Anal. Chem. **1994,** *66,* 988–993

Nonlinear Multicomponent Kinetic Analysis for the Simultaneous Stopped-Flow Determination of Chemiluminescence Enhancers

Aurora Navas Díaz* and José Antonio González García

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

Protocatechuic and caffeic acids were determined from kinetic data of luminol chemiluminescence induced by hexacyanoferrate(III) enhanced by phenolic acids. The phenolic acids could be quantified both separately and together using the stopped-flow technique and monitoring intensity at 0.3 s and the initial rate. Synergy was observed when two or more enhancers acted simultaneously. The interaction between components was analyzed by surface response methodology and modeled by least-squares matrix methodology that used as the independent variable a measurement parameter (chemiluminescence intensity or initial rate) proportional to the reaction product. We used a two-factor factorial design to obtain the experimental response, and a first-grade equation was constructed to fit as closely as possible the experimental responses. Analytical accuracies showed good agreement between the theoretical model and the behavior of the protocatechuic–caffeic–luminol system.

Chemiluminescence (CL) is a very sensitive technique, but it suffers a lack of selectivity.[1] Several techniques have been used to increase the specificity of CL analysis, and a detection of separated species has been suggested. In addition, mathematical treatment of chemiluminescence data to achieve multicomponent assays has been tried to resolve inorganic and organic mixtures.[2,3]

For linear systems, in which the species behave independently, there are several different approaches to resolve one or more components in mixtures that employ the differences between the kinetics of the components.[2-9]

For many nonlinear multicomponent systems, the problematical interferences arising from the synergism between components can be avoided by limiting the analyses to the linear working range of the calibration curve by successively diluting the samples. Curve-fitting methods can also be used to fit mathematical models to experimental data that show deviations from linearity. The usual methods are the least-squares method and the Kalman filter. To use linear expressions like the linear Kalman filter, or the classical least-squares method (also known as the K matrix method[10]) to calculate a nonlinear function, one must calculate the rate constants exactly and these must not change from run to run; but this is difficult because velocity constants depend on temperature, pH, ionic forces, and a number of other factors. However, for independent and nonsynergic reactions, the problem has been recently resolved by using a nonlinear or extended Kalman filter.[11]

In kinetic analysis, such as CL analysis, synergic phenomena and reactions between components occur quite often. This present work applies the least-squares P matrix method[12] to multicomponent kinetic analysis. The main advantage of this method is that one does not have to calculate beforehand the rate constants of the reactions, and this permits the resolution of two-component mixtures even though the reactions may be synergic.

Kinetic analysis usually employs concentration as the independent variable in equations that express the relationships between the parameter being measured and initial concentrations of the components. For multicomponent analyses that use the classical least-squares method (the K matrix method),[10] the measurement parameter is a function of concentration, but, in this case, the fact that the concentration is the independent variable presents a number of disadvantages for nonlinear multicomponent analyses.[12] On the other hand, the problem is simplified if the measured parameter is used as the independent variable, and moreover, this method resolves for the concentration of the components of interest being measured as a function of a measurable quantity. This model can be used to fit data that are far from linear.

The usefulness of this new approach was demonstrated by applying it to simultaneous determination of protocatechuic and caffeic acids, two CL enhancers, by following the kinetics of luminol light emission. Phenolic acids like these are natural biological products found in plant tissues. The ability to carry out simultaneous determinations of protocatechuic and caffeic acids is useful since these acids are involved in plant growth. High-performance liquid chromatography[13-15] and gas chromatography[16] permits analysis of these acids with low

