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Improved ultraviolet detection in high-temperature open-tubular liquid chromatography

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ABSTRACT

Different ways to increase the sensitivity of UV detection in high-temperature open-tubular liquid chromatography are addressed. A micro flow (Z-shaped) cell was evaluated for the possibility of enhancing the signal-to-noise ratio in high-temperature open-tubular liquid chromatography. For the same chromatographic technique, a novel approach was taken with on-column detection. Optical fibre bundles were employed, yielding a significant increase in sensitivity with only a small decrease in efficiency. This approach is also feasible for other micro-separation techniques, *e.g.*, capillary electrophoresis. Sample preconcentration and a gradient elution are also shown to be a powerful means of increasing detection limits in high-temperature open-tubular liquid chromatography.

INTRODUCTION

High-temperature open-tubular liquid chromatography (HT-OT-LC) suffers from the same problems as all the other micro-separation techniques *viz.*, a lack of sensitivity owing to reduced column loadability and limited light path length when on-column UV detection is employed. Although, fluorescence and electrochemical detection are feasible detection modes in OT-LC [1–4], UV detection is still preferred.

Whenever high efficiency is required, on-column UV detection is used [5–7]. On-column detection reduces the contribution of the detector cell volume to the peak band broadening. However, owing to the limited light path length, with capillary columns with I.D. < 75 μm, the sensitivity of on-column UV detection is poor. The development of the small-volume Z-shaped micro flow cell [8–10] was a significant contribution to UV detection in micro separation techniques. In HT-OT-LC, detection has to be performed at the same temperature as the separation in order to avoid the cold spots, which have an adverse effect on the chromatographic efficiency [7,11] and may cause sample precipitation. This problem can be solved by placing the detector cell together with optical fibres in the column oven. On-column UV detection using either a single optical fibre or a bundle of optical fibres, was assessed for its usefulness in HT-OT-LC. Further, the Z-shaped micro flow cell was also tested for its applicability in HT-OT-LC. A sample focusing technique and gradient elution were tried as means of increasing detection in HT-OT-LC.

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EXPERIMENTAL

The instrumental set-up is shown in Fig. 1. A high-temperature liquid chromatograph (consisting of two Model 420 pumps, a Model 460 autosampler and a Model 450 PC/AT data station was obtained from Kontron (Zurich, Switzerland). To generate a micro flow through the capillary column, a split T-piece [Valco, 1/16 in. × 0.01 in. bore (1 in. = 2.54 cm)] was used in combination with a relief valve (R3A series; Swagelok, Willoughby, OH, USA). Open-tubular capillary columns were obtained

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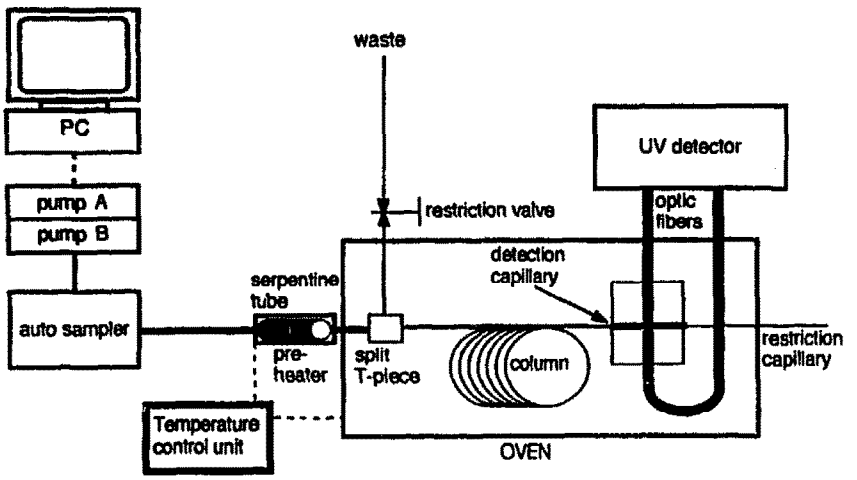

Fig. 1. High-temperature chromatograph.

