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Kinetic Method for the Quantitative Resolution of Structural Isomers Based on the Catalytic Properties of β-Cyclodextrin

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This paper describes a new approach for the quantitative resolution of mixtues of structural isomers. The method is based on the observation that rate constants for the cyclodextrin-catalyzed hydrolysis of selected structural isomers are significantly different. By using cure-fitting methods, it is possible to use these differences in rate constants to resolve kinetic responses for mixtures into the responses for the individual components. The new approach is evaluated for the ortho-, meta-, and para-isomers of nitrophenyl acetate. At pH 10, with β-cyclodextrin as catalyst, ratios of rate constants for the three isomers differ by ratios of 1:6.7:1.6 in the order mentioned above. Results are reported for both two- and three-component mixtures. For two-component mixtures of the ortho- and para-isomers which have rate constants differing by only 1.6-fold, linear least-squares slope and intercept of determined vs prepared concentrations for the ortho-isomer were 1.00 ± 0.02 and 2 ± 2.2 μmol/L for three runs on each of five samples in the concentration range from 22 to 176 μmol/L. The pooled standard deviation for these 15 runs was 3.7 μmol/L, corresponding to a relative standard deviation of 3.7% for the average concentration. Similar results were obtained for other two- and three-component mixtures.

Cyclodextrins are macrocyclic compounds consisting of 6–12 glucose units joined at the α-1,4 positions. The most common forms consist of 6, 7, and 8 glucose units and are commonly identified as the α-, β-, and γ-cyclodextrins (α-, β-, and γ-CDs).[1] An interesting property of cyclodextrins is that they tend to mimic enzymes in the sense that they catalyze certain types of reactions such as hydrolysis of esters.[1-3] Although the catalytic properties of CDs have been studied extensively,[1-8] prior to our own studies,[9] there have been no attempts to exploit these properties for analytical purposes.

In a recent paper from this laboratory, it was shown that the catalytic properties of cyclodextrins can indeed be used to quantify selected species on the basis of selective catalysis of reactions that can be monitored continuously with appropriate detection systems.[9] The study focused on the use of error-compensating data processing methods for single-component (*p*-nitrophenyl acetate) determinations. In the course of that study, it was confirmed that the catalytic activity of any particular cyclodextrin was different for different structural isomers of any given compound. For example, it was confirmed that the catalytic activity of β-CD was significantly different for the ortho-, meta-, and para-isomers of nitrophenyl acetate (NPA)[1]. This observation opened the possibility of simulatneous quantitation of these isomers in mixtures based on differences in kinetic behavior.

Several papers from this and other laboratories describing simultaneous determinations based on kinetic differences among different compounds undergoing the same or similar reactions have been described in several monographs[10-12] and reviews.[13-15] Most of these studies have focused on entirely different compounds that undergo similar reactions to produce a common product. However, there have been a few studies, dating as far back as the 1930s, applied to the simultaneous quantitation of isomers of the same compound.[16-18] The present study was undertaken to determine if the catalytic properties of a cyclodextrin could be used to resolve mixtures of structural isomers quantitatively. The example chosen for study was quantitative resolution of the ortho-, meta-, and para-isomers of nitrophenyl acetate based on catalysis by β-CD of the hydrolysis of the isomers to produce the respective nitrophenolates (NP^-). The nitrophenolates were monitored by their absorption in the near-ultraviolet region.

EXPERIMENTAL SECTION

Instrumentation. Preliminary studies involving development of conditions for the measurement step were done by using a diode array-based rapid-scanning spectrophotometer (Model 8450A, Hewlett-Packard Co., Palo Alto, CA). Quantitative results for one-, two-, and three-component samples were obtained by using a multichannel centrifugal mixing/measurement system (Rotochem IIa, Travenol, Deerfield, IL). Data for absorbance vs time were recorded by

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on-board computers with each system and then transferred to a high-speed microprocessor system (Model 4DX-33, Gateway 2000, North Sioux City, SD) for processing.

Gas chromatography (Model 3700, Varian Associates, Sugerland, TX) was used to test the purity of the nitrophenyl acetates.

Reagents. All aqueous solutions were prepared in doubly distilled (Corning megapure distillation apparatus, Corning Inc., Corning, NY) water and filtered through 0.2-μm pore size membrane filter (Nylon-66, Ranin Instrument Co., Inc., Woburn, MA).

