Study of the enhanced chemiluminescence from the luminol–horseradish peroxidase–hydrogen peroxide–p-coumaric acid system at very short times: stopped flow selective determination of p-coumaric acid in beers

F. García Sánchez *, A. Navas Díaz, J.A. González García

Departamento de Química Analítica, Facultad de Ciencias, Universidad de Málaga, 29071 Málaga, Spain

Received 13 October 1994; revised 25 January 1995; accepted 6 February 1995

Abstract

p-Coumaric acid has a greater enhancing effect on the chemiluminescence of the luminol–H₂O₂–horseradish peroxidase system, at low concentration, than other phenolic acids studied. We have used this effect to study the variations of the chemiluminescent signal with luminol, hydrogen peroxide, p-coumaric acid, horseradish peroxidase concentrations and pH, using the stopped-flow technique, by monitoring the initial reaction rate. The interference effects of other phenolic acids on the enhanced chemiluminescence with p-coumaric acid (25 nM) were negligible at similar concentrations of phenolic acid. We monitored the chemiluminescence intensity at 10 s for the determination of p-coumaric acid in beers. The detection limit was ca. 0.7 nM and the linear range was 0–12.5 nM. The precision of the method, expressed as a relative standard deviation, was 2.5%.

Keywords: Chemiluminescence; p-Coumaric acid; Luminol; Phenolic acids; Horseradish peroxidase

1. Introduction

Numerous compounds increase or decrease the chemiluminescence of the luminol–H₂O₂–horseradish peroxidase system [1–7]. These compounds have been used in immunoassay [8–10] and other analytical determinations [11].

Phenolic acids are a group of numerous organic compounds which have one or more hydroxyl functions and a carboxylic acid function at the benzene ring. These compounds increase or decrease the chemiluminescence of the luminol–H₂O₂–horseradish peroxidase system. These effects are associated with the position and nature of the different substituents on the ring and with the redox character of the phenolic acid in each case [12]. A mechanism was proposed by Thorpe et al. in 1987 and by Lundin et al., also in 1987, to explain these effects [13–15] (Ref. [13] describes a mechanism for the luminol–H₂O₂–horseradish peroxidase reaction without enhancer). Phenolic acids are natural products which influence the growth of plants. They
interact with certain vegetable hormones. Thus, it was suggested that there is an interaction between phenolic acids and indole 3-acetic acid oxidase (IAA-oxidase) [16]. Some of these compounds cause colour changes and adverse flavours in foods and are responsible for the loss of a protein’s nutritional value [17]. Phenolic acids were also found in numerous drinks, however, only small quantities are free. Normally they are bound as phenolic glycosides.

Hydroxycinnamic acids and other phenolic compounds have been analyzed usually using chromatographic techniques, i.e., thin-layer chromatography [18–20], column liquid chromatography [21], gel filtration [22] and gas chromatography [23,24]. However, the most used technique is high resolution liquid chromatography (LC), generally with spectrophotometric detection [25–29] or with electrochemical detection [30,31].

Phenolic compounds are specially important in oenology because they influence the colour, bitter flavour and astringency of wines. These compounds are related to the clouding and the browning of wines during their ageing and fermentation.

Interest in the phenolic constituents of beers is generally centered in those polyphenols implicated in clouding phenomena, however, recently the incidence of various phenolic compounds on the flavour, stability and organoleptic characteristics of beers have been studied. As a consequence, in recent years a number of methods have been developed for the determination of phenolic compounds in wines [32–34] and beers [35–37], using LC in all cases.

p-Coumaric acid (4-hydroxycinnamic acid) is a phenolic acid. The determination of p-coumaric acid in real samples is very difficult without chromatographic techniques, because the other phenolic acids interfere. Nevertheless, some methods permit the determination of certain phenolic acids without chromatography [38–41]. Previous studies made by us with ten phenolic acids showed that only p-coumaric acid had an important enhancing effect, at low concentration, on the luminol–H₂O₂–horseradish peroxidase system [12]. This enhancing effect of p-coumaric acid has been used in immunoassay [42]. In this paper, we describe a study of the enhanced chemiluminescence emission over a short period of time during the luminol–H₂O₂–horseradish peroxidase reaction using a stopped-flow technique and monitoring the initial rate between 0 and 0.08 s. When this method was used to determine p-coumaric acid in beers matrix problems arose with such initial rates so the emission was monitored over longer time periods. We determined p-coumaric acid in beers without the need for chromatographic separation.

