CHROMATOGRAPHIC STUDIES

Personal Overpressured-Layer Chromatography (OPLC) Basic System 50, Flexible Tool in Analytical and Semipreparative Work

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A new automated overpressured-layer chromatographic (OPLC) system called the Personal OPLC Basic System 50 is suitable for analytical and semipreparative separations. The automatic microprocessor-controlled system ensures rapid and reproducible off-line isocratic and stepwise gradient separations. High external pressure (5 MPa) makes the sorbent layer more homogeneous, yielding more efficient off-line separation compared with those by early Chrompres chambers. A theoretical plate height of 10–30 μm can be achieved on an analytical high-performance thin-layer chromatographic (HPTLC) layer made of irregular silica gel with an average particle size of 5 μm if an optimal linear velocity (20–40 mm/min) and a nonviscous solvent system are used. On an analytical layer of 3 μm spherical silica gel, a theoretical plate height of 6–15 μm can be reached. Rapid analytical separations of resveratrol (1555 s) and xanthine by one- (498 s) and two-directional (274 s) off-line developments were accomplished. On-line separation and detection combined with off-line sample application and fully on-line processing (including on-line sample application, separation, and detection) were fulfilled with a TLC plate for xanthine separation. Semipreparative isolation of xanthines was achieved through a fully on-line OPLC operating mode and scaled-up chromatography.

Overpressured-layer chromatography (OPLC) refers to techniques involving a pressurized ultramicro (UM) chamber and a pump system that delivers eluent into the chamber, which contains analytical or preparative chromatographic plates under pressure. The ancestor of this technique is the UM chamber (1), from which we have developed the experimental pressurized UM chamber. Here, an external pressure is applied to the surface of the sorbent layer by means of a cushion system, forcing the eluent to flow (by overpressure) through the sorbent layer (2–4).

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The first commercially available OPLC instrument (Chrompres 10) is a completely off-line system. All the principal chromatographic steps, such as sample application, separation, quantitative evaluation, and preparative isolation, are performed independently. The advantages of OPLC are revealed by this system (5–10).

The second-generation instrument (Chrompres 25) is suitable for both off-line and on-line separations. With a selective eluent to increase the resolution of compounds running with low R_f values, continuous development can be used. Separated components are evaluated quantitatively by densitometry on the plate. If the eluent outlet of the chamber is connected to a flow-cell detector, eluting solutes can be detected on-line, and fractions can be collected. The entire chromatographic process can be performed by a fully on-line mode connecting a loop injector to the eluent inlet and a UV detector to the eluent outlet, as in column liquid chromatography (LC; 11–14).

Different combinations of off-line and on-line steps can be used within this system (15), and it can be applied to various analytical and preparative applications (16–25). For example, separations of ascorbigens, amino acids, biogenic amines, and steroids demonstrate the efficacy of the personal OPLC system (25–28). Furthermore, the combination of OPLC and digital autoradiography is a powerful method in metabolite research (29).

Experimental

Materials

Mobile phases were made from chromatographic-grade chemicals (LiChrosolv, Merck Darmstadt, Germany).

Stationary phases of analytical TLC, HPTLC, and 0.5 mm thick preparative sorbent layers (20 × 20 cm) with irregular silica gel 60 and Raman plates (10 × 10 cm) made of 3 μm, spherical, superfine silica gel 60 (Merck) were used. The layers were sealed on 4 sides by a robot (OPLC-NIT Ltd., Budapest, Hungary) to ensure perfect closing of the planar sorbent bed during separation.

PTH-Valine (phenylthiohydantoin derivatives of Valine; Pierce Chemical, Rockford, IL) dissolved in eluent (dichlo-

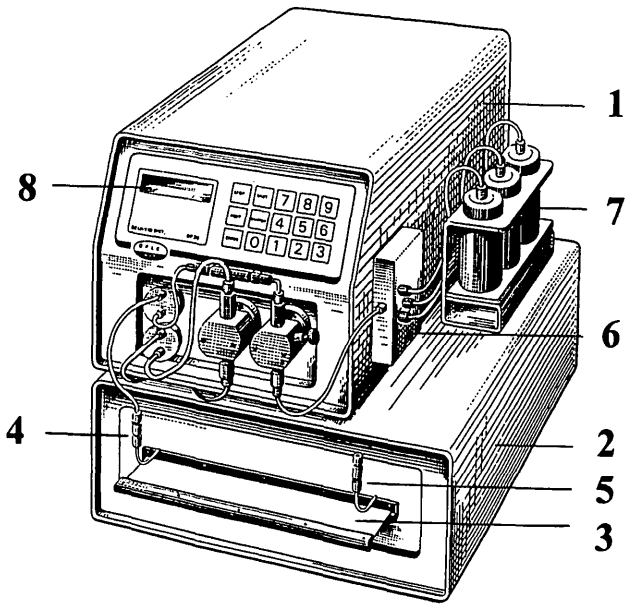

Figure 1. Personal OPLC BS-50 3SG system: 1, liquid delivery system; 2, separation chamber; 3, cassette; 4, eluent inlet; 5, eluent outlet; 6, eluent switching valve; 7, eluent reservoirs; 8, liquid crystal display.

romethane–ethyl acetate, 92 + 8, v/v, 0.4 mg/mL) was the standard for theoretical work.

A mixture of xanthines (Merck) dissolved in eluent (chloroform–acetic acid, 6 + 4, v/v; theophylline, 2.96 mg/mL; caffeine, 1.75 mg/mL; and theobromine, 1.04 mg/mL) was used as standard for separation of tea xanthines.

Extraction

For xanthine separation, commercial tea leaves (1.5 g) were extracted with 5 mL eluent (chloroform–acetic acid, 6 + 4, v/v). The extract was applied to the layer by off-line or on-line sample application (concentration, 2.6 mg/mL).

For resveratrol separation, fresh grape leaves irradiated with UV were homogenized with liquid nitrogen and extracted with methanol (300 mg + 600 μL) for 5 min in an ultrasonic bath. The mixture was centrifuged, and the supernatant was applied to the layer.

Sample Application

Off-line application of sample to the dry sorbent layer was done with either the Nanomat or Linomat III sample applicator (Camag, Muttenz, Switzerland). In the fully on-line operating mode, samples were applied to the wet sorbent layer by a loop injector delivering 20 μL for an analytical layer and 1750 μL for a preparative layer.

Instrumentation

The new, 2-module Personal OPLC Basic System BS-50 3SG (OPLC-NIT Ltd.) consists of the liquid delivery system and the separation chamber. The microprocessor-controlled liquid delivery system includes a hydraulic liquid delivery pump and an eluent delivery pump. The separation chamber has 2 eluent connections and can withstand the maximum 5 MPa external pressure that can be applied (Figure 1).

