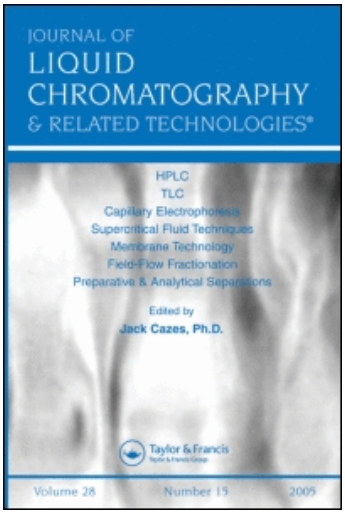

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Single- and Multi-channel OPLC Separation on Non-segmented Sorbent Bed Using Flowing Eluent Wall for Operating Segmentation

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ABSTRACT

A new overpressured layer chromatography (OPLC) separation procedure has been developed for single- and multi-channel separation using a non-segmented sorbent bed and flowing eluent wall (FEW) for operating segmentation. The FEW detaches the sorbent bed into active and non-active parts regarding separation during the process. Only mobile phase is introduced into the non-active part, while, for the active part, eluent and

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also the sample can be admitted, thus the non-homogeneous part of the sorbent bed is excluded from the separation process. The FEW helps the elimination of the edge effect of OPLC in case of single sample injection and abolition of the sample mixing effect of neighboring lanes in the case of a multi-channel separation process. In the case of dirty samples, the one channel FEW-OPLC system is well suited for quick isolation in different preparative ranges using preparative chromatoplates. The multi-channel solution will be a tool for high throughput analysis using efficient fine, superfine, or monolithic layers. The four-channel version can be applied for high throughput multi (parallel) analysis, as well as micro- and semi-preparative parallel isolation using efficient analytical or preparative layers. The FEW provides the possibility for real multi-channel liquid chromatographic separation on a non-segmented sorbent bed.

Key Words: OPLC; Overpressured layer chromatography; Optimum performance laminar chromatography; FEW; Flowing eluent wall; Multi-channel OPLC; Ascorbigen.

INTRODUCTION

It is known, that in overpressured layer chromatography (a new term: optimum performance laminar chromatography; in short form, OPLC), an external pressure is applied to the sorbent layer surface by means of a cushion system ensuring conditions under which the mobile phase can be forced through the sorbent layer by means of a pump. In this system the vapor phase above the sorbent layer is eliminated, and, therefore, OPLC corresponds to high performance liquid chromatography (HPLC) on a column having a very thin but wide cross section.[1–5]

Owing to the high flexibility, the conventional OPLC instrument equipped with an inlet and outlet for mobile phase manipulation is suitable for both off-line and on-line OPLC separations on analytical and preparative chromatoplates. If the mobile phase outlet of the chamber is connected to a flowcell detector, eluting solutes can be detected on-line, and fractions can also be collected. The entire chromatographic process can be performed in a fully on-line operating mode by connecting a loop injector to the mobile phase inlet and a flowcell [e.g., ultraviolet (UV)] detector to the mobile phase outlet, in much the same way as in HPLC.[6–9] The large number of analytical and preparative applications reported in the literature shows the potential of OPLC.[10–14] These conventional OPLC instruments and methodological solutions were suitable in general for illustration of the advantages of OPLC over thin layer chromatography (TLC) and high performance TLC (HPTLC).[15–18]

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A new automated microprocessor-controlled separation system, which represents new technology in the field of forced-flow layer liquid chromatography, ensures rapid and reproducible off-line and on-line isocratic and stepwise gradient separations.[19–21] The efficiency and resolution of separations is considerably increased in the newly developed OPLC instrument and can further be increased in new potential versions. As a result of the sharper, more compact spots or bands, purer compounds can be isolated than from TLC, HPTLC, or conventional OPLC. Owing to the openable sorbent layer, off-line operation is suitable for the application of different detection, as well as biological reagents, which is recognized as an objective advantage of off-line liquid chromatography in comparison with column techniques. The on-line mode of operation of OPLC is better than off-line operation for preparative applications because time-consuming scraping and extraction can be eliminated.[9,22–24] On-line OPLC, a genuine layer chromatographic version of HPLC, is suitable for direct (as well as indirect) coupling to other chromatographic, electrophoretic, and/or spectroscopic techniques (e.g., OPLC-FTIR, OPLC-MS), and these combinations will play a determining role in many future analyses.[14,25–27]

