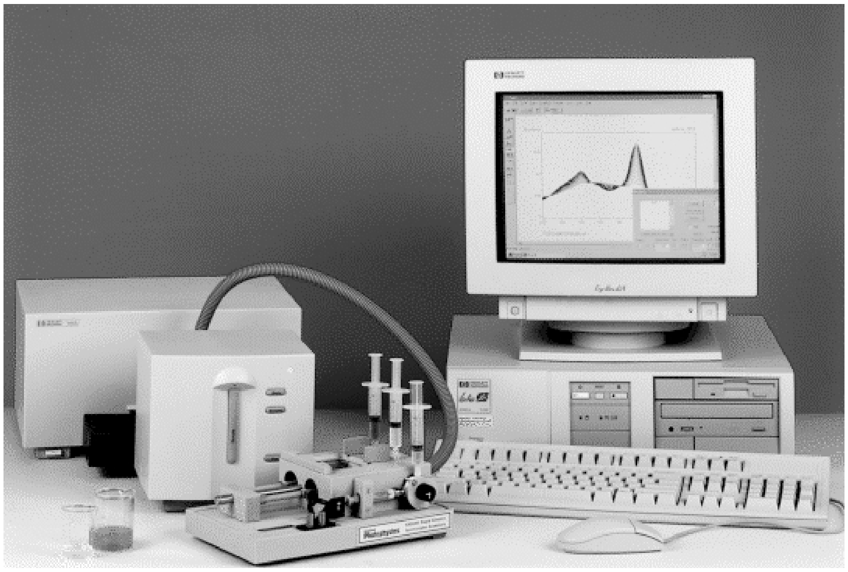
Measuring Reaction Kinetics with the Agilent 8453 Spectrophotometer and the Pro-K.2000 System

Technical Note



Introduction

The study of reaction kinetics is one of the key approaches to elucidate reaction mechanisms in the chemical and biochemical sciences. The objective is to monitor reaction intermediates and measure the dynamics of their formation and decay as a function of time during the period after the start of the reaction and before attainment of steady-state conditions.

However, many laboratories neglect the study of reaction kinetics because of the perceived expense and technical difficulties of obtaining results. Justifying the purchase of dedicated machinery may be difficult in laboratories where reaction kinetics measurements represent only part of the work done or where budgets are limited. Further, kinetic studies present certain technical challenges. The most significant of these are controlled and reproducible initiation of the reaction at the point of observation (typically a spectrophotometer) and reliable triggering of accurate, time-resolved data acquisition. Subsequent data analysis is often perceived to be a daunting task, particularly if the reaction of interest is complex.

This note describes the combination of an Agilent 8453 spectrophotometer and Pro-K.2000 rapid kinetics system (Applied Photophysics Ltd., Leatherhead, UK), which provides a complete solution for kinetics measurements of complex chemical reactions.

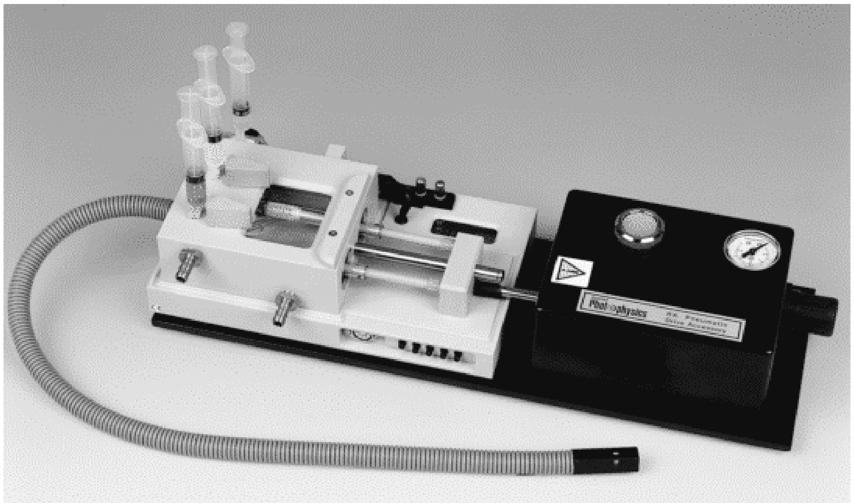


Figure 1
Pro-K.2000 stopped-flow accessory with pneumatic drive

Pro-K.2000 Rapid Kinetics System

The Pro-K.2000 rapid kinetics system comprises a stopped-flow mixing device and kinetic analysis software. This package enables the multiwavelength capability of the Agilent 8453 spectrophotometer to be exploited.

