

of 400 μm and 600 μm , respectively. The fiber optic cables were spaced 3.5 mm apart, resulting in a flow cell volume of 6.8 μL (Fig. 1). The entire system was controlled by a personal computer running FIALab software, version 5.9.137.

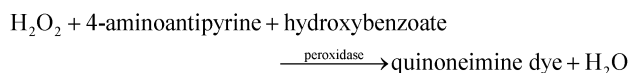
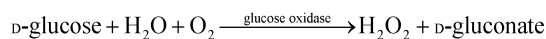
For the ethanol assay, a deuterium lamp (Model D 1000, Analytical Instrument Systems, Inc., Flemington, NJ, USA, <http://www.aishome.com/>) was used as the ultraviolet light source. A 400 μm fiber optic cable was connected to the light source, and a 200 μm fiber optic cable was connected to the spectrophotometer (S2000, Ocean Optics, Inc.). The flow cell volume and configuration remained the same as for the glucose assay.

Reagents and standards

Glucose

One vial of dry glucose oxidase reagent (G7519, Pointe Scientific, Inc., Lincoln Park, Michigan, USA, <http://pointescientific.com/>) was dissolved in 2.5 mL DI water prior to use. The reconstituted reagent contained glucose oxidase $>60 \text{ U mL}^{-1}$, peroxidase (horseradish) 4.8 U mL^{-1} , 4-aminoantipyrine 1.52 mM, phosphate buffer, pH 7.5 ± 0.1 , sodium *p*-hydroxybenzoate 40 mM, sodium 0.4% and non-reactive stabilizers and fillers.

The assay is based on two enzymatic reactions:



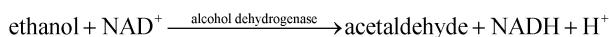
In the first step glucose oxidase catalyzes the oxidation of glucose by oxygen to produce hydrogen peroxide. Subsequently, peroxidase catalyzes hydrogen peroxide to react with the other reagents and produce quinoneimine dye, which is detected spectrophotometrically at 500 nm. A single mixture of reagent was used for glucose assay.

Glucose standards were made by serial dilution of 1000 ppm D-glucose (G-8270, Sigma, St. Louis, MO, USA, <http://www.sigma.com/>) stock solution. The carrier solution was 0.4% v/v commercial detergent Joy (Procter & Gamble, Cincinnati, OH, USA) in DI water. All solutions were stored in the refrigerator at 2–8 °C and were warmed in the incubator to reaction temperature (22 °C or 37 °C) prior to use.

Ethanol

One vial of dry reagent was dissolved in 5 mL of DI water. Reconstituted ethanol reagent (A7504, Pointe Scientific, Inc.) contained alcohol dehydrogenase (yeast) 600 U mL^{-1} , NAD 8.4 mM, buffer, pH 9.2 ± 0.1 , surfactant and preservative.

The assay is based on the enzymatic reaction:



Alcohol dehydrogenase catalyzes the oxidation of alcohol to acetaldehyde with the associated reduction of NAD^+ to NADH. The change in absorbance at 340 nm is directly proportional to the ethanol concentration in the sample. Similar to the glucose assay, a single mixture of reagent was used.

Ethanol standards were made by serial dilution of 1000 ppm ethanol standard (A7504-STD, Pointe Scientific, Inc.) stock solution. The carrier solution was 0.01% v/v P20 surfactant (BR-1000-54, Biacore, Inc, Piscataway, NJ, USA, <http://www.biacore.com/>) in DI water. All solutions were stored in the refrigerator at 2–8 °C and were warmed in the incubator to reaction temperature (22 °C or 37 °C) prior to use.

Assay protocol

The protocol for a SI stopped-flow assay typically consists of aspiration of sample, reagent(s), and often a spacer into the holding

coil, followed by a flow reversal that dispenses a desired section of the sequentially stacked zones to the flow cell for kinetic measurement. The function of the spacer, which is composed of the carrier solution, is to increase the amplitude of zone dispersion and thus promote mutual penetration of sample and reagent zones and their mixing. A selected section of the stacked zones can be captured in the flow cell by stopping the flow, and an increase in signal is recorded in the presence of analyte. The stopped-flow technique measures the rate of chemical reaction of an analyte with its reagent, eliminates the contribution of background color, and yields optimized signal to noise ratio by allowing longer time for the reaction to proceed.⁴ Stopped-flow SI also reduces sample and reagent consumption and waste generation. A typical response curve (Fig. 2) contains an initial peak when the reacting zones enter the flow cell. Following a flow stop, a short period (about 5 s) of residual mixing takes place during which the reaction mixture settles within the flow cell and the monitored signal is noisy. After the solution settles, a linear portion of the reaction rate curve (AB in Fig. 2) is recorded while data is collected.

