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Design and implementation of an array of micro-electrochemical detectors for two-dimensional liquid chromatography—Proof of principle

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ABSTRACT

Simultaneous two-dimensional liquid chromatography (2D-LC) is an implementation of two-dimensional liquid chromatography which has the potential to provide very fast, yet highly efficient separations. It is based on the use of time × space and space × space separation systems. The basic principle of this instrument has been validated long ago by the success of two-dimensional thin layer chromatography. The construction of a pressurized wide and flat column (100 mm × 100 mm × 1 mm) operated under an inlet pressure of up to 50 bar was described previously. However, to become a modern analytical method, simultaneous 2D-LC requires the development of detectors suitable for the monitoring of the composition of the eluent of this pressurized planar, wide column. An array of five equidistant micro-electrochemical sensors was built for this purpose and tested. Each sensor is a three-electrode system, with the working electrode being a 25 μm polished platinum micro-electrode. The auxiliary electrode is a thin platinum wire and the reference electrode an Ag/AgCl (3 M sat. KCl) electrode. In this first implementation, proof of principle is demonstrated, but the final instrument will require a much larger array.

1. Introduction

The purpose of liquid chromatography is the separation of the components of a plug of a sample into a string of bands of its components during the migration of this plug along a porous bed that is percolated with a suitable solution. This separation requires the proper selection of the material for making of the porous bed and of the solution. Plates covered with a thin porous layer and columns filled with a porous bed have successively been the preferred tool to implement liquid chromatography [1–8]. Both approaches have been successfully used to achieve the analysis of most complex mixtures, using appropriate methods of two-dimensional chromatography [9,10]. Today, however, the field is dominated by column-based separations and planar chromatography is no longer the widely used method as it was 50 years ago.

The main reasons for the preference of column over plate chromatography are (1) the fact that columns are eluted, a process easier than plate development; (2) the detection and quantitation of the bands of separated compounds exiting the columns with the stream of mobile phase is much easier than that of bands spread on plates of porous materials that scatter light; and (3) column separations were easily instrumentalized while considerable difficulties are still encountered to do so for plate separations [10,11]. These considerable advantages have obscured the potential qualities of planar separations. The latter explain why two-dimensional thin layer chromatography [12] has been reduced to practice 60 years before two-dimensional liquid chromatography [10,11]. While only one single sample can be separated at any given time on a given column, numerous samples can be separated in the same time on a given plate, which could provide a considerably higher analytical throughput.

Although thin layer chromatography (TLC) was the first practical and for a long time the most popular method of liquid chromatography, its traditional implementation suffered from serious drawbacks: (1) capillary forces acting between the mobile phases and the dry particles of the layer cause this liquid to percolate through the bed. With passing time, however, the hydraulic resistance increases while the capillary forces remain constant and the mobile phase velocity decreases. This does not affect the retention factor nor the rate at which bands broaden [2,4,5,13]. So, the velocities of the bands decrease and, eventually, band broadening becomes faster than the differential migration rate between adjacent zones, limiting the separation performance [13,14] and (2) the thin layer is in contact with a gas phase that promotes evaporation of the mobile phase in some regions of the plate and adsorp-

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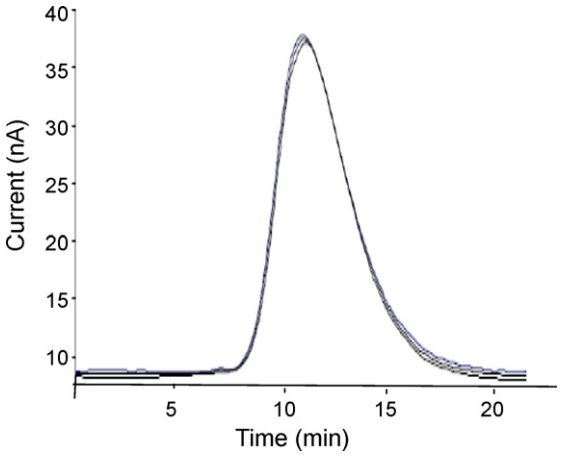

Fig. 1. Overlay of four chromatograms recorded at 2 h intervals, at the center of the exit of the flat wide column; flow rate of 1.0 mL/min.

tion in others, affecting the separation [4,13,15]. To palliate these drawbacks and those listed above overpressurized thin layer chromatography (OPTLC) methods were developed [15–17,9,18–20]. In these new approaches, the mobile phase flow rate is precisely controlled. Eventually, the design of the bed evolved toward a thin wide column operated much like traditional cylindrical columns. The bed is fully wet and there is no exchange of eluent components with a gas phase. Bands migrate at a constant velocity and resolution increases with passing time. However, on-line detection remains a serious problem.

