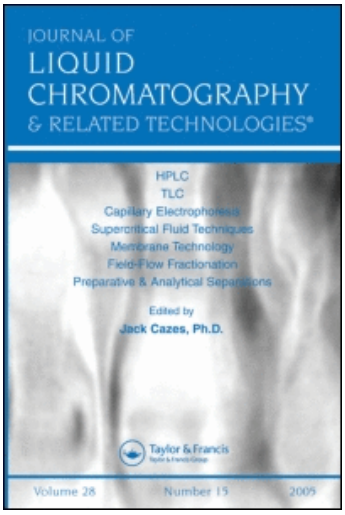

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IN-COLUMN AND POST-COLUMN PHOTOCHEMICAL FLUORESCENCE ENHANCEMENT IN PACKED CAPILLARY LIQUID CHROMATOGRAPHY

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ABSTRACT

A photochemical reaction detection system coupled with a 320-μm I.D. packed fused-silica HPLC analytical column is described. This reaction detection system does not require the addition of reagent, hence it is suitable for combination with a miniaturized analytical column. As a model system, the separation and detection of some dansylated chlorophenols was chosen.
In the photochemical reactor, the main reaction product is 5-dimethylaminonaphthalene-1-sulphonic acid (dansyl-OH), which is determined by fluorescence monitoring. Both the photochemical reaction after the column and in the end part of the column ('in-column') were investigated and a comparison between the two approaches was made. In both cases, band broadening due to the photochemical reaction detection system was less than 0.1 μl.
In-column detection of the photochemical reaction product was investigated in order to study the possibility of carrying out separation, derivatization and detection in the analytical column. An increase in signal-to-noise ratio of more than an order of magnitude was obtained when compared with detection after the column.

INTRODUCTION

In the past years, next to conventional scale (3-5 mm I.D.) analytical columns, columns with a smaller inner diameter have gained some popularity in high-performance liquid chromatography (HPLC). Two main types of these miniaturized columns can be distinguished: (i) packed stainless-steel or fused-silica columns and (II) open tubular columns. Several books and review papers covering the field of miniaturized HPLC have appeared in the past years [1-5]. Distinct

advantages of miniaturized columns include the saving in solvents and packing material, the small sample volumes and the possibility of splitless combination with detection modes which require low flow-rates, e.g., the mass spectrometer [6], flame-based detectors [7] or an electron capture detector when using partly aqueous eluents [8].

Low-volume detection devices, tuned to the volumes of the peaks, must be used in miniaturized HPLC. It is generally known that with conventional optical detectors such as the UV-VIS and fluorescence detector, this reduction of cell volume causes a decrease in sensitivity. One way to compensate, at least partly, for this drawback is the use of post-column reaction detection, although this is not widely used in miniaturized HPLC up to now [9].

Another interesting development in the field of optical detection is the use of in-column detection. For in-column detection, at the end of the fused-silica column just upstream from the frit, a detection cell is created by removing the polyimide coating. In-column detection will usually yield higher sensitivity since now, more concentrated peaks are detected [10-12]. The sensitivity gain will be discussed in the present paper.

The present paper describes the work done to explore the possibility of using in-column detection as part of a reaction detection system. The photochemical decomposition of dansylated chlorophenols as described by De Ruiter et al. [13] for conventional scale HPLC was chosen as the model reaction. This photochemical reaction was carried out in the post-column mode and also directly in the packed 320 μm I.D. fused-silica analytical column, just upstream from the frit, i.e., as an in-column instead of a post-column reaction. Hence, the possibility of carrying out the separation, the photochemical reaction and the detection in the analytical column, thus avoiding any connection and merging column, reactor and detector into one system will be discussed. Factors influencing band broadening when a reaction is carried out on a solid phase [14] will be discussed.

More generally, the present work is intended as a pilot study for the combination of in-column reactions with relatively fast kinetics and in-column detection. These reactions include those originating from loop injected reagent plugs, analoguous to the solvent plugs injected in the transient mobile phase approach [15]. A further extension of in-column detection may be laser scanning along the entire (transparent) column, so that the chromatogram can be monitored at any place along the column, i.e., at any point during separation ('whole column detection' [16]).