(1) Nieman, T. A. In *Luminescence Techniques in Chemical and Biochemical Analysis*; Baeyens, W. R. G., De Keukeleire, D., Korkidis, K., Eds.; Marcel Dekker: New York, 1991; pp 523–565.
(2) MacDonald, A.; Chan, K. W.; Nieman, T. A. *Anal. Chem.* **1979,** *51,* 2077–2082.
(3) Veazey, R. L.; Nieman, T. A. *Anal. Chem.* **1979,** *51,* 2092–2096.
(4) Mark, H. B.; Rechnitz, G. A. *Kinetics in Analytical Chemistry*; Interscience Publishers: New York, 1968; pp 142–231.
(5) Pardue, H. L. *Anal. Chim. Acta* **1989,** *216,* 69–107.
(6) Rutan, S. C.; Brown, S. D. *Anal. Chim. Acta* **1985,** *167,* 23–27.
(7) Fitzpatrick, C. P.; Pardue, H. L. *Anal. Chem.* **1989,** *61,* 2551–2556.
(8) Schechter, I.; Schröder, H. *Anal. Chem.* **1992,** *64,* 325–329.
(9) Cladera, A.; Gómez, E.; Estela, J. M.; Cerdá, V. *Anal. Chem.* **1993,** *65,* 707–715.
(10) Brown, C. W.; Lynch, P. F.; Obremsky, R. J.; Lavery, D. S. *Anal. Chem.* **1982,** *54,* 1472–1479.
(11) Quencer, B. M.; Crouch, S. R. *Analyst* **1993,** *118,* 695–701.
(12) Maris, M. A.; Brown, C. W.; Lavery, D. S. *Anal. Chem.* **1983,** *55,* 1694–1703.
(13) Vioque, E.; Millan, F. *Grasas Aceites* **1985,** *36,* 325–327.
(14) Nagels, L. J.; Creten, W. L. *Anal. Chem.* **1985,** *57,* 2706–2711.
(15) Yan, X.; Barlow, P. J. Craven, C. *Food Chem.* **1991,** *40,* 93–99.
(16) Elujoba, A. A.; Nagels, L.; Sofowora, A.; Van Donge, W. *Int. J. Pharm.* **1989,** *53,* R1–R3.

0003-2700/94/0366-0988$04.50/0

sensitivity. In this work, their simultaneous detection is performed with high sensitivity.

Luminol CL is a complex multistep reaction[17,18] that creates several sites at which improved efficiency can increase light emission. Enhancers may increase light emission by accelerating some steps. To monitor the effect of enhancers, one needs a technique that can follow the first milliseconds of the reaction. Mechanical mixing of solutions is satisfactory for selective reactions that have long lifetimes or give high quantum yields, but is unsuitable for fast (<15 s) CL reactions. Various flow systems have been devised to mix samples and reagents quickly and reproducibly,[19] and among these, stopped flow has a number of attractive features. For CL monitoring, it gives rapid mixing, and unlike continuous-flow procedures, the emitting solution is retained in the detector flow cell permitting measurements of CL intensity against time.

THEORETICAL CONSIDERATIONS

For a mixture of n compounds reacting to yield products proportional to some parameter S, the dependency on the concentration of all the analytes present can be described by a mathematical function of the form

$$S_{pi} = K_p + \sum K_{pj} C_{ji}$$

$$i = 1, ..., m, \quad p = 1, ..., l, \quad j = 1, ..., n$$

where S_{pi} is some parameter or a pth property directly proportional to yield products measured at ith mixture, C_{ji} are the initial concentrations of the jth species in the ith mixture, K_p is a correction factor for the reaction blank, and K_{pj} are constants corresponding to the slopes of plots of S_{pi} against concentration for each jth component. In the case of simple first- or pseudo-first-order kinetic reactions they can be expressed as $K_{pj} = s_j r_j(1 - \exp(-k_{pj}t)$. s_j expresses the stoichiometry of the reaction. The r_j are proportionality constants that associate S_{pi} with concentration, and k_{pj} are the first- or pseudo-first-order rate constants. To calculate the unknown parameters (K_{pj}), n observations are made of the variable S_{pi} for n different combinations of the m controlled variables C_{ji}.

Resolving these equations simultaneously for C_{ji} in terms of S_{pi} gives new equations of the type

$$C_{ji} = L_j + \sum L_{jp} S_{pi}$$

in which L_{jp} are constants that associate the concentration of the jth compound with the measured parameter and L_j is a correction factor for the blank signal.

The above derivation assumes that the measured parameter–concentration relationships for all components of the system are linear; if they are not, large analytical errors occur. Consequently, when the system does not behave independently, the nonlinearity can be expressed by resolving equations of the type

$$C_{ji} = L_j + \sum L_{jp} S_{pi} + L_{jl+1} S_{1i} S_{2i} + L_{jl+2} S_{1i} S_{3i} + ...$$

Another possible model incorporates exponential terms like those described for measurements of absorbance.[20]

(17) Merenyi, G.; Lind, J.; Eriksen, T. E. *J. Biolumin. Chemilumin.* **1990**, *5*, 53–56.
(18) Hodgson, E. K.; Fridovich, I. *Photochem. Photobiol.* **1973**, *18*, 451–455.
(19) Townshend, A. *Analyst* **1990**, *115*, 495–500.