Hardware

The stopped-flow mixing accessory, shown in figure 1, is used to initiate a reaction and trigger data gathering in one step. The accessory rapidly mixes the two reagents and triggers the spectrophotometer. The mixing and observation cell is mounted on the end of a flexible tube. The cell provides for absorbance light path lengths of 2 and 10 mm. The two fluid samples are held in separate syringes and are pushed through a mixing chamber, where the reaction starts, and then into an observation cell. The solution previously in the cell is displaced into another syringe, the movement of which is limited by a back stop. Filling this syringe operates a trigger mechanism which activates data acquisition by the spectrophotometer for the sample now in the observation cell.

The stopped-flow mixing accessory is constructed from chemical-resistant materials, making it robust and shock resistent. The PTFE sample flow circuit is fully biocompatible, allowing almost any samples to be loaded without danger of contamination or loss of activity. The circuit is also chemically inert, allowing a wide range of both inorganic and organic chemical reactions, to be carried out on a routine basis as well as rigorous cleaning programs. All parts of the flow circuit may be bathed in thermostating fluid from an external water bath and the accessory features a built-in digital thermometer, allowing the exact temperature of the samples to be controlled.

A 60-µl fused silica observation cell provides 2 and 10 mm absorbance light paths and incorporates an sample mixing chamber. The design of the cell allows volumes of less than 100 µl (per sample) to be mixed efficiently and observed. The cell is mounted on the end of a flexible tube that fits directly into any Agilent cell holder designed for 1 cm cuvettes (including the multi-cell transport).

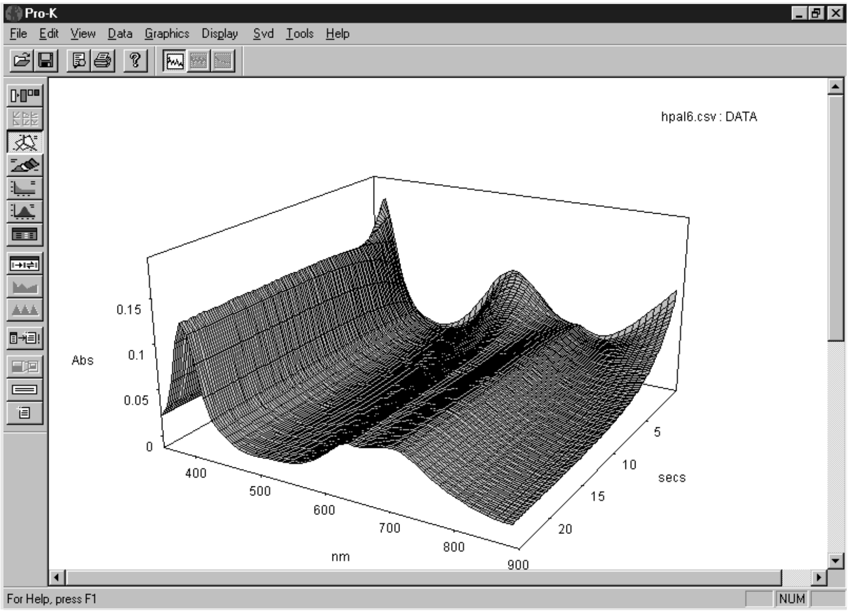


Figure 2
3-D wireframe of the acid hydrolysis of 50 mM tris(ethylamine)nickel(II) ion by 1 M hydrochloric acid

The stopped-flow accessory can be operated either by hand or pneumatically using the drive unit included as part of the system.
A cable between the accessory and the Agilent 8453 spectrophotometer's general purpose input/output (GPIO) connector provides coordinated triggering of data acquisition when a stopped-flow drive is performed.

