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A capillary electrophoresis chip for the analysis of print and film photographic developing agents in commercial processing solutions using indirect fluorescence detection

The separation and detection of both print and film developing agents (CD-3 and CD-4) in photographic processing solutions using chip-based capillary electrophoresis is presented. For simultaneous detection of both analytes under identical experimental conditions a buffer pH of 11.9 is used to partially ionise the analytes. Detection is made possible by indirect fluorescence, where the ions of the analytes displace the anionic fluorescing buffer ion to create negative peaks. Under optimal conditions, both analytes can be analyzed within 30 s. The limits of detection for CD-3 and CD-4 are 0.17 mM and 0.39 mM, respectively. The applicability of the method for the analysis of seasoned photographic processing developer solutions is also examined.

Keywords: Capillary electrophoresis / Microchip / Indirect fluorescence / Photographic developer

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1 Introduction

The concept of a miniaturised total (chemical) analysis system (μ-TAS) was introduced by Manz and co-workers over a decade ago [1, 2]. Since then, chip-based analytical systems have been developed and applied to a variety of fields such as separation science, chemical production, DNA analysis, drug discovery, pharmaceutical screening, medical diagnostics, and environmental analysis [3–6]. The advantages of shrinking analytical systems lie in improved efficiency with respect to sample size, response time, cost, throughput and automation. Ideally, all steps of a complete analytical procedure (including sample handling, chemical reaction, sample pretreatment, analytical separation, analyte detection, product isolation) should be performed on a single, integrated device.

Analytical techniques such as gas chromatography [7], liquid chromatography [8–10], flow injection analysis [11], and capillary electrophoresis (CE) [12–17] have all been successfully transferred to chip-based formats. In particular, CE is ideally suited to application in planar environments due to facile electrokinetic manipulation and reproducible injection of small amounts of sample. Both optical and electrochemical detection methods have proved popular when performing CE in microfabricated structures [18, 19]. However, fluorescence detection has been most widely applied due to extremely high sensitivities and low limits of detection. Unfortunately, few analytes are intrinsically fluorescent, and consequently alternative optical detection methods are often needed. Indirect fluorescence detection (IFD) is a promising alternative detection protocol and was first applied to conventional CE by Kuhr and Yeung in 1988 [20].

Recently, we demonstrated the feasibility of using IFD as a detection method for on-chip CE analysis [21]. In those studies, simple microfluidic networks were utilised to separate and detect the colour developer, 4-amino-3-methyl-*N*-ethyl-*N*-(β-methane sulfonamidoethyl)aniline (CD-3) in a commercial developer solution. Results obtained from seasoned developer solutions were promising in terms of both analysis time and limit of detection. Subsequently, IFD has been used by others to detect explosive compounds after separation by micellar electrokinetic chromatography on microfabricated glass structures [22].

In the current paper, we develop the utility of IFD as a detection method to create a chip-based CE system for the analysis of both photographic film and print developing agents using identical experimental conditions. This approach allows for the simultaneous assessment of 'developer quality' in both stages (photographic film development and photographic print formation) of a conventional photographic process. Specifically, we focus on complex solution systems containing both CD-3 (the active agent in commercial RA-4 developer solutions) and

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Abbreviation: IFD, indirect fluorescence detection

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4-(*N*-ethyl-*N*-2-hydroxyethyl)-2-methylphenylenediamine (CD-4: the active agent in commercial C-41 developer solutions). The chemical structures of both molecules are shown in Fig. 1. Analytical parameters were optimized using 'fresh' commercial developing solutions and then applied to 'seasoned' commercial developer solutions from both RA-4 and C-41 processes.

2 Materials and methods

2.1 Microfabrication

The glass microchip (3 × 3 cm) was made in-house using direct write laser lithography (DWL), wet chemical etching and bonding techniques as previously described [21]. Briefly, a positive photoresist (S 1818; Shipley Corporation, Whitehall, PA, USA) was spun onto the surface of a glass substrate, and the channel design transferred to the substrate into the photoresist using a DWL system (DWL2.0; Heidelberg Instruments, Heidelberg, Germany). After the photoresist was developed (Microposit 351; Shipley Europe, Coventry, UK), the channels were etched into the glass substrate using a buffered oxide etching solution (HF/NH_4F). A glass cover plate, with holes drilled for external access, was cleaned in H_2SO_4/H_2O_2 and aligned before contact with the etched glass substrate. To form enclosed channels, the plates were thermally bonded in an oven. Small pipette tips were connected to the drilled holes using epoxy resin, and used as reservoirs to contain sample or buffer solution.

