α-Cyclodextrin as a restricted access mobile phase for reversed-phase liquid chromatography with fluorimetric detection of phenolic compounds

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Abstract

A reversed-phase liquid chromatographic (LC) method to determine the phenolic derivatives, p-hydroxybenzoic (PHB), ferulic and vanillic acids, and methyl paraben (MPB), was developed. The mobile phase used was $1 \times 10^{-2}$ M α-cyclodextrin/acetate buffer pH 4.6 (1:1), that forms inclusion complexes with the analytes. The inclusion process enhances the fluorescence properties of the phenolic compounds, allowing detection limits in the 1–5 ng ml$^{-1}$ levels. Formation constants were calculated from retention parameters. The method was applied to the determination of the phenolic compounds in beer by applying the method of standard additions.

Keywords: Liquid chromatography; Fluorimetry; Phenolic compounds; Cyclodextrins

1. Introduction

Fluorescence detection usually enhances sensitivity and selectivity over UV absorbance detection, so a method that fully exploited the fluorescence properties of the analytes in a liquid chromatographic (LC) assay would be advantageous. Generally, the ways used to enhance or promote the fluorescence properties of analytes in LC methods are related to pre- or post-column derivatization steps. However, these approaches comport several risks that affect the final performances of the analytical method, e.g., contamination from the derivatization reagents used, time-consuming steps and complication of the instrumental configuration. In this work we examine the usefulness of a mobile phase in LC that improves two key aspects of the analytical performances: selectivity and sensitivity. Cyclodextrin (CD) accomplishes both objectives by means of the microencapsulation of the analytes in the internal cavity of the mobile phase; selectivity, because the particular fit of each compounds with the CD, mainly directed by size and polarity considerations; and sensitivity, because the inclusion process enhances fluorescence emission as a consequence of the protected micro-
environment that precludes non-radiative deactivation pathways of the excited state.

α-, β- and γ-CDs and CD derivatives have been widely used as stationary phases, linked to the support bed, in LC [1–4], existing in commercial chromatographic columns. However, few applications of CDs in a mobile phase have been described [5–7], and mainly focused on the separation power promoted by the differential capacity of the guest compounds to fit into the cavity of CD. Also, the chiral recognizing ability of CD has been exploited to obtain good separations of very similar compounds [8–10]. The capability of α-, β- and γ-CDs to form inclusion compounds, modify capacity factors and improve detection limits, is related to the size of the internal cavity and the size, shape and polarity of the guest compounds. From this, it can be deduced that CD as a mobile phase shows an attractive behaviour to separate and detect closely related compounds, such as isomers.

The phenolic derivatives studied in this work are structurally related compounds that, by size and polarity, form inclusion complexes better fitted to α-CD than to β-CD [11].

Phenolic compounds have received considerable attention as some of them cause adverse tastes and colour changes in food products or decrease protein nutritive value [12]. Sensitive analytical methods permitting the evaluation of substances that may adversely affect beer flavour would have great practical interest, especially if it was simple and reliable. Phenolic compounds are usually analyzed by one or several chromatographic methods: LC [10,13,14], gas chromatography [15,16], paper and thin-layer chromatographies [17,18], fluorimetry [19] and chemiluminescence [20].

In the present work the sensitivity of LC determination of ferulic, vanillic, p-hydroxybenzoic (PHB) acids and methyl paraben (MPB) in beer is studied. Also, we propose a simple method to correct proportional error when the method of standard additions is applied to samples showing calibration slopes depending on the sample size. Sample preparation is simple, only requiring degassification, and application of a modified method of standard additions give the endogenous phenolic compounds content in commercial beer with relative standard deviations below 5%.

2. Experimental

2.1. Materials and apparatus

HPLC gradient grade solvents (Lichrosolv) were supplied by Merck (Darmstadt, Germany). Aqueous standard solutions of PHB and MPB, and ethanolic solutions of ferulic and vanillic acids (Sigma, Madrid, Spain) were prepared weekly at concentrations of 1 mg ml⁻¹. Working standard solutions were prepared daily from these by appropriate dilution. α- and β-CDs (Amaizo, USA) were recrystallized once from boiling water. Solutions of 1 x 10⁻² M were prepared in distilled demineralized water and stored in amber coloured bottles at 4°C. A pH 4.60 buffer solution was prepared by mixing appropriate volumes of 0.2 M acetic acid and sodium acetate (Merck).

Fluorescence spectra were obtained with a Perkin-Elmer LS-50 luminescence spectrometer (Beaconsfield, UK), fitted with a xenon lamp (9.9 W) pulsed at line frequency, 1 x 1 cm fused-silica cells and a R928 Hamamatsu photomultiplier tube. The LC equipment (Merck-Hitachi, Darmstadt, Germany) included a L-6200 pump, L-4250 UV-Visible detector and AS-4000 autosampler. The LS-50 spectrofluorimeter was used as a detector (cell volume 25 μl) placed in series after the UV-Visible detector.