EXPERIMENTAL

Chemicals

HPLC-grade water was produced in a Milli-Q system (Millipore, Bedford, MA, U.S.A.) and was used throughout this work. The eluent was made by mixing a 25 mM aqueous sodium phosphate buffer (pH 7.0) and HPLC-grade methanol in a 20:80 (v/v) ratio, and was degassed in vacuo in an ultrasonic bath prior to use. 2,3,4-Trichlorophenol and 2,3,5,6-tetrachlorophenol (pro analysi quality) were obtained from Aldrich (Beerse, Belgium); pentachlorophenol (pro analysi) and *dansyl chloride* (98%) were obtained from Merck (Darmstadt, F.R.G.). All other chemicals and solvents were of pro analysi quality.

For the preparation of the dansyl derivatives, 100 µl of a chlorophenol solution ($3.3 \cdot 10^{-3}$ M in acetone) were mixed with 900 µl of a dansyl chloride solution ($3.7 \cdot 10^{-3}$ M in acetone) and 100 µl of a sodium carbonate solution (0.1 M in water) in a screw-cap reaction vial. This mixture was allowed to react for 30 min at 50°C. A 200-µl aliquot was spotted on a 5 cm long amino-bonded phase TLC plate (Merck, Darmstadt, F.R.G.) and was eluted with chloroform till the top edge of the plate was reached by the solvent. After drying, the unretained fraction following the solvent front was scraped off and dissolved in 300 µl of methanol-water (70:30,m v/v). 600-nl aliquots (corresponding to ca. 100 pmol chlorophenol) were injected in the HPLC system.

Apparatus

A schematic diagram of the experimental set-up is given in Fig. 1. The mobile phase pump was a syringe pump with 5 ml capacity (LDC Milton Roy, Riviera Beach, FL, U.S.A.). The injection valve with an internal loop of 600 nl was laboratory-built. The 182 mm long analytical column was made from 320-µm I.D. fused silica (Scientific Glass Engineering, Melbourne, Australia). The frit was made from PTFE and prepared as follows: A porous PTFE frit was soaked in xylene for 24 h. This was followed by soaking in molten paraffin and subsequent storage at 50°C for 24 h. After cooling, 30-µm cuts were made. Then, the paraffin was removed with toluene in an ultrasonic bath. The frit was inserted into the column by turning the latter with its sharp-cut end on the PTFE-cut. The 75-µm I.D. fused-silica (Scientific Glass Engineering) exit tubing which supports the frit was glued to the column using an epoxy resin as shown in the insert in Fig. 1.

The column was packed with a slurry of 50-µm Spherisorb ODS-1 (Phase-Sep, Queensferry, U.K.) in 0.25 ml of methanol using methanol at 300 bar as packing solvent and was directly connected to the injection valve using a laboratory-built Kel-F nut-ferrule combination. In the column and in the exit tubing, windows were made by removing the polyimide coating (see Fig. 1). This was done by using a small flame for the exit tubing (windows 2 and 3). For the column (wondow 1), this procedure was found to destroy the packing, hence hot concentrated sulphuric acid was used instead.

In the experiments, two systems were compared. In the first system window 2, immediately downstream from the analytical column, was used for UV irradiation and window 3 for detection. This is the system used for post-column photochemical reaction. The other system, developed for in-column photochemical reaction was the same as the above system, except that window 1 instead of window 2 was used for UV irradiation. UV irradiation was done on either window 1 or window 2 with a mercury lamp (93110 E; Philips, Eindhoven, The Netherlands), the irradiated length being 15 mm with window 1 and 22 mm with window 2. Fluorescence was detected with a Fluorichrom (Varian, Walnut Creek, CA, U.S.A.) filter fluorimeter with the original conventional-scale flow cell replaced by the fused-silica tubing with removed coating (window 3). The uncoated tubing was illuminated over a 5-mm length, which resulted in a 0.02-µl geometrical cell volume.