A cassette containing the sorbent layer can be inserted into the chamber (Figure 2). Linear, one- and two-directional, and

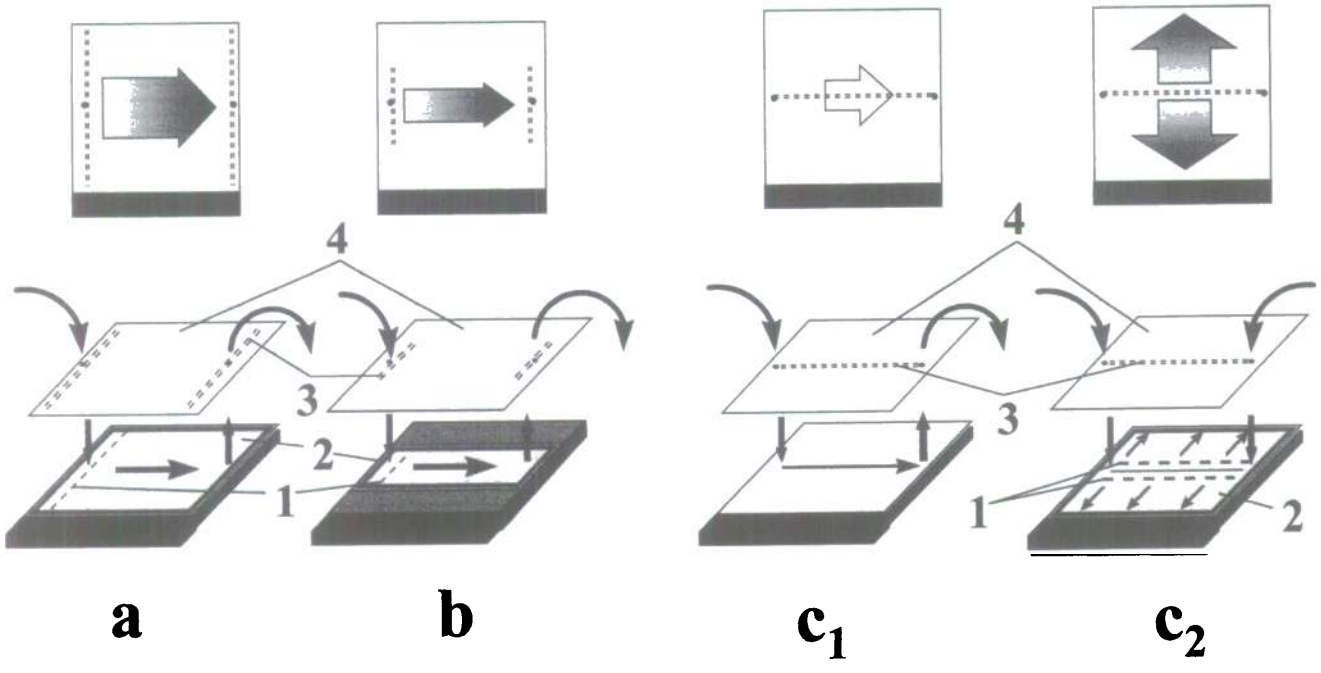

Figure 2. Cassettes: (a) cassette of 20 × 20 cm layer for linear one- and two-dimensional development; (b) cassette of 10 × 20 cm glass-backed layer for linear one-dimensional development; (c) cassette for system rinsing (C_1) and two-directional development (C_2) of 20 × 20 cm layer. Arrows represent the eluent movement. Upper pictures are overviews of cassettes. In the lower pictures, the polytetrafluoroethylene (PTFE) cover plates are in floating position. 1, sample; 2, sorbent layer; 3, eluent-directing trough (on back side of cover plate); 4, PTFE cover plate involves eluent directing trough and hole.

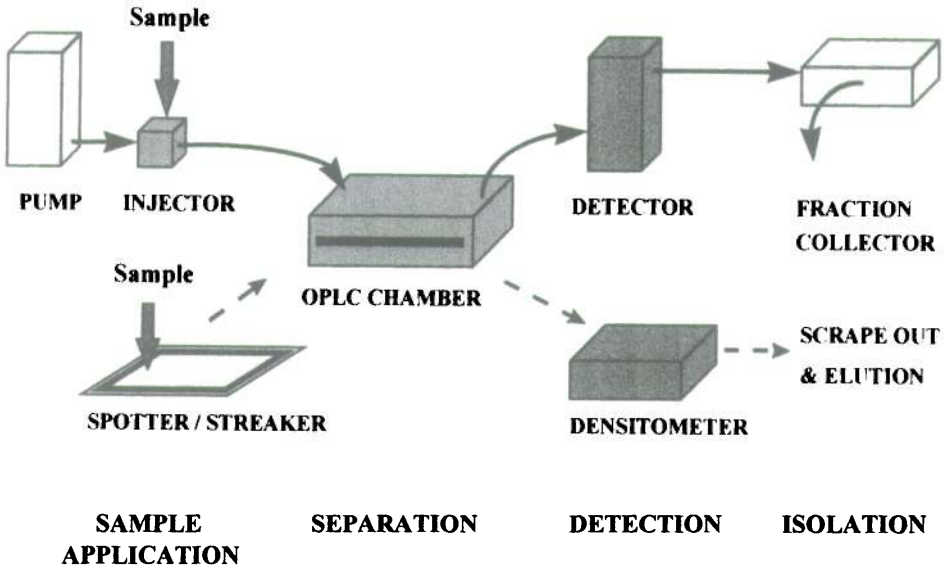

Figure 3. Scheme of off-line (dotted arrows) and on-line (solid arrows) OPLC processes.

two-dimensional off-line and linear one-directional on-line separations are suitable for isocratic as well as two- and three-step gradient developments. Both semipreparative isolation and analytical separation are possible with the appropriate cassette. The maximum migration distance between the inlet and the outlet is 183 mm (standard size). The distance between the eluent-directing troughs of the polytetrafluoroethylene cover sheet of the cassette corresponds to this value. A 10 mm distance separates the inlet trough and the edge of the cassette.

Instrument control, system rinsing, eluent change, and on-line separations for isolation can be tuned under the TEST function. All the steps are manual with this menu.

Automatic development can be managed under the PARAMETERS and DEVELOPMENT menus. Every step can be monitored through a liquid crystal display panel on the front of the liquid delivery system. In the PARAMETERS menu, the following parameters can be adjusted for isocratic and stepwise gradient separations: external pressure (EXT PRESS), eluent flow rate (E FLR), rapid volume (VOL R), eluent volume A (A VOL), eluent volume B (B VOL), eluent volume C (C VOL) and eluent dead volume (VOL*, which is suggested for repetitive stepwise gradient separation).

External pressures can range from 0 to 50 bars. The eluent flow rate is adjustable in the range 10–10 000 μL/min; A, B, and C solvent systems can be chosen for isocratic or stepwise gradient runs. Delivery of eluent volumes, rapid volume, and dead volume can be varied between 0 and 63 000 μL.