Software

To obtain a set of time-dependent spectra in combination with the Pro-K.2000 stopped-flow accessory, the kinetics mode of the Agilent ChemStation's biochemical analysis software is used with the external trigger (GPIO) activated.

Pro-K.2000 provides analysis and reaction simulation facilities which complement Agilent ChemStation, both online and offline. The software is designed for use by scientists with all levels of kinetic experience.

Data transfer is automated by a file conversion macro which is accessed from the Agilent ChemStation command line.
Both new data and data loaded into Agilent ChemStation from disk may be wholly or partially converted for analysis. Once converted the data can be resaved as a separate file (CSV format) leaving the original file untouched.

The analysis software can run alongside Agilent ChemStation or run as a standalone program.

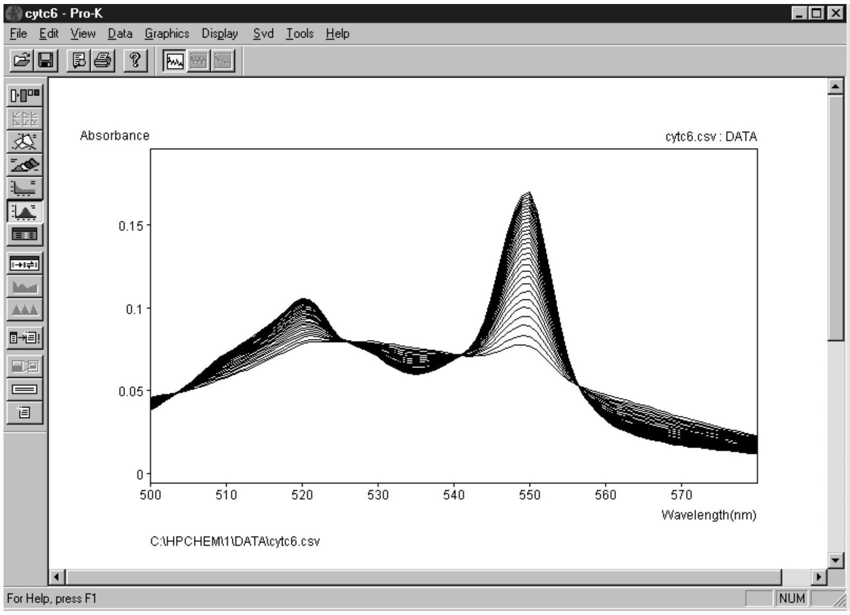


Figure 3
2-D spectra of the reduction of 6.5 µM cytochrome c by 1 mM ascorbate in Tris buffer, pH 7.0