We believe that the new technology of automated OPLC, and its potential new versions, will, because of their analytical and preparative potential, be leading techniques. However, present OPLC systems generate certain limitations because of the sorbent bed and instrumentation, alike. In the present form of on-line OPLC, one sample can be used, which is enough in the case of preparative separation and isolation; however, there is a demand for multichannel and multi-detection systems, also. If we use special conventional techniques for segmentation of the sorbent bed, these sealing methods are problematic. Segmentation of sorbent bed with polymer solution is possible, however, this method soils the sorbent bed at the edges, and the impurities released by the polymer during the separation may disturb the combination of OPLC with mass spectrometry (MS) and other sensitive techniques. For the elimination of these disturbing factors, a technical breakthrough is necessary.

In the present paper, we introduce for first time single- and multi-channel OPLC separations on non-segmented sorbent bed using a flowing eluent wall (FEW) for operating segmentation.

EXPERIMENTAL

Materials and Methods

Chromatographic grade solvents (LiChrosolv, Merck Co., Darmstadt, Germany) were used for mobile phases as well as for extraction. Fine particle

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irregular silica gel 60 layers (20 × 20 cm) sealed on four sides (HTSorb, Bionisis, Le Plessis Robinson, France) were applied for OPLC separations. Dye mixture III was purchased from CAMAG (Muttenz, Switzerland). For sample injection, the original solvent was changed to the actual eluent.

Ascorbigen standard was kindly donated by Mr. György Kátay (Plant Protection Institute, Hungarian Academy of Sciences, Budapest, Hungary). Fresh commercial cabbage was homogenized in methanol–water mixture (5 g in 15 mL; 100%, 90%, 75%, 60%, and 50% of methanol) and filtered. Methanol was evaporated under vacuum, and the aqueous phase was extracted with ethyl acetate. The ethyl acetate was evaporated to dryness and redissolved in a known amount of ethyl acetate (0.5 mL). Ascorbigen separation was accomplished by off-line OPLC using an overrun with chloroform–methanol–acetic acid eluent (90 : 10 : 1). The following program was applied: 5 MPa external pressure, 600 μL/min flow rate, 400 μL rapid eluent volume, and 7000 μL eluent volume. The development time was 706 sec. Separation was monitored visually after derivatization (10% molybdatophosphoric acid in *n*-propanol; 120°C, 5 min). On-line separation-detection was carried out with 1500 μL/min flow rate at 5 MPa external pressure using either off-line or on-line sample application. Ultraviolet detection was accomplished at 285 nm.

Off-line sample application was carried out onto the dry sorbent layer by means of LINOMAT III sample applicator (CAMAG). Band sizes of 40 μL/10 mm and 25 μL/40 mm of cabbage extract were applied for off-line and on-line separation/detection, respectively.

Instrumentation

The automated OPLC 50 System, as well as the new OPLC Purification Unit (Bionisis-OPLC, Le Plessis Robinson, France), have been modified by building more inlet and outlet connections into their hydraulic unit. Both systems are suitable for the formation of 5 MPa external pressure.

The OPLC 50 consists of a liquid delivery system and separation chamber. The microprocessor controlled liquid delivery system includes hydraulic liquid delivery and eluent delivery pumps. The hydraulic unit of the chamber has been changed to an experimental one suitable for four parallel fully on-line separations. The system has five inlet and four outlet connections. One of the inlets was applied for the formation of the FEW and others for parallel sample injection. In the PTFE cover sheet of the cassette, 4 × 40 mm long injection troughs and 4 × 44 mm collection troughs have been formed. The distance between inlets and outlets is 170 mm. The sample application inlets were connected with an HPLC injector through a four channel

distributor, and one of the outlets was coupled to the Liquodet 308 UV (Labor MIM, Budapest, Hungary) detector having an 8 μL cell volume. The other three outlets were extended with tubes, resulting in the same pressure drop for each as the line equipped with the detector cell. Automatic development can be managed under the PARAMETERS and DEVELOPMENT menu points. Every step can be monitored through the LCD display. External pressure can be selected between 0 and 50 bars. The eluent flow rate is adjustable in the range of 10–10,000 μL/min, and A, B, and C solvent systems can be chosen for isocratic or step-wise gradient runs. The DEVELOPMENT menu automatically integrates separation steps of off-line or on-line developments. The process begins with external pressure formation. To ensure a straight front line, at the starting period of development a rapid eluent flush is performed and, just before reaching the starting zone, the eluent devoted to separation starts traveling slower at constant optimum velocity. After delivering the full volume of eluent needed for the separation, an automatic termination is followed by the release of external pressure. An end-signal warns that the process is over, and the cassette can be pulled out.