The DEVELOPMENT menu automatically integrates the separation steps of developments. After the blanks of PARAMETERS menu are filled, the development time is automatically calculated. By selecting DEVELOPMENT and pressing START, the process begins immediately, providing external pressure for separation. To ensure a straight front line, a rapid eluent flush is given at the beginning of development. Just before it reaches the starting zone, the eluent devoted to separation starts traveling slowly at constant optimum velocity. After the full volume of separation has been let out, automatic termination of eluent delivery is followed by release of external pressure. An end signal warns that the process is over. The cassette enveloping the layer can be pulled out in pressure-release mode (EXT PRESS, 0 bar).

Built-in self-control of the system deletes unacceptable parameters that may threaten successful development and instrumental safety.

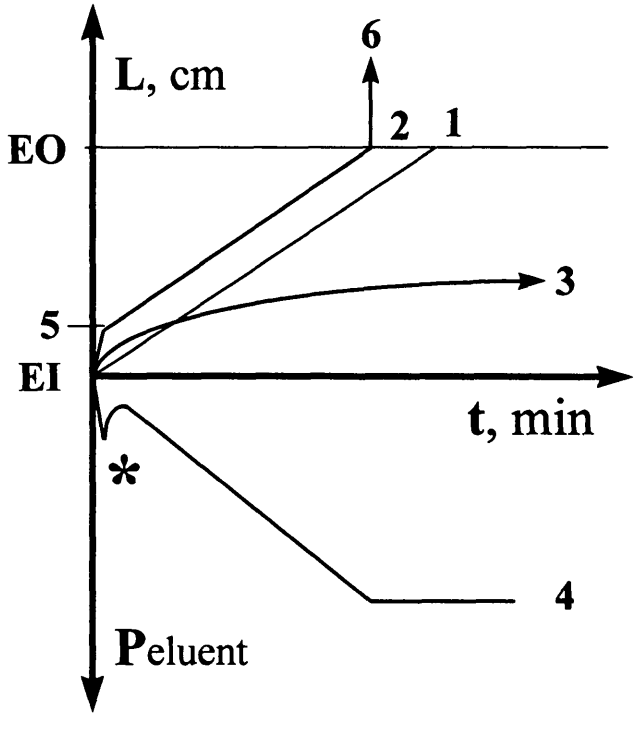

Figure 4. Variation of eluent front distance and inlet pressure during development: 1, theoretical front line; 2, front line of personal OPLC; 3, front line of conventional TLC development; 4, eluent inlet pressure; 5, break point of front line; 6, eluent outflow; EI, eluent inlet; EO, eluent outlet; *, transition period.

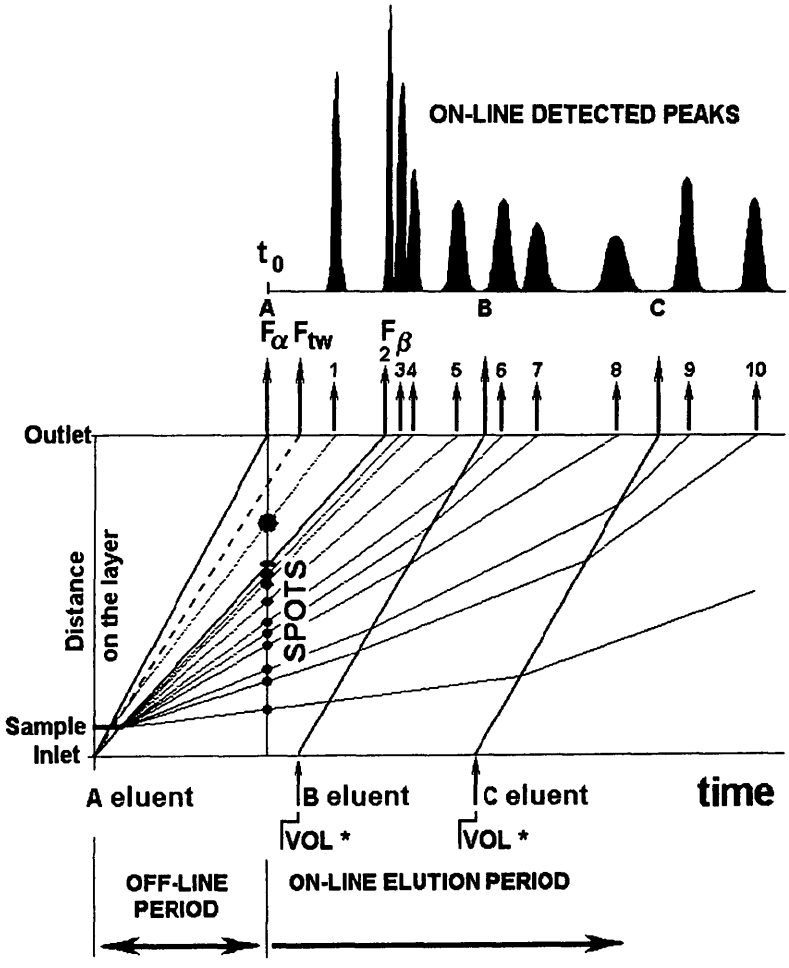

Figure 5. Scheme of continuous, stepwise gradient OPLC development: F_α, alpha front; F_{tw}, front of total wetness; F_β, beta front; 1–10, eluted spots; VOL*, dead volume.

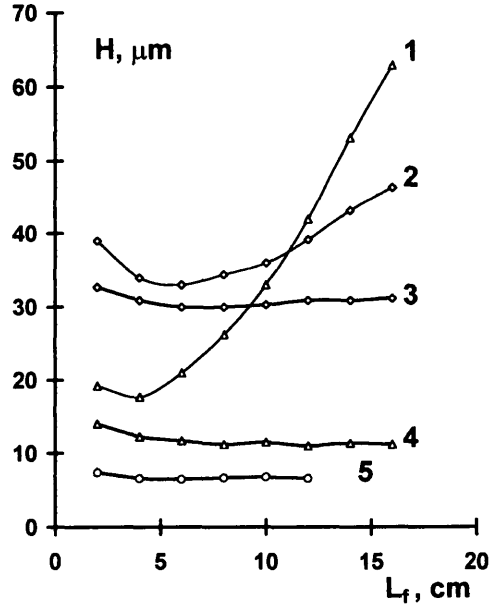

Figure 6. Variation of theoretical plate heights of PTH-valine along the plate with a conventional normal unsaturated chamber and the personal OPLC system: 1, HPTLC silica gel, normal unsaturated chamber; 2, TLC silica gel, normal unsaturated chamber; 3, TLC silica gel, OPLC, 5 MPa; 4, HPTLC silica gel, OPLC, 5 MPa; 5, Raman silica gel, OPLC, 5 MPa. Sample application: TLC silica gel (thickness, 0.220 mm), 0.2 μL; HPTLC silica gel (thickness, 0.160 mm), 0.1 μL; Raman silica gel (thickness, 0.095 mm), 0.05 μL; H theoretical plate height; and L_f, front distance.