The microchip layout is presented in Fig. 2a. All channels were approximately 10 μm deep and 40 μm wide (the isotropic etching procedure results in a rounded channel profile, with a channel bed width of 20 μm). The channel connecting reservoirs 2–4 has a total capillary length of 5 cm, the channel connecting reservoirs 2–6 has a total capillary length of 6 cm, and the channel connecting reservoirs 2–7 has a total capillary length of 9 cm. A double-T injector design [23] was fabricated to allow injection of either 20 pL of sample (using a voltage between reservoirs 1 and 3) or 40 pL of sample (using a voltage between reservoirs 1 and 5). Figure 2b shows a photomicrograph of the intersection cross used for defining injected volume of the sample.

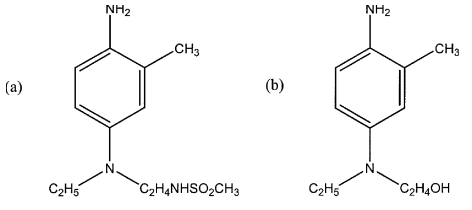

Figure 1. Chemical structures of the colour-developing agents. (a) CD-3 (b) CD-4.

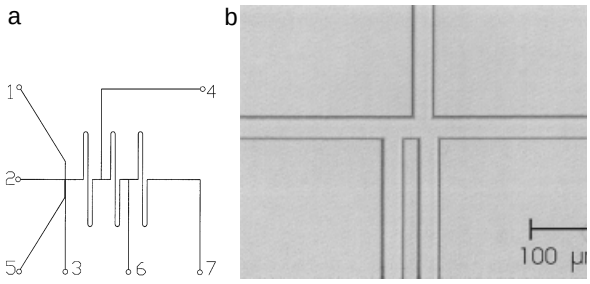

Figure 2. (a) Schematic of the glass microchip used for all electrophoretic separations. Reservoirs labelled 1–7: (1) sample inlet, (2) buffer solution inlet, (3) and (5) sample outlet, (4), (6) and (7) buffer solution outlet. (b) Photomicrograph of the intersection cross used for defining injection volume of the sample. The widths at the top and the bottom of all channels are 40 and 20 μm, respectively.

2.2 Instrumentation

Electrophoretic separations were observed on microchip *via* fluorescence and indirect fluorescence, using an inverted microscope (DMIL; Leica, Milton Keynes, UK) and filter cube consisting of an excitation filter (BP 450–490), a dichroic mirror (RKP 510), and a suppression filter (BP 515–560). Specifically, excitation light from a 50 W mercury lamp (Leica) was passed through an excitation filter, reflected by the dichroic mirror and focused onto the microchip. The fluorescence emission was collected by a 10 × microscope objective (0.42 NA; Newport, Irvine, CA, USA), passed through the dichroic mirror, a suppression filter, and 10 μm × 150 μm viewing window. A photomultiplier tube (MEA153; Seefelder Messtechnik, Germany) operating in current mode was employed to detect fluorescence photons. Data were acquired at a rate of 10 Hz and stored using a PC data acquisition program (PicoLog; Pico Technology, Hardwick, Cambridge, UK) and processed in Origin 6.0 program (Microcal Software, Northampton, USA). The in-house power supply used for electrophoresis was operated between 0 and +3 kV relative to ground and controlled by a programme written under the LabView 3.0 graphical programming environment (National Instruments, Austin, TX, USA). Prior to experiments, the channels were flushed sequentially with NaOH, 18 MΩ Millipore water, and running buffer solution for 10 min. The microchip was operated in either a 'sample loading' or a 'separation' mode. Electrical contact between the high voltage leads and reservoirs was made using platinum electrodes. In all experiments, sample in sample reservoir 1 was injected *via* the double-T injector through sample waste reservoir 3 by applying voltages between both reservoirs. The sample plug at the double-T injector was then introduced onto the separation channel by applying voltages across buffer reservoir 2 and

buffer waste reservoir 4. During the separation mode, the sample and the sample waste are maintained at 58% of the potential applied to the buffer reservoir. This prevents leakage of sample into the separation channel from the sample and sample waste channels.