A LiChrospher (Merck) 100 RP8 (200 x 4.6 mm i.d., 10 μm particle size) reversed phase column was used as stationary phase. The mobile phase was buffer pH 4.6/α-CD (50:50, v/v) at a flow rate of 1.5 ml min⁻¹.

2.2. LC method

Inject 10 μl of aqueous standard solution or analytical sample into the LC working under isocratic conditions: buffer (pH 4.6): α-CD (50:50, v/v). Program the fluorimetric detector to set the wavelengths pair (excitation/emission) to 0 s, 260/335 nm; 180 s, 340/430 nm; 350 s, 260/350 nm and 540 s, 260/335 nm. Measure peak area response at retention times of PHB acid (1.96 min), ferulic acid (3.49 min), vanillic acid (7.24 min) and MPB (9.98 min), for each sample. Use the monitored responses to construct a linear regression calibration plot to determine concentration of benzyl derivatives in experimental samples.
3. Results and discussion

3.1. Inclusion compounds

The structures of the four phenolic derivatives studied in this work are given in Fig. 1. These compounds have a size that favours better inclusion and fit in α-CD than in β-CD. When included in CDs, the small "non-polar" emission from aqueous solutions is enhanced [19] and this effect has been exploited to propose fluorimetric analytical methods with enhanced sensitivity. On the other hand, the size and structural configuration of the host compound promotes steric effects in the inclusion process that precludes the inclusion of similar compounds but with branched configuration. This capability of CDs, general to restricted access systems, provides the tool to select the compounds to be included in the interior cavity and to improve the selectivity of the analytical method. These two aspects are especially important in the case of chromatographic methods in which both selectivity in the separation step and sensitivity in detection can be attained by using CD in the mobile phase.

Excitation and emission spectra of the phenolic derivatives in water, α-CD and β-CD show (Fig. 2), as expected, that there is better inclusion and fit occurs in α-CD, and this promotes three to six times more intense emission in α-CD than in β-CD. Also, the wavelengths pair (excitation/emission) recommended for setting the fluorimetric detector were

- COOCH₃
  - CH₂=CHCOOH
  - OH
  - OH

Methyl paraben

- COOH
  - OCH₃
  - OH
  - OH

Ferulic acid

- COOH
  - OH
  - OCH₃
  - OH

Vanillic acid

- p-hydroxybenzoic acid

COOCH₃

OH

Fig. 1. Structure of the phenolic compounds studied.

260/335 nm (PHB acid), 340/430 nm (ferulic acid),
260/350 nm (vanillic acid) and 260/335 nm (MPB)
(Fig. 3).

Inclusion constants in α-CD display the trend of retention times to be expected in the chromatographic elution. However, because of the acidic character of the phenolic derivatives, acidity of the elution solvent
Table 1
Effect of pH on relative fluorescence intensity (RFI) at the recommended wavelengths

<table>
<thead>
<tr>
<th>pH</th>
<th>PHB</th>
<th>MPB</th>
<th>Ferulic acid</th>
<th>Vanillic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.6</td>
<td>909</td>
<td>705</td>
<td>150</td>
<td>800</td>
</tr>
<tr>
<td>4.5</td>
<td>831</td>
<td>670</td>
<td>160</td>
<td>720</td>
</tr>
<tr>
<td>7.0</td>
<td>320</td>
<td>670</td>
<td>290</td>
<td>240</td>
</tr>
<tr>
<td>9.0</td>
<td>583</td>
<td>635</td>
<td>630(^a)</td>
<td>390</td>
</tr>
</tbody>
</table>

*\(\lambda_{em}=450\). must be taken into account. In this regard, ionogenic species, predominant at pH below the pK\(_a\) of the phenolic derivatives, can be expected to be accepted more easily into the CD internal cavity than the basic forms. In Table 1, Relative Fluorescence Intensity of the studied phenolic compounds at several selected pH values reveal a strong pH dependence of the compounds under study, except in the case of methyl paraben, in which this dependence is not so pronounced, as expected, because of its methyl ester character. Inclusion of a buffer solution of pH 4.6 in the mobile phase guarantees adequate extension of the inclusion process in all cases.

Preliminary assays from isocratic elution profiles of the phenolic derivatives, obtained by changing the composition of the mobile phase, indicates that addition of an organic modifier (methanol) does not improve peak resolution or signal intensity, but only decreases retention times. A plot of logarithm of capacity factor (log \(k'\)) against mobile phase composition (buffer pH 4.6/\(\alpha\)-CD 1\(\times\)10\(^{-2}\) M) shows that with 20–50% \(\alpha\)-CD in the mobile phase, all the compounds are well separated. However, sensitivity and column efficiency increased with increasing \(\alpha\)-CD concentrations (Table 2). From this Table it can be seen that increasing \(\alpha\)-CD concentration promoted both high intensity signals and better column efficiency. A mobile phase of buffer pH 4.6/\(\alpha\)-CD 1\(\times\)10\(^{-2}\) M (50:50, v/v) have been selected for the analytical method.