An instrument for planar chromatography was recently described that includes a pressurized column with a rectangular cross-section (100 mm × 1 mm) and methods to access the bed (for sampling and detection), to pressurize the column, and to control the mobile phase flow rate [21]. This instrument did not incorporate an on-line detector. The purpose of this work was to design and build an array of micro-electrode sensors and to assess its potential performance for on-line detection of eluent at the exit of a pressurized flat wide column.

2. Experimental

2.1. Materials

HPLC grade water and methanol were purchased from Fisher Scientific (Fair Lawn, NJ, USA). The samples used in this study, *p*-benzoquinone, epinephrine and norepinephrine, were purchased from Aldrich (Milwaukee, WI, USA). Unless otherwise stated, the mobile phase flow rate was 0.50 mL/min. The operation of the instrument was investigated primarily with samples of 0.04 M solution of *p*-benzoquinone dissolved in the mobile phase; this solute had a retention factor of 1.75. The sample injection size was 10.0 μL. This sample was stable and the injection reproducible (see Fig. 1).

The column was packed with silica gel (average particle size, 5 μm; surface area 500 m^2/g; pore volume, 0.75 cm^3/g; average pore size, 60 Å) purchased from Sigma (Sigma–Aldrich, St. Louis, MO, USA). Calcium sulfate hemihydrate from Sigma (Sigma–Aldrich, St. Louis, MO, USA) was added to consolidate the bed during operations.

2.2. Instrumentation

The instrument, which is similar to one previously described [17,18,21] was built in-house. It incorporates some key features of instruments developed for OPTLC [15,19,20]. It differs from our earlier instrument [21] by the addition of a detector, which consists of an array of electrochemical microsensors placed across the exit slit of the mobile phase stream. Analyte signals are acquired using a CHI900B scanning electrochemical microscope (CH Instruments, Austin, TX, USA) operated in the amperometric mode. An HP1050 microprocessor-controlled standalone pump unit with full programming capabilities (Agilent Technologies, Palo Alto, CA, USA) was used to deliver the mobile phase.

2.2.1. Column housing

The instrument is made of two 15 cm × 15 cm × 2.5 cm blocs separated by a 0.24 mm Mylar sheet purchased from Fisher Scientific. Schematics of these blocs are shown in Fig. 2a and b. The system is designed to control the mobile phase stream through very thin and wide channels, for which there is no convenient valves. The top plate is made of plexiglass (Fig. 2a), the bottom one of stainless steel (Fig. 2b).

The top plate contains three chambers used for pressurizing the Mylar sheet against the lower plate [21]. Applying pressure to the column bed by pumping water into the central cavity of the top bloc (Fig. 2a) compresses strongly the Mylar sheet against the packing material contained in the central chamber of the lower bloc, prevents particles to drift downstream, and, by sealing the packing material inside its cavity, forces the mobile phase to percolate through the bed in the central cavity of the lower bloc during the whole experiment (Fig. 2b), avoiding that it by-passes the bed. The flow of mobile phase in and out of the flat wide column is controlled by applying pressure in the other two chambers (inlet and outlet chambers) of the top bloc, which function as valves. Applying pressure in these chambers compresses the Mylar sheet against the bottom plate, preventing the mobile phase from flowing through the bed. When the pressure is relieved, the Mylar sheet is pushed back into the compression chamber and the mobile phase flows freely. A Gilson pump model 302 (Fisher Scientific) was used to deliver the water needed to pressurize the column bed and to switch the mobile phase stream on and off through the valves V_{inlet}, $V_{central}$, and V_{outlet}.

The mobile phase is delivered by an HP1050 microprocessor-controlled standalone pump, into the column through an inlet groove parallel to the column edge, through the valve $V_{solvent\ inlet}$ in Fig. 2b. The packing material is contained in a 10 cm × 10 cm × 0.1 cm cavity, with two thin, long rectangular frits inserted in the steel block, at the opposite sides of this cavity, one at the inlet, the other at the outlet, to avoid that any particle of the bed move downstream. The mobile phase entering the inlet groove flows first through the inlet frit, which has a relatively low permeability (compared to those of the inlet valve and the column bed). This enables a constant pressure to be established along the groove, which ensures a uniform flow velocity across the bed. The distribution of the mobile phase velocity across the bed width and depth is comparable to that achieved in the stream of mobile phase entering a standard 4.6 mm i.d. packed column [21]. Then the mobile phase percolates through the exit frit.