from Lee Scientific (Salt Lake City, UT, USA) and Macherey–Nagel (Oensingen, Switzerland). A serpentine tube (400 mm × 0.2 mm I.D.) from Scientific Systems (State College, PA, USA) was fitted between the injection valve and the T-splitter in a separate heating body. The preheating temperature was usually set 5–10°C higher than the column temperature, enabling eluents to reach the column temperature before entering the separation column. To maintain the mobile phase in the liquid state, for temperatures above the boiling point of the eluent, a pressure restriction was necessary. The pressure restriction at the end of the analytical column was achieved by using a fused-silica tube (2 m × 18.5 μm I.D.). The split T-piece, the capillary column and the detector cell were placed in an oven. The oven and the solvent preheating unit were controlled by a temperature regulator from Zahner Electronic (Kaltbrunn, Switzerland). A Spectroflow 783 UV detector (Kratos, Ramsey, NJ, USA) was modified to allow the use of optical fibres and thus could provide on-column detection in the column oven itself. The original detector flow cell was removed from the detector. One end of the optical fibre (or the round end with the bundle of fibres) was placed in front of the monochromator to direct the light to the capillary, which was placed in the holder depicted in Fig. 2. The equivalent fibre was placed across the capillary to collect light transmitted through the capillary and guide the light to the photomultiplier. The bundles of fibres, with a round-end I.D. of 1.57 mm and a line-end bundle length and width of 8.8 and 0.23 mm, respectively, were obtained from Luxmatic (Baar, Switzerland). A schematic diagram of the detection set-up, with the bundle of fibres, is shown in Fig. 2. The single optical fibres, with core size 200 μm, were also from Luxmatic. The Z-shaped flow cell (10 mm × 25 μm) was purchased from LC Packings (Amsterdam, Netherlands) and was installed in a Model 432 UV detector (Kontron), between the column outlet and the flow restriction fused-silica tube.

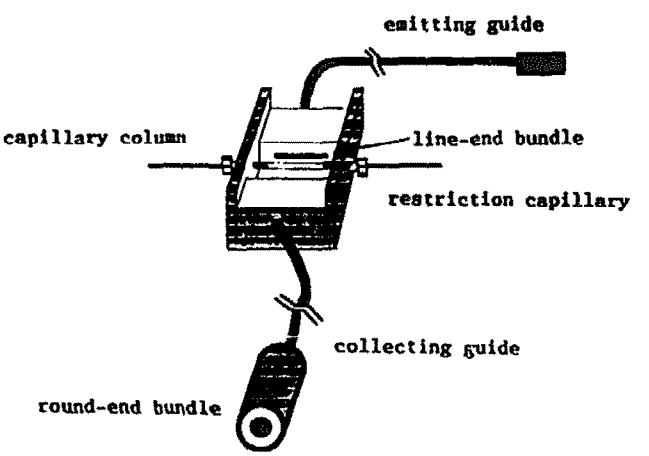

Fig. 2. Set-up for on-column detection with the bundle of optical fibres.

All solvents were of HPLC grade from Rathburn (Walkerburn, UK). Cyclosporin A was obtained from Sandoz Pharma (Basle, Switzerland). All other chemicals were from Fluka (Buchs, Switzerland).

RESULTS AND DISCUSSION

Sample focusing and gradient elution

Various methods were tested in order to find the most suitable way to increase detectability and at the same time maintain a high system efficiency. One way to improve the detection limit of a minor sample component, without losing efficiency, is to focus the sample on the top of the column. This can be done according to the same principle as in conventional reversed-phase column liquid chromatography. Sample injection (sample loading) is performed with a weak mobile phase, so that the sample is strongly retarded on the top of the column. When this procedure is followed by a gradient elution, a significant improvement in detectability can be accomplished.

Generally, cyclosporins (cyclic peptides) lack a chromophore that yields a low signal in the UV absorption range. In order to overcome this problem, the amount of substance injected has to be increased. To minimize the contribution of the injection volume to the total peak broadening, especially for the early-eluting peaks [7], and thus maintain a high system efficiency, the sample focusing technique is preferred to the injection of the large volume in one stroke. A solution of cyclosporin A in methanol (1.25 mg/ml) was prepared. The sample solution was injected (injection volume 80 μl) and split (splitting ratio = 1:750) before entering the analytical column. Because cyclosporins have a pronounced hydrophobic character, sample loading of cyclosporin A (several consecutive injections) was performed utilizing a mobile phase that had a low solvent strength. When the injections were completed, the solute was eluted under gradient conditions. Signals generated with on-column detection, using single optical fibres, for (A) ten and (B) five consecutive injections per minute are presented in Fig. 3. Sample loading was performed under isocratic conditions [acetonitrile–water (15:85)] followed by the gradient run (from 15% to 50% acetonitrile in water in 14 min). Doubling the peak area counts was accomplished without a decrease in efficiency.