3.2. Quality parameters

Calibration graphs were obtained under the optimum conditions in the range 0–250 ng ml\(^{-1}\). Peak area were used as the detector response and the correlation coefficients were better than 0.990. Nine replicate determinations of 100 ng ml\(^{-1}\) of each phenolic acid were carried out under the optimum conditions to determine the precision in the analysis. Relative standard deviations (RSD) in the range 2.2–4.3% based on peak area were obtained. The RSD values of retention times were within 0.2–1.2%. Detection limits (3\(\sigma\)) ranged from 1.4 to 5.3 ng ml\(^{-1}\). Table 3 summarizes the quality parameters obtained.

3.3. Inclusion constants

Inclusion constants have been calculated by applying the equation proposed by Fujimura et al [21] and based on the pseudophase theory developed by Armstrong[22]

\[
\frac{1}{k'} = \frac{1}{k'_0} + \frac{[CD]_T}{K_D k'_0},
\]

where \(k'\) is the capacity factor of the sample solute, \(k'_0\) is the capacity factor obtained in absence of CD, \([CD]_T\) is the CD concentration in the mobile phase and \(K_D\) is dissociation constant of the inclusion compound.

Table 2
Effect of concentration on sensitivity and column efficiency

<table>
<thead>
<tr>
<th>[CD] (mM)</th>
<th>PHB</th>
<th>RFI</th>
<th>N</th>
<th>MPB</th>
<th>RFI</th>
<th>N</th>
<th>Ferulic acid</th>
<th>RFI</th>
<th>N</th>
<th>Vanillic acid</th>
<th>RFI</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>100</td>
<td>553</td>
<td>40</td>
<td>3586</td>
<td>50</td>
<td>4427</td>
<td>25</td>
<td>346</td>
<td>95</td>
<td>4831</td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>110</td>
<td>553</td>
<td>50</td>
<td>3586</td>
<td>30</td>
<td>379</td>
<td>30</td>
<td>438</td>
<td>95</td>
<td>5707</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>110</td>
<td>553</td>
<td>55</td>
<td>4427</td>
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<td>553</td>
<td>60</td>
<td>3973</td>
<td>30</td>
<td>438</td>
<td>95</td>
<td>6846</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(N\) is the plate number.
Table 3

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Detection limit C&lt;sub&gt;L&lt;/sub&gt; (ng ml&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Quantitation limit C&lt;sub&gt;Q&lt;/sub&gt; (ng ml&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Analytical sensitivity S&lt;sub&gt;A&lt;/sub&gt; (ng ml&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>RSD&lt;sup&gt;a&lt;/sup&gt; (%)</th>
<th>Retention time T&lt;sub&gt;R&lt;/sub&gt; (min)</th>
<th>RSD of T&lt;sub&gt;R&lt;/sub&gt; n=4 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHB</td>
<td>1.4</td>
<td>4.7</td>
<td>0.5</td>
<td>3.6</td>
<td>1.96</td>
<td>0.28</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>5.3</td>
<td>17.8</td>
<td>1.8</td>
<td>4.3</td>
<td>3.49</td>
<td>0.23</td>
</tr>
<tr>
<td>Vanillic acid</td>
<td>3.0</td>
<td>10.0</td>
<td>1.0</td>
<td>4.3</td>
<td>7.24</td>
<td>1.19</td>
</tr>
<tr>
<td>MPB</td>
<td>4.2</td>
<td>14.1</td>
<td>1.4</td>
<td>2.2</td>
<td>9.98</td>
<td>0.45</td>
</tr>
</tbody>
</table>

<sup>a</sup> measurements at 100 ng ml<sup>-1</sup>.

For the determination of the complex formation constants, different concentrations of CD in the mobile phase were used.

Ideally, a plot of 1/k' vs. [CD]<sub>T</sub> would give a straight line of slope 1/(k'<sub>0</sub>K<sub>D</sub>) and intercept 1/k'<sub>0</sub>. By taking the ratio of slope over the intercept, the k'<sub>0</sub> values cancel. Thus, one can easily determine K<sub>D</sub>, provided k' is known. Table 4 compares the dissociation constants for each phenolic compound as determined by the elution profiles and application of Eq. (1) As can be seen, some relationship between structure of the compounds and dissociation constant can be established. Thus, PHB acid having the highest value constant, elutes rapidly and its structure allows (at pH 4.6) good fit in the interior cavity of CD. MPB with a low dissociation constant, elutes the last and its structure precludes a good fit into the cavity.