2.3. Column bed preparation

The procedure for preparing the flat bed is similar to that previously described [21]. The bed cavity is entirely filled with a thick slurry of the packing material, mechanically homogenized and composed of 15.0 g of silica gel with an average particle size of 5 μm, 2.0 g of a gypsum binder (calcium sulfate hemihydrate), and 32.0 mL of water. A thick flat metal bar is slid over the bloc to eliminate the excess of slurry while making sure that the cavity is filled. After a few minutes the slurry solidifies, and left overnight to dry, and dried for 30 min in an oven at 110 °C, to activate the silica.

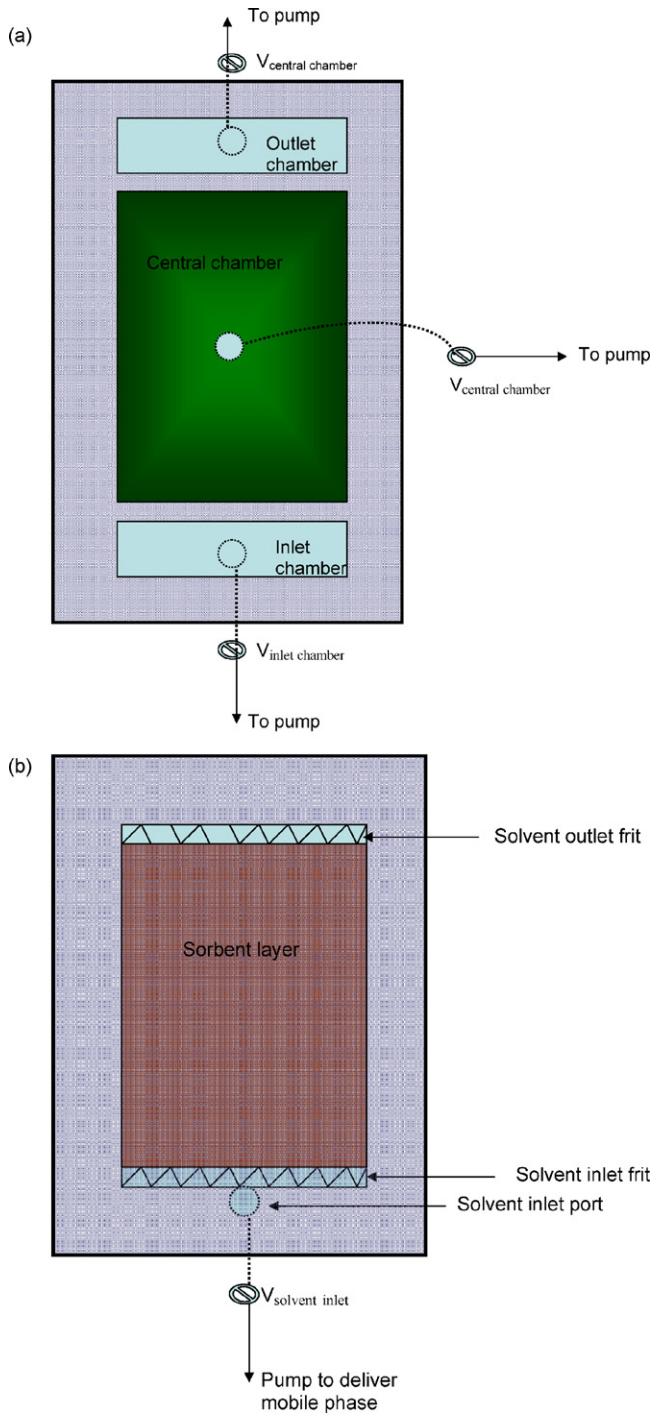

Fig. 2. (a) A 1D view of the top plate used for pressurizing the flat bed, and (b) a 1D view of the bottom plate used to contain the sorbent layer.