EXPERIMENTAL SECTION

Instrumentation. An SLM-Aminco 48000S fluorometer (Urbana, IL) equipped with a MilliFlow stopped-flow reactor was employed with the light source turned off and no filtering before the photomultiplier. The cell volume was 32 μL. Equal volumes of the two reagent solutions were introduced into the cell when a force of 4.5 bar was applied on the two supply syringes. The dead time was 1.0 ms, flow velocity 20 mL·s^{-1}, and mixing efficiency better than 98%. The intensity (in mV) was collected throughout the reaction at a rate of 10 ms per point for 3 s. Signal values were measured directly as chemiluminescence intensity at 0.3 s; rate data dI/dt were obtained by subtracting signal values at 0.07 s from that at 0 s and dividing by 0.07. All measurements were carried out at 20 ± 0.1 °C.

Batch chemiluminescence experiments were carried out in a Perkin-Elmer LS50 spectrofluorometer (Beaconsfield, UK) with the light source turned off and the bandwidth of the emission monochromator at 20 nm. The sample was placed in a quartz cuvette fitted with a magnetic stirrer, and the chemiluminescence reaction was started by adding the luminescent reagent manually with a syringe through a septum. Kinetics of light emission were recorded.

The calculations were made with PC-MATLAB software (MatWorks Inc., Sherborn, MA). To resolve the linear system with more equations than unknowns, we used orthogonal factorization that serves for both square and rectangular matrices. Of the many solution vectors, orthogonal factorization found the best solution in a least-squares sense. Surface graphs were obtained by using Surfer software (Golden Software, Golden, CO).

Reagents and Solutions. Standard protocatechuic acid (3,4-dihydroxybenzoic acid) and caffeic acid (3,4-dihydroxycinnamic acid) (Sigma, St. Louis, MO), 10^{-3} M, were prepared daily by weighing 0.0154 and 0.0180 g, respectively, and diluting to 100 mL with bidistilled water. A 5×10^{-4} M stock solution of luminol (Sigma) was prepared by dissolving 0.0091 g of the compound (5-amino-2,3-dihydro-1,4-phthalazinedione) in a few drops of 2 M NaOH, and the volume was adjusted to 100 mL with 0.2 M tris(hydroxymethyl)aminomethane to give a final pH of 8.6. This solution was renewed weekly.

Methods. In the batch chemiluminescence experiments, a solution of NaOH, hexacyanoferrate(III), and EDTA was placed in a quartz cuvette and one of luminol and protocatechuic acid or caffeic acid was added manually with a syringe. The final concentrations in the cuvette for all experiments were 2×10^{-4} M hexacyanoferrate, 1×10^{-4} M EDTA, and 3.33×10^{-6} M luminol. For the protocatechuic acid experiments, the NaOH concentration was 0.27 M and protocatechuic acid concentrations ranged from 6.7×10^{-8} to 66.7×10^{-8} M. For caffeic acid the NaOH concentration was 0.47 M and caffeic acid concentrations ranged from 13.3×10^{-8} to 133.3×10^{-8} M.

In the stopped-flow CL experiments, one syringe was filled with 0.9333 M NaOH, 2×10^{-4} M EDTA, and 4×10^{-4} M hexacyanoferrate(III) and the other with the CL reagent luminol (6.7×10^{-6} M) and either protocatechuic acid, or caffeic acid. Protocatechuic acid concentrations ranged from

(20) Barnett, H. A.; Bartoli, A. *Anal. Chem.* **1960**, *32*, 1153–1156.

I; $R_1 = R_4 = OH$, $R_2 = R_3 = H$, II; $R_1 = R_4 = H$, $R_2 = R_3 = OH$, III; $R_1 = R_2 = OH$, $R_3 = R_4 = H$,

IV; $R_1 = R_4 = H$, $R_2 = OCH_3$, $R_3 = OH$ V; $R_1 = H$, $R_2 = R_4 = OCH_3$, $R_3 = OH$,

VI; $R_1 = R_2 = R_4 = H$, $R_3 = OH$, VII; $R_1 = R_3 = OH$, $R_2 = R_4 = H$,

VIII; $R_5 = H$, $R_6 = OH$, IX; $R_5 = OCH_3$, $R_6 = OH$, X; $R_5 = OH$, $R_6 = OH$.

Figure 1. Structures of phenolic acids derived from benzoic acid and cinnamic acid tested for enhancement of light emission from the hexacyanoferrate(III) oxidation of luminol.