Separation

Off-line or on-line analytical and semipreparative separations were performed. In the OPLC system, the eluent inlet was connected to a loop injector, and the outlet was connected to a UV detector for on-line separation.

For theoretical work, PTH-valine was separated with dichloromethane–ethyl acetate, 92 + 8 v/v.

Separation of resveratrol from grape leaf extract was accomplished in fully off-line operating mode with stepwise-gradient air elimination. The following program was used for repetitive separation: EXT PRESS, 5 MPa; E FLR, 450 μL/min; A VOL R, 1000 μL; A VOL, 3000 μL; B VOL, 7700 μL, VOL*, 800 μL. Calculated separation time was 1555 s. Eluent A, isooctane; eluent B, dichloromethane–ethyl acetate–methanol, 90 + 5 + 5, v/v.

Chloroform–acetic acid, 60 + 40, v/v, was used to separate xanthine. The one-directional, linear, fully off-line separation was accomplished with the following conditions: EXT PRESS, 5 MPa; E FLR, 500 μL/min; B VOL R, 300 μL; B VOL, 4100 μL. Calculated separation time was 498 s. For the two-directional, fully off-line separation, the following parameters were applied: EXT PRESS, 5 MPa; E FLR, 900 μL/min; B VOL R, 350 μL; B VOL, 4050 μL. Calculated separation time was 274 s (layer thickness, 0.160 mm).

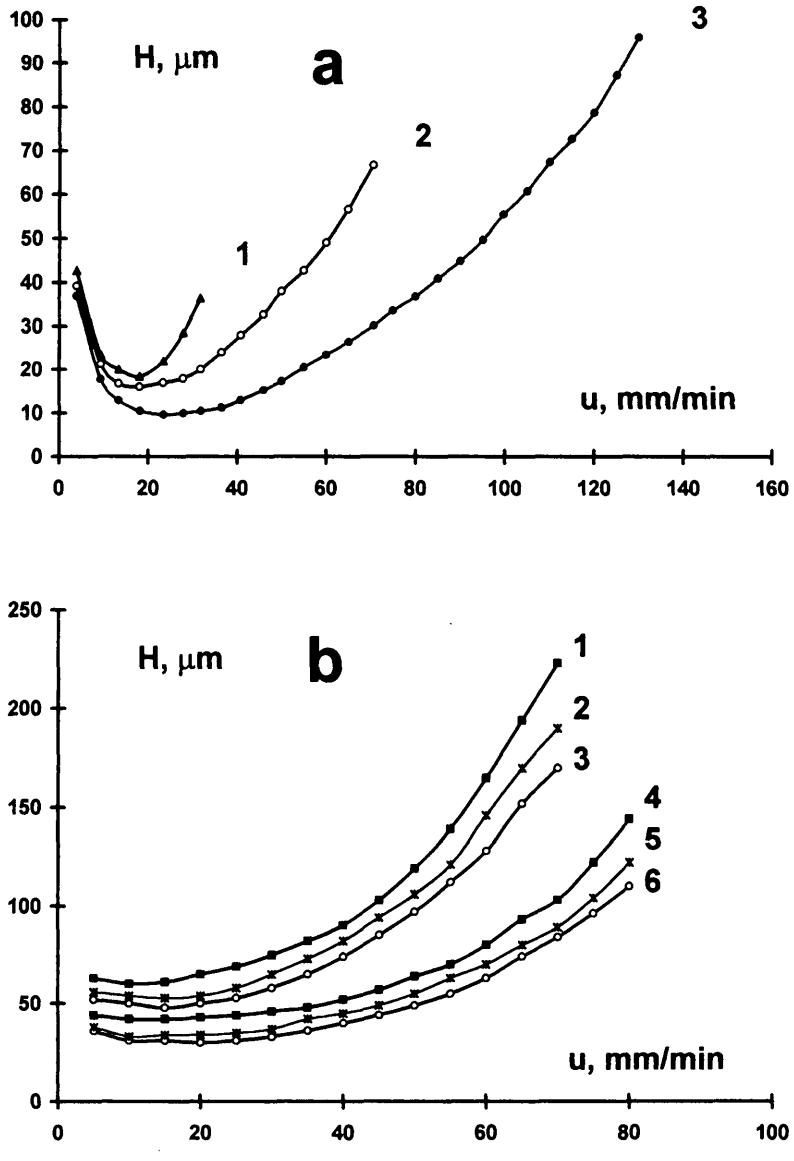

Figure 7. Effect of linear velocity (U) on theoretical plate height (H) of various plates under different OPLC operating conditions: (a) HPTLC silica gel, fully off-line OPLC, different external pressure: 1, 1 MPa; 2, 2.5 MPa; 3, 5 MPa; sample, PTH-valine, 5 μL/10 mm band. (b) 1, fully on-line OPLC, preparative layer; 2, off-line sample application and on-line separation and detection, preparative layer; 3, fully off-line OPLC, preparative layer; 4, fully on-line OPLC, TLC layer; 5, off-line sample application and on-line separation and detection, TLC layer; 6, fully off-line OPLC, TLC layer. Off-line sample applications, 20 μL of 4× diluted standard/10 mm band; on-line sample injection, 20 μL of 4× diluted standard.

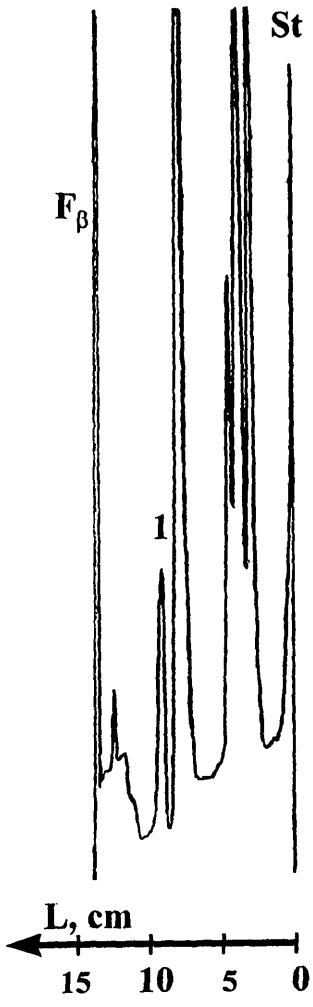

Figure 8. Fully off-line separation of resveratrol from grape leaf extract on TLC silica gel layer: F_β, beta front; St, sample origin; 1, resveratrol. Sample application, 10 μL grape leaf extract/6 mm band.