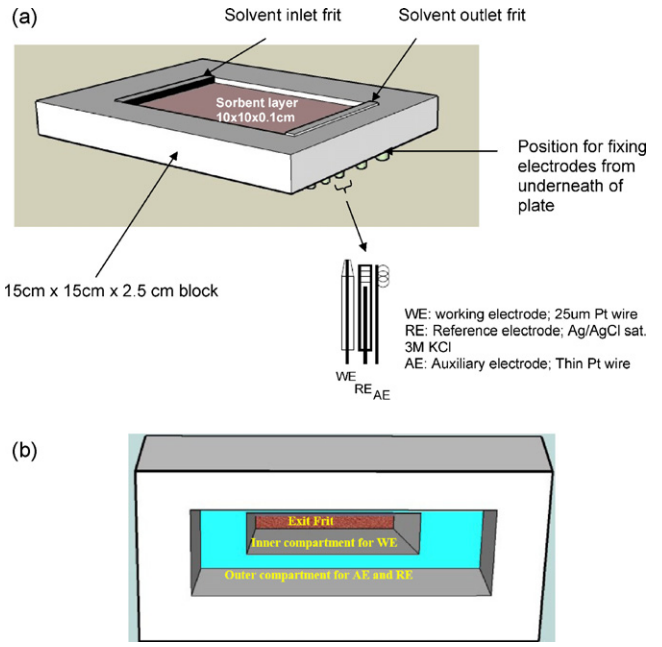

Fig. 3. A 3D cross-section view of the bottom plate showing (a) the positioning of the electrochemical microsensors, and (b) a compartment for electrode placement.

2.4. Micro-electrochemical detector setup

The electrochemical detector responds to compounds that exhibit some redox property. The electrical output results from the electron flow caused by the chemical reaction that takes place at the surface of the electrodes. Our detector cell consists of three electrodes, the working electrode (where the oxidation or reduction takes place), the auxiliary electrode and the reference electrode (which compensates for any change in the electrical conductivity of the mobile phase).

Directly after the exit frit, a narrow slit perpendicular to the column bed was machined through the bottom plate, so that the mobile phase flows out of the instrument as a liquid curtain. Five special compartments were designed to accommodate the micro-electrochemical detector. These electrodes were placed at equidistant 1, 3, 5, 7, and 9 cm from the side of the bed. A 3D cross-section view of the bottom plate showing the placement of these microsensors is shown in Fig. 3a and b. The tip of the working electrode was inserted immediately after the exit slit while the other two electrodes were placed at 2 cm downstream. As long as there is some solute present between the electrodes, a current will be maintained. This arrangement ensures that the current generated by the redox reaction involving the analyte is immediately measured as the analyte exits the flat wide column. This also eliminates the band broadening which could arise from extra column contributions.

3. Results and discussion

3.1. Operation of the instrument

The mode of operation of this instrument was previously described elsewhere [21]. The pivotal difference is that our instrument is now operated more like a conventional HPLC instrument than like a thin layer chromatography (TLC) or planar chromatography (PC) one. It is operated in the on-line mode, with the array of micro-electrochemical sensors serving as detectors. Unless otherwise stated, the sample (10.0 μL aliquot) dissolved in the mobile phase was injected through the inlet port into and through the sorbent layer. When pressure is released from the two side chambers of the top bloc, the inlet valve port opens, allowing the establishment of a stable pressure distribution across and along the column bed. The flow velocity of the mobile phase is constant in the lateral direction. At the exit of the bed, the sample components pass through the detector array, point at which they are detected (see Fig. 3). Unlike the previous instrument [21], an array of micro-electrode sensors is incorporated and is used for on-line detection of eluent at the exit of the pressurized flat wide column.

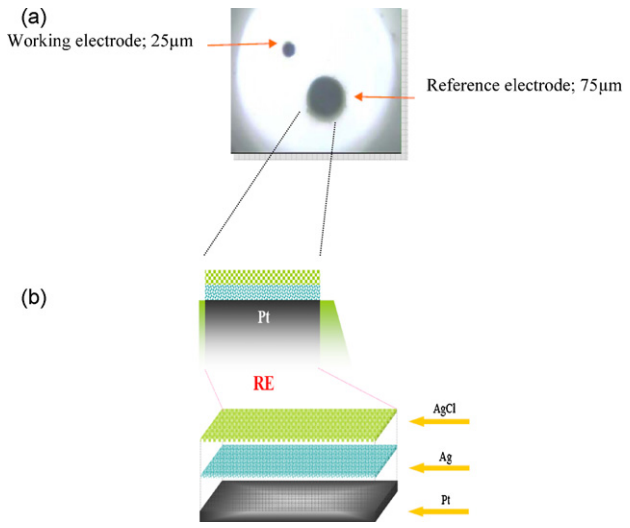

Fig. 4. (a) Optical image of the combination electrode surface platform, and (b) cross-section view of the reference electrode showing layers of Ag/AgCl deposition.