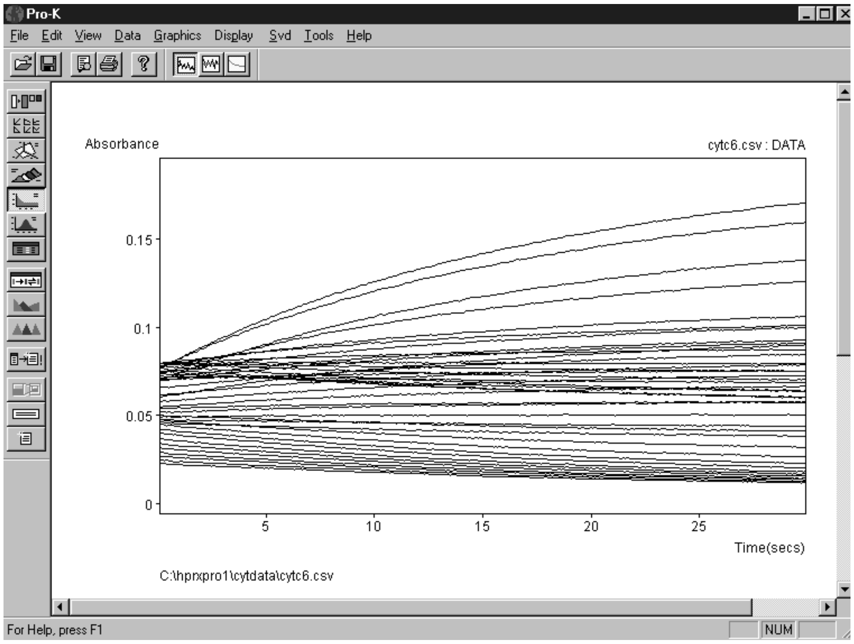


Figure 4
2-D kinetic traces of reaction in figure 3

Once loaded, data sets can be viewed and manipulated in a number of ways: they can be viewed as spectra (absorbance against wavelength), optionally overlaid together, as a subset, or individually. These views can easily be switched to kinetic mode (absorbance against time). 3-D surface display modes (with tilt and rotate functions) can be helpful and instructive, allowing inspection and clarification of all the data. A selection of the display modes available in Pro-K.2000 is shown in figures 2, 3 and 4.

Singular value decomposition (SVD) is a useful mathematical tool which is often the first Pro-K.2000 facility used to examine spectral data. A set of time-resolved spectra can be arranged as a very large matrix. SVD decomposes this matrix and

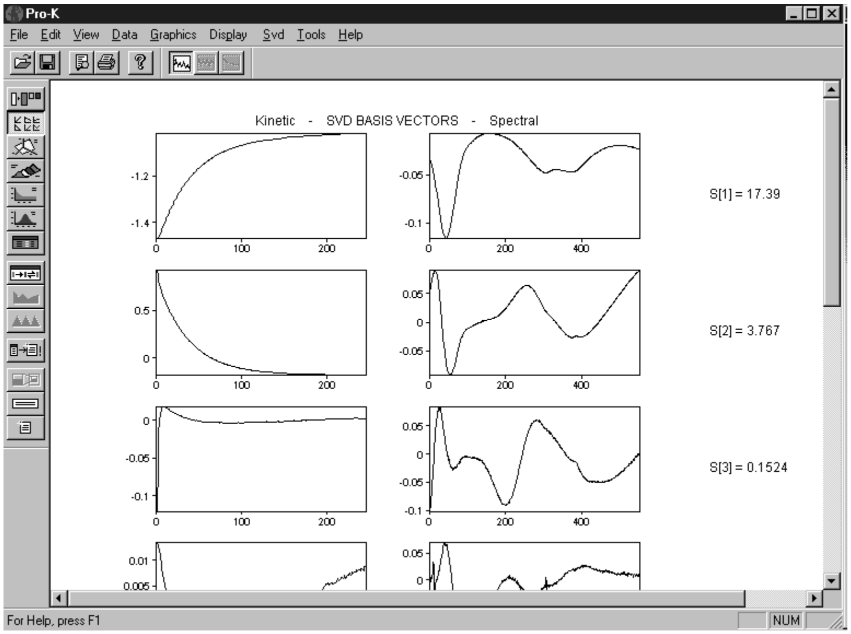
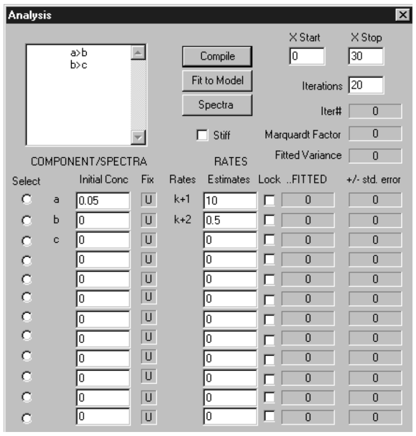


Figure 5
SVD vectors for the acid hydrolysis of tris(ethylamine)nickel(II) ion



a>b
b>c

X Start: 0
X Stop: 30
Iterations: 20
Iter#: 0
☐ Stiff
Marquardt Factor: 0
Fitted Variance: 0

Select		Initial Conc	Fix	Rates	Estimates	Lock	..FITTED	+/- std. error
○	a	0.05	U	k+1	10	☐	0	0
○	b	0	U	k+2	0.5	☐	0	0
○	c	0	U		0	☐	0	0
○		0	U		0	☐	0	0
○		0	U		0	☐	0	0
○		0	U		0	☐	0	0
○		0	U		0	☐	0	0
○		0	U		0	☐	0	0
○		0	U		0	☐	0	0
○		0	U		0	☐	0	0
○		0	U		0	☐	0	0
○		0	U		0	☐	0	0