2.3 Chemicals

The colour developing agents, 4-amino-3-methyl-*N*-ethyl-*N*-(β-methane sulfonamidoethyl)aniline and 4-(*N*-ethyl-*N*-2-hydroxyethyl)-2-methylphenylenediamine, were obtained as a gift from Kodak (Harrow, Middlesex, UK). Kodak Ektacolor RA developer solution consisting of three parts (Part A, triethanolamine, *N,N*-diethylhydroxylamine, substituted stilbene; Part B, CD-3, lithium sulphate, potassium sulphite; Part C, potassium carbonate, potassium bicarbonate, potassium chloride) and Kodak Flexicolor developer solution consisting of three parts (Part A, potassium carbonate, potassium bicarbonate, penetetic acid, pentasodium salt, potassium sulphite, sodium bromide; Part B, hydroxylamine sulphate, Part C, CD-4, sodium bisulphite) were obtained from Kodak. Fluorescein di-sodium salt (Fluka, Dorset, UK), disodium hydrogenphosphate (BDH-Merck, Poole, Dorset, UK) and Calcein (Fisher Scientific, Leicestershire, UK) were used as received. All other chemicals were of analytical grade and prepared using high resistivity (18 MΩ), deionized water and filtered using 0.45 μm filters before introduction into the microchip. The pH of the running buffer containing fluorescein was adjusted by addition of NaOH.

3 Results and discussion

The microchip fabricated by means of the procedure above was first characterised by separating two fluorescent dyes, calcein and fluorescein. As expected, separation of the two dyes could be reproducibly achieved within a few tens of seconds of injection (using moderate electric fields). In the presence of electroosmotic flow, the efficiency of an electrophoretic separation can be defined in terms of *N* (number of theoretical plates) as shown in Eq. (1) [24]

$$N = \frac{(\mu_{ep} + \mu_{eo})V}{2D} \quad (1)$$

Here μ_{ep} and μ_{eo} are the electrophoretic mobility ($cm^2\ V^{-1}\ s^{-1}$) and electroosmotic mobility ($cm^2\ V^{-1}\ s^{-1}$), respectively, V is the total applied voltage (V), and D is the specific molecular diffusion coefficient ($cm^2\ s^{-1}$). In addition, the time t it takes an ion migrate the entire length of the capillary L is given by Eq. (2) [24]

$$t = \frac{L^2}{(\mu_{ep} + \mu_{eo})V} \quad (2)$$

Figure 3 illustrates the variation of the number of theoretical plates (*N*) as a function of the applied voltage (between 1.5 to 3.0 kV) for injections of 200 μM fluorescein in 60 mM phosphate buffer at pH 11.9. It can be seen that the relationship is essentially linear, indicating that under the experimental conditions used there is no significant temperature variation within the channel during separation. Furthermore, Fig. 4 shows the variation of reciprocal time against the applied voltage. Again, the expected linear relationship is observed.

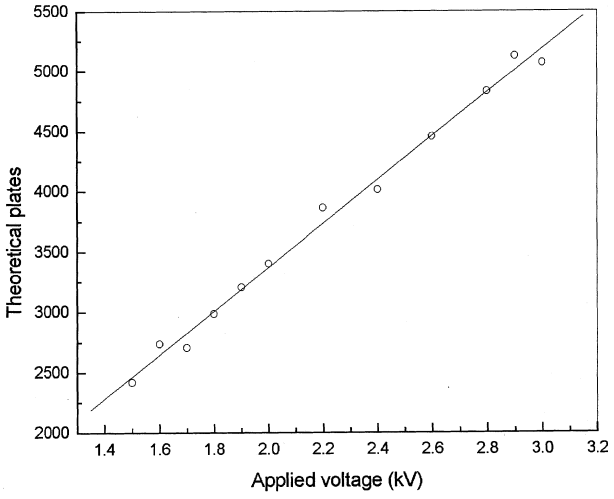

Figure 3. Variation of the number of theoretical plates (*N*) with applied voltage for injections of 200 μM fluorescein in 60 mM phosphate buffer at pH 11.9. Injection voltages were applied between reservoirs 1 and 3, and separation voltages were applied between reservoirs 2 and 4.