2. Experimental

2.1. Reagents

All reagents were of analytical grade. The water was doubly distilled and demineralized. Luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) (Sigma, St. Louis, MO), 0.01 M, was prepared by dissolving 0.0913 g of luminol 97% in a small amount of NaOH, the final volume was made up to 50 ml with Tris–HCl buffer, 0.1 M (pH 8.6). Peroxidase from horseradish Type VI-A, RZ apr. 3.1 (Sigma, St. Louis, MO) was used.

p-Coumaric acid (4-hydroxycinnamic acid), gentisic acid (2,5-dihydroxybenzoic acid), 2,3-dihydroxybenzoic acid, caffeic acid (3,4-dihydroxycinnamic acid), protocatechuic acid (3,4-dihydroxybenzoic acid), ferulic acid (4-hydroxy-3-methoxycinnamic acid), syringic acid (4-hydroxy-3,5-dimethoxybenzoic acid), vanillic acid (4-hydroxy-3-methoxybenzoic acid), p-hydroxybenzoic acid (4-hydroxybenzoic acid) were supplied by Sigma and β-resorcylic acid (2,4-dihydroxybenzoic acid) by Merck. All the phenolic acid solutions were prepared from 0.01 M or 0.001 M stock solutions.

2.2. Instruments

A Perkin-Elmer LS-50 (Beaconsfield, UK) luminescence spectrometer with the light source switched-off was used. The apparatus was set in the phosphorescence mode with 0.00 ms delay time and 60 ms gate time. The slit-width of the emission monochromator was set at 20 nm with λem – 425 nm and photomultiplier voltage set manually to 700 V. The samples were placed in a quartz cuvette continuously stirred with a magnetic stirrer. The chemiluminescent reaction was triggered by injecting
horseradish peroxidase solution with a syringe, through a septum.

The study of the p-coumaric emission at short times was carried out in an SLM-Aminco 48000S fluorometer (Urbana, IL) equipped with a MilliFlow stopped-flow reactor with the light source turned off and no optical filter before the photomultiplier. The cell volume was 32 μl. Equal volumes of the two reagent solutions were introduced into the cell when a pressure of 4.5 bar was applied on the two supply syringes. The dead time was 1.0 ms, flow velocity 20 ml/s, and mixing efficiency was > 98%. The intensity (in V) was collected throughout the reaction at a rate of 10 ms per point with a photomultiplier gain of 10 and a voltage of 650 V. All measures were carried out at 25 ± 0.1°C.

2.3. Study of the chemiluminescence with p-coumaric acid at long time

The concentrations of luminol, peroxidase and H₂O₂ were chosen to give a chemiluminescence signal detectable in the absence of p-coumaric acid so as to study the effects of the addition of this phenolic acid. Solutions of Tris-HCl buffer (0.1 M, pH 8.3), luminol, hydrogen peroxide and p-coumaric acid were introduced into a quartz cuvette by pipette and up to 2950 μl with doubly distilled water. The chemiluminescence reaction was triggered by the injection of 50 μl of horseradish peroxidase solution (73 U ml⁻¹). The progress of the light emission was recorded between 0 and 300 s. The final concentrations of the reagents in the cuvette for all the experiments were: Tris–HCl buffer 33.3 mM, luminol 66.7 μM, hydrogen peroxide 2 mM, p-coumaric acid (16.7–333.3 nM) and horseradish peroxidase 1.22 U ml⁻¹.