In order to accelerate the assay, the stopped-flow period needs to be utilized for preparation of the next run and the mixing of sample/reagent zones has to be accelerated. Since the flow cell can be isolated from the rest of the system by turning the groove of the multi-position valve away from the flow cell port (Fig. 1), the sample, reagent, and spacer of run #2 can be stacked into the holding coil, while the reacting mixture from run #1 is being monitored in the flow cell (Fig. 3). By choosing a large volume of

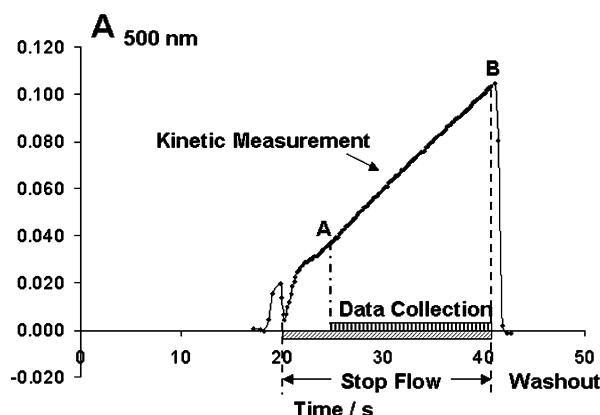


Fig. 2 Stopped-flow reaction based assay of glucose. The response curve was recorded as the stacked zones entered the flow cell, followed by stopped-flow and washout. When the flow was resumed, the signal returned to baseline as the flow cell was washed out. (400 ppm glucose, sample 30 μL , reagent 80 μL , spacer 100 μL , dispensed volume 210 μL , flow rate 200 $\mu\text{L s}^{-1}$, stopped-flow 20.8 s, 22 °C.) Note that in all subsequent figures except Fig. 4, data were only collected for the A–B section of the reaction rate curve for simplicity of graphical presentation.

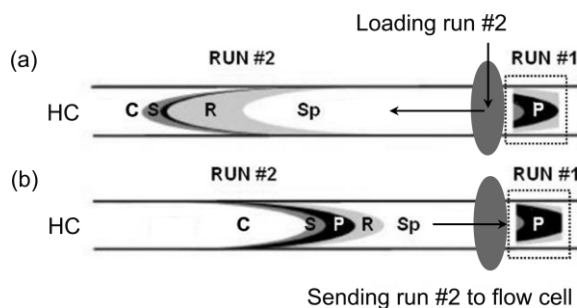


Fig. 3 Accelerated $\mu\text{SI-LOV}$ protocol. C: Carrier, S: sample, R: reagent, Sp: spacer, HC: holding coil, FC: flow cell. (a) Stacking sample, reagent, and spacer of run #2 into the holding coil during the stopped-flow measurement of run #1. (b) Sending the stacked zones of run #2 to flow cell, to wash out the flow cell and start the measurement of run #2. Note that the extended volume of the spacer prevents intermixing of sample/reagent zones of run #1 and run #2, and assists in washing out the flow cell.

spacer, intermingling of zones from run #1 with run #2 is prevented, and the flow cell is well flushed, before monitoring of run #2 takes place.

The accelerated protocol (Table 1) is comprised of the following steps: (1) Metering of sample solution by aspiration into the holding coil, followed by metering and aspiration of reagent and spacer solutions. (2) Dispensing of a desired volume of the stacked zones to the flow cell and stopping the flow. (3) After a brief delay (5 s), during which the solution within the flow cell has settled, absorbance measurement and data collection starts. At the same time, the syringe pump starts to send the remaining part of the stacked zones from run #1 to the waste port. (4) The syringe pump initiates run #2 by aspirating sample, reagent and spacer into the holding coil, then absorbance measurement of run #1 stops. Next, step 2 is repeated, where the valve turns to the flow cell port and a certain volume is dispensed to the flow cell followed by stopped-flow. In this process, the material from run #1 is flushed out to waste, and the desired section of stacked zones of run #2 is transported into the flow cell and stopped there for kinetic measurement. Steps 2–4 are in this way repeated for subsequent runs. In the last run, the processed sample/reagent zones are flushed to waste through flow cell after the data collection period. See Table 1 for more detail.