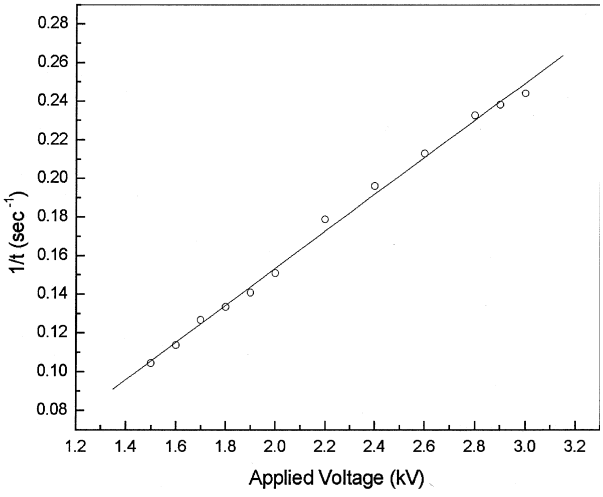

Figure 4. Variation of analysis time with applied voltage for injections of 200 μM fluorescein in 60 mM phosphate buffer at pH 11.9. Injection voltages were applied between reservoirs 1 and 3, and separation voltages were applied between reservoirs 2 and 4.

As stated previously, in IFD fluorescing ions in the running buffer create a constant fluorescence background. A (negative) signal is obtained when fluorescing ions are displaced by nonfluorescent analyte ions. In this work, a high pH buffer was used to increase analyte deprotonation and enhance displacement-based detection. All experiments were performed using a pH 11.9 phosphate buffer. Above pH 9, the fluorescence intensity of fluorescein is constant. Furthermore, the $pK\alpha_3$ of CD-3 and $pK\alpha_2$ of CD-4 are 11.6 and 9.82, respectively. Consequently, the experimental conditions ensured that both CD-3 and CD-4 are fully deprotonated and exist as anions. It is also noted that because both CD-3 and CD-4 are strong reducing agents they are extremely sensitive to the presence of molecular oxygen in solution. To minimize oxidation, the addition of an antioxidant is essential [25]. Therefore, 0.1 M sodium sulphite was incorporated into running buffer solutions.

Initially, electrophoretic separations of CD-3 and CD-4 were performed at varying concentrations of fluorescein in the running buffer. Figure 5 shows the effect of fluorescein concentration on the signal-to-noise (S/N) ratio for CD-3 and CD-4. It is observed that an increase in concentration of fluorescein between 1 and 5 mM resulted in an increase in the S/N ratio for both analytes. Above 5 mM, any further increase in fluorescein concentration results in an insignificant increase in the S/N ratio for both CD-3 and CD-4.

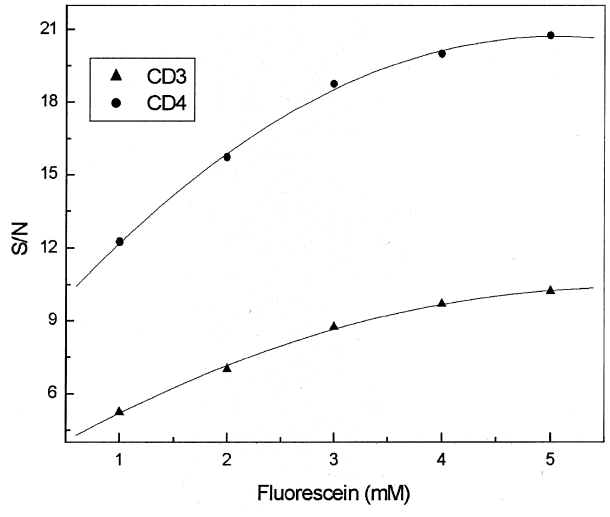

Figure 5. Effect of fluorescein concentration in running buffer on S/N ratio of CD-3 and CD-4. The detection volume is located 12 mm downstream from the point of injection. Electric field strength, 600 V cm^{-1}; total capillary length, 5 cm; buffer composition, 5 mM fluorescein, 60 mM phosphate and 0.1 M Na_2SO_3 (pH 11.9).