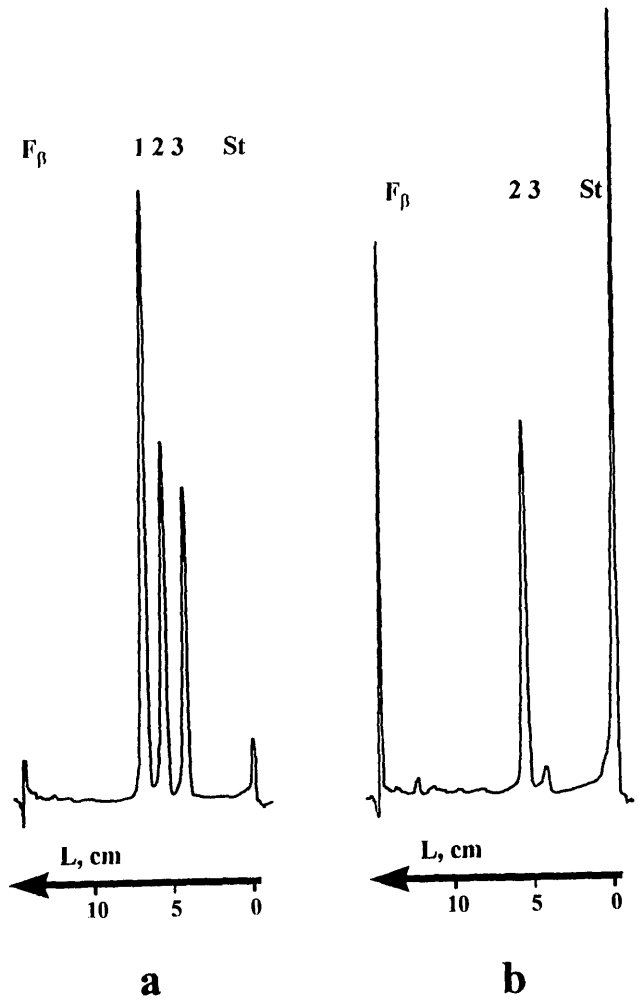

Figure 9. Fully off-line one-directional separation of xanthine standards (a) and xanthines of tea leaf extract (b) on HPTLC silica gel layer: F_β, beta front; St, sample origin; 1, theophylline; 2, caffeine; 3, theobromine; a, 0.25 μL standard mixture; b, 2 μL tea leaf extract.

Detection

Off-line quantitative evaluation of spots or bands was accomplished with the CS-920 TLC and HPTLC scanner (Shimadzu, Kyoto, Japan).

On-line detection was performed with a Liquodet 308 UV detector (Labor MIM, Budapest, Hungary) having 8 μL cell volume.

With both off-line and on-line modes, analytes were detected at the following wavelength: 275 nm for PTH-valine, 305 nm for resveratrol, and 280 nm (analytical) and 300 nm (semipreparative) for xanthines.

Results and Discussion

The OPLC system is highly flexible. If it is equipped with a loop injector and a flow-cell detector, different combinations of off-line and on-line procedures can be applied that are between the fully off-line and fully on-line modes (Figure 3; 11, 17).

Theoretical and Practical Aspects of Use

The first step in development is to optimize the starting separation conditions. A rapid eluent flush ensures a straight front line at the beginning of fully off-line development (*see* the break point, No. 5, in Figure 4). It is followed by a lower flow rate required for separation. In the starting period, the curve of the theoretical front versus time for OPLC (No. 1) crosses the corresponding curve for conventional development (No. 3). The rapidly admitted starting volume quickly fills the layer pores around the trough, overcoming capillary action. The eluent moves 6 times faster during the rapid period than it does in the separation period. Samples should be applied over that distance (No. 5). During development, the eluent inlet pressure increases proportionally with the front distance except during the transition period (indicated by *). When the eluent leaves the layer, eluent pressure becomes constant.

During automatic development, the first step is eluent selection for rapid delivery, which results in admission of the first eluent. For isocratic separation, let us suppose the use, e.g., eluent B. The parameters of external pressure, eluent flow rate,

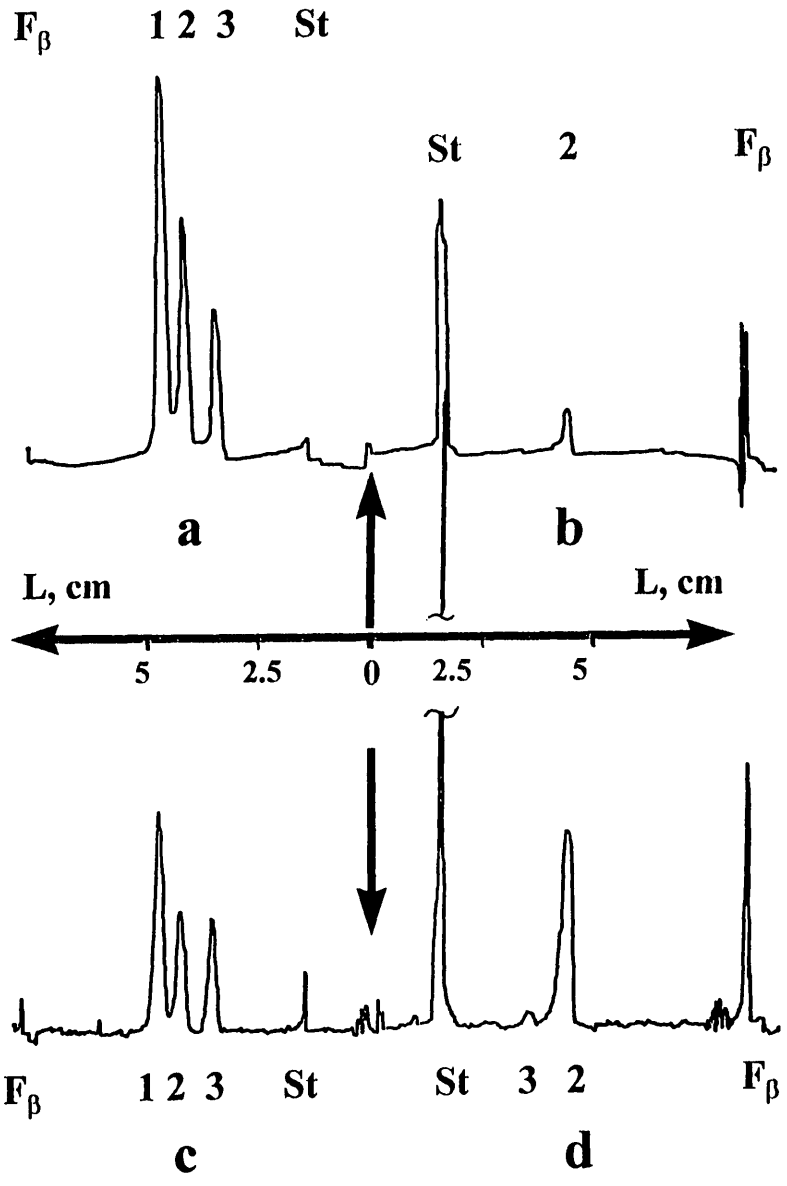

Figure 10. Rapid, fully off-line, two-directional separation of xanthine standards (a and c), caffeine of cola (b), and xanthines of tea leaf extract (d) on HPTLC silica gel layer: F_β, beta front; St, sample origin; 1, theophylline; 2, caffeine; 3, theobromine; a, 0.2 μL standard mixture; b, 2.5 μL cola; c, 0.1 μL standard mixture; d, 1 μL tea leaf extract.

rapid volume, and volume of eluent B should be entered in the blank windows of the PARAMETERS menu. During development, eluent B is automatically selected for rapid admission.