Results and discussion

The accelerated μ SI protocol has been optimized to allow the segment of sample/reagent zones that yields the steepest reaction rate slope to be arrested within the flow cell. To achieve this, two factors were investigated: (1) the volume of sample/reagent zones dispensed by flow reversal from the holding coil to the flow cell and (2) the flow rate at which the reacting zones are transported to the flow cell. While the dispensed volume defines the ratio of analyte to reagent, the flow rate influences the mixing of sample with reagent. As the dispensed volume was varied from 160 to 260 μ L with flow rate of 200 μ L s^{-1} , the observed maximum reaction rate was achieved by delivering 210 μ L (Fig. 4). At lower dispensed volumes, the monitored section of sample/reagent zones contains lower analyte/reagent ratios, while at higher volumes the section becomes reagent deficient. The glucose and ethanol assays both yielded maximum slope responses using a dispensed volume of 210 μ L, confirming the consistency and repeatability of this parameter.

The flow rate at which the sample/reagent zones are dispensed to the flow cell has an effect on the magnitude of the signal.⁸ As the flow rate increases, better mixing leads to steeper response signals (Fig. 5) and a maximum signal was achieved using a flow rate of 200 μ L s^{-1} . Remarkably, acceleration of the flow rate above 50 μ L s^{-1} did not significantly alter the position of the maximum signal yield, which remained positioned around 210 μ L of dispensed volume. Since the same segment of sample/reagent zones is comprised of the same analyte/reagent ratio,^{1,4} this observation confirms that perfect control of sample zone dispersion and positioning within the flow cell can be reproducibly maintained at both low and high flow rates.

An additional factor that allows shortening of the assay cycle is related to flushing of the LOV module ports between subsequent assays. In Wu *et al.*'s work,⁶ the sample port was flushed between different samples to eliminate carryover from the dead volume of the flow-through port, and sample and reagent ports were flushed between runs to eliminate carryover from the LOV ports. In this work, the monitored section of the stacked zones was further away from its tail section where solution from the dead volume of the flow-through port would cause cross contamination. This is why larger sample, reagent, and spacer volumes were used, as it allows the tail section to be diverted to waste in step 3, before the next run starts. This assumption was validated by performing accelerated glucose assays alternately for the highest concentration sample (1000 ppm), followed by the lowest concentration (100 ppm), a

Table 1 Accelerated μ SI-LOV protocol

	Notes
Syringe pump valve in Syringe pump flow rate 400 μ L s^{-1} Syringe pump aspirate 500 μ L Syringe pump delay until done —Sample—	
Syringe pump valve out Valve sample Syringe pump flow rate 30 μ L s^{-1}	Step 1 Stacking sample, reagent, and spacer into holding coil.
Syringe pump aspirate 30 μ L Syringe pump delay until done —Reagent— Valve reagent Syringe pump flow rate (μ L s^{-1}) 100 Syringe pump aspirate 80 μ L Syringe pump delay until done —Spacer— Valve spacer Syringe pump aspirate 100 μ L Syringe pump delay until done	
Loop start (#) 4 —To flow cell— Valve flow cell Syringe pump flowrate 200 μ L s^{-1}	Loop start Step 2 Dispensing the desired section of stacked zones to flow cell by flow reversal, which also washes out flow cell.
Syringe pump dispense 210 μ L Syringe pump delay until done Delay 5 s Spectrometer reference scan Spectrometer absorbance scanning Valve waste Syringe pump flow rate 400 μ L s^{-1} Syringe pump empty	Step 3 Absorbance monitoring starts. Sending remaining part of the stacked zones to waste.
Syringe pump delay until done Syringe pump valve in Syringe pump aspirate 500 μ L Syringe pump delay until done —Sample— Syringe pump valve out Valve sample Syringe pump flow rate 30 μ L s^{-1}	Step 4 Stacking sample, reagent, and spacer into holding coil for the next run, while data collection continues.
Syringe pump aspirate 30 μ L Syringe pump delay until done —Reagent— Valve reagent Syringe pump flow rate 100 μ L s^{-1} Syringe pump aspirate 80 μ L Syringe pump delay until done —Spacer— Valve spacer Syringe pump aspirate 100 μ L Syringe pump delay until done Spectrometer stop scanning Loop end —To flow cell— Valve flow cell Syringe pump flow rate 200 μ L s^{-1}	Absorbance monitoring stops. Loop end Last run Dispensing the desired section of stacked zones to flow cell by flow reversal, which also washes out the flow cell.
Syringe pump dispense 210 μ L Syringe pump delay until done Delay 5 s Spectrometer reference scan Spectrometer absorbance scanning Delay 15.8 s Spectrometer stop scanning Syringe pump flow rate 400 μ L s^{-1} Syringe pump empty	Absorbance monitoring starts. Absorbance monitoring stops. End of the assay, washing out flow cell.
Syringe pump delay until done	

