Journal of Chromatography, 475 (1989) 229–235
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 533

IMPROVED METHOD FOR CONTINUOUS UV MONITORING IN HIGH-SPEED COUNTER-CURRENT CHROMATOGRAPHY

HISAO OKA[a] and YOICHIRO ITO*

Laboratory of Technical Development, National Heart, Lung, and Blood Institute, Building 10, Room 5D12, 9000 Rockville Pike, Bethesda, MD 20892 (U.S.A.)

(First received January 25th, 1989; revised manuscript received March 28th, 1989)

SUMMARY

Continuous UV monitoring of the effluent in high-speed counter-current chromatography often encounters difficulty mainly due to the thermolabile nature of the mobile phase which tends to develop turbidity in the flow cell under a slight shift of the ambient temperature. This problem was effectively solved by inserting a fine PTFE tube (3 m × 0.46 mm I.D.) between the column outlet and the UV monitor and immersing a large portion of the tube into a waterbath heated at 30°C. A similar tube was applied at the outlet of the UV monitor to create back pressure which suppressed gas bubble generation from the mobile phase. By the combined use of these devices, noiseless UV tracing was successfully demonstrated in two model experiments using thermolabile two-phase solvent systems: separation of flavonoids from the sea buckthorn ethanol extract with chloroform methanol–water (4:3:2, v/v/v) and separation of bacitracin components with chloroform–ethanol–water (5:4:3, v/v/v).

INTRODUCTION

High-speed counter-current chromatography (HSCCC) using a multi-layer coiled column is a unique liquid–liquid partition technique that does not require the use of solid supports[1]. The use of two immiscible solvent phases in an open column free of solid support matrix can eliminate various complications associated with conventional liquid chromatography such as tailing of solute peaks, adsorptive sample loss and deactivation, and sample contamination. On the other hand, it is usually difficult to obtain stable continuous UV monitoring of the effluent from a CCC separation and, therefore, the elution curve is usually drawn manually by the spectrophotometric analysis of individual fractions after the effluent is fractionated with a fraction collector[2–4]. In order to avoid the above laborious procedure, it is highly desirous to establish a CCC monitoring system which produces stable UV tracing of the elution curves comparable to those obtained from other chromatographic methods.

Problems in direct UV monitoring of the effluent in CCC may be classified into

[a] Visiting scientist from Aichi Prefectural Institute of Public Health, Nagoya, Japan.

0021-9673/89/$03.50

the following four categories: (1) steady carryover of the stationary phase due to an improper choice of the elution mode and/or the application of an excessively high flow-rate of the mobile phase; (2) migration of the stationary phase into the flow cell which is caused by various conditions such as fluctuations of revolutional speed, vibration of the centrifuge system, and overloading of the sample (which may cause local alteration of the phase volume ratio and the physical properties of the two phases); (3) turbidity of a thermolabile mobile phase in the flow cell due to altered ambient temperature; (4) gas bubble formation from the effluent under reduced pressure in the periphery of the flow passage. Among those, the first two problems can be avoided by a proper choice of the experimental conditions, whereas a suitable modification of the UV monitoring system is essential to overcome the remaining problems[5].

The present paper describes a simple and effective method for continuous UV monitoring of the effluent in HSCCC to yield noiseless tracing comparable to that in high-performance liquid chromatography.

EXPERIMENTAL

Apparatus

The high-speed counter-current chromatograph used in this study was the commercial model of a flow-through coil planet centrifuge called "Ito Multi-layer Coil Separator-Extractor" (P.C. Inc., Potomac, MD, U.S.A.). The column holder is positioned at a distance of 10 cm from the central axis of the centrifuge. The separation column was prepared by winding a long piece of PTFE tubing, 1.6 mm I.D. and 0.3 mm wall thickness, directly onto the holder hub of 10 cm diameter making multiple coiled layers. The β value (the ratio of the rotational radius to the revolutional radius) ranges from 0.5 at the internal terminal to 0.8 at the external terminal. The total capacity of the multi-layer coil measures about 280 ml. This apparatus is equipped with an ACCU-FLO-pump (Beckman, Palo Alto, CA, U.S.A.) and a speed controller (Bodine, Chicago, IL, U.S.A.). Continuous UV monitoring was performed with an LKB 2138 Uvicord S UV monitor (LKB, Bromma, Sweden) operated at 254 nm and a Pharmacia 482 recorder (Pharmacia, Uppsala, Sweden). On the flow line between the coiled column and the UV monitor, a fine PTFE tube of 3 m × 0.46 mm I.D. (Zeus, Raritan, NJ, U.S.A.) was inserted, which can be heated in a water bath at a desired temperature. A similar tube was also applied at the outlet of the UV monitor to prevent a sudden pressure drop which would generate gas bubbles from the mobile phase.