Figure 6
The Pro-K.2000 analysis dialog box

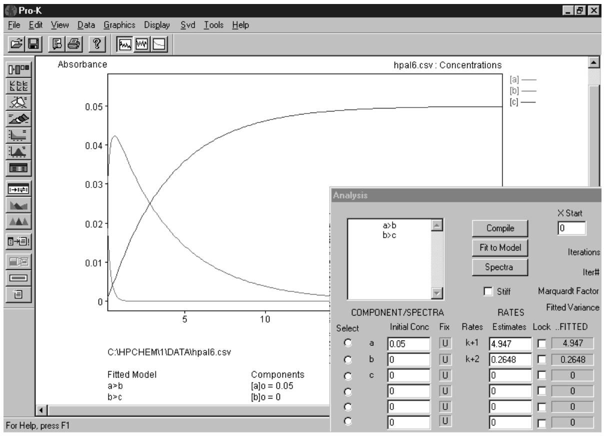
extracts a model-free indication of the minimum number of independent absorbing components present (a so-called rank analysis). In so doing it provides an indication of the minimum complexity of the underlying reaction without making any assumptions as to the mechanism. The SVD output, as shown in figure 5, consists of basis vectors related to time and wavelength and a weighting factor which shows the relative significance of each SVD component. The contribution of noise is quantified by SVD and can be reduced or eliminated if desired, for example, for presentation purposes. The translation of raw data into its SVD equivalent often yields a much smaller matrix providing savings in disk space, memory and analysis time.

The next stage of data analysis is to enter a reaction scheme or model. A minimal model may be suggested by the SVD or established by other methods. The Pro-K.2000 reaction scheme editor allows the user to enter a reaction scheme for simulation by the built-in numerical integrator. This, when provided with rate estimates, generates intermediate concentration profiles which are used as the starting point for a least-squares fit. Known parameters, such as rate constants or spectra, can be loaded and locked. Any reaction scheme can be entered in the form of single steps.

All of these functions are encapsulated in the single analysis window shown in figure 6. The reaction model entered is a two-step process with estimated rates of 10 s^{-1} and 0.5 s^{-1} which is suitable for the analysis of the acid hydrolysis of tris(ethylamine)nickel(II) ion.

Global fitting of time-resolved spectral data requires that the data satisfy a kinetic model simultaneously at all wavelengths and all times. Pro-K.2000 uses an algorithm to iteratively improve the reaction model's fit to the data. The fitting process generates a set of concentration profiles and spectra for each reaction component yielding a best fit data set and corresponding residuals. Global fitting calculates accurate and robust results. In many situations analysis at only one wavelength can miss at least part of a reaction and cause poor discrimination of multiple steps. By applying global fitting over a wide wavelength range minor intermediates are better resolved and more complex kinetics can be analysed with confidence.

In Pro-K.2000 all the results of fitting are available for visual inspection (figures 7, 8, 9 and 10 give a selection) and with just one or two mouse clicks printouts of every stage of display and analysis data can be made. Global analysis produces a fit which satisfies the



C:\HPCHEM\1\DATA\hpal6.csv

Fitted Model
a>b
b>c

Components
[a]o = 0.05
[b]o = 0

a>b
b>c

X Start 0

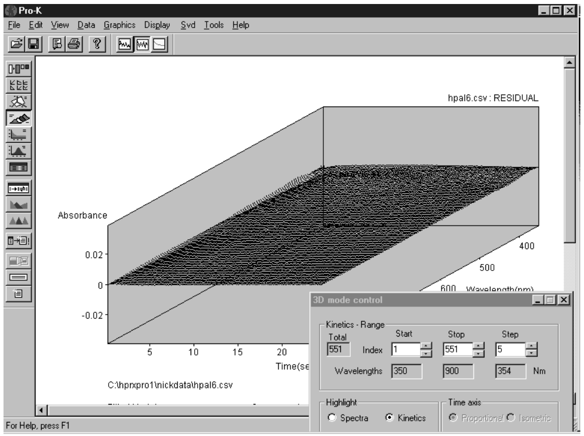
☐ Stiff

COMPONENT/SPECTRA

Select		Initial Conc	Fix	Rates	RATES Estimates	Lock	..FITTED
○	a	0.05	U	k+1	4.947	☐	4.947
○	b	0	U	k+2	0.2648	☐	0.2648
○	c	0	U		0	☐	0
○		0	U		0	☐	0
○		0	U		0	☐	0
○		0	U		0	☐	0

