous phase was again extracted with 2 × 25 mL portions of ethyl acetate. The ethyl acetate extract was evaporated to dryness under reduced pressure at 25°C, and then the methanol was added to give 5 mL. The resultant mixture was shaken until dissolved. Aliquots of this solution were then analyzed by the general method.

**RESULTS AND DISCUSSION**

Relative fluorescence intensity was used as a qualitative measure of the complexing ability of PHB with cyclodextrins. Separate experiments were conducted to study the behaviour of PHB in water, employing α-CD and β-CD solutions. In Fig. 1 the excitation and emission spectra in these media are presented. A fluorescence intensity enhancement in cyclodextrin media compared with water is observed because cyclodextrin offer a protective, more constrained microenvironment to an electronically excited lumiphore and so the resulting fluorescence is enhanced. The maximum fluorescence obtained from a molecule is subjected to the aqueous medium surrounding the cyclodextrin. So, the
greater enhancement with α-CD probably results from a better fit of the PHB molecule in the α-CD cavity, whereas the diameter of β-CD (7.8 Å) is too large to fit the PHB molecule and the fluorescence intensity is lower.

To describe quantitatively complex formation between the α-CD and PHB the binding constant was obtained by the Benesi-Hildebrand method [Benesi and Hildebrand 1949; Kondo et al. 1976], using the following expression:

$$\Delta F^{-1} = (\kappa[\text{PHB}]_o K[\alpha-\text{CD}])^{-1} + (\kappa[\text{PHB}]_o)^{-1}$$
where $\Delta F$ is the change of fluorescence intensity upon addition of $\alpha$-CD, $[\text{PHB}]_o$ is the initial concentration of $p$-hydroxybenzoic acid (mol L$^{-1}$), and $\kappa$ is proportional constant. The linear relationship between $\Delta F^{-1}$ and $([\alpha-\text{CD}])^{-1}_o$ gives $K$, the host guest association constant. Figure 2 shows a plot of the linear dependence over the $\alpha$-CD concentration range studied ($10^{-2}$–$10^{-3}$ M). The binding constant was determined at $2.72 \times 10^{-5}$ M initial concentration of PHB. A value of $967 \pm 14$ M$^{-1}$ for PHB was obtained; the uncertainty is the standard error estimate calculated from the scatter of the observed points from the least squares Benesi-Hildebrand plot lines. Previous reported values for the association constant are $K = 2000$ M$^{-1}$ and $1130$ M$^{-1}$ [Connors et al. 1982].

When the pH of the PHB in $\alpha$-CD solutions varied in the range of 3–9, a change in the excitation and emission wavelengths and intensity was observed. At a pH $< 4.2$ the nonionized form of $p$-hydroxybenzoic acid is predominate in solution, with $\lambda_{\text{exc}} = 263$ nm and $\lambda_{\text{em}} = 333$ nm. The monoprotonated form is predominant between pH 4.2–4.6 with the same excitation and emission wavelengths. At a pH $> 4.6$ the two

![Figure 2](image-url)
functional groups are ionized, the species display $\lambda_{\text{exc}} = 287 \text{ nm}$ and $\lambda_{\text{em}} = 338 \text{ nm}$, and the emission intensity falls with respect to that in acid media. At high pH the molecule is ionized, and as size-added hydrophobicity establishes the strength of the complex, this more hydrophilic species forms weaker complexes and produces faint emission signals. As result of the preceding, a pH of 3.6 was selected for fluorimetric measurements. The maximum relative fluorescence (RFI) was achieved in $10^{-2} \text{ M}$ solutions of $\alpha$-CD, but a slight modification in this concentration had little effect on fluorescence intensity (Fig. 2); a final $\alpha$-CD concentration of $8 \times 10^{-3} \text{ M}$ decreased a 0.3% of fluorescence intensity.

**Quantitative Analysis**

A linear calibration graph was obtained by plotting the fluorescence intensity against standard PHB concentrations between 0.1 and 1 $\mu$g mL$^{-1}$. The regression curve obtained by the least-squares treatments obtained the following:

$$I_f = 164.1[\text{PHB}] - 4.8(r = 0.9992, n = 8)$$

where $I_f$ is the relative fluorescence intensity, $r$ the correlation coefficient, and [PHB] is in $\mu$g mL$^{-1}$. Table 1 gives the analytical parameters.

To evaluate the selectivity of the method, the effect of some other food additives on the determination of 0.5 $\mu$g mL$^{-1}$ of PHB were studied. The additives selected were sweeteners usually found in food, such as saccharin; coloring, such as $\beta$-carotene; and substances such as ferulic acid, $p$-coumaric, and caffeic acid, which are widespread in fruits and vegetables. Various volumes of stock solutions of the different potential interferents were added to PHB standard solution in order to obtain different interferent to analyte ratios in the final solution. The results (Table 2) show that good recoveries are obtained in the presence of

<table>
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<th>Table 1. Analytical parameters</th>
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<td>Analytical sensitivity, $S_A (\mu g \text{ mL}^{-1})$</td>
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<tr>
<td>Detection limit$^a (\mu g \text{ mL}^{-1})$</td>
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<td>Relative standard deviation (%, n = 8)</td>
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$S_A = s_a / m$ (s$_a$ is the standard deviation of the analytical signal, m is the slope of calibration graph).

$^a$for a signal-to-noise ratio = 3.

$^b$for signal-to-noise ratio = 10.
saccharin and β-carotene, but the method fails at high ratios of ferulic, 
*p*-coumaric, and caffeic acid to PHB.

Phenolic compounds are especially important in enology, because they influence the colours, bitter flavors, and astringency of wine. These compounds are related to the clouding and discoloration of wines during aging conditioning. The interest of phenolic constituents in beer is generally centered on those polyphenols implicated in clouding phenomena, but lately there have been studies on the effects of various phenolic compounds on the flavor, stability, and organoleptic characteristics. As a consequence, various HPLC methods have developed for the determination of phenolic compounds in wines and beers.

### Analysis of Beer Samples

The usefulness of the method developed here was evaluated by applying it to the determination of PHB in beer (García-Sánchez, Carnero, and
Heredia 1988). To determine recoveries the samples were spiked with 0.3 and 0.5 \( \mu \text{g mL}^{-1} \) of PHB standard solution, filled to 10 mL with different extract sample volumes (0.3 and 0.5 \( \mu \text{g mL}^{-1} \)) to ensure a final \( \alpha \)-CD concentration near \( 8 \times 10^{-3} \) M, and treated as described in the experimental section. The samples were sonicated for 20 min and submitted to general procedure. Table 3 gives the results obtained. As can be seen, recovery values are low (between 80–84\%\), probably because of the negative interference caused by other phenolic compounds in the matrix (see Table 2). The endogenous PHB found in beer are 1.33 and 1.20 \( \mu \text{g L}^{-1} \) for 0.3 and 0.5 aliquots of beer, respectively.

### CONCLUSIONS

By means of the fluorescence technique, PHB can be quantified at ng \( \text{mL}^{-1} \) levels in beer after extraction. The assay is quick and the improved sensitivity of fluorimetric measurements (because of the cyclodextrin inclusion process) extends the proposed procedure to trace analysis. Recovery values from fortified beer samples were measured to evaluate the extraction procedure and the analytical method. Endogenous PHB content in beer samples has been determined.

### REFERENCES