A solvent gradient is a useful method for optimizing the resolution and analysis time and at the same time achieve a higher relative peak sensitivity (reciprocal of peak width) in liquid chromatography. An increase in the mobile phase strength during the separation greatly decreases the retention time and increases the relative peak sensitivity. There are several mechanisms responsible for sample retention in reversed-phase HPLC (hydrophobic interactions, adsorption with residual silanols and solute solubility in the mobile phase) [12]. The equilibria of the processes indicated are altered when the gradient run is performed. Further, solute mass transfer in the mobile and stationary phases (kinetic processes) is also changed in the gradient mode. Combined thermodynamic and kinetic effects in the gradient

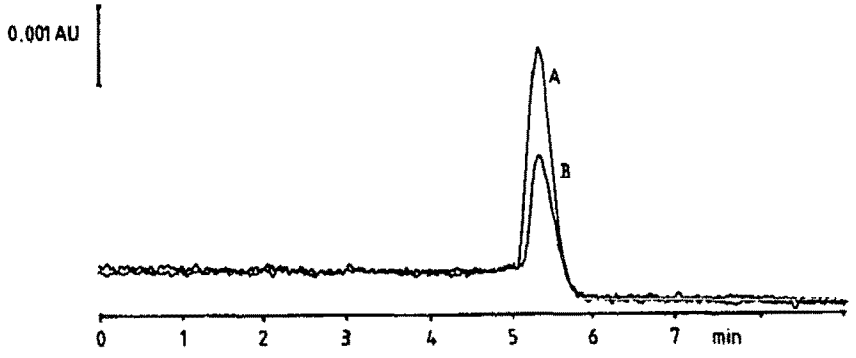

Fig. 3. Chromatogram of cyclosporin A (1.25 mg ml). Injection volume, 80 μl with a splitting ratio of 1:750. Sample loading was done with (A) ten and (B) five consecutive injections per minute with acetonitrile in water (15:85). Elution was done with a gradient from 15% to 50% acetonitrile in water in 14 min. Analytical column, SB-Methyl-100 (1.5 m × 50 μm I.D.), film thickness 0.25 μm; column temperature, 100°C; flow-rate, 1.5 μl min.

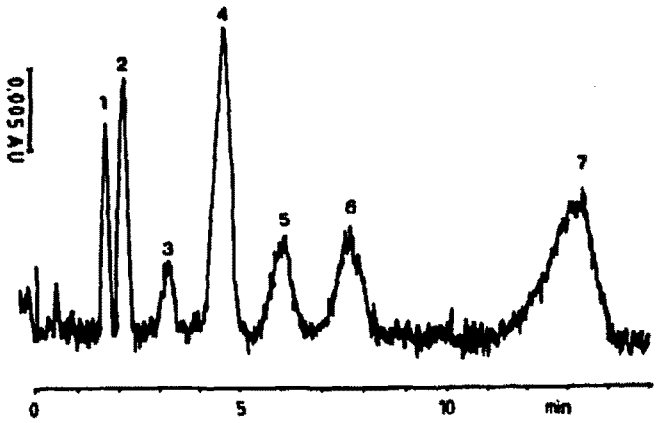

Fig. 4. On-column detection with single optical fibres. Separation column, SB-Methyl-100 (1.5 m × 50 μm I.D.), film thickness 0.25 μm; column temperature, 150°C; mobile phase, methanol–water (50:50); flow-rate, 1.5 μl/min; injection volume, 50 μl with a splitting ratio of 1:670. Peaks: 1 = tropolone; 2 = benzene; 3 = chlorobenzene; 4 = 1,4-dichlorobenzene; 5 = 1,3,5-trichlorobenzene; 6 = 1,2,4,5-tetrachlorobenzene; 7 = pentachlorobenzene.