second run at the highest concentration, and a blank (Fig. 6), using a protocol that eliminated flushing of the flow-through port between samples and flushing of the sample and reagent ports between individual runs.

Using the optimized accelerated protocol (Table 1), assays of glucose and ethanol were performed at 22 °C and 37 °C. The reaction rate curves for several concentrations of glucose are presented in Fig. 7. At 22 °C, the glucose calibration yielded a linear response ($y = 9.57 \times 10^{-6}x + 9.84 \times 10^{-5}$, $r^2 = 0.9999$) for the concentration range of 100–1000 ppm (Fig. 7a). At 37 °C, the calibration was linear ($y = 1.91 \times 10^{-5}x + 1.23 \times 10^{-4}$, $r^2 = 0.9996$) for 100–600 ppm, but was a second order polynomial curve ($y = -6.07 \times 10^{-9}x^2 + 2.34 \times 10^{-5}x - 4.10 \times 10^{-4}$, $r^2 = 0.9996$) for 100–1000 ppm (Fig. 7b), most likely due to the saturation of the enzymes or depletion of oxygen. For ethanol, at both 22 °C and 37 °C, the calibrations were linear ($y = 1.02 \times 10^{-4}x + 1.88 \times 10^{-3}$,

$r^2 = 0.9994$ and $y = 1.47 \times 10^{-4}x + 5.93 \times 10^{-5}$, $r^2 = 0.9995$, respectively) for the concentration range of 50–250 ppm, showing no limitation due to reagent availability. The relative standard deviations were below 3% for both the glucose and ethanol assays. These results were obtained from *initial* reaction rate slope measurements using data collected from 3 to 7 s. It should be noted, however, that reaction rate responses in Fig. 7 are shown recorded for 15.8 s for the clarity of graphical presentation. Since the pump and valve operations required for washing the system and loading the solutions for a subsequent run require a minimum of 15.8 s, shortening the data collection period will not accelerate the assay any further.

Influence of temperature

The rate constant k of a reaction is dependent on temperature (T , in K) and the activation energy (ΔG^*):

$$k = (k_B T/h) e^{-\Delta G^*/RT}$$

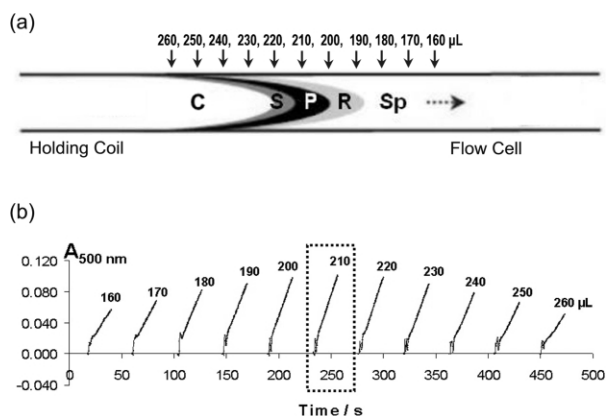


Fig. 4 Flow reversal volume optimization (a) The stacked zones of sample, reagent and spacer are shown in the holding coil. C: Carrier, S: sample, R: reagent, Sp: spacer, P: product. Different dispensed volume would place different segment of the stacked zones into the flow cell. (b) Delivery of different volumes of the reacting zones to the flow cell, with flow rate of 200 $\mu\text{L s}^{-1}$. Data were collected during the entire stopped-flow period. (400 ppm Glucose, sample 30 μL , reagent 80 μL , spacer 100 μL , stopped-flow 20.8 s, 22 °C.)