Reagents

Organic solvents including *n*-hexane, ethyl acetate, chloroform, *n*-butanol, *sec.*-butanol, and methanol were all of glass-distilled chromatographic grade (Burdick and Jackson Labs., Muskegon, MI, U.S.A.) while 95% ethanol (Warner-Graham, Cockeysville, MD, U.S.A.) and glacial acetic acid (J. T. Baker, Phillipsburg, NJ, U.S.A.) were of reagent grade. Dried sea buckthorn ethanol extract was obtained from China by the courtesy of Professor Tian You Zhang at Beijing Institute of New Technology Application, Beijing, China, and bacitracin was purchased from Sigma, St. Louis, MO, U.S.A.

Procedure for thermostability test of solvent systems

From the above organic solvents, 11 pairs of solvent systems with a broad spectrum in hydrophobicity (see Table I for their composition) were examined for their thermostability. Each solvent mixture, ranging 3–5 ml in volume, was delivered in a test tube (100 mm × 13 mm O.D.) and a polyethylene plug was applied to the tube. Then, the contents were thoroughly mixed to bring the phases to equilibrium at room temperature (*ca.* 22°C). The mixing was repeated until two clear layers were obtained.

TABLE I

EFFECTS OF TEMPERATURE ON TWO-PHASE EQUILIBRIUM

No.	*Solvent systems*	*Effects of temperature*[a]			
		Cooling		*Warming*	
		UP	*LP*	*UP*	*LP*
1	Hexane methanol	+	+	−	−
2	Hexane–methanol–water (2:1:1, v/v/v)	−	±	−	−
3	Hexane–ethyl acetate–methanol–water (1:1:1:1, v/v/v/v)	±	+	−	−
4	Ethyl acetate–water	+	−	−	+
5	Ethyl acetate–acetic acid–water (4:1:4, v/v/v)	±	−	−	−
6	Chloroform–water	−	+	−	−
7	Chloroform–methanol water (5:4:3, v/v/v)	+	+	−	−
8	Chloroform–acetic acid–water (2:2:1, v/v/v)	+	+	−	−
9	*n*-Butanol–water	+	−	−	+
10	*n*-Butanol–acetic acid–water (4:1:5, v/v/v)	+	−	−	−
11	*sec.*-Butanol water	−	−	+	+

[a] UP = upper phase; LP = lower phase; + = development of turbidity; − = no change in transparency.

In the first series of experiments, each tube was immersed in ice water for 5–10 s to observe turbidity in the upper and/or the lower phases. The second series of experiments was similarly performed with the same set of solvent systems pre-equilibrated at room temperature by immersing each tube into warm water (*ca.* 40°C) for 5–10 s to observe development of turbidity in each phase. All experiments were repeated at least twice to ensure reproducibility of the results.

Preparation of solvent systems and sample solutions

Two different two-phase solvent systems were prepared: chloroform–methanol–water (4:3:2, v/v/v) for separation of flavonoids from the sea buckthorn ethanol extract and chloroform–ethanol–water(5:4:3, v/v/v) for separation of bacitracin components. Each solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature by repeated vigorous shaking and degassing by opening the stopcock, and the two phases were separated shortly before use.

The sample solutions of sea buckthorn ethanol extract and bacitracin were similarly prepared by dissolving 50 mg of each sample in 4.5–4.8 ml of the above solvent mixture used for the separation.

Separation procedure

In each experiment, the coiled column was first entirely filled with the upper aqueous stationary phase, and the sample solution containing 50 mg of the sample was injected into the head of the column through the sample port. (The head–tail relationship of the rotating coil is conventionally defined by an Archimedean screw force which acts on each phase to different extents but in the same direction, namely, towards the head of the coil) Then, the coil planet centrifuge was rotated at the optimal speed of 800 rpm, while the mobile phase was pumped into the head of the column at a flow-rate of 180 ml/h. Effluent from the outlet of the column was continuously monitored with a Uvicord S at 254 nm to record the elution curve. In order to prevent trapping the stationary upper phase within the flow cell, the effluent (lower phase) was passed through the flow cell in an upward direction. During the elution, the fine tube on the flow line between the column outlet and the monitor was immersed in a water bath at the desired temperature which was maintained with a heating rod and a thermal controller (Fisher, Pittsburgh, PA, U.S.A.).