The effect of buffer concentration on the resolution between CD-3 and CD-4 and migration time of the analytes is shown in Fig. 6 and Fig. 7. Resolution R is calculated according to the following standard equation [26],

$$R = \frac{2(t_{M_2} - t_{M_1})}{W_{b_1} + W_{b_2}} \quad (3)$$

Here, t_M is the migration time of a given component and W_b is the peak width at the base of a given peak. It can be

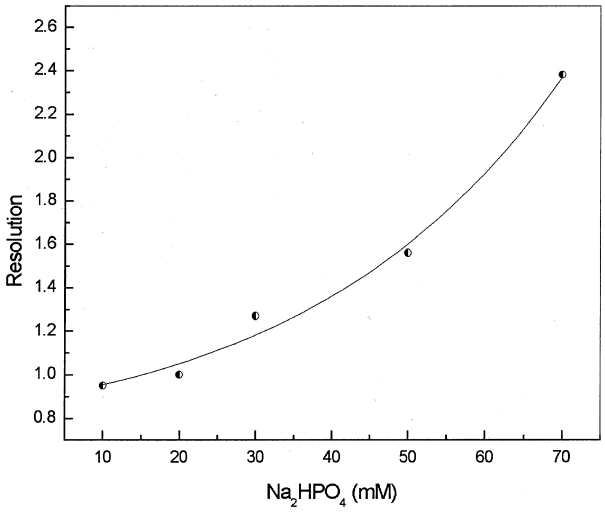

Figure 6. Effect of running buffer concentration on resolution of CD-3 and CD-4 peaks. The detection volume is located 12 mm downstream from the point of injection. Conditions as in Fig. 5.

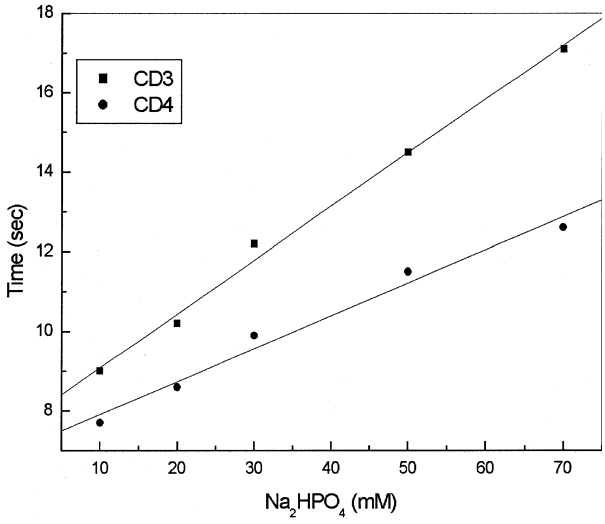

Figure 7. Effect of buffer concentration on migration time of CD-3 and CD-4. The detection volume is located 12 mm downstream from the point of injection. Conditions as in Fig. 5.

seen that at a constant fluorescein concentration, the resolution between the CD-3 and CD-4 peaks increases with higher buffer concentrations. However, the total analysis time also increases at higher buffer concentrations. As a result of these studies, a running buffer electrolyte of 5 mM fluorescein in 60 mM phosphate buffer at pH 11.9 was chosen for further analyses of C-41 and RA-4 developer solutions. These conditions yield fast analysis times and sufficient resolution of components.