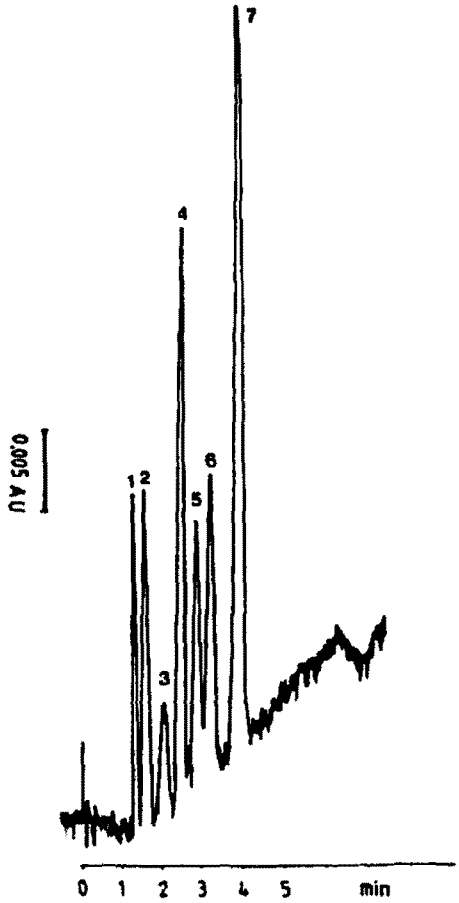

Fig. 5. Gradient elution separation with column and temperature as in Fig. 4. Mobile phase: A = methanol and B = water with a gradient from 30 to 60% B in 5 min; flow-rate, 1.5 μl/min; injection volume, 50 μl with a splitting ratio of 1:670. Peak identification as in Fig. 4.

mode lead to peak compression. The concept of the gradient separation in HT-OT-LC is the same as the gradient strategy for column liquid chromatography. The usual shortcoming with a mobile phase gradient is mobile phase mixing. In our experimental set-up, static mixing was utilized together with thermal mixing. Thermal mixing was induced by preheating the mobile phase before it entered the analytical column. A temperature increase leads to an increase in the kinetic energy of the molecules in the mixtures, resulting in rapid mixing. This was an additional benefit, apart from higher efficiency, of working at higher temperatures. Signals obtained with on-column detection employing single optical fibres with isocratic and gradient elution are shown in Figs. 4 and 5, respectively. There was a significant improvement in the relative peak sensitivity in the gradient mode compared with isocratic elution.

On-column detection

On-column detection is usually performed to minimize the peak broadening cause by the detector cell volume. Single optical fibres are commonly used for on-column detection in micro separation techniques. We investigated the use of round to line-end bundle optical fibres in on-column detection. A segment (15 mm) of the polyimide coating was burned off the analytical column to make a window for UV detection. The window was placed in the specially made cell holder, between the line-ends of the bundles, as depicted in Fig. 2. The length of the optical window is determined by the length of the line-end of the bundle (8.8 mm), while its width was limited by the diameter of a single fibre in the bundle (0.23 mm). The detection window for on-column detection utilizing a single optical fibre is limited by the fibre diameter (0.20 mm). The light path length in on-column detection is equal by the capillary column I.D. (50 μm). The separation of chlorobenzenes on a biphenyl column was studied with the single and bundle optical fibres and is shown in Figs. 6 and 7, respectively. The bun-

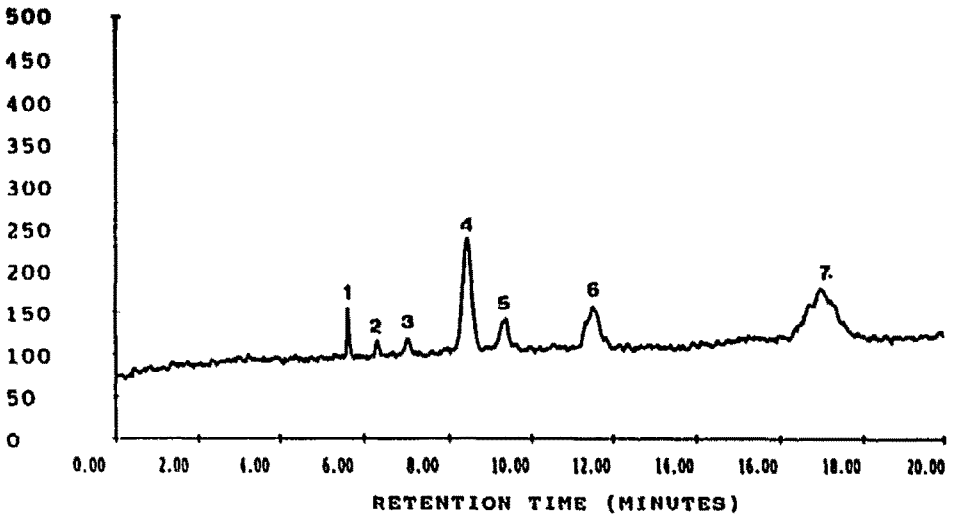

Fig. 6. On-column detection with single optical fibres. Separation column, biphenyl (10 m × 50 μm I.D.), film thickness 0.25 μm; mobile phase, acetonitrile–water (40:60); flow-rate, 4 μl/min; injection volume, 10 μl with a splitting ratio of 1:900. Peak identification as in Fig. 4. y-Axis is detector response (10 μV).

dled fibres provided a tenfold increase in signal-to-noise ratio (S/N) compared with single fibres. With the bundle of fibres a *ca.* 15% decrease in efficiency was observed owing to the large detection window. This decrease in efficiency is tolerable in most routine separations.