RESULTS AND DISCUSSION

Phase compositions of the two-phase solvent system used in CCC are in a subtle equilibrium at room temperature. Any change in the ambient temperature may cause one or both phases to develop a cloudy appearance; when this occurs in the flow cell of

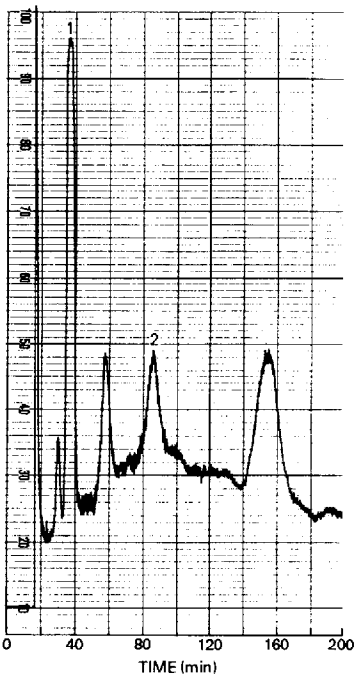


Fig. 1. UV tracing chart in flavonoid separation obtained with the conventional monitoring for HSCCC. Experimental conditions: Monitor, LKB Uvicord S; flow cell, rectangular type, 2.5-mm light path, 10 μl capacity; wavelength, 254 nm; recorder, Pharmacia Model 482 recorder; chart speed, 0.5 mm/min. Peaks: 1 = isorhamnetin; 2 = quercetin.

the UV monitor, it results in an intensive noise and raised base line in the elution curve tracing. The effects of cooling and warming on each phase of 11 selected solvent systems are summarized in Table I where positive signs indicate development of turbidity and negative signs, no change in transparency. The results clearly show that in the majority of these solvent systems cooling tends to develop turbidity in the organic phase, *i.e.*, the lower phase in the chloroform systems, both phases in the non-aqueous hexane–methanol system, and the upper phase in the remaining solvent systems. On the other hand, warming gives no change in transparency except for some binary systems including *sec.*-butanol–water, *n*-butanol–water and ethyl acetate–water. These results strongly suggest that warming the effluent not only prevents development of the turbidity in the effluent, but it may also enable the mobile phase to absorb some amounts of the stationary phase carried over from the separation column. Hence, warming serves to maintain high transparency of the effluent passing through the flow cell in the UV monitor.

In the present studies, these observations were applied to the separation of natural products on two different chloroform solvent systems both utilizing the thermolabile lower non-aqueous phase as the mobile phase. Fig. 1 shows a typical chromatogram of flavonoids in the sea buckthorn ethanol extract with a solvent system of chloroform–methanol–water (4:3:2, v/v/v) obtained by the conventional monitoring method for HSCCC. The effluent was continuously passed upward through a straight standard flow cell held vertically in the Uvicord S where the absorbance was monitored at 254 nm. The thermolabile nature of the lower mobile

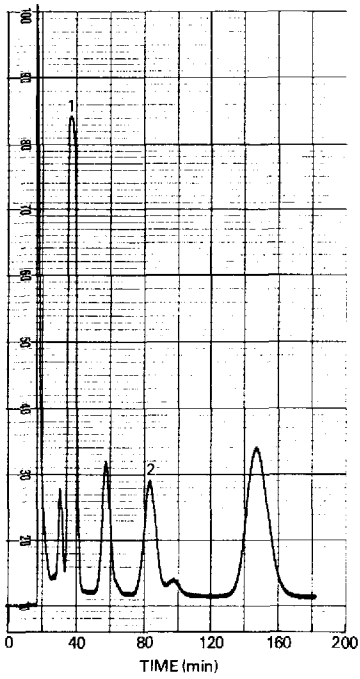


Fig. 2. UV tracing chart in flavonoid separation obtained by heating the effluent. The effluent from the separation column was passed through a fine tube immersed into a water bath maintained at 30°C. Other experimental conditions as in Fig. 1.

phase led to a UV tracing of the elution curve which was disturbed by intensive noise and irregular elevation of the base line. The overall effect was to obscure a minor peak in the chromatogram.

Fig. 2 shows a chromatogram obtained under similar experimental conditions except that the effluent from the separation column was first passed through a narrow tube heated to 30°C in a water bath before entering the UV monitor. The results clearly demonstrate a radical improvement in UV tracing as evidenced by a stable flat base line and smooth tracing of the elution curve. A minor peak, which was obscured by noise in Fig. 1, is now clearly observed in the chromatogram. A slight thickening of the base line was found to be caused by periodical passage of gas bubbles through the flow cell.

Formation of gas bubbles in the peripheral portion of the separation column is a common complication in both liquid chromatography and CCC. This undesirable phenomenon can be effectively controlled by applying a narrow-bore tube at the outlet of the monitor to maintain sufficient back-pressure. A chromatogram of flavonoids shown in Fig. 3 was obtained from a Uvicord S UV monitor equipped with a fine tube (3 m × 0.46 mm I.D.) at the outlet without heating the effluent. This simple method produced a substantial improvement in tracing over the control run (Fig. 1) by eliminating high-frequency noise which was apparently caused by the passage of gas bubbles through the flow cell.