Table 1. Relative Intensities of Light Emission from the Hexacyanoferrate(III) Oxidation of Luminol by Addition of Phenolic Acids

compound	I^a	A^b
none	43	479
gentisic acid (**I**)	32	574
protocatechuic acid (**II**)	135	1200
2,3-dihydroxybenzoic acid (**III**)	49	774
vanillic acid (**IV**)	96	544
syringic acid (**V**)	0	0
p-hydroxybenzoic acid (**VI**)	48	448
β-resorcilic acid (**VII**)	37	760
p-coumaric acid (**VIII**)	107	562
ferulic acid (**IX**)	34	162
caffeic acid (**X**)	77	891

[a] Intensity at maximum of chemiluminescence with relative standard deviation (RSD) in the range 2–13%. [b] Area under the light emission curve with RSD in the range 3–17%. Experimental conditions: [luminol] = 1.3×10^{-6} M, [hexacyanoferrate(III)] = 1×10^{-4} M, [NaOH] = 0.27 M, [EDTA] = 1×10^{-4} M, [phenolic acid] = 6.7×10^{-6} M.

5×10^{-8} to 50×10^{-8} M. Caffeic acid concentrations ranged from 10×10^{-8} to 100×10^{-8} M. Mixtures of both phenolic acids were analyzed by decreasing the concentration of luminol to 2.5×10^{-6} M.

RESULTS AND DISCUSSION

Enhanced CL reactions of luminol form the basis of rapid and sensitive assays.[21] They give more intense, longer, and more stable light emissions than those of unenhanced reactions. A number of substituted phenols are reported to be enhancers of luminol chemiluminescence in reactions with hexacyanoferrate(III).[22] The present work studied the behavior of other phenolic derivatives as enhancers of this reaction. Figure 1 gives the structures of 10 phenolic acids derived from benzoic acid and cinnamic acid selected for their possible capacity to enhance light emission from the hexacyanoferrate(III) oxidation of luminol. Table 1 lists the responses obtained from the oxidation of luminol by addition of phenolic acids. These data did not allow us to make generalizations about the relation between structure and enhancement of the phenolic derivatives. However, the data show that protocatechuic and caffeic acids were among the best enhancers and they increased the maximum intensities and durations of chemiluminescence signals.

(21) Kricka, L. J.; Stott, R. A. W.; Thorpe, G. H. G. In *Luminescence Techniques in Chemical and Biochemical Analysis*; Baeyens, W. R. G., De Keukeleire, D., Korkidis, K., Eds.; Marcel Dekker: New York, 1991; pp 599–635.

(22) Kalinichenko, I. E.; Tkachuk, T. M.; Pilipenko, A. T. *Zh. Anal. Khim.* **1988**, *43*, 2074–2078.

The general features of luminol chemistry and CL are quite clear.[17,18] However, the enhancers' behavior is far from being understood. For the luminol–H_2O_2–peroxidase–enhancer system[23] a mechanism has been suggested, but the data obtained for the luminol–hexacyanoferrate(III)–enhancer system are not fit to this mechanism; instead the pathways could be

1. $LH_2 + NaOH \rightarrow LH^-$ (1)

2a. unenhanced

$LH^- + Fe^{3+} \rightarrow L^{\bullet -} + Fe^{2+} + H^+$ (2)

$L^{\bullet -} + O_2 \rightarrow L + O_2^{\bullet -}$ (3)

$L^{\bullet -} + O_2^{\bullet -} \rightarrow LO_2^{2-}$ (4)

2b. enhanced

$EH_2 + NaOH \rightarrow EH^-$ (5)

$EH^- + Fe^{3+} \rightarrow E^{\bullet -} + Fe^{2+} + H^+$ (6)

$E^{\bullet -} + O_2 \rightarrow E + O_2^{\bullet -}$ (7)

$E^{\bullet -} + O_2^{\bullet -} \rightarrow EO_2^{2-}$ (8)

$EO_2^{2-} + L^{\bullet -} \rightarrow LO_2^{2-} + E^{\bullet -}$ (9)

3. $LO_2^{2-} \rightarrow N_2 + AP^{2-*}$ (10)

$AP^{2-*} \rightarrow AP^{2-} + h\nu$ (11)

Figure 2 presents the structural formulas of LH_2, LH^-, $L^{\bullet -}$, L, LO_2^{2-}, AP^{2-}, EH_2, EH^-, $E^{\bullet -}$, E, and of EO_2^{2-}. The symbols Fe^{3+}, Fe^{2+}, $O_2^{\bullet -}$, and AP^{2-*} refer to hexacyanoferrate(III), hexacyanoferrate(II), superoxide radical, and excited aminophatalate dianion, respectively.