Buffer Solutions. The two primary buffers used in this study were a phosphate buffer at pH 8.2 and a carbonate buffer at pH 10.0. The phosphate buffer (2 mmol/L) was prepared by dissolving 0.272 g of potassium phosphate (Aldrich Chemical Co., St. Louis, MO) in water, adjusting the pH to 8.2 with concentrated sodium hydroxide (18 mol/L), and diluting to 1.0 L. The carbonate buffer (0.13 mol/L) was prepared by dissolving 11.1 g of sodium carbonate (Aldrich) and 18 g of sodium chloride (Mallinckrodt Chemical Works, St. Louis, MO) in water, adjusting the pH to 10.0 with concentrated sodium hydroxide, and diluting to 1.0 L. Each buffer was filtered through a 0.2 μm pore size membrane filter (Nylon-66, Ranin) prior to use.

Substrates. The *o*- and *p*-nitrophenyl acetates (Aldrich) were recrystallized from hexane and tested for purity by using gas chromatography; the impurity level was judged to be less than 0.1%. The *m*-nitrophenyl acetate was synthesized according to a published method,[19] and the purity was tested as for the other isomers. Stock and diluted samples of all the isomers were prepared in freshly distilled acetonitrile (Fisher Scientific, Chicago, IL) and stored at 4 °C.

Cyclodextrin. The β-cyclodextrin (Aldrich) was used as received. Solutions were prepared in appropriate buffers each day. The β-CD for quantitative determinations was prepared in carbonate buffer (pH 10.0) containing 0.3 mol/L sodium hydroxide.

Procedure. A variety of common procedures were used for preliminary experiments designed to establish appropriate conditions for the quantitative determinations. For the quantitative determinations, 500 μL of 10 mmol/L β-CD in carbonate buffer was injected into the middle well of each position on the rotor of the centrifugal mixing system, 500 μL of phosphate buffer (2 mmol/L, pH 8.2) was injected into the inner well of each rotor channel, and then 10.0 μL of each sample in acetonitrile was injected into the phosphate buffer in each inner well of the rotor. This latter step was completed as rapidly was possible for all samples, usually within 4 min, and the rotor and measurement process was started as soon as the last sample was injected. Data acquisition was initiated 6.7 s after the rotor was started, and data points were collected at 6-s intervals; a total of 200 data points were collected for each sample.

Data Processing. Multicomponent mixtures were resolved by fitting models for two and three simultaneous first-order processes to data for absorbance vs time. The equations and general approach have been discussed elsewhere[20] and are not repeated here. The principal changes from earlier procedures involved the manner by which initial estimates were obtianed and the fact that rate constants were permitted to vary during the final fitting process.

The curve-fitting method is an iterative process which requires initial estimates of the fitting parameters, namely the rate constant and the equilibrium absorbance corresponding to each component in each sample. Average values of rate constants determined for several concentrations of each isomer were used as initial estimates of rate constants. A method called successive integration was used to obtain initial estimates of absorbances used in the iterative process. This method has been described elsewhere.[21] The important point here is that it gives much more reliable initial estimates than procedures used previously. This permits more rapid convergence and, more importantly, more rugged resolution of data for mixtures.

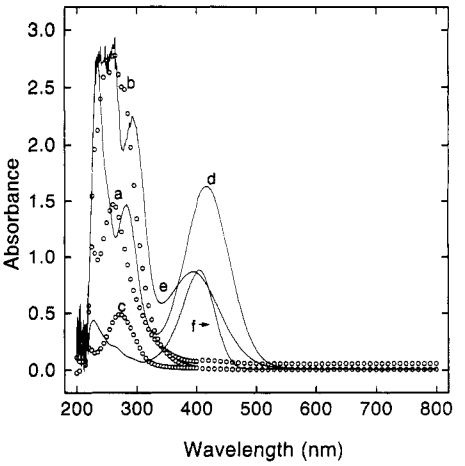

Figure 1. Spectra for the isomers of nitrophenyl acetates and the nitrophenolates: (a–c, O) *o*-, *m*-, and *p*-nitrophenyl acetates; (d–f, —) *o*-, *m*-, and *p*-nitrophenolates. Conditions: pH = 10, 25 °C, 0.15 mol/L NaCl. Concentrations: 200, 600, and 50 μmol/L for the ortho-, meta-, and para-isomers, respectively.