Finally, the experiment was performed by applying both modifications, *i.e.*, heating the effluent to 30°C near the inlet of the monitor and attaching a narrow-bore

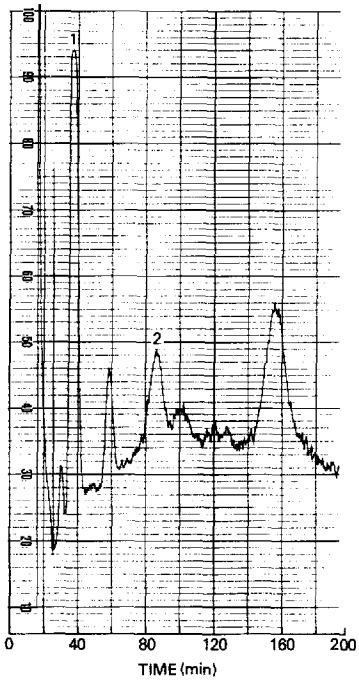
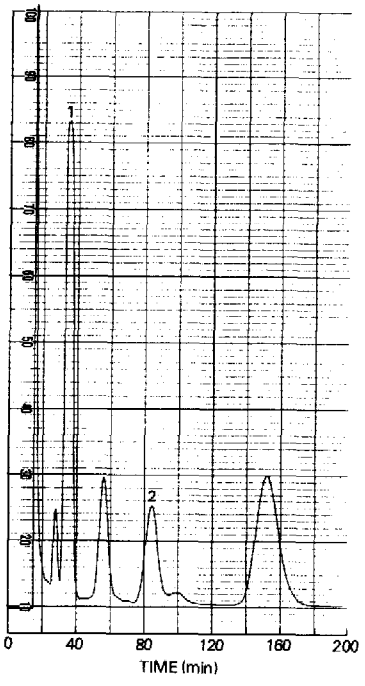


Fig. 3. UV tracing chart in flavonoid separation obtained by applying a fine tube at the outlet of the monitor. Other experimental conditions as in Fig. 1.



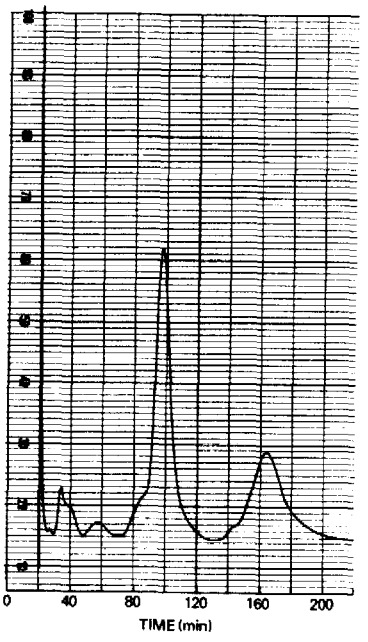


Fig. 4. UV tracing chart in flavonoid separation obtained by the present method. The effluent from the separation column was heated to 30°C before entering the monitor, and a fine tube was applied to the outlet of the monitor to create back pressure.

Fig. 5. UV tracing chart in bacitracin separation by the present method. UV monitoring conditions as in Fig. 4.

tube at the outlet of the monitor. Fig. 4 shows the counter-current chromatogram of flavonoids which was obtained by the present method. As expected, the method yielded a noiseless UV tracing of the elution curve that is comparable in quality to those obtained from HPLC. The chromatogram was found to be almost identical to the elution curve manually drawn after the spectrophotometric analysis of individual fractions without the use of the present device. The above method was also successfully applied to the separation of bacitracin with a two-phase solvent system composed of chloroform–ethanol–water (5:4:3, v/v/v) using the lower nonaqueous phase as the mobile phase (Fig. 5).

As described above, we have established a simple method for stable continuous UV monitoring of effluent from HSCCC. The method may also be effectively applied for other CCC schemes. All elements used in the present device, including fine PTFE tubing, a heater, and a thermoregulator, etc., will be incorporated in the future design of a UV monitor for CCC.

REFERENCES

1 Y. Ito, *CRC Crit. Rev. Anal. Chem.*, 17 (1986) 65.
2 Y. Ito, J. Sandlin and W. G. Bowers, *J. Chromatogr.*, 244 (1982) 247.
3 T. Y. Zhang, D. G. Cai and Y. Ito, *J. Chromatogr.*, 435 (1988) 159.
4 T. Y. Zhang, X. Hua, R. Xiao and S. Kong, *J. Liq. Chromatogr.*, 11 (1988) 233.
5 A. Berthod and D. W. Armstrong, *J. Liq. Chromatogr.*, 11 (1988) 1457.