When the reaction was unenhanced,[17,18] the oxidation of luminol by hexacyanoferrate(III) in basic media took place at six stages: 1–4, 10, and 11. We believe that the phenolic acids increase light emission by increasing the rate constant for the CL pathway, the conversion of luminol to endoperoxide (LO_2^{2-}). When the reaction was enhanced, probably oxidation in basic media took place in stages 5–9. Comparison of the spectra in enhanced and unenhanced reactions, plus the fact that the light emission spectrum was independent of the enhancer, revealed that the emitter was the luminol reaction product and not the phenolic acid enhancers.

The reactions between hexacyanoferrate(III) and the enhancers at stages 5 and 6 and those with luminol are redox processes that took place as the hexacyanoferrate(III) was consumed because the intensity of its absorption spectra decreased in the presence of luminol and also of the enhancers.

Table 2 shows that signals of enhanced reaction appear at hexacyanoferrate(III) concentrations higher than those of the enhancers. When concentrations of hexacyanoferrate(III) were below this, the emission disappeared in the presence of

(23) Cormier, M. J.; Prichard, P. M. *J. Biol. Chem.* **1968**, *243*, 4706–4714.

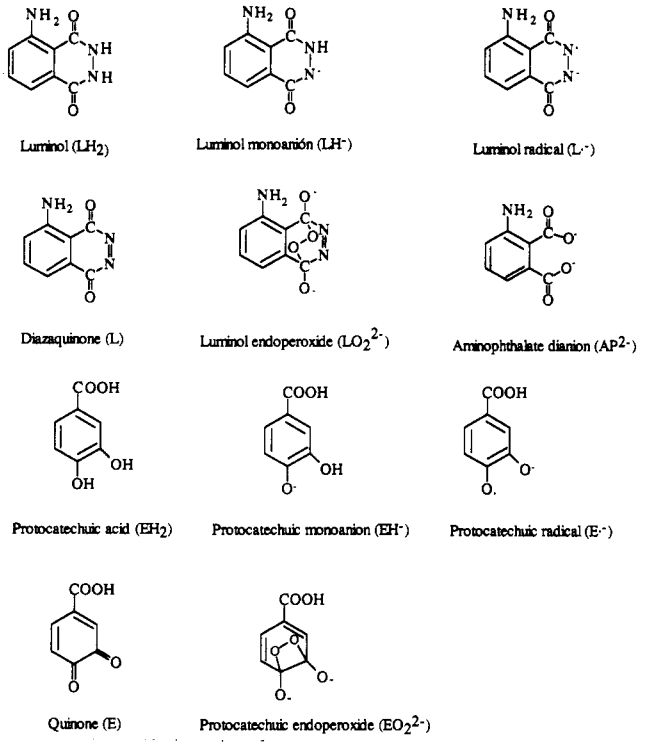

Figure 2. Structural formulas.

Table 2. Influence of Hexacyanoferrate(III) on Light Emission in the Presence and Absence of Protocatechuic Acid

hexacyanoferrate(III) concn, μM	blank reaction		enhanced reaction	
	I^a	A^b	I	A
1	4	93	0	0
3.3	8	831	0	0
8.3	16	1319	0	0
13.3	24	1612	8	290
20	24	1257	31	1436
30	32	833	54	2373
50	41	633	110	2071

[a] Intensity at maximum of chemiluminescence with relative standard deviation (RSD) in the range 2–20%. [b] Area under the light emission curve with RSD in the range 3–20%. Experimental conditions: [luminol] = 1.7 μM, [protocatechuic acid] = 6.7 μM.

enhancers; however, emission of the blank was noted. This circumstance can only be explained by the reaction with enhancers (6) being faster than the reaction with luminol (2), and by the fact that in (6) the hexacyanoferrate(III) was consumed before it reacted with luminol, and moreover, the protocatechuic or caffeic radicals ($E^{\cdot-}$) produced would not act on the luminol (LH^-) ion to convert it into a luminol ($L^{\cdot-}$) radical, as occurs in the luminol–H_2O_2–peroxidase–enhancer system.[23] If this were not so, one would see chemiluminescence emission with low concentrations of hexacyanoferrate(III).