RESULTS AND DISCUSSION

Unless stated otherwise, all random uncertainties are reported at the level of one standard deviation (±1 sd).

Absorption Spectra. Most applications of simultaneous kinetic determinations have involved similar reactions that produce a common product such that only one sensitivity factor for signal vs concentration is involved. The present study was complicated by the fact that the spectra for the different phenolate isomers were all different.

Absorption spectra for the esters and hydrolysis products are shown in Figure 1. The absorptivities for all the esters are very small near 400 nm, where the spectra for all the hydrolysis products have absorption maxima. Accordingly, the measurement region was selected in this range, namely 406 nm. At this wavelength, the molar absorptivities of the phenolate ions for the different isomers are quite different. At pH 10, the values (10^3 L/mol cm) for the ortho-, meta-, and para-isomers are 3.5, 1.1, and 14.4, respectively. Concentra-

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Table 1. Apparent First-Order Rate Constants at 25 °C for the Hydrolysis of the Ortho-, Meta-, and Para-Isomers of Nitrophenyl Acetate

apparent rate constants (10^{-3} s^{-1})

	o-NPA[a]		*m*-NPA[a]		*p*-NPA[a]	
pH	without β-CD	with β-CD[b]	without β-CD	with β-CD[b]	without β-CD	with β-CD[b]
8.2[c,e]	0.01	0.01	0.02	0.04	0.02	0.02
9.5[c,e]	0.7	2.9	0.5	24	1.2	5.1
9.5[c,f]	0.6	2.3	0.45	16	0.9	3.5
10.0[d,f]	1.6 ± 0.01	8.2 ± 0.03	1.4 ± 0.002	53 ± 1.4	2.2 ± 0.01	12.6 ± 0.1

[a] Concentrations of ortho-, meta-, and para-isomers were 0.2, 0.6, and 0.05 mM, respectively. [b] pH 8.2, 9.5; 10 mM β-CD. pH 10; 5 mM β-CD. [c] One determination each. [d] Averages of three determinations on one concentration without β-CD and three determinations on each of five concentrations (*o*-NPA, 0.002–0.02 mM; *m*-NPA, 0.06–0.6 mM; *p*-NPA, 0.005–0.05 mM) with β-CD. [e] No control of ionic strength. [f] Ionic strength controlled at 0.15 M.

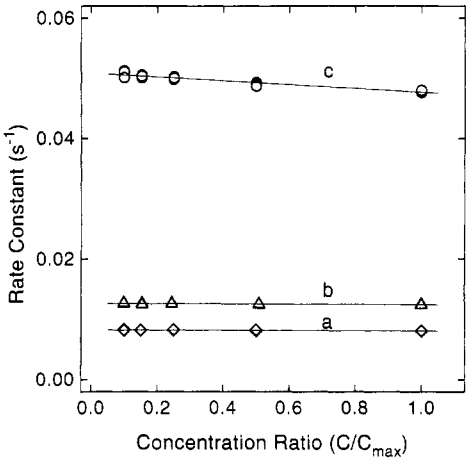

Figure 2. Dependencies of the apparent rate constants on concentrations of the esters. Conditions and maximum concentrations as in Figure 1.