A study of two different detection modes, on-column detection with the single optical fibres and the Z-shaped micro flow cell, was also performed. The separation of chlorobenzenes was done at 100°C utilizing a SB-Methyl-100 capillary column (19.5 m × 5 μm I.D.) with acetonitrile–water (50:50) as the mobile phase. A very long column was used in order to minimize the contribution of the cell volume to the overall band broadening [7]. The length of the column outlet section (outside the oven) was 4 cm

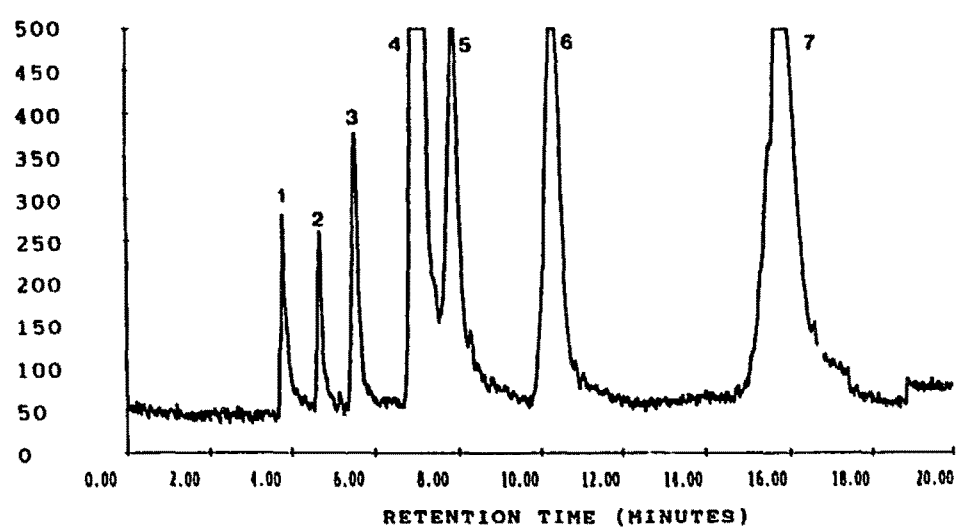

Fig. 7. On-column detection with the bundle of optical fibres. Separation conditions (except flow-rate, 5 μl min) and peak identification as in Fig. 6. y-Axis is detector response (10 μV).