It is more likely that in the luminol–hexacyanoferrate(III)–enhancer system the reaction of the phenolic acid enhancer radicals with molecular oxygen took place during stages 7–9. Previous works show that superoxide anion ($O_2^{\cdot-}$) is the only reactive form of oxygen that may play an important role under alkaline conditions.[24,25] As to luminol, it is proposed that an intermediate is formed between it and the protocatechuic radical ($E^{\cdot-}$) in step 8 that can act on the luminol

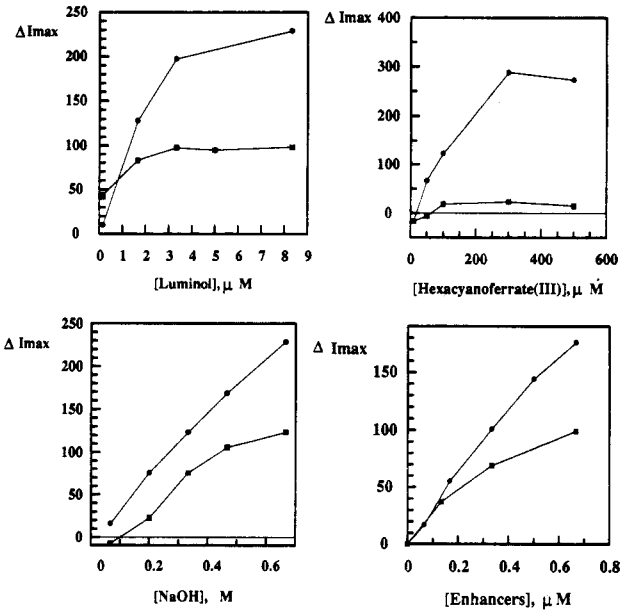

Figure 3. Influence of reagent concentration on chemiluminescence: (a) luminol; (b) hexacyanoferrate(III); (c) NaOH; (d) phenolic acids.

radical ($L^{\cdot-}$), accelerating the emission steps. An intermediate similar is proposed for the luminol–Co(II)–H_2O_2–penicillin system.[26]

Reaction Conditions. The influence of reaction variables was studied separately for each phenolic acid by using a batch technique; these data form the base of the stopped-flow experiments of this present work. The parameters chosen were intensity at maximum of CL (peak height) in batch experiments, CL intensity at 0.3 s, and initial reaction rate in stopped-flow experiments. Figure 3 shows the effects of the concentrations of hexacyanoferrate(III), NaOH, luminol, protocatechuic acid, and caffeic acid on peak height. The reaction variables were chosen because they had high sensitivity for the acids and their blank signals were weak. Both characteristics were satisfied by 2×10^{-4} M hexacyanoferrate(III), 0.47 M NaOH, and 3.33×10^{-6} M luminol. In addition, 1×10^{-4} M EDTA was added to chelate metal ions, particularly, Co, Cu, and Fe, because these are efficient catalysts of luminol CL and are often present as impurities in many analytical reagents.

The degree of enhancement was closely correlated with enhancer concentration, but the relationship could not be determined with precision from batch measurements because of the high standard deviation of the measured values. Relative standard deviations (RSD) of the peak height signals ($n = 6$) were between 3 and 20% for concentrations that ranged between 7×10^{-8} and 1.3×10^{-6} M. These data indicate that the batch measurements introduce high error in the calibration.

Because of the high RSD obtained with static measurements, calibration was performed by the stopped-flow technique, which give accurate, reproducible, and complete detection of fast reaction courses. The effects of the concentrations of either protocatechuic acid in the range 2.5×10^{-8}–1×10^{-6} M, or of caffeic acid in the range 5×10^{-8}–2×10^{-6} M, on the initial reaction rate were studied by stopped flow. For protocatechuic acid, the linear range was 2.5 ×

(24) Rabani, J.; Nielsen, S. O. *J. Phys. Chem.* **1969**, *73*, 3736–3744.
(25) Sawyer, D. T.; Valentine, J. S. *Acc. Chem. Res.* **1981**, *14*, 393–400.
(26) Chen, S.; Yan, G.; Schwartz, M. A.; Perrin, J. H.; Schulman, S. G. *J. Pharm. Sci.* **1991**, *11*, 1017–1019.