3.2. Implementation of the micro-electrochemical detector

The use of an array of micro-electrochemical detectors enables the operation of the instrument in an on-line mode, similar to that of any HPLC instrument. The working electrodes are placed at selected spots at the edge of the exit slit, so that the sample components could be detected as they exit with the mobile phase, through the slit parallel to the edge of the bed exit.

Each micro-electrochemical detector cell consists of three electrodes; a 25 μm diameter platinum wire, which is the working electrode (WE), a Ag/AgCl sat. 3 M KCl reference electrode (RE), and a thin platinum wire, which serves as an auxiliary electrode (AE) [22]. The RE and AE are placed about 2.5 mm downstream the tip of the WE. Each set of a WE and its corresponding RE and AE were carefully inserted into a specially designed cavity or compartment drilled from the underneath section of the bottom bloc, so that these micro-detector cells were fixed into position.

The three electrodes of each detector are connected to an electrochemical analyzer, and are operated in the amperometric mode. The electrochemical analyzer used in this work may acquire the signals of only two detector cells at the same time. However, we could use two such electrochemical analyzers, so we were able to operate a maximum of four detector cells simultaneously.

The procedure for fabricating the WE was described elsewhere [24,25] but an alternate fabrication procedure was used to make the micro-electrochemical detector. The dual working and the reference electrodes were combined on the same platform. To make this combined electrode, two Pt wires (a 25 and a 75 μm diameter Pt wires) are inserted each into a separate barrel of a septum theta borosilicate glass capillary to prevent direct electrical contact between the WE and the RE. For the construction of the reference electrode, a thin silver layer was deposited on the surface of a 75 μm diameter Pt wire, using an electrodepostion bath composed of 30 g/L silver chloride, 500 g/L sodium thiosulfite, and 30 g/L potassium metabisulfite at a current density of 0.5 A/dm^2 for 3 min. Then, the electrode tip is immersed in a saturated ferric chloride solution for 10 min to form a Ag/AgCl layer which will serve as the reference electrode. Fig. 4a shows the optical image of this combination electrode at the point of completion. The optical image was acquired using a stereomaster zoom light microscope (Fisher Scientific, Fair Lawn, NJ, USA). Fig. 4b is the cross-section view of the reference electrode showing the layers of deposition.

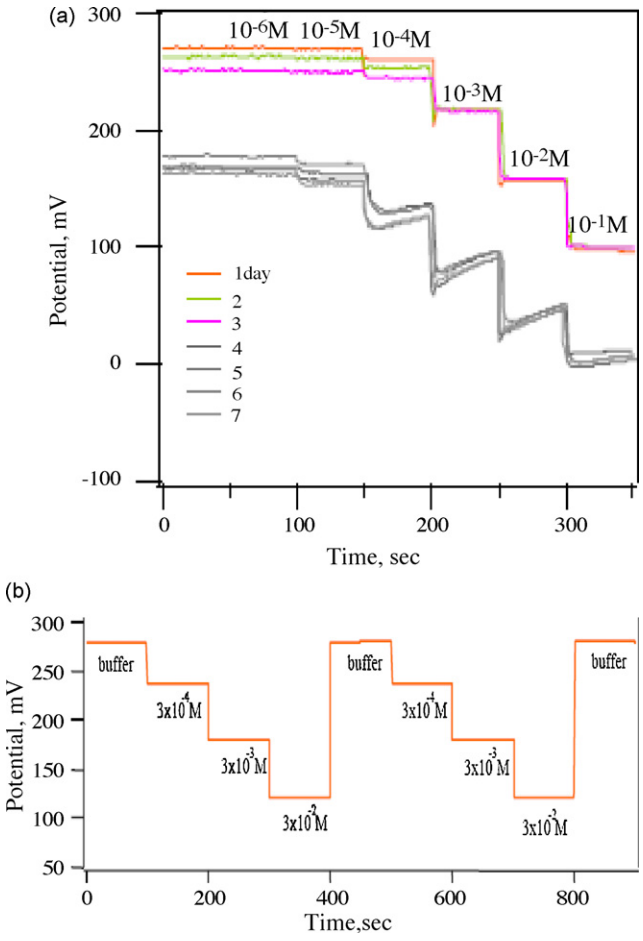

Fig. 5. Chloride response using the combination electrode showing (a) the useful lifetime, and (b) its reproducibility. Background electrolyte is a 0.05 M Tris–H_2SO_4, pH 7.4 buffer.