Generally 200–300 μL of a rapid eluent flush is suggested for off-line, one-directional development of a 200 × 200 × 0.2 mm analytical layer (the surface used is only 193 × 193 mm). The suggested distance for off-line sample application is 20–30 mm from the lower edge of the layer (the distance of the inlet trough from the layer bottom is 10 mm). For two-directional runs, samples should be applied at both sides of the central line of the layer (*see* Figure 2, c_2, No. 1). The proposed distance for sample application is 10–15 mm measured from the central line. The suggested rapid volume is 300–500 μL for the above-mentioned layer. (The flow-rate for a two-directional development should be about double that for a one-directional run.) This volume varies proportionally with the thickness of the layer.

The hypothetical chart for continuous development using off-line sample application and stepwise gradient separation can be seen in Figure 5. The first period of the development is fully off-line OPLC, with components moving with constant R_f values. The separation is over when the alpha front reaches the top of the layer or it flows out from the layer (in continuous development). The separated components remain on the layer, and they can be measured in situ by densitometry.

With this system, separation starts on a dry sorbent layer. The diameter differences of the internal and external pores of the layer cause partially and totally wetted zones to appear. The eluent penetrates the pores of particles with low pore diameter more slowly than it fills external pores. Between these zones is the front of total wetness (F_{tw}). (After development, the difference in the refractive indexes of the 2 zones clearly show the boundary.) Above this front is the partially wetted zone, which

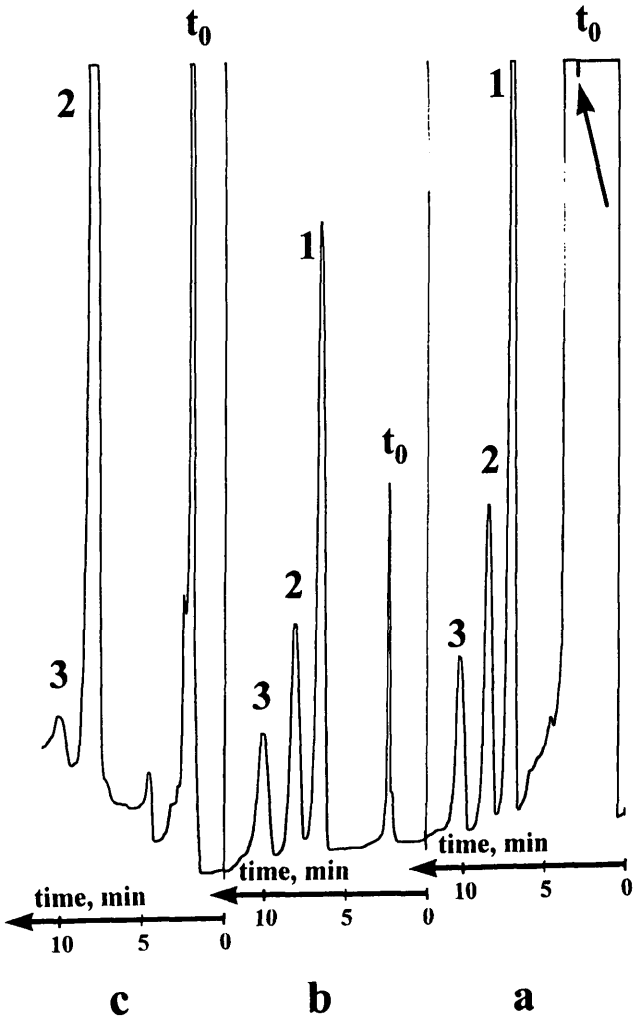

Figure 11. Off-line sample application and on-line separation and detection of xanthine standards (a) followed by fully on-line separation of standard mixture (b) and tea leaf extract (c). 1, theophylline; 2, caffeine; 3, theobromine; flow rate, 2000 μL/min; a, 20 μL of 4× diluted standard mixture/10 mm band; b, 20 μL injection of 4× diluted standard mixture; c, 20 μL injection of tea leaf extract.

is a mixture of eluent and air. Below F_{tw}, pores of the sorbent are totally filled by the eluent (11). The F_{tw} has a flow-rate-dependent R_f value and a zig-zag shape (representing the inhomogeneity of external porosity), which may spoil the separation of this area at low external pressure (<3 MPa). The retention factor of F_{tw} is constant along the plate at constant flow-rate, but the R_f value of F_{tw} depends on the eluent speed (30).

This disturbing effect is slight with the personal OPLC basic system at an external pressure of 5 MPa. Increasing the external pressure from 0.3 to 5 MPa yields a high R_f and a nearly straight-line F_{tw}. The total volume of the layer (V_0, given by the elution volume of benzene using dichloromethane–ethyl acetate, 92 + 8, v/v, and different external pressure) can be reduced by increasing the external pressure. If the external pressure is increased from 0.3 to 5 MPa, the total volume reduction is about 4–5%. This volume difference can be measured also during pressurization, when the eluent flows out from the chamber. Prior to this measurement, the layer should be perfectly filled by the eluent. In this pressure range, this volume increases linearly with increasing external pressure. Assuming a constant,

Table 1. Comparison of xanthine capacity factors for different OPLC processes

OPLC Process	Capacity factor		
	Theophylline	Caffeine	Theobromine
Fully off-line[a]	1.46	1.95	3.07
Off-line sample application and on-line separation and detection	1.67	2.25	3.00
Fully on-line	1.71	2.38	3.21

[a] Calculated according to the method of Geiss (32): $k = (1 - R_f)/R_f$.