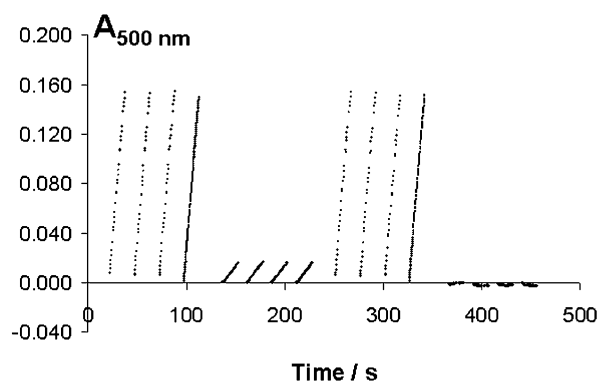


Fig. 6 Carryover test. Accelerated glucose assays were done for the highest concentration sample (1000 ppm), followed by the lowest concentration (100 ppm), highest concentration, and blank, with a protocol that eliminated flushing of the flow-through port between samples and flushing of sample and reagent ports between individual runs. (Sample 30 μL , reagent 80 μL , spacer 100 μL , dispensed volume 210 μL , flow rate 200 $\mu\text{L s}^{-1}$, stopped-flow 20.8 s, data collection 15.8 s, 22 °C.)

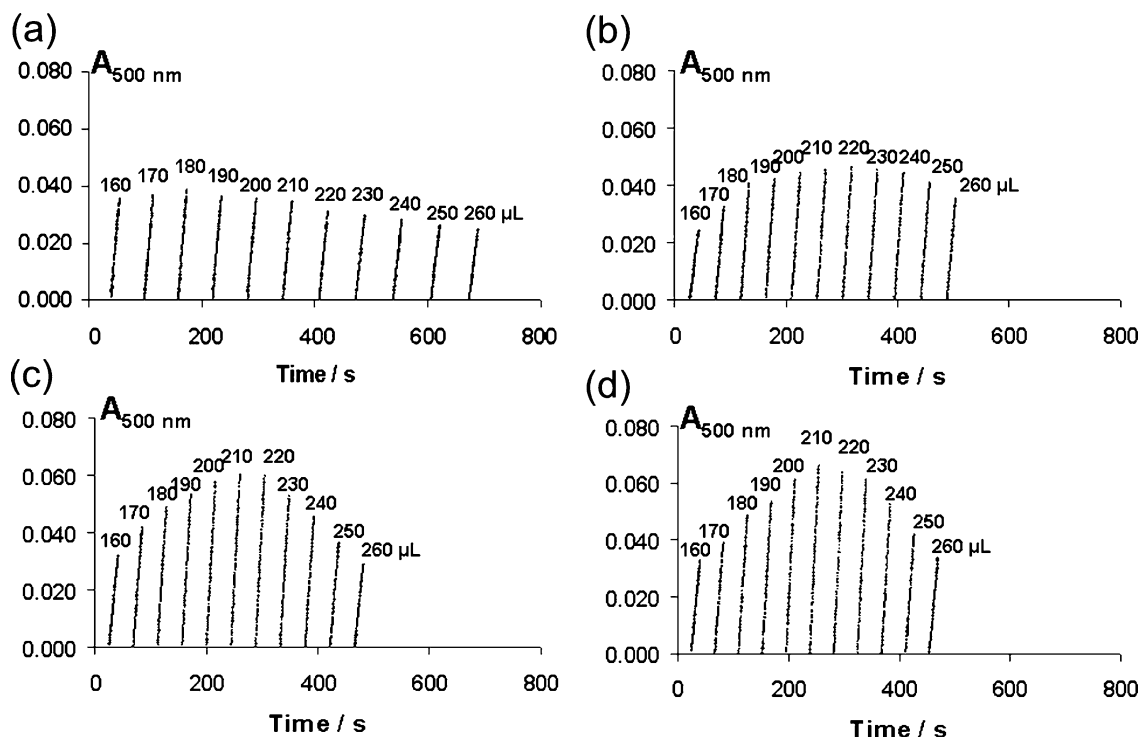


Fig. 5 Flow rate optimization, with dispense flow rate 10 $\mu\text{L s}^{-1}$ (a), 50 $\mu\text{L s}^{-1}$ (b), 100 $\mu\text{L s}^{-1}$ (c), and 200 $\mu\text{L s}^{-1}$ (d). Data were collected after a 5 s delay in the stopped-flow period. (400 ppm Glucose, sample 30 μL , reagent 80 μL , spacer 100 μL , stopped-flow 20.8 s, data collection 15.8 s, 22 °C.)