The useful lifetime of the combination Ag/AgCl electrode is shown in Fig. 5a; and it was found to be 3 days. During this time, the electrode exhibited a highly linear response (r^2 = 0.996) to Cl^- concentrations between 1.0×10^{-4} and 1.0×10^{-1} M, with a slope of 55.2 mV/pCl^- in a background electrolyte of 0.05 M Tris–H_2SO_4, pH 7.4 buffer. As shown in Fig. 5b, the combination reference electrode showed excellent reproducibility and a high response (a signal-to-noise ratio of 95 was obtained) equivalent to that of a commercial Ag/AgCl sat. 3 M KCl reference electrode. The homemade combination electrode can easily be renewed by gently polishing the surface on a filter paper impregnated with methanol, then re-depositing the Ag/AgCl layer.

The main drawback for the combination electrode was its short life time. On the other hand, commercial Ag/AgCl sat. 3 M KCl reference electrode exhibits long term stability, and would thus be the obvious choice for all data acquisition in this work. The homemade combination electrode was fabricated and tested in an effort to miniaturize the detector cell as it may become even more necessary in future instrument designs. The surface diameter of the combined electrode ranges from 300 to 500 μm, depending on the extent of polishing of the glass around the active electrode surfaces, whereas the diameter of commercial reference electrode alone is 4.5 mm.

A potential of −0.30 V vs Ag/AgCl was applied to the WE for the detection of the analyte. Before the first injection of the analyte in an experiment, a sufficient time (typically ∼3–5 min) was allowed for a stable background signal to be achieved. Once a stable background signal is achieved, however, it is not necessary to wait for some time

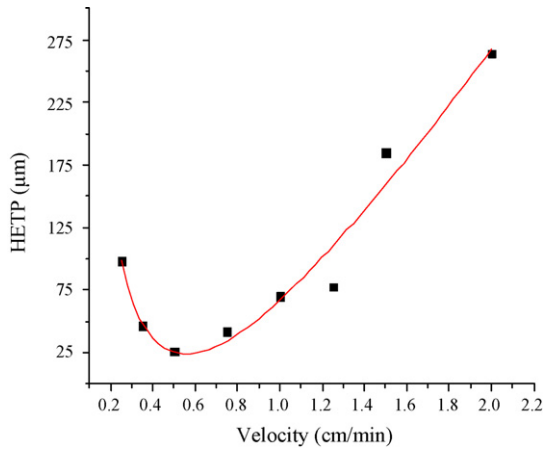

Fig. 6. Average HETP plots simultaneously recorded by four micro-electrochemical detectors.

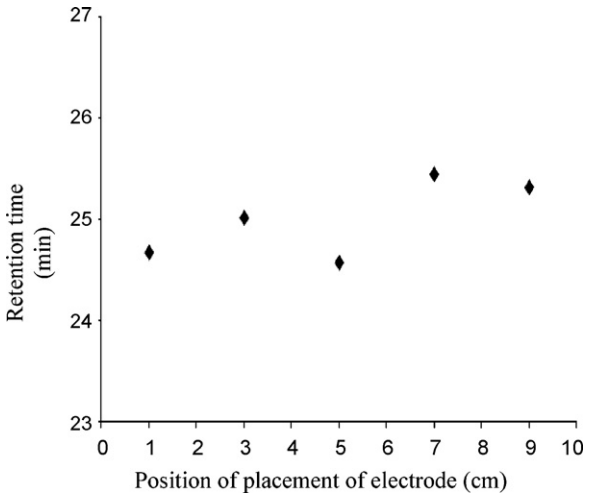

Fig. 7. Distribution of retention time for *p*-benzoquinone across the exit of the flat column bed.

before injecting another sample. The sample solution was stable throughout the whole series of measurements. The signal was also highly reproducible during the entire experiments. For example, Fig. 1 shows the overlay of four chromatograms, each one recorded at a 2 h interval from the previous and following ones. All chromatograms were recorded at the center of the exit of the flat wide column, using the central one of the five micro-electrochemical detectors, at a flow rate of 1.0 mL/min. All four chromatograms are well overlaid. The same results are achieved when chromatograms from different sets of experiments are overlaid showing that the instrument is stable and that the detector signal is reproducible.