Fig. 4 shows the change of 1/k' versus [CD]<sub>T</sub> for the four phenolic derivatives, with a concentration of CD ranging from 20 to 100 mM. The linear plot fit well with Eq. (1) with r values better than 0.96 in all instances. This implies that the stoichiometry of the inclusion of four compounds is 1:1 [23].

These results indicated that the decrease in k' values caused by the addition of α-CD is based on the weakening of the hydrophobic interaction between solutes and the stationary phase.

Table 4

| Compound | Dissociation constant K<sub>D</sub> (mol<sup>-1</sup>) | Regression Coefficient (n=5) |
|----------|---------------------------------|------------------|------------------|----------------|------------------|------------------|
| PHB      | 0.4027                          | 0.97             | 0.97             |
| Ferulic acid | 0.0717                     | 0.99             | 0.99             |
| Vanillic acid | 0.0818                 | 0.97             | 0.97             |
| MPB      | 0.0430                          | 0.97             | 0.97             |

3.4. Determination of phenolic compounds in beer

The usefulness of the method developed was evaluated by applying it to the determination of phenolic compounds in beer. Aliquots of degassed beer were injected and their endogenous content of phenolic acids was quantified by applying the method of standard additions.

Table 5 summarizes the results obtained including the parameters used to correct constant and proportional errors. As pointed out elsewhere [24] the method of standard additions correct constant errors but not proportional errors if a significant difference between the standard and standard additions slopes occurs. By linear regression of the sample (non spiked) data, the intercept (termed the total Youden blank) represent the constant error of the method.
Table 5
Endogenous phenolic contents determined in beer

<table>
<thead>
<tr>
<th>Sample Vol (ml)</th>
<th>PHB (ng ml⁻¹)</th>
<th>MPB (ng ml⁻¹)</th>
<th>Vanillic acid (ng ml⁻¹)</th>
<th>Ferulic acid (ng ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>1</td>
<td>1.301</td>
<td>1.294</td>
<td>0.265</td>
<td>0.258</td>
</tr>
<tr>
<td>3</td>
<td>1.241</td>
<td>1.220</td>
<td>0.275</td>
<td>0.255</td>
</tr>
<tr>
<td>5</td>
<td>1.296</td>
<td>1.259</td>
<td>0.297</td>
<td>0.261</td>
</tr>
<tr>
<td>x</td>
<td>1.279</td>
<td>1.257</td>
<td>0.279</td>
<td>0.258</td>
</tr>
<tr>
<td>σₜₙ₋₁</td>
<td>0.033</td>
<td>0.037</td>
<td>0.016</td>
<td>0.003</td>
</tr>
<tr>
<td>TYB</td>
<td>-35.17</td>
<td>-0.955</td>
<td>-15.9</td>
<td>0.11</td>
</tr>
<tr>
<td>m_M</td>
<td>-0.0575</td>
<td>0.31</td>
<td>0.0011</td>
<td>0.19</td>
</tr>
</tbody>
</table>

A - method of standard additions.
B - modified method of standard additions.

extrapolated to the zero sample level (the true sample blank) [25]. The first column in Table 5 shows the results obtained after applying the classical method of standard additions, in which only constant errors are corrected. However, if the slopes corresponding to different sample amounts spiked with standards differs considerably, a noticeable proportional error occurs and this must be corrected. Fig. 5 shows the slope change that different volumes of matrix (beer) produced in the calibration graph of ferulic acid.

We propose in this paper a correction of this error based on the equation:

\[ y = (A + TYB) + (m_m - m_M V)x, \]  

in which A is the intercept of the standard additions plot at each sample volume, TYB is total Youden blank, \( m_m \) is slope of the standard additions curve, \( m_M \) is slope of the regression plot of the different slopes at each sample volume, V is sample volume and x is analyte concentration.

Fig. 5. Standard and standard additions plots corresponding to standard ferulic acid (●), and 1 (△), 3 (■) and 5 ml (▼) of beer sample.
This equation allows the proportional error to be corrected. This kind of error affects the slopes of the standard additions plots and is the function of sample volume. It can produce severe errors in the method of standard additions. As indicated in column 2 of Table 4, Eq. (2) gives a better standard deviation of the results obtained in all cases.

As can be seen, the proportional error correction factor, \( m_M \), differs from one analyte to another and is dependent of the kind of interaction between the matrix and the analyte. Thus, this correction is noteworthy in the case of MPB and ferulic acid \( (m_M = 0.31 \) and \( 0.19 \), respectively). In these cases, excellent \( \sigma_{n+1} \) values between phenolic compounds found in different matrix volumes were obtained.

Acknowledgements

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References