2.4. Signal at short times and determination of p-coumaric acid in beers

For the study of the p-coumaric acid signals at short times and the determination of p-coumaric acid in beers, a syringe of the stopped-flow apparatus was filled with horseradish peroxidase in a 0.02 M buffer solution and the other with a mixture of luminol, hydrogen peroxide and p-coumaric acid in the same buffer solution, at the same concentration. The initial rates, dI/dt, were measured by subtracting the signal values at 0.08 s from that at 0 s and dividing by 0.08 for the studies of pH, luminol, horseradish peroxidase and p-coumaric acid concentrations. The chemiluminescence intensity at 10 s was measured for the determination of p-coumaric acid in beers to avoid matrix effects.

2.5. Effect of reagent concentrations and pH

We studied the effects of the different reactants (peroxidase, luminol, hydrogen peroxide and pH) on the initial rate (between 0 and 0.08 s) of the chemiluminescent emission. The assays were carried out in the following buffer solutions for the following pH values: potassium dihydrogen phosphate buffer (pH 6.5), tris(hydroxymethyl)aminomethane buffer (pH 7.5 and 8.5), borax buffer (pH 8.5 and 9.5), sodium hydrogen carbonate buffer (pH 10.5), disodium hydrogen phosphate buffer (pH 11.5). As a result of these studies we chose these conditions to study the variation in chemiluminescence with the concentration of p-coumaric acid: [luminol] = 3 × 10⁻⁵ M, [H₂O₂] = 2 mM, [peroxidase] = 8.51 U/ml, [tetraborate buffer] = 0.02 M at pH 8.5 and [p-coumaric acid] = 0–100 nM. We studied the different results obtained at different initial rates or slopes of the chemiluminescent curve between 0 s and 0.08 s, 0.125 s, 0.140 s and 0.2 s, respectively.

2.6. Determination of p-coumaric acid in beers

A beer sample was degasified by shaking and slight heating (between 30 and 35°C) and the pH was adjusted to 2 by adding 1 M HCl. We added 25 ml of n-hexane to 25 ml of this acidified sample. After vigorous agitation the aqueous phase was separated and extracted twice with 25 ml of ethyl acetate. The ethyl acetate was evaporated at reduced pressure between 30 and 35°C and the residue was diluted to 100 ml with doubly distilled water. A small volume of this solution (30 or 60 μl) was mixed in a 10 ml volumetric flask with a luminol–H₂O₂–p-coumaric acid mixture. This solution was introduced into one syringe, and a peroxidase solution in the other syringe. The chemiluminescent reaction was triggered
with the stopped-flow technique and the intensity at 10 s was measured. A calibration graph of chemiluminescence intensity at 10 s against \( p \)-coumaric acid concentration was prepared, and also, a standard addition method was applied to determine \( p \)-coumaric acid in beer extracts.

3. Results and discussion

3.1. Study of the chemiluminescence with \( p \)-coumaric acid at long time

Using the Perkin-Elmer spectrometer conditions the chemiluminescence emission of the luminol–horseradish peroxidase–\( H_2O_2 \) system between 0 and 300 s was studied. We observed that under these conditions the representation of maximal intensity against the \( p \)-coumaric acid concentration (in nM) was linear in the range 16.7 to 333.3 nM. The linear fit was: 

\[
I_{\text{max}} = \left(24.5094 \pm 5.0540\right) + \left(1.2344 \pm 0.0330\right)C \quad (C \text{ is the } p \text{-coumaric acid concentration, in nM}), \text{ with a correlation coefficient of 0.9989 for } n = 5. \text{ The limit of detection was 29.5 nM, and a 1\% determination error for } n = 3.
\]

Though \( p \)-coumaric acid is the only phenolic acid found that is an important enhancer at low concentration [12], other non-enhancing phenolic acids could attenuate the enhanced chemiluminescence of \( p \)-coumaric acid in real samples or mixtures. Such effects were observed, for example, for the enhanced chemiluminescence of 4-iodophenol by other phenols such as phenol, 4-methylphenol, 4-fluorophenol, 3-hydroxybenzaldehyde and 4-phenolsulphonic acid [43].