3.3. Efficiency of the column bed

Similar definitions apply to the efficiencies of columns and TLC plates [23], assessing in the present case their capacity to maintain narrow and short zones and to restrain the axial and lateral dispersions of a solute migrating along the plate/column. Plate height equations that explicitly describe axial and lateral dispersions in TLC have not have been developed [23]. Since our setup allows a precise adjustment of the mobile phase velocity and the recording of the profiles of elution peaks, it makes possible to assess the efficiency of thin wide columns in much the same way as it is conventionally done in HPLC.

For the sake of simplicity and although the recorded peaks tail slightly (see Fig. 1), the efficiency was assumed to be given by $N = 5.54(t_R/w_{1/2})^2$, with N being the number of theoretical plates of the column, t_R the retention time of the band, and $w_{1/2}$, its width at half height. The height equivalent to a theoretical plate (HETP) is given by $H = L/N$; with L the column length. Fig. 6 shows a plot of the average HETP obtained as a function of the mobile phase velocity. Each data point is the average of the value obtained for the four detectors used. The mobile phase flow velocity was gradually increased from 0.25 to 2 cm/min, and the highest efficiency was obtained at a velocity of 0.50 cm/min. At the flow rate of maximum efficiency, the lateral variation of efficiency of the plate was obtained and was found to be fairly constant with a random fluctuation of less than about 12%. While this is significant, it is less than similar variations recorded for some analytical columns used in HPLC [24].

3.4. Homogeneity of the column bed

A key property of the bed of any column, whatever its shape and dimensions, is the degree of its radial and lateral heterogeneities. Because the bed of a wide, thin column is thinner and much broader than the diameter of conventional column beds, the homogeneity of the flat wide beds that we need must be carefully monitored.

It is necessary to prepare column beds through which the flow velocity of the mobile phase is uniformly distributed, so that the front of a breakthrough band would be flat. This makes it easy to calculate and compare the retention factors of different compounds. Because our instrument uses localized micro-electrochemical detectors, it is easy to measure and compare the lateral variations of the mobile phase velocities. Fig. 7 shows the lateral distribution of the retention times of *p*-benzoquinone across the exit of the flat column bed. This distribution of the retention times across the bed is not flat, but with a random fluctuation of less than about 4%, it is comparable to the results obtained for the relative amplitude of the radial velocity distribution observed for some typical analytical columns used in HPLC [24,25].

3.5. Further evaluation of performance of the on-line micro-electrode detector

To further test the performance of the electrode array as a detector, the determination of the neurotransmitters epinephrine ($K' = 2.05$) and norepinephrine ($K' = 2.14$) (both from Fisher Scientific, Fair Lawn, NJ, USA) was investigated at room temperature. The hydrodynamic voltammograms and the combined chromatograms for these compounds with isocratic elution of 30 pg separate injection of each sample are shown in Fig. 8. These compounds are often used to study the performance of electrochemical detectors, since the determination of neurotransmitters is one of the major applications of these detectors in HPLC [26]. The detector array was again operated in the amperometric mode and the current responses of the electrodes at the maximum of the chromatoamperometric curves for both compounds were investigated. A flow rate of 0.5 mL/min was used to elute the compounds in the mobile phase (composed of 5.0 g/L of NaCl, 3.0 g/L acetic acid and 0.25 g/L of ethylendiaminetetraacetic acid in 80% methanol and 20% water). Based on the hydrodynamic voltammograms, an applied voltage of +0.75 vs Ag/AgCl was selected and used to investigate the dynamic range of the detectors. All detectors gave a linear response between 4.0 pg and 20.0 ng for both compounds injected separately. Using the average value for the five detectors, the limit of detection in both cases was ~2.0 pg. The reproducibility of the detectors was checked by using eight successive 20.0 pg injection of both compounds, and the coefficients of variation were found to be 2.4% and 2.0% for norepinephrine and epinephrine respectively.