Using the optimised experimental parameters described above, reproducibility, linearity and detection limits were investigated. Run-to-run reproducibility on the microchip was very good. The standard deviation of the migration times, based on five replicate injections of CD-3 and CD-4 were 0.31 and 0.12%, respectively. Furthermore, calibration graphs were linear over the proposed concentration range of use (2.5–4.5 mM) for CD-3 with a correlation coefficient of 0.9981, and 0.4–5.5 mM for CD-4 with a correlation coefficient of 0.9934. Based on a signal-to-noise ratio of three, the detection limit for the current system was 0.28 mM for CD-3 and 0.31 mM for CD-4.

Figure 8 shows the results of an electrophoretic analysis of fresh Kodak Ektacolor RA developer solution (RA-4). This is made up from three individual aqueous solutions: Part A, triethanolamine, *N,N*-diethylhydroxylamine, substituted stilbene; Part B, CD-3, lithium sulphate, potassium sulphite; Part C, potassium carbonate, potassium bicarbonate, potassium chloride. Figure 9 shows results from a similar analysis of fresh Kodak Flexicolor developer (C-41) made from three individual aqueous solutions: Part A, potassium carbonate, potassium bicarbonate, pentetic acid, pentasodium salt, potassium sulphite, sodium bromide; Part B, hydroxylamine sulphate, Part C, CD-4, sodium bisulphite. In the case of fresh RA-4 developer solution, two peaks were detected under all conditions. The identity of the CD-3 peak was verified by spiking sample solutions with a standard solution of CD-3. However, the molecular origin of the additional peak has yet to be elucidated. Analysis of reference samples demonstrates that the additonal peak originates from the RA-4 solution matrix and only coincidentally has the same retention time as that observed for CD-4 in C-41 developer solutions (Fig. 9).

Figures 10a and b illustrate electrophoretic analyses of 'seasoned' RA-4 and C-41 developer solutions, respectively. It is clearly observed that the fluorescence baseline signal is unstable for both RA-4 and C-41 developer solutions. The 'seasoned' solutions originated from trade photographic processing systems and contain a range of development side products and material that has been leached out of film/paper. The resulting complex sample matrix clearly reduces detection sensitivity. However, the identity and concentration of both developing agents can be elucidated with reference to the standard sample analysis (Figs. 8 and 9) within the necessary concentration range.

4 Concluding remarks

The studies presented in this paper demonstrate that chip-based CE analysis with IFD can be used to rapidly monitor two colour-developing agents in commercial photographic processing solutions. Current laboratory tech-

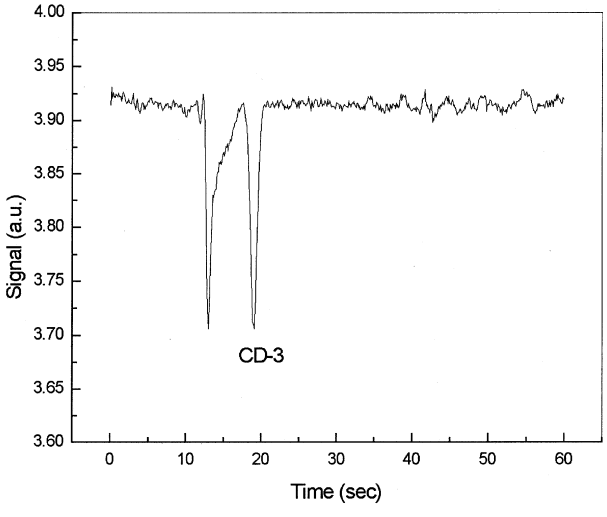

Figure 8. Electrophoretic analysis of a fresh RA-4 colour photographic developer solution. Conditions as in Fig. 5; separation length, 12 mm.

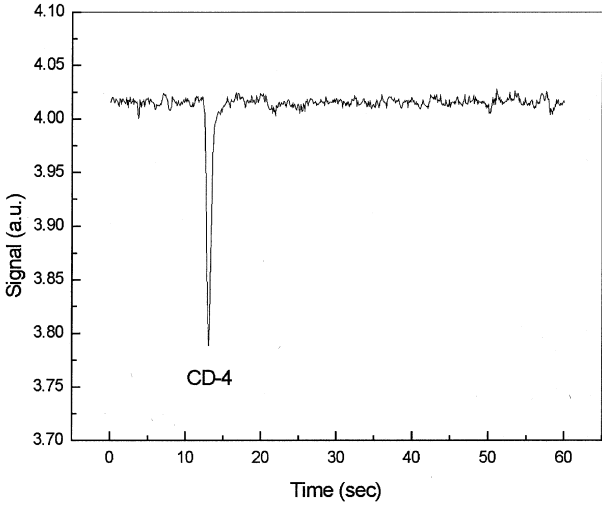

Figure 9. Electrophoretic analysis of a fresh C-41 colour photographic developer solution. Conditions as in Fig. 5; separation length, 12 mm.