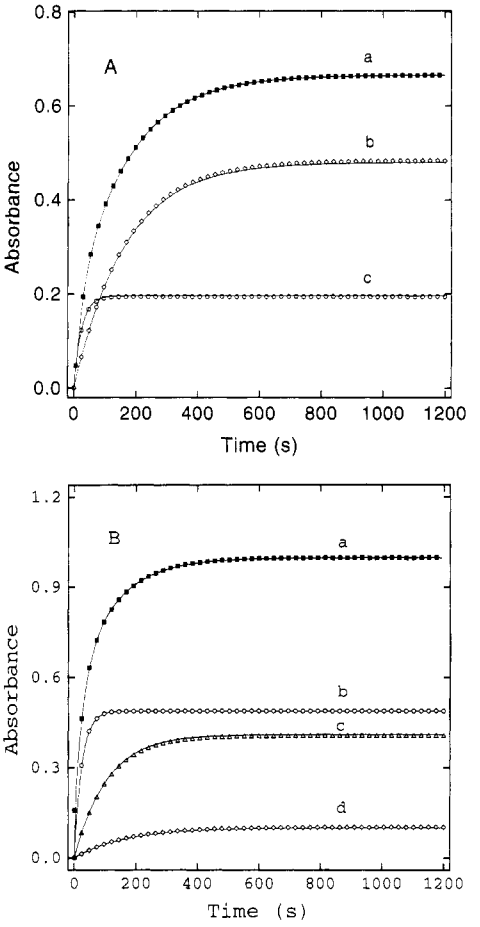

Figure 3. Experimental and fitted data for a two- and three-component mixture of the nitrophenyl acetates. Conditions as in Figure 1. (A) Curve a, experimental (■) and best-fit (solid curve) results for the mixture. Curves b and c, best-fit values for individual components (b, 154 μmol/L *o*-NPA; c, 198 μmol/L *m*-NPA) and expected values based on prepared concentrations, rate constants, and molar absorptivities (solid curves). (B) Same as A except for the components: b, 594 μmol/L *m*-NPA; c, 28 μmol/L *p*-NPA; d, 28 μmol/L *o*-NPA.

tions of the different isomers in mixtures were adjusted to give similar absorbance changes for each isomer.

Rate Constants. Preliminary studies were done to establish conditions for which the catalyzed hydrolyses of the *p*-nitrophenyl acetates followed pseudo-first-order kinetics and to compare rate constants for a range of pH values for the different isomers. Kinetic behavior was tested by using plots of $\ln(A_\infty - A_t)$ vs t and by fitting a first-order model to data for absorbance vs time. Both tests confirmed good pseudo-first-order behavior for all conditions reported in this paper. Moreover, there was good agreement among apparent first-order rate constants determined by the two data processing options. Rate constants obtained in these studies are summarized in Table 1. Results at pH 8.2 and 9.5 are reported only for the record because subsequent studies were done at pH 10 to take advantage of the faster rates and correspondingly shorter measurement times. In the course of these studies it was discovered that ionic strength had some small effects on rate constants (see data for pH 9.5). Accordingly, ionic strength was controlled for all subsequent studies.

Having chosen pH 10 for subsequent studies, rate constants were evaluated in more detail at this pH. Values and uncertainties at pH 10 in Table 1 are based on three runs each at each of five different concentrations for each isomer. Ideally, the rate constants should be independent of concentration. As shown in Figure 2, apparent rate constants obtained with β-CD as catalyst tended to decrease slightly with concentration for each isomer. Although the meta-isomer appears from the figure to exhibit the largest change, it should be noted that the concentration range for the meta-isomer is significantly larger than those for the other isomers. Linear least-squares slopes of rate constants (10^{-3} s^{-1}) vs concentration (μmol/L) for the ortho-, meta-. and para-isomers were −0.9, −5.3, and −3.4, respectively (last column in Table 2). Although this behavior is not ideal, the curve-fitting process used to resolve mixtures is expected to compensate for such nonidealities because the rate constants are used as fitting parameters.

Other features of the rate constants at pH 10 merit comment. Ideally, the differences between rate constants for catalyzed and uncatalyzed reactions should be large so that

Table 2. Calibration Results[a] and Concentration Dependencies of Rate Constants for Single-Component Samples

ester	range (mM)	slope (sd) (10^{-3} $\Delta A/\mu M$)	intercept (sd) (ΔA)	std error (ΔA)	correlation coefficient	slope k vs C (10^{-6} $s^{-1}/\mu M$)
o-NPA	0.002–0.2	3.54 (0.04)	0.006 (0.004)	0.011	0.999	–0.9 ± 0.2
m-NPA	0.06–0.6	1.11 (0.01)	0.004 (0.002)	0.007	0.999	–5.3 ± 0.5
p-NPA	0.005–0.05	14.4 (0.21)	0.003 (0.005)	0.016	0.998	–3.4 ● 1.3

[a] Three runs on each of five concentrations.