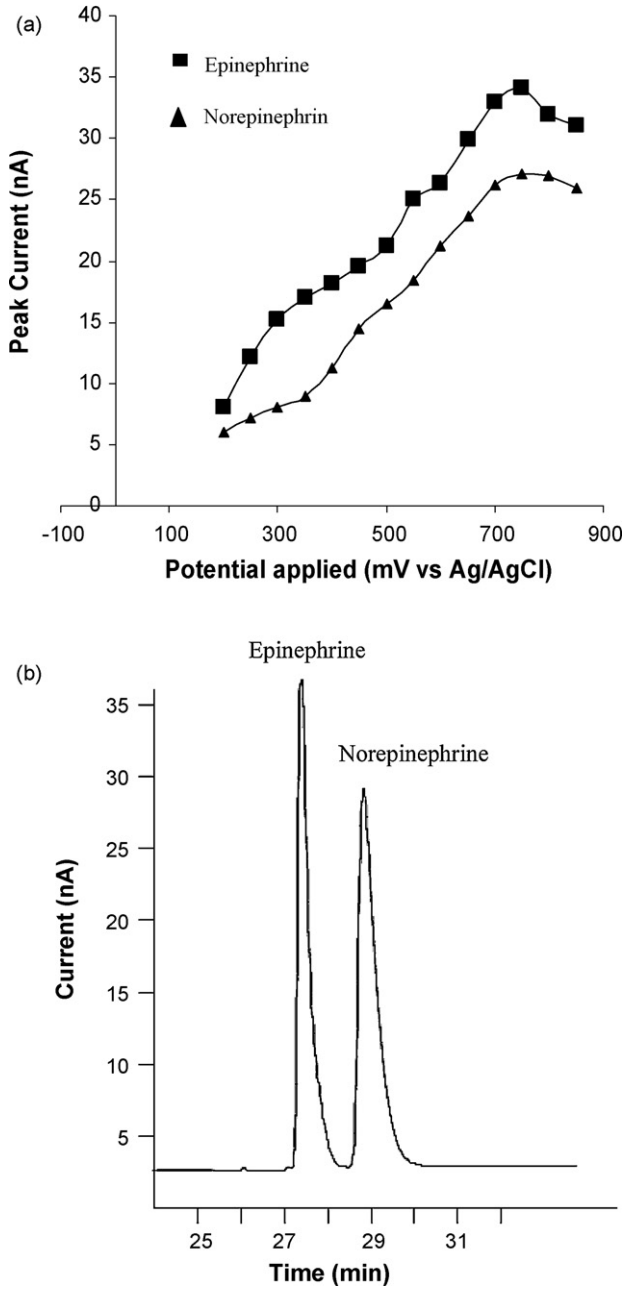

Fig. 8. Hydrodynamic voltammograms for epinephrine and norepinephrine with isocratic elution (a) and combined chromatograms of both analytes recorded at +750 mV vs Ag/AgCl (b). 30 pg of each sample were injected (for other conditions, see Section 3.5).

4. Conclusion

In traditional analyses made by TLC or OPTLC, the detection of the sample components is made by scanning the bed using a spectrophotometer. The sample components are adsorbed in the porous particles with which the layer is made and light is scattered by these particles, making the relationship between the component concentrations and the signals detected quite complex. An approach to detection that is far more practical than scanning the plate consists in eluting the separated components from the flat wide column and in detecting them in an on-line mode, using an array of detectors, as exemplified in this work. The mobile phase flows out of the instrument through a slit parallel to the bed exit edge, as a thin liquid curtain. The detectors are placed at this edge, across the mobile phase stream.

The instrument developed enables an easy and accurate monitoring of the performance of the bed, its lateral homogeneity and the quality of the bed packing. This quality was assessed by measuring the retention times of the same analyte (*p*-benzoquinone) at different locations and calculating the relative percent difference in retention times. The column bed was found to be fairly homogeneous and to compare quite reasonably to those of columns used in HPLC [24,25].

The availability of a suitable detector for flat wide columns would allow the development of new, improved methods of implementing two-dimensional chromatography. Means have been suggested to perform a first dimension separation and spread the sample components along an edge of a plate/column similar to the one described here [21].

Because the detectors used in this work are electrochemical in nature and as is the case with any electrochemical detector, only compounds that are capable of conducting an electrical current, can be dissociated into ions or those that can undergo redox reactions under appropriate conditions can be detected. Although their number is large, this limits the applicability of the method. Research toward the development of a UV diode-array detector that would permit the monitoring of the UV absorbance of the effluent stream using a monochromatic UV beam is ongoing.

Acknowledgments

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