Table 3. Figures of Merit for the Individual Determination of Protocatechuic Acid and Caffeic Acid

	protocatechuic acid	caffeic acid
linear range, μM	0.025–0.25	0.05–0.5
limit of detectn,[a] μM	0.001 56	0.004 39
sensitivity,[b] μM	0.001	0.0174
regression eq	$S_2 = (4.9605 \pm 0.2544^c) + (73.8190 \pm 1.8110)C_{prot}$	$S_2 = (4.8806 \pm 0.2282^c) + (26.2953 \pm 0.8806)C_{caff}$
regression coeff	0.9985 (n = 7)	0.9972 (n = 7)

[a] Based on three times the standard deviation of the blank. [b] Calculated as the standard deviation of sample divided by the slope of calibration. [c] Standard deviation of three determinations, S_2 initial reaction rate; C_{prot} and C_{caff} are the concentration of the acids.

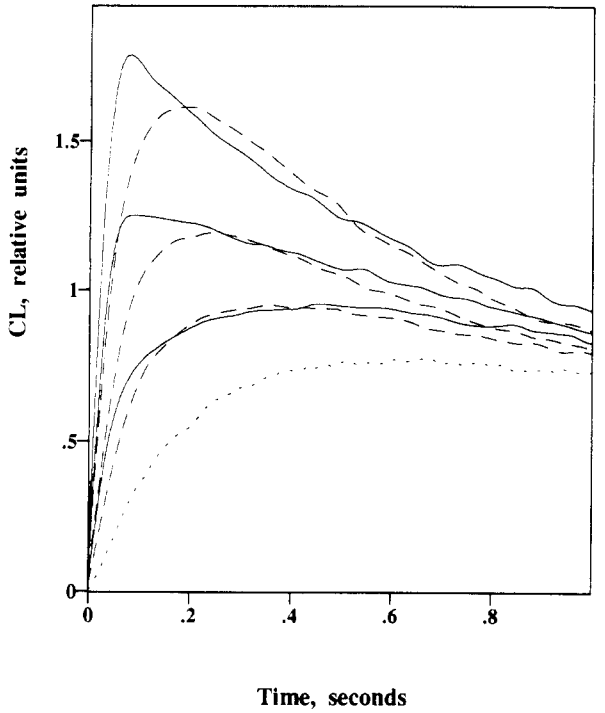

Figure 4. Time–intensity curves as a function of protocatechuic acid concentration (50, 150, and 250 nM, connected line) and caffeic acid concentration (100, 250, and 500 nM, dashed line). Reaction blank, dotted line.

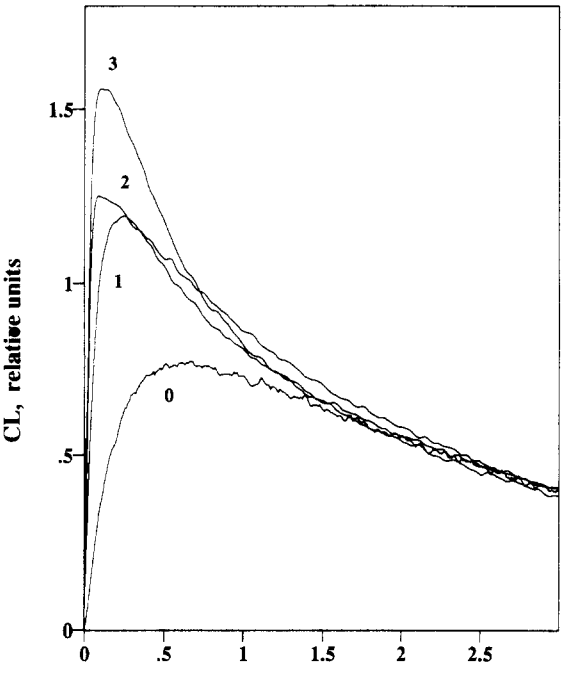

Figure 5. Time–intensity curves for the blank reaction, enhanced by protocatechuic acid and caffeic acid alone and by a mixture of both: (0) blank reaction; (1) 25 × 10^{-8} M caffeic acid; (2) 15 × 10^{-8} M protocatechuic acid; (3) 25 × 10^{-8} M caffeic acid, 15 × 10^{-8} M protocatechuic acid .

10^{-8}–2.5 × 10^{-7} M, and for caffeic acid, it was 5 × 10^{-8}–5 × 10^{-7} M. RSD (n = 3) of each measurement extended from a minimum of 0.2 to a maximum of 1.9%. Figure 4 shows the modifications of the time–intensity curves as a function of the concentrations of protocatechuic and caffeic acids. Typical analytical figures of merit for protocatechuic and caffeic acid determinations are shown in Table 3.