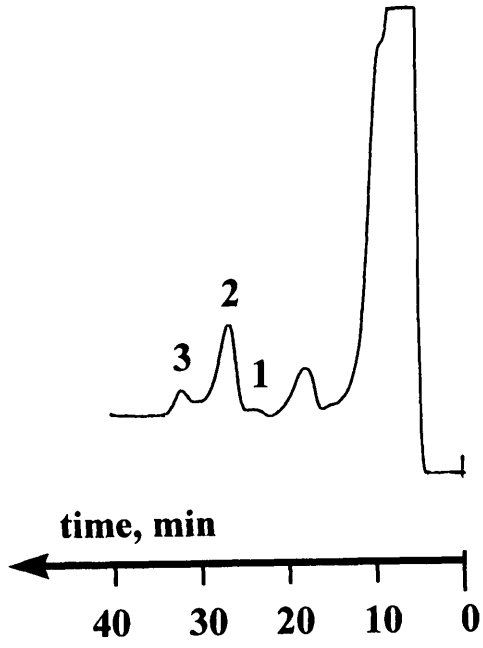

Figure 12. Fully on-line separation of tea leaf extract on 0.5 mm thick preparative plate: 1, theophylline; 2, caffeine; 3, theobromine; flow rate, 1500 μL/min.

pressure-independent volume of internal porosity in this pressure range, external pressure can influence the volume of external porosity and the volume of the empty sorbent bed (V, computed from the physical parameters of the layer at pressure-free conditions; at higher external pressure, it is calculated from the volume reduction through the above-mentioned linear relationship). A 7–9% reduction of external porosity volume was found for TLC and HPTLC silica gel 60 layers. The packing density (ρ) of the planar sorbent bed was calculated from the weight of silica gel (W) and from the volume of the empty sorbent bed, according to LC practice (ρ = W/V; 3% binder content was subtracted from W). Under pressure-free conditions, 0.45–0.46 g/cm^3 and 0.42–0.43 g/cm^3 packing densities were found for TLC and HPTLC silica gel layers, respectively. These densities are slightly higher at 5 MPa external pressure. The total porosity of the layer was calculated from the volume of the empty sorbent bed and from the elution volume of benzene. The external pressure influences the total (ε_T), the internal (ε_i), and the external (ε_e) porosities of the layer. The volume of internal porosity of the layer was calculated from the weight of silica gel and from the specific pore volume of the silica gel (the certified data from the layer producer is 0.74 mL/g). The external volume is the difference between the total and the internal volumes. The porosity data are as follows: ε_T: TLC, 0.78 and 0.77; HPTLC, 0.79 and 0.78; ε_i: TLC, 0.34 and 0.35; HPTLC, 0.32 and 0.33; and ε_e: TLC, 0.44 and 0.42; HPTLC, 0.47 and 0.45, measured at 0.3 and 5.0 MPa external pressures, respectively. The data are slightly higher than previously reported (31).

The total volume (V_0) of a 1 × 193 × 0.2 mm layer at completely filled conditions is 29–30 μL at 5 MPa external pressure. The homogeneity of wetting and the level of filling along the layer were measured by densitometry using fat red (R_f = 1 in the eluent) containing ethyl acetate, according to the method described by Geiss (32). The concentration profile of fat red indicates that in any zone (partially and totally wetted ones) the fat red concentration is constant. In the partially wetted zone, this level was 70–80% of the level for totally wetted conditions at 5 MPa external pressure and 20 mm/min front velocity. Over that, the specific eluent-filling volume of the partially wetted zone can be calculated. In this case, a measured volume of toluene was introduced into the layer for development. After the run, the distance of the alpha front (F_α) and the F_{tw} was measured from the sealed edge. For a 0.200 mm thick, 200 × 200 mm TLC layer, the following parameters were used: EXT PRESS, 5.0 MPa; FLR, 500 μL/min; A VOL R, 0 μL; A VOL, 4750 μL, development time, 570 s. The specific eluent-filling volume of the partially wetted zone was calculated with the above-mentioned V_0 (29 μL/mm) and distances (F_α, 183 mm, and F_{tw}, 97 mm): [4750 μL – (97 mm × 29 μL/mm)]/(183 mm – 97 mm) = 22.5 μL/mm. This volume is 78% the volume of the totally wetted zone (22.5/29 = 0.776) at a linear velocity of 19.3 mm/min. This result shows a good correlation with the above-mentioned densitometric data. Generally, in the partially wetted zone, the specific eluent-filling volumes of a 1 × 193 × 0.2 mm layer are 21–23 μL and 20–22 μL, respectively, with TLC and HPTLC layers.

A rough calculation related to volume can be made for fully off-line, one-directional development by using a factor of 0.9 (considering the incomplete filling conditions of the layer, cf. the above measured data, 4750 μL/[183 mm × 29 μL/mm] = 0.895). For a 185 mm long development (the distance between the sealed edge and the front), roughly 4100 μL (185 × 29 × 0.9 × 0.85 = 4104) is needed for a 0.170 mm thick layer. (The volume varies proportionally with the layer thickness.)

The disturbing effect of F_{tw} can be completely eliminated by using a stepwise gradient separation (30). In this case, the first eluent should be weak, not able to elute the separated components but able to remove the air from the layer. The second eluent should be the solvent system for separation. This version is suggested for off-line sample application and on-line separation and detection to eliminate difficulties with the flow-cell detector caused by eluted air bubbles.

In a fully off-line OPLC process, separation of sample components occurs by elution chromatography while the constituents of the mobile phase (mixture of nonpolar and polar solvents) move according to the principles of frontal analysis. The polar constituent sorbed strongly in sorbent sites can cause secondary fronts (F_β, F_γ, ...) and zones with different solvent strengths (11). In one zone, the solvent strength and polarity are practically the same. The retention factor of a secondary front increases with increasing concentration of the polar modifier (17). A similar effect was found in TLC using a sandwich chamber (33, 34), but because of vapor-phase action, this effect is less pronounced in a normal TLC tank (32). The retention of secondary fronts is constant along the sorbent layer during isocratic development. The dramatic change of solvent strength between the zones can disturb the separation. The secondary front collects the slowly moving components in a preceding zone. The change of sample origin, the change of development distance, and the use of double development are the simplest

ways to eliminate this disturbing effects. This transition period is not used for separation in column LC and fully on-line OPLC because the equilibrium is not perfect.

With stepwise gradient separation analogous to TLC (35), admission of the first eluent is followed by admission of the second and then the third. The elution power of the eluent must be increased, ranked as A, B, and C. The front velocities of the first, second, and third eluents are nearly the same (Figure 5). Different wetting conditions below the first front line (when pore filling is not complete) result in a lower speed of the fronts for the second and third eluents. The introduction times of the B and C eluents can be shifted by the volume of the mobile phase. This shift can lead to the appearance of new parallel fronts. At these front lines, the drop of retention results in peak compression. If the most polar constituent is present in every mobile phase, no more secondary front formation occurs when the B and C eluents are introduced.