3.2. Enhanced chemiluminescence at short times

These studies were carried out in a SLM-Aminco apparatus, using the stopped-flow technique. We preferred to work at short times to determine \( p \)-coumaric acid in real samples in an attempt to eliminate interferences. Thus, we measured initial rates between 0 and 0.08 s, and studied \( pH \) and reagent concentration effects on the initial rates.

In the stopped-flow experiments, one syringe was filled with a solution of luminol and \( H_2O_2 \) (with or without \( p \)-coumaric acid) in a buffer solution, and the other syringe with a solution of horseradish peroxidase (with or without buffer solution). The results showed that when the borate buffer concentration in the syringe with peroxidase was less than the buffer concentration in the syringe with luminol and \( H_2O_2 \), the initial rate was very large and similar for the non-enhanced and the enhanced emissions. Much less intense emissions were observed when each syringe had an equal buffer concentration (Fig. 1). Equal buffer concentrations were used in all further experiments.

Fig. 2 shows the variation of the initial rate (between 0 and 0.08 s) with the luminol, peroxidase and hydrogen peroxide concentrations and \( pH \). We found differences in the results when the buffer solutions at \( pH \) 8.5 were tetraborate buffer, 0.02 M (circle), or Tris–HCl, 0.02 M (triangle in Fig. 3c).

After this study, we chose the \( pH \) and reactant concentrations for the calibration curve of initial rate against \( p \)-coumaric acid concentration. Fig. 3 shows calibration graphs of initial rate measured between 0 and
Fig. 3. Initial rates plotted against p-coumaric acid concentration for different time interval: (A) between 0 and 0.08 s; (B) between 0 and 0.125 s; (C) between 0 and 0.040 s; (D) between 0 and 0.2 s. Experimental conditions: [pH 8.5 tetraborate buffer] = 0.02 M in syringe; [horseradish peroxidase] = 1.21 U/ml; [luminol] = 3 x 10^{-5} M; [H_2O_2] = 2 mM; [p-coumaric acid] = 2 x 10^{-7} M.

Regression equation after subtracting the blank intensity at 10 s was: \( \Delta I_{10 s} = (-0.0049 \pm 0.0051) + (0.017 \pm 0.0007)C; \ r = 0.9973, \ n = 5 \) without beer. The limit of detection was 0.871 nM, and the precision of the method, expressed as relative standard deviation was 2.5%. For the standard addition procedure applied to 30 \( \mu \)l with diluted extract of Cruzcampo beer (Spain) the equation was: \( \Delta I_{10s} = (0.0451 \pm 0.0026) + (0.018 \pm 0.0004)C; \ r = 0.9993, \ n = 5 \). The concentration of p-coumaric acid in the Cruzcampo beer was 1.1 \( \mu \)g/ml. We measured these signals with an increment of 0.01 s per point.

We measured p-coumaric acid in other beers; in these cases we measured the signal with an increment of 0.1 s per point. The calibration graph was linear in the range 0–12.5 nM p-coumaric acid, the regression equation was \( \Delta I_{10s} = (-0.0119 \pm 0.0190) + (0.021 \pm 0.001)C; \ r = 0.991, \ n = 5 \) without beer. The limit of detection was 0.7 nM, and the

Table 1
Interference effects on the initial rate between 0 and 0.125 s for the enhanced chemiluminescence of the luminol–H_2O_2–peroxidase–p-coumaric acid system by other phenolic acids, for different p-coumaric acid to phenolic acid ratios in the mixtures

<table>
<thead>
<tr>
<th>Interferent</th>
<th>Ratio</th>
<th>%Recovery</th>
<th>Ratio</th>
<th>%Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protocatechuic acid</td>
<td>1:1</td>
<td>106</td>
<td>1:2</td>
<td>95</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>1:1</td>
<td>94</td>
<td>1:2</td>
<td>66</td>
</tr>
<tr>
<td>Syringic acid</td>
<td>1:1</td>
<td>101</td>
<td>1:2</td>
<td>66</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>1:1</td>
<td>104</td>
<td>1:2</td>
<td>88</td>
</tr>
<tr>
<td>Vanillic acid</td>
<td>1:1</td>
<td>103</td>
<td>1:2</td>
<td>84</td>
</tr>
<tr>
<td>p-Hydroxybenzoic acid</td>
<td>1:1</td>
<td>103</td>
<td>1:2</td>
<td>93</td>
</tr>
</tbody>
</table>