Multicomponent Determination. Synergy was observed when two or more enhancers acted simultaneously. In the presence of the two phenolic acid enhancers, light emission was not the sum of the individual emissions of the two enhancers. Figure 5 shows the time–intensity curves for the blank reaction, the protocatechuic acid enhanced reaction, the caffeic acid enhanced reaction, and the reaction enhanced by a mixture of both phenolic acids. It can be seen that the intensity response of the mixture was lower than the sum of the intensities recorded for each individual acid.

The results of simultaneous determination with the proportional equations method[4] showed up to 100% inaccuracy because synergism caused a considerable degree of error in the concentration measurements. Because the two enhanced reactions were not independent we decided to use surface response methodology,[27] an efficient optimization method. We proposed an empirical model that might describe well the experimental data. System behavior was studied by first carrying out a series of predesigned experiments, and models were constructed to simulate the experimental results. Factorial design was used because it gives a large amount of useful information from a small number of experiments and can help estimate the effects of interactions commonly found in kinetics. The two variables that determined the response outputs in this case were the protocatechuic and caffeic acid concentrations. The two-level two-factor factorial design centered on values of 60 nM protocatechuic acid and 90 nM caffeic acid. The design also included points for concentrations of 20 and 100 nM for protocatechuic acid, and of 35 and 140 nM for caffeic acid. The five different experimental conditions suggested by this design were used to calculate the four coefficients that can be used to fit a model that contains first-order effects and interaction effects.

The classical least-squares method was used to obtain the experimental responses as a function of the acid concentration.

(27) Massart, D. L.; Vandeginste, B. G. M.; Deming, S. N.; Michotte, Y.; Kaufman, L. *Chemometrics: A Textbook*; Elsevier: Amsterdam, 1988; pp 255–290.

Table 4. Simultaneous Determination of Protocatechuic and Caffeic Acids

added concn, nM		found concn,[a] nM			
protocatechuic acid	caffeic acid	protocatechuic acid	rec, %	caffeic acid	rec, %
40.0	115.0	40.3	100.7	117	101.7
60.0	35.0	61.6	102.6	37.5	107.1
60.0	140.0	56.5	94.2	133.2	95.1
80.0	115.0	78.3	97.9	129.3	112.4
80.0	60.0	82.6	103.2	67.6	112.6
100.0	90.0	93.3	93.3	100.5	111.7

[a] Mean of three determinations.

The values of C_{prot} and C_{caff}, the concentration of the phenolic acids (in nM), were used to construct the K matrix. S_1 and S_2 are the CL intensity at 0.3 s and initial reaction rate, respectively. The experimental responses are described by the equations

$$S_1 = 24.442 + 1.203C_{prot} - 0.006C_{prot}C_{caff} + 0.984C_{caff}$$

$$S_2 = 13.388 + 3.545C_{prot} - 0.058C_{prot}C_{caff} + 1.737C_{caff}$$

The interaction effects were interpreted by rearranging the fitted models. Thus, within the domain of the experiments in the present work, increasing protocatechuic or caffeic concentration decreased the effect of the other acid; however, in every case, the overall experimental response increased. We observe that the interactions between the enhancers were stronger in initial rate measurements and concluded that will be even stronger for the higher values of the associated variable, in this case, the concentration of the phenolic acids.

To obtain concentration as a function of the experimental responses the least-squares P matrix method[10] was applied. The values of CL intensity at 0.3 s and initial reaction rate, S_1 and S_2, respectively, were used to construct the P matrix. The results of this study show that the concentration of acids, as a function of the two parameters, gave the equations

$$C_{prot} = 295.085 - 146.071S_1 + 41.163S_2 + 8.011S_1S_2$$

$$C_{caff} = -541.602 + 252.580S_1 - 52.917S_2 - 6.964S_1S_2$$

If our fitted model has an appropriate form, and if the parameters have been estimated reasonably precisely, there will be very little difference between the values predicted by the model and the values obtained experimentally from the system at factor combinations not too far removed from our original area of experimentation. The results are shown in Table 4. The theoretical model fitted well the behavior of the protocatechuic–caffeic–luminol system. The mean recoveries of both acids were close to 100%.

Received for review August 17, 1993. Accepted December 22, 1993.[⊕]

[⊕] Abstract published in *Advance ACS Abstracts*, February 1, 1994.