The VOL* function in the operation window is used to fill the dead volume of valves and tubes between the switching valve and layer surface. This function is suitable for stepwise gradient separation. The suggested minimum volume is generally 750–770 μL. When the stepwise gradient development is finished, the dead volume is filled up by the most polar eluent. Thus, the instrument should not be used for repetitive separation without rinsing the dead volume by the first eluent applied. To eliminate this difficulty, the VOL* function is applied as the last step of separation. For serial separation, the volume corresponding to VOL* must be subtracted from the volume of the most polar eluent. This newly calculated volume is required for subsequent repeated development. Consequently, the dead volume is filled by the first eluent applied for separation, and the instrument is ready to repeat the separation. For example, the following are parameters for an ideal separation: EXT PRESS, 5 MPa; E FLR, 500 μL/min; A VOL R, 250 μL; A VOL, 1000 μL; B VOL, 2500 μL; and C VOL, 2000 μL. For repetitive separation, the parameters would be: EXT PRESS, 5 MPa; E FLR, 500 μL/min; VOL R, 250 μL; A VOL, 1000 μL; B VOL, 2500 μL; C VOL, 1200 μL; and VOL*, 800 μL (* corresponds to eluent A).

The second period of continuous development is on-line elution (Figure 5). It starts when alpha front leaves the layer and the eluent appears in the connected detector. Because of the partially wetted zone above F_{tw}, the effluent initially contains the mixture of air and eluent that causes detection problems. These difficulties can be eliminated by stepwise gradient separation.

Efficiency

The efficiencies of different sorbent layers were measured. Under fully off-line operating conditions, the correlation between the theoretical plate height (H) and the eluent front distance (L_f) can be seen in Figure 6 for silica gel of various particle sizes developed in normal unsaturated chamber and in personal OPLC system (3). The volume of sample applied was adjusted to the layer thickness. For OPLC measurements at 5.0 MPa external pressure, the following optimal linear velocities were applied: 1.1 cm/min for TLC, 2.4 cm/min for HPTLC, and 3.9 cm/min for Raman plate. (These optimal velocities correspond to reduced velocities of 0.92, 0.90, and 1.08 respectively, and reduced theoretical plate heights at the minimum of 2.6, 2.3, and 2.1, respectively. These reduced velocities are the same as those reported earlier [31], but the reduced plate heights are lower than those reported earlier.) With an HPTLC layer developed under conventional conditions in a tank, efficiency decreases dramatically with development distance. With OPLC, separation remains efficient even with extended development, and the theoretical plate height is practically constant along the plate. (In a conventional tank, the maximum development distance for an HPTLC layer is only 6–8 cm.) Use of sorbent layer made up of superfine particles yields especially efficient separation in OPLC.

Figure 7a demonstrates the differences in the efficiencies of earlier OPLC instruments (Chrompres 10 and Chrompres 25) and the Personal OPLC BS 50 system operating at fully off-line operation mode and with HPTLC silica gel. The H versus u curves hit the ones of column LC. The high external pressure in OPLC significantly increases efficiency. Furthermore, the optimal range of linear velocity becomes broader.

Figure 7b shows the H versus u relationship for TLC layers and preparative layers at different OPLC operating modes. For correct comparison of off-line and on-line separations, the migration distance of a standard sample under fully off-line condition is 170–173 mm. For fully on-line OPLC, the inlet and the outlet sides significantly increase the band broadening. Off-line sample application and on-line separation and detection result in an intermediate height equivalent to theoretical plate (HETP) value, because extra band broadening occurs nowhere but at the outlet side. Because of the excluded extra band broadening, the fully off-line operation yields the most efficient separation. In agreement with early experiences, the TLC layer yields a more efficient separation than does the preparative layer.

Analytical, Fully Off-Line Separations

The fully off-line OPLC is useful for quick analytical separations using either TLC and HPTLC layers. As with conventional layer chromatography, parallel sample application can be used; 17–18 spots and 12–14 bands can be separated on one 200 × 200 mm layer through one-directional, 16–18 cm development. For short development with proper resolution, two-directional separation can be applied. Because of the lower lateral band broadening of shorter development, samples can be applied closer to each other. For example, samples can be spotted at 5 mm intervals instead of 10 mm intervals; 68–70 spots and 34–38 bands can be applied in one plate for two-directional development.

Unlike in column LC where the column can be used for consecutive separations, the layer in OPLC can be used only once. Consequently, the layer is not sensitive to dirty samples, and plant extracts can be applied without preliminary purification. For example, in the fully off-line OPLC separation of resveratrol from grape leaf extract (Figure 8), extracts were applied directly to the silica gel TLC sheet. A stepwise gradient was applied to eliminate air (using isooctane). Neither the pore-filling phenomenon nor the high concentration of contaminants

disturbed the separation of resveratrol. This separation is also suitable for determination of resveratrol in wines.

An HPTLC layer was used for analytical separation of theophylline, caffeine, and theobromine. Figure 9 shows fully off-line isocratic separation of xanthine standards and xanthines of tea leaf extract. The extract was spotted without any purification. The eluent-related retention factor of the beta front was 0.89. The separation of 17 samples required only 8.3 min. Figure 10 shows a rapid two-directional separation of xanthines in 70 different samples. Despite the shorter development, the two-directional separation yielded powerful separation within 5 min.

On-Line Separation and Detection, Semipreparative Scaleup

Off-line sample application and on-line separation and detection of xanthine standards on a TLC silica gel layer is illustrated in Figure 11. Figure 11 shows that off-line sample application yields narrower bands compared with on-line sample application. Furthermore, elution distances for off-line and on-line sample applications vary even though retention times are similar. For example, the distances between the outlet trough and the origin are 173 mm for off-line application and 183 mm for on-line application.

The retention times of xanthines under fully off-line, fully on-line, and off-line sample applications combined with on-line separation and detection are very similar (Table 1).

The linear capacity of loading or semipreparative scaleup separation can be modeled by fully off-line OPLC on a TLC layer and different sample volumes of tea leaf extract. The selected volume of linear capacity measured at a 10 mm band size is 40 μL. (The linear capacity of loading is 0.54 mg extract/g sorbent.) This volume can be converted to the band size of 175 mm, which is the usual band size for off-line sample application prior to isolation. The TLC sampling volume for a 175 mm band is 700 μL (= 40 μL × 175 mm/10 mm), and this can be transferred to the 0.5 mm thick preparative layer. The transfer factor calculated from the thickness ratio of preparative and analytical layers is 2.5. Consequently, the sampling volume of the preparative layer is 1750 μL. Figure 12 shows a fully on-line xanthine separation of tea leaf extract using a 1750 μL (4.55 mg) injection. The quantity of isolated caffeine is 0.6 mg. To remove contaminants remaining on the layer after several repeated injections, a rinse of 25 mL ethanol is applied. Instead of a relatively long equilibration, the layer is dried to remove methanol. When dry, the layer is ready for the next isolation.

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