Experimental conditions: [tetraborate buffer, pH 8.5] = 0.02 M; [peroxidase] = 8.5 U/ml; [H_2O_2] = 2 mM; [luminol] = 3 x 10^{-5} M; [p-coumaric acid] = 2 x 10^{-7} M.

Fig. 4. Interference effect of ferulic acid on the enhanced chemiluminescence of the luminol–H_2O_2–horseradish peroxidase–p-coumaric acid system, for different concentrations of ferulic acid: (A) 0 M; (B) 5 x 10^{-8} M; (C) 10 x 10^{-8} M; (D) 20 x 10^{-8} M; (E) 50 x 10^{-8} M; (F) 100 x 10^{-8} M; (G) blank. Experimental conditions: [tetraborate buffer] = 0.02 M, pH 8.5, [horseradish peroxidase] = 8.5 U/ml; [luminol] = 3 x 10^{-5} M; [H_2O_2] = 2 mM; [p-coumaric acid] = 25 nM.
0.08 s, 0 and 0.125 s, 0 and 0.140 s, and between 0 and 0.2 s, respectively, against p-coumaric acid concentration. The initial rate measured between 0 and 0.125 s was chosen for subsequent calibration and interference studies, because in this interval the graph was linear. The linear fit was: \( \frac{dI}{dt} (\text{initial rate}) = (10.0895 \pm 0.3267) + (0.3249 \pm 0.0063)C \), where \( C \) is the concentration of p-coumaric acid between 5 and 100 nM, the correlation coefficient was 0.9991 for \( n = 7 \). The objective was to apply this calibration curve to the determination of p-coumaric acid in beers.

The results of interference studies on other phenolic acids are shown in Table 1 and Table 2. These effects were very significant when the concentration of p-coumaric acid was 100 nM (at a ratio of 1:1 between p-coumaric acid and interferent) but were negligible when the concentration of p-coumaric acid was 25 nM (p-coumaric to interferent ratio, 1:1). Fig. 4 shows the interference effects of a mixture of p-coumaric acid and ferulic acid at different concentrations of ferulic acid.

### 3.1. Determination of p-coumaric acid in beers

When the initial rate method was applied to beers, the sample matrix decreased the initial rate of the enhanced chemiluminescence from the luminol–\( \text{H}_2\text{O}_2 \)–peroxidase system. The matrix effect was very important over short periods of time, but at a longer period of time (10 s) the p-coumaric acid signals with and without beer extract were more similar (Fig. 5).

A calibration graph was prepared for the determination of p-coumaric acid in beers. Plotting differences between the sample intensities (with p-coumaric acid or beer extract) at 10 s and the blank intensity at 10 s (\( \Delta I_{10} \)), against p-coumaric acid concentration added, the calibration graphs were linear in the range 0–12.5 nM for p-coumaric acid. The
Fig. 5. Time–intensity curves for the blank reaction (A), and for increasing volumes of extract of beer sample (B, C, D).

Mid-range precision of the method, expressed as relative standard deviation, was 8%. For the standard addition method applied to 60 μl of diluted extract of Carlsberg beer (Denmark) the equation was: $A_{I_{09}} = (0.0620 \pm 0.0092) + (0.0226 \pm 0.0012)C$; $r = 0.9943$, $n = 6$. The concentration of p-coumaric acid was found 0.6 μg/ml.

There was a problem when applying this method to determine p-coumaric acid in Guinness stout (Ireland), associated with the extraction procedure with ethyl acetate.

Acknowledgements

We thank the Comisión Interministerial de Ciencia y Tecnología (Project PB93-1006).

References