For some experiments, no UV irradiation was carried out, and window 1 was used for fluorescence detection (5 mm illumination length, in-column detection). Filters suitable for the detection of dansyl compounds (λ_{ex} = 330 nm and λ_{em} = 470-500 nm [13]) were used. All

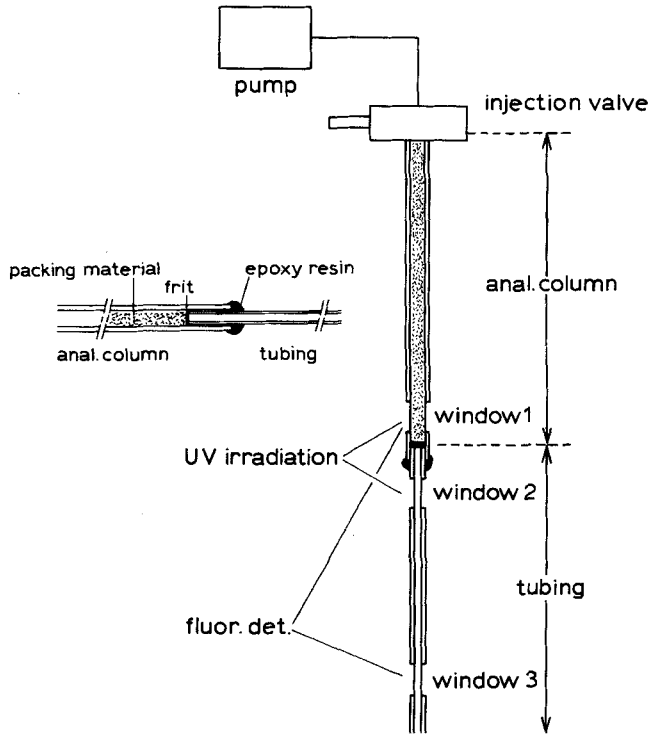

FIGURE I. Schematic diagram of the experimental set-up. For details, see text. Insert: detail of the connection of the analytical column and exit capillary.

chromatograms were registered by a BD-40 strip-chart recorder (Kipp & Zonen, Delft, The Netherlands).

RESULTS AND DISCUSSION

Post-Column Versus in In-Column Photochemical Reaction

Three chlorophenols, i.e., 2,3,4-trichlorophenol, 2,3,5,6-tetrachlorophenol and pentachlorophenol, were off-line dansylated as described in the experimental section and mixtures of the three compounds were injected in the HPLC system using either post-column or in-column photochemical reaction conditions. The injected amount was ca. 100 pmol for each of the chlorophenols. Different flow-rates were used to vary the irradiation time. Typical chromatograms obtained at a flow-rate of 1 µl/min are given in Fig. 2a for post-column and in Fig. 2b for