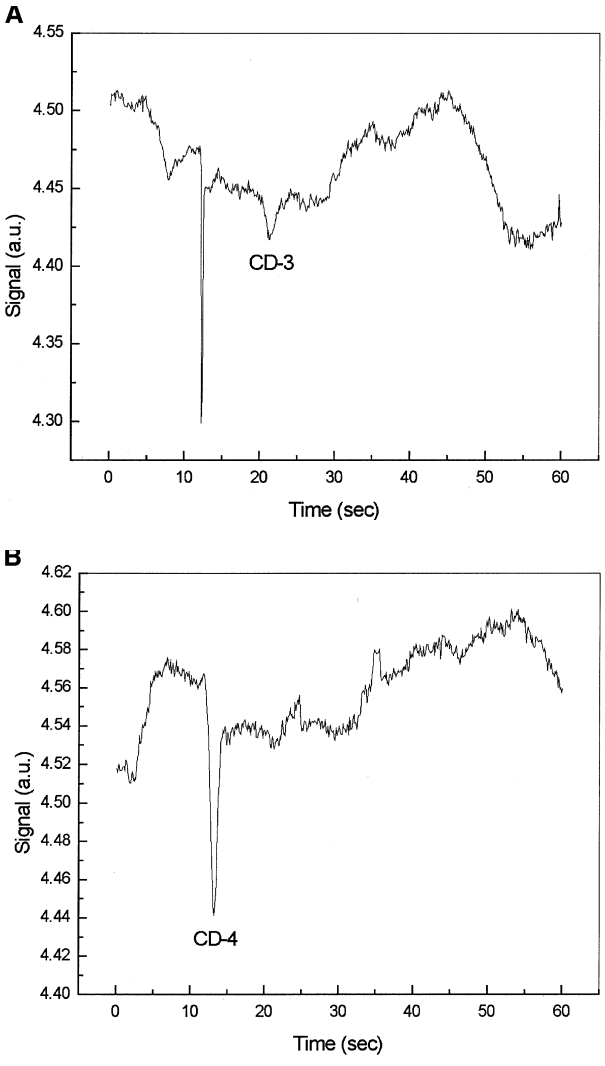

Figure 10. Electrophoretic analysis of (a) a seasoned RA-4 colour photographic developer solution; (b) a seasoned C-41 colour photographic developer solution. Conditions as in Fig. 5; separation length, 12 mm.

niques used in the analysis of CD-3 and CD-4 in photographic colour developers are based on HPLC protocols employing columns with octadodecyl (C_{18}) stationary phases (Bumfrey, T., personal communication). In the current device, sample preparation prior to analysis is trivial, and only requires dilution of the process solution prior to analysis. Results indicate that for the current system (using a mercury lamp as an excitation source) a high background fluorophore concentration is required for optimum sensitivity. Currently, detection limits in the region of ppb level cannot be achieved, and this limitation is being addressed by replacing the lamp with an argon ion laser source. The 488 nm line of the argon ion laser is closely matched to the primary absorption band of fluorescein in aqueous solution. Furthermore, the argon ion laser will undoubtedly allow for highly stable and intense excitation. Nevertheless, it should be emphasied that for analysis of the commercial processing solutions discussed detection limits and sensitivity are perfectly adequate, and indeed sample dilution is a prerequisite in any analysis. Importantly, the planar glass chip described herein allows for the simultaneous analysis of both print and film developing agents under identical experimental conditions. This firmly establishes the feasibility of using a single device for analysis of 'developer' quality in commercial print and film photographic processing solutions.

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