where k_B is the Boltzmann constant and h is Planck's constant. It can be seen that the higher the temperature, the higher the reaction rate constant. In the case of glucose assays, at higher temperature and higher reaction rate, the enzymes are saturated quickly or oxygen is depleted quickly, so that reaction rate is no longer proportional to higher analyte concentrations. On the other hand, an increase in reaction temperature helps to increase sensitivity and improve the detection limit of the assay.

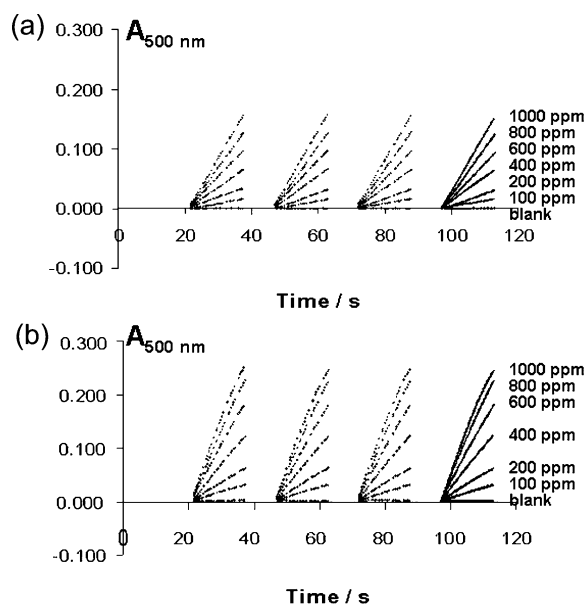


Fig. 7 Assay of glucose in the range of 0–1000 ppm at 22 °C (a) and 37 °C (b). Each concentration had four replicate runs. (Sample 30 μL , reagent 80 μL , spacer 100 μL , dispensed volume 210 μL , flow rate 200 $\mu\text{L s}^{-1}$, stopped-flow 20.8 s, data collection 15.8 s.)

Conclusion

Sequential injection is being increasingly accepted as an efficient tool for automation and micro-miniaturization of reagent-based assays, both in research and industrial settings. Its wide scope of applications have been recently summarized in a review by Lenehan *et al.*,³ where the conventional SI format was discussed.

The impact of miniaturization and protocol acceleration can be appreciated by comparing the enzymatic assay carried out in traditional SI format⁷ that required 200 s to complete a single run, with the work of Wu *et al.*⁶ that completed an enzymatic assay in the LOV format within 100 seconds, and with the present work, which takes ≈ 30 s to complete an assay cycle. This improvement highlights advantages of sophisticated flow programming as compared to more traditional and widely used flow injection mode that uses continuous forward flow and, as a result, consumes more reagents and generates correspondingly larger volumes of waste.

Acknowledgments

The authors wish to express their gratitude to Center for Process Analytical Chemistry (CPAC) for financial support, to Dr Andrea Carroll, Dr Holger Erxleben, and Dr Louis Scampavia for critical comments, and to Dr Richard Chao-Hsiang Wu for assistance and comments at the outset of this work.

References

- 1 J. Ruzicka and E. H. Hansen, *Flow Injection Analysis*, Wiley, New York, 2nd edn. 1988.
- 2 J. Ruzicka and G. Marshall, *Anal. Chim. Acta*, 1990, **237**, 329–343.
- 3 C. Lenehan, N. Barnett and S. Lewis, *Analyst*, 2002, **127**, 997–1020.
- 4 J. Ruzicka, *Flow Injection Tutorial cd-rom*, FIALab Instruments, Inc., Bellevue, WA, 2nd edn., 2002, <http://www.flowinjection.com>.
- 5 J. Ruzicka, *Analyst*, 2000, **125**, 1053–1060.
- 6 C. Wu, L. Scampavia, J. Ruzicka and B. Zamost, *Analyst*, 2001, **126**, 291–297.
- 7 J. Ruzicka and T. Gubeli, *Anal. Chem.*, 1991, **63**, 1680–1685.
- 8 C. Schulz and J. Ruzicka, *Analyst*, 2002, **127**, 1293–1298.