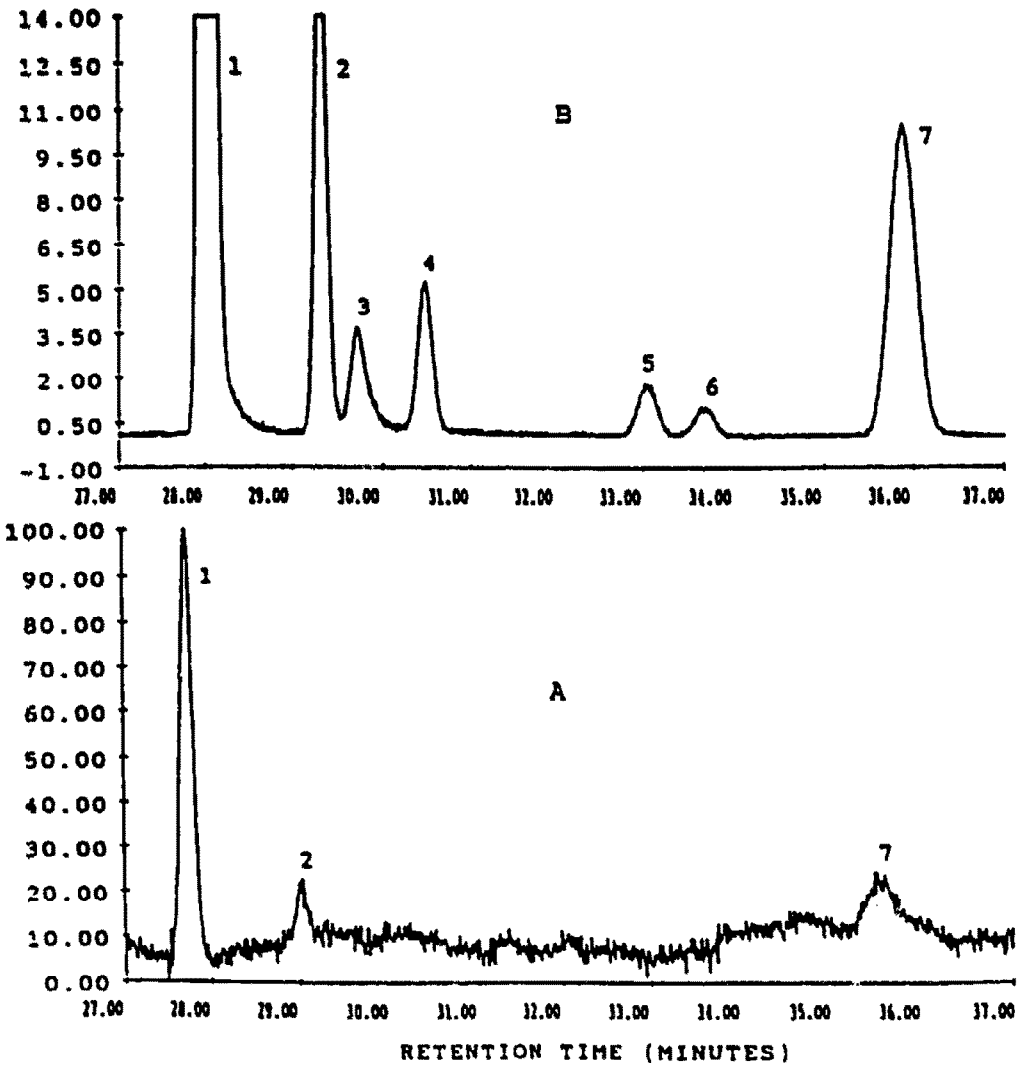

Fig. 8 (A) On-column detection with the single optical fibres. Detector wavelength, 250 nm; separation column, SB-Methyl-100 (19.5 m × 50 μm I.D.), film thickness 0.25 μm; column temperature, 100°C; mobile phase, acetonitrile–water (50:50); flow-rate, 1 μl/min; injection volume, 10 μl with a splitting ratio of 1:900. Peak identification as in Fig. 4. Signals are normalized to the highest signal. (B) Detection with the Z-shaped cell. Separation conditions as in (A). Peak identification as in Fig. 4. The y-axes represent detector responses: (A) 10 μV and (B) %.

and that of the cell inlet section was 40 cm. The cell and its holder were placed in the detector, which was outside the column oven at room temperature. Chromatograms obtained with on-column detection with a single optical fibre and the Z-shaped micro flow cell are shown in Fig. 8A and B, respectively. The S/N with the Z-shaped cell is twenty times higher than with single fibre on-column detection. As the outlet section of the analytical column was at a lower temperature, the decrease in efficiency of more retained solutes was expected to be greater at elevated column temperatures owing to the "cold spot" effect [7]. The decrease in efficiency for pentachlorobenzene becomes significant with increase of the column temperature. This obviously results from the additional retardation of the more retained solute on the cold stationary phase at the outlet section. If the Z-shaped cell is to be used in HT-OT-LC, the connection between the column and the cell should be as short as possible and, if possible, heated. In addition to the "cold spot", the detector cell volume (5.0 nl) can also lead to an inferior efficiency.

CONCLUSION

Gradient elution could be easily and successfully performed with the HT-OT-LC system. Indeed, the sample focusing provided by this method may prove to be very important when improved detection limits are required. When seeking the optimum efficiency, one should also take into consideration the detection sensitivity. The sensitivity gain with the Z-shaped flow cell is impressive. The Z-shaped micro flow cell should be applied for separations in which detection is the first priority and problems associated with some decrease in efficiency due to the "cold spot" and a prolonged analysis time (long column) could be tolerated. Use of an optical fibre bundle also significantly enhanced the detection limit of the system. This is a very promising mode for on-column detection which can be applied to some other micro separation techniques. The bundled fibres give the best compromise between increased sensitivity, decreased efficiency and speed of analysis, and therefore are the best choice for detection in HT-OT-LC.

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