Table 3. Least-Squares Results[a] and Pooled Standard Deviations for Two- and Three-Component Mixtures

isomer	range (μM)	slope (sd)	intercept (sd) (μM)	std error (μM)	correlation coefficient	pooled sd[b] (μM)
			o-NPA + *m*-NPA			
o-NPA	22–176	1.00 (0.02)	2 (2.2)	4.4	0.997	3.7
m-NPA	132–593	1.00 (0.02)	–22 (7.6)	13.5	0.997	10.9
total	307–615	1.00 (0.03)	20 (14)	14.0	0.993	9.0
			o-NPA + *p*-NPA			
o-NPA	22–198	1.00 (0.02)	–9 (2.5)	4.8	0.997	4.2
p-NPA	5.7–52	1.00 (0.02)	0.5 (0.6)	1.4	0.996	1.0
total	74–204	1.00 (0.03)	–8 (4.7)	5.9	0.992	4.7
			o-NPA + *m*-NPA + *p*-NPA			
o-NPA	30–178	1.00 (0.02)	–3 (2.5)	6.3	0.995	3.6
m-NPA	198–594	1.00 (0.02)	–19 (7.5)	16	0.997	7.1
p-NPA	10–59	1.00 (0.02)	–2 (0.6)	1.5	0.997	0.84
total	336–653	1.00 (0.02)	16 (7.8)	15	0.997	7.8

[a] Determined vs prepared concentrations. [b] Pooled standard deviations based on three runs on each of five concentrations. Conditions: pH 10.0, 25 °C, 0.15 M NaCl, all fits over 10.5 half-lives of slower reaction.

the uncatalyzed reaction does not complicate the kinetic behavior. The ratios of rate constants for catalyzed vs uncatalyzed reactions for the ortho-, meta-, and para-isomers are 5, 38, and 5.7, respectively. Despite the small ratios for the ortho-, and para-isomers, kinetic responses for catalyzed reactions showed very close to first-order behavior. Accordingly, no attempt was made to compensate for the uncatalyzed reactions by using more complex curve-fitting models. However, the relatively large rates of the uncatalyzed reactions at pH 10 complicated the sample handling procedure. To avoid problems associated with the volatility of the organic solvent in which the esters were dissolved initially, it was desirable to prepare dilutions in aqueous media. To avoid excessive hydrolysis prior to the measurement step, samples in acetonitrile were injected into a buffer solution at pH 8.2 just before the mixing and measurement steps were begun. The buffer capacity of the solution was made low enough that it did not interfere with the adjustment to pH 10 in the final mixing and measurement step.

Because the resolution of the different isomers was to be based on kinetic differences, it is necessary that rate constants for the different isomers be different. For the catalyzed reactions at pH 10, the ratios of rate constants for the ortho-, meta-, and para-isomers respectively are 1:6.8:1.6. Thus, the ortho- and para-isomers are the most difficult to resolve in both two- and three-component mixtures.

Single-component Calibrations. Data used to evaluate apparent rate constants at pH 10 were also used to test linearity of absorbance changes vs concentration. All reactions were monitored for at least eight half-lives. Equilibrium absorbances were obtained by direct measurement and by a predictive method.[22] Direct measurement results were obtained by averaging several values near eight half-lives of each response. Predictive results were obtained by fitting a first-order model to data for absorbance vs time over selected data ranges (2–5 half-lives) and extrapolating the fits to infinite time.

All calibration plots were linear for both data processing options and there was excellent agreement between calibration results obtained by the direct measurement and predictive options. Some typical least-squares results for the predictive option are given in Table 2. Intercepts, standard deviations of slopes, and standard errors of the estimates are all small, and correlation coefficients are near unity, reflecting the good linearity observed visually. Very similar results were obtained with the direct measurement option. These results support the use of a first-order model for individual components and parallel first-order models for mixtures.

Mixtures. Results are reported here for two- and three-component mixtures. Two sets of two-component mixtures were examined. One set involved the ortho- and meta-isomers with relatively large differences between rate constants, and one set involved the ortho- and para-isomers with relatively small differences between rate constants. Concentrations of the different isomers used in the various studies are given in appropriate tables and figure legends below. All sets of mixtures used for quantitative determinations included low/low, low/high, high/low, and high/high concentration ratios of the different isomers. For example, for mixtures of the ortho- and meta-isomers containing 22–176 μM *o*-NPA and 132–593 μM *m*-NPA, the concentration ratios of the ortho-isomer to the meta-isomer varied from 22:593 to 176:132.