Figure 7
Fitted concentration profiles for the reaction components in the acid hydrolysis of tris(ethylamine)nickel(II) ion

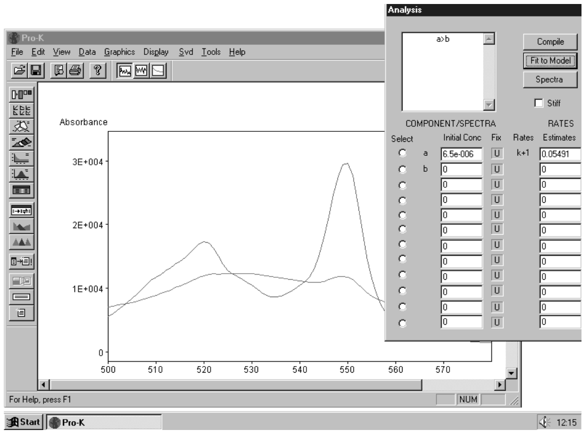
data simultaneously at all wavelengths and times. The rate constant for each step and concentration profiles and predicted spectra for each component in the reaction model are calculated. Residual plots are available to judge the quality of fit. Results can be exported directly from Pro-K.2000 into your word processor for inclusion in reports.



C:\hpnxpro1\nickdata\hpal6.csv

Kinetics - Range
Total 551
Index Start 1 Stop 551 Step 5
Wavelengths 350 900 354 Nm
Highlight ○ Spectra ● Kinetics
Time axis ● Proportional ○ Isometric

Figure 8
3-D display of residuals to kinetic fit in figure 7



a>b

☐ Stiff

COMPONENT/SPECTRA

Select		Initial Conc	Fix	Rates	RATES Estimates
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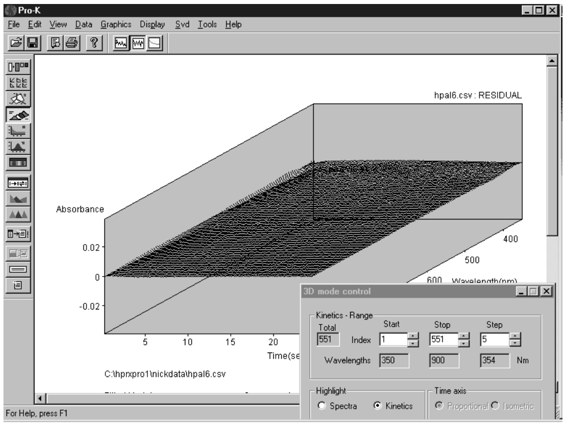
○	b	0	U		0
○		0	U		0
○		0	U		0
○		0	U		0
○		0	U		0
○		0	U		0
○		0	U		0
○		0	U		0
○		0	U		0
○		0	U		0
○		0	U		0

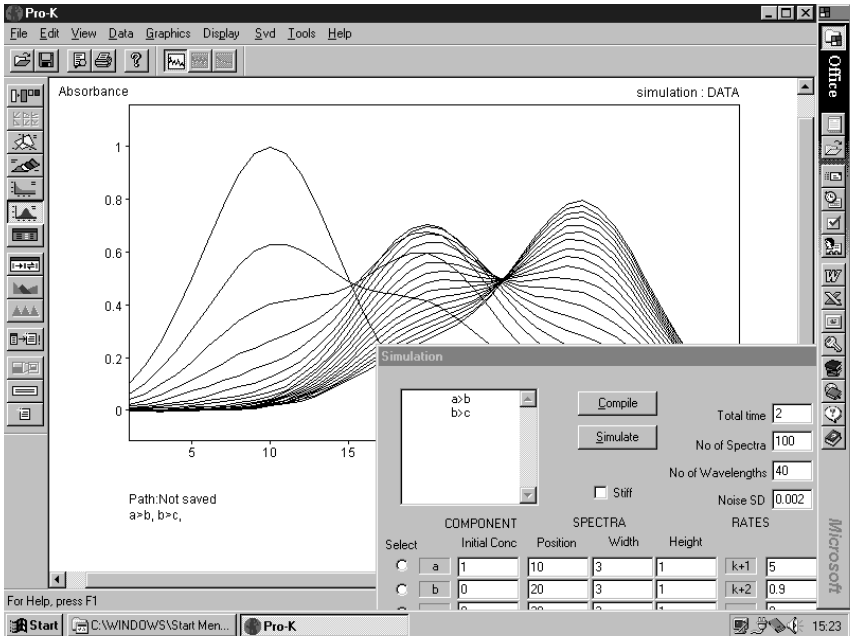
Figure 9
Calculated spectra of oxidised and reduced cytochrome c from global fit