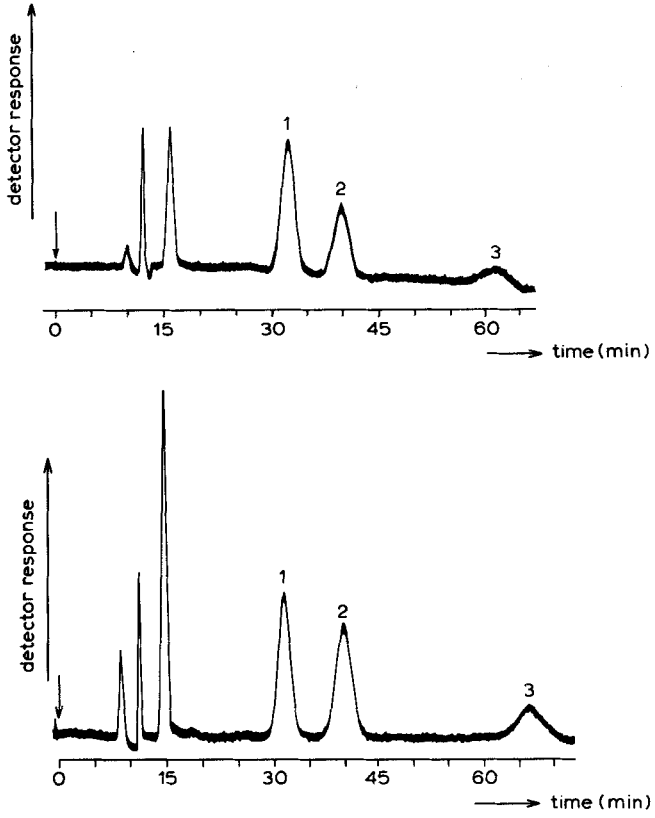

FIGURE II. Chromatogram of three dansylated chlorophenols with (a) post-column photochemical reaction and (b) in-column photochemical reaction. 1: 2,3,4-trichlorophenol, 2: 2,3,5,6-tetrachlorophenol, 3: pentachlorophenol, ca. 100 pmol each. Conditions: Flow-rate of mobile phase (methanol-25 mM sodium phosphate buffer, pH 7.0, 80:20, v/v), 1 µl/min; column 182 mm x 320 µm I.D. fused-silica, packed with 5-µm Spherisorb ODS-1; fluorescence detection at 330 (ex) and 470-500 (em) nm; same detector sensitivity in a and b. Other experimental details are given in the text.

in-column photochemical reaction. In both cases, the peaks for the chlorophenols disappeared from the chromatogram when the UV irradiation was switched off, which is in accordance with the observations reported by De Ruiter et al. [13].

Data on peak areas (height multiplied by width in volume units) at different flow-rates are collected in Table I. From this table, it can be concluded that in the post-column irradiation mode, an irradiation time of ca. 3 s yields the highest peak areas, which agrees well with results published before [13]. The situation was different when in-column photochemical reaction was carried out. The dependence of peak area on flow-rate was less pronounced than with post-column reaction. One should note that in this mode, irradiation times are influenced by both the flow-rate and the capacity factors (see also foot note in Table I) of the dansylated chlorophenols and are all much larger than with post-column reaction. Because of the relatively small dependence of peak area on flow-rate it is not possible to clearly determine an optimal irradiation time for in-column reaction. However, with one exception, all the experiments on in-column reaction yielded peak areas higher than those obtained at the optimal irradiation time of ca. 3 s for post-column irradiation. The irradiation times in the two different approaches are not directly comparable. In the case of

TABLE I

Irradiation Times and Peak Areas of Dansylated Chlorophenols at Various Flow-Rates

Conditions as in Fig. 2.
t_{irr} = Irradiation time, PA = peak area (arbitrary units)

	Flow-rate, 1 µl/min		Flow-rate, 2 µl/min		Flow-rate, 3 µl/min		Flow-rate, 4 µl/min	
	post-column reaction	in-column reaction	post-column reaction	in-column reaction	post-column reaction	in-column reaction	post-column reaction	in-column reaction
	t_{irr}* PA	t_{irr}** PA	t_{irr}* PA	t_{irr}** PA	t_{irr}* PA	t_{irr}** PA	t_{irr}* PA	t_{irr}** PA
Compound	(s)	(s)	(s)	(s)	(s)	(s)	(s)	(s)
2,3,4-Trichloro-phenol	5.9 72	167 76	3.0 89	84 95	2.0 63	56 100	1.5 45	42 100
2,3,5,6-Tetra-chlorophenol	5.9 43	210 74	3.0 56	105 92	2.0 46	70 100	1.5 38	53 92
Pentachloro-phenol	5.9 23	361 40	3.0 27	181 38	2.0 32	120 40	1.5 22	90 47

*t_{irr} = volume of window 2/flow-rate

**t_{irr} = retention time · length of window 1/length of column upstream from window 1

in-column photochemical decomposition, the reaction takes place in a packed reactor in which it is unlikely that the light penetrates completely. Another important point is that part of the molecules to be reacted are adsorbed on the stationary phase, which may have an influence on reaction kinetics.

A direct comparison between peak areas obtained with post-column and in-column photochemical reaction at each of the flow-rates used shows the in-column approach to yield larger peak areas at equal noise level (see Fig. 2). The highest peaks in both approaches were found when the flow-rate was 1 µl/min (data not included in Table I). The reason for this was that the plate height (obtained for 2,3,4-trichlorophenol) with our relatively low efficiency column increased from 130 to 350 µm when the flow-rate was increased from 1 to 4 µl/min. With a freshly packed column, a plate height increase of 25 to 70 µm was found when going from 1 to 4 µl/min. A steep increase in plate height upon increasing the flow-rate was reported before for packed capillary columns [10].