Response Curves. Figure 3A contains typical response data for a two-component sample. The top plot (curve a) represents the unresolved response for the mixture. The points represent experimental data, and the solid curve represents a

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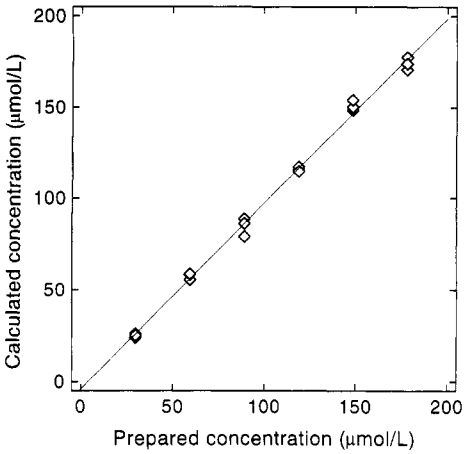

Figure 4. Comparison of prepared and determined concentrations for individual component in a three-component mixture. *o*-NPA with *m*- and *p*-NPA (see seventh row of Table 3).

fit of a model for two simultaneous first-order processes to the data. The model fits the data very well.

The two lower plots (curves b and c) represent the resolution of the overall response into the individual components of which it is composed. The absorbance change for the ortho-isomer (smaller rate constant) is about 2.5-fold that of the meta-isomer. The data points in these plots are the computed values based on the fit of the parallel first-order model to the overall response. The lines represent values computed by using the determined values of rate constants, prepared values of concentrations, and molar absorptivities of each component. Good agreement is obtained among determined (points) and expected (lines) results.

Figure 3B contains typical response data for a three-component sample. The different plots are as described above for two-component samples. The top plot represents the unresolved response, and the lower plots represent the contributions of the individual components. The model for three simultaneous first-order processes fits the overall data very well, and there is good agreement among the determined and expected results for the individual components.

Quantitative Results. A typical plot of determined vs prepared concentrations is given in Figure 4, and results for linear least-squares fits of determined vs prepared concentrations for several two- and three-component samples are summarized in Table 3. The plot respresents results for the component (*o*-NPA) with the smallest rate constant in several three-component mixtures. Both the plot and the least-squares results in the seventh row of Table 3 confirm good agreement among determined and prepared concentrations.

Plots of determined vs prepared concentrations for other mixtures were similar to the plot in Figure 4, and least-squares results in Table 3 permit quantitative comparisons. The components which are most difficult to resolve in both two- and three-component mixtures are the ortho- and para-isomers because the rate constants for these isomers differ by less than a factor of 2. Even so, least-squares statistics in rows 4, 5, 7, and 9 of Table 3 and results in Figure 4 confirm good performance for these components.

Pooled standard deviations are included in the last column of Table 3. Absolute values are in the range of 1–10 μmol/L. Values relative to average concentrations in each range at 3.7% or less.

Discussion. This study demonstrates the feasibility of resolving structural isomers quantitatively on the basis of differences in kinetic behavior. Reasonably good results were obtained despite some potential problems. The features expected to cause the most serious problems were the small differences among rate constants for the different components and between catalyzed and uncatalyzed reactions for each component. It is possible that other forms of cyclodextrins could enhance differences among rate constants for different isomers, but additional studies are necessary to determine if this is the case. Unmodified β-CD was used in this study because it is readily available. However, the effects of the uncatalyzed reactions could be reduced significantly by using a modified form of cyclodextrin with enhanced catalytic activity, such as that described in our earlier paper.[9]

It is possible that other curve-fitting methods such as the Kalman filter might give improved results. However, our focus was not on the curve-fitting method but on the potential use of the cyclodextrins as analytical reagents. In any event, the most serious limitation is that imposed by the large differences in molar absorptivities for the different isomers. These differences make it difficult to impossible to resolve similar concentrations of all the isomers by using a single wavelength. One possible solution to that problem would be to combine the kinetic-based resolution method with multi-wavelength data processing methods as used previously with phosphorescence[23] and other[15] data. For wavelengths longer than 450 nm, the absorptivities of the ortho- and meta-isomers approach and exceed those of the para-isomer, which should permit resolution of all the isomers in nearly equimolar concentrations. We did, in fact, intend to apply multiwavelength methods with these reactions. However, because the mixing times we were able to achieve in our multiwavelength instrumentation were too long for the faster-reacting components, we opted for the shorter mixing times achievable with the centrifugal mixing system which, unfortunately, does not have multiwavelength capability.

All results for mixtures reported above were based on fitting ranges corresponding to six or more half-lives of the component with the smallest rate constant. However, other data ranges were examined, and it was found that results degraded by factors of 2–4-fold relative to those reported above could be obtained by using fitting ranges as short as three half-lives of the component with the smallest half-life.

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