C:\hpnxpro1\nickdata\hpal6.csv

Kinetics - Range
Total 551
Index Start 1 Stop 551 Step 5
Wavelengths 350 900 354 Nm
Highlight ○ Spectra ● Kinetics
Time axis ● Proportional ○ Isometric

Figure 10
Single fitted kinetic trace from Pro-K.2000 for reduction of cytochrome c



Path:Not saved
a>b, b>c,

a>b
b>c

Total time 2
No of Spectra 100
No of Wavelengths 40
Noise SD 0.002
☐ Stiff

Select		Initial Conc	Position	Width	Height		
○	a	1	10	3	1	k+1	5
○	b	0	20	3	1	k+2	0.9

Figure 11
The data shown are for a two-step reaction with rate constants of 5 s-1 and 0.9 s-1, 100 spectra were simulated
(for clarity only 20 are shown)

Under certain circumstances it may prove very difficult to obtain a reasonable or unique answer when analyzing data. It is often possible to solve the problem by fixing a known parameter (spectrum or rate) in a model, but frequently the simulation facility in Pro-K.2000 may be the best way forward, an example of which is shown in figure 11. Simulation is often useful when planning further experiments. Teaching kinetics is also aided. Simulated data generated in Pro-K.2000 can be analysed like real data. Simulation is not only invaluable in clarifying and modelling experimental behavior, but will also predict model behavior which can be confirmed by further experiments, for example, by changing the concentration of a reactant.

These simulated data sets, including variable added noise, can also be subjected to all the probes available for analysing real data, making them an invaluable tool for learning about or teaching kinetics without resorting to lab work.

Summary

- The Pro-K.2000 rapid kinetics system configured with the Agilent 8453 spectrophotometer can perform and analyse rapid reaction kinetic experiments without the need to dedicate the spectrophotometer to this purpose.

- The pneumatically-driven stopped-flow accessory allows two samples to be rapidly and reproducibly mixed and automatically triggers data acquisition by the spectrophotometer.

- The special micro-volume cell on the accessory offers versatile 2 and 10 mm absorbance path lengths.

- The file conversion macro running in Agilent ChemStation makes it easy to select data for analysis while leaving the original data file untouched.

- Global kinetic analysis and simulation software runs in parallel with Agilent ChemStation or runs as a standalone program.

- Singular value decomposition can save disk space and reduce analysis time. An indication of minimum reaction complexity is made available and noise can be reduced for presentation purposes.

- Any reaction model can be compiled using the reaction scheme editor with numerical integration. No knowledge of analytical rate equations is required. Known parameters can be incorporated into a model.

- Global fitting generates reliable results. The concentration profile and predicted spectrum of each reaction component are calculated.

- Data simulation may suggest new experimental strategies and is a good way to teach kinetics.

- Advanced graphics can be used to view the data and results in a wide variety of ways.

- Exporting data and results for inclusion in reports is easy.

Applied Photophysics

Pro-K.2000 Rapid Kinetics
System is available direct from:

Applied Photophysics Limited,
203-205 Kingston Road
Leatherhead
KT22 7PB
United Kingdom

In UK call 01372 386537
or fax 01372 386477

In US call 800 543 4130

Email: sales@apltd.demon.co.uk

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