Band Broadening

When comparing band broadening in each of the two approaches, no significant differences were observed (cf. Figs. 2a and b). The influence of extra-column effects, including the photochemical reaction, on total band broadening was determined by plotting the squared total band broadening in time units against the squared retention time of the dansylated chlorophenols on the column. Band broadening due to extra-column effects, found by extrapolating the curve to a retention time of zero, was less than 0.1 µl when expressed in volume units (σ_V) in both systems. This means that, even at the low-volume scale of packed capillary liquid chromatography, reaction detection is possible without deteriorating the separation.

Since in the in-column photochemical reaction system part of the reactant (i.e., the dansylated chlorophenol) is adsorbed on the stationary phase, additional band broadening due to the reaction (reaction band broadening) may occur [14]. In the present study, band broadening in both set-ups in essentially negligible (cf. above); i.e., reaction band broadening does not play a role.

For the in-column reaction it is important that the main reaction product, dansyl-OH, was not retained by the column since it is in the anionic form at the pH of the mobile phase used, i.e., pH 7.0 [13], so that the band broadening between the point of formation, i.e., window 1, and the detector cell is minimal.

In-Column Detection of Dansyl-OH

In order to study the possibility of in-column fluorescence detection of dansyl-OH which is the main product of the photochemical reaction, the HPLC column was connected with the detector such that window 1 (cf. Fig. 1) was in the excitation light beam permitting in-column detection. Dansyl-OH was prepared by reacting an excess of dansyl chloride with an aqueous sodium hydroxide solution of known concentration. An aliquot of the reaction mixture was injected and the peak height measured. This was compared with the peak height obtained when the reaction mixture was injected in a system with detection in the conventional way, i.e., via window 3, and the UV irradiation switched off. An increase in signal intensity by a factor of 20 and an increase in noise by

a factor of 1.5 were found when in-column detection of dansyl-OH was made via window 1, i.e., an increase in signal-to-noise ratio of 13. Band broadening, σ_V, was the same, viz. 0.17 µl, in both approaches. This demonstrates that the increase in signal-to-noise ratio was not obtained at the expense of chromatographic resolution.

In general, several factors can influence the signal when in-column detection is used: (i) the fluorescence signal from the analyte is measured in a more concentrated peak; this should, theoretically, provide an increase by a factor (k' + 1) [10-12]; (ii) the fluorescence quantum yield can change upon adsorption [12,17,18]; (iii) the fluorescence wavelengths can be shifted upon adsorption [18] and (iv) the cell geometry (diameter) and the cell as such (packed vs. open) differs in the two approaches. In our system, dansyl-OH was found not to be retained by the column, as was mentioned above. As a result, the factors i, ii and iii should have no influence on the fluorescence intensity, and the increase recorded should be attributed to factor iv.

CONCLUSIONS

The study reveals that in-column photochemical reaction yields good results when compared with results obtained in the post-column irradiation mode. It is also important to emphasize that, although the extra-column volume should be kept as low as possible in miniaturized HPLC (≤ 1 mm I.D. analytical column), reaction detection at this small scale is possible as has also been found by Van Vliet et al. [19] for even smaller (25 µm I.D.) columns.

If, as with the present model system, the conditions apply that (i) the in-column photochemical reaction is rapid and as least as efficient as the post-column photochemical reaction, (ii) the photochemical reaction product is not retained under the HPLC conditions used and (iii) the in-column detection of this product yields an over 10-fold increase in signal-to-noise ratio, one can predict that combined separation, photochemical reaction and detection on the analytical column will give good results. In such an approach, two windows must be made on the column, one for the photochemical reaction and one for detection; no connections and diameter changes, well known sources of band broadening, will be needed.

In order to construct a compact analytical system and to improve the low-ng but ppm detection limits, lasers seem to be attractive as the light source for photochemical reaction and for detection because of their high intensity and their easy optical manipulation [20]. This will be a research project for the near future.

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