The new Purification Unit is an OPLC chamber having a built-in hydraulic pump to form 50 bars of external pressure. For eluent delivery, an independent pump, e.g., an HPLC pump or the pump of an OPLC 50 instrument, can be connected into the inlet tee of the chamber. The hydraulic unit of the separation chamber is equipped with two eluent inlet connections, one for sample injection and the other for the FEW formation. The outlet is capable for flowcell detector and/or fraction collector connection. The cassette containing the sorbent layer can be inserted into the chamber. The maximum migration distance between the inlet and outlet is 178 mm. This value corresponds to the distance between the eluent-directing troughs of the PTFE cover sheet of the cassette. The troughs for injection and collection are 178 and 185 mm long, respectively.

RESULTS AND DISCUSSION

In the case of parallel off-line OPLC separation, where the samples are applied separately onto the localized area of the dry sorbent layer, the flow is homogeneous, resulting in the same migration distance for appropriate sample components in the separation field as can be seen in Fig. 1. In contrast, close to the sealed edges the mobile phase velocity in the direction of development is distinct from the homogeneous bulk velocity. This anomaly is valid for the component bands as well, and this impairs the separation quality. The effect is called the side or edge effect. Generally, this band part of the sorbent layer is not used for sample application and off-line OPLC separation. As can be seen,

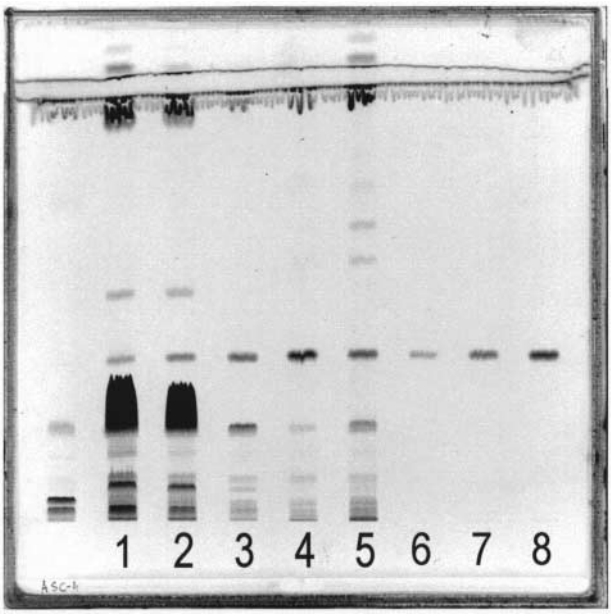

Figure 1. Chromatogram of fully off-line OPLC separation of cabbage extracts and ascorbigen standard visualized by phosphomolybdic acid reagent. 1–5, extracts of methanol–water mixture of 100%, 90%, 75%, 60%, and 50% methanol, 40 μL/10 mm; 6–8, ascorbigen standard of 1, 2.5, 5 μg/10 mm, respectively.

the lateral band broadening is small, and no mixing effect occurs between neighboring lanes. In the case of 10 mm starting sample bands, the final sizes of the component bands after separation are less than 13 mm, as is visible on the picture.

Based on that knowledge and taking also into consideration the results of HPLC,[28] a new general concept has been developed for single-channel (Fig. 2a) and multi-channel (Fig. 2b) OPLC separations using a non-segmented sorbent bed and FEW system for operational segmentation. According to this new concept, in case of single on-line sample application–separation and on-line and/or off-line detection, the sample, as well as the eluent, can be introduced into the same place of the sorbent layer. For mobile phase administration, a pump (item 1) can be used. The mobile phase distributor (item 6) scatters the stream of eluent (item 5) to the mobile phase line of the FEW (item 7) and to the mobile phase line of sample application (item 8), where sample is applied into the eluent stream by the injector (item 2). The distributor space of the eluent to form the FEW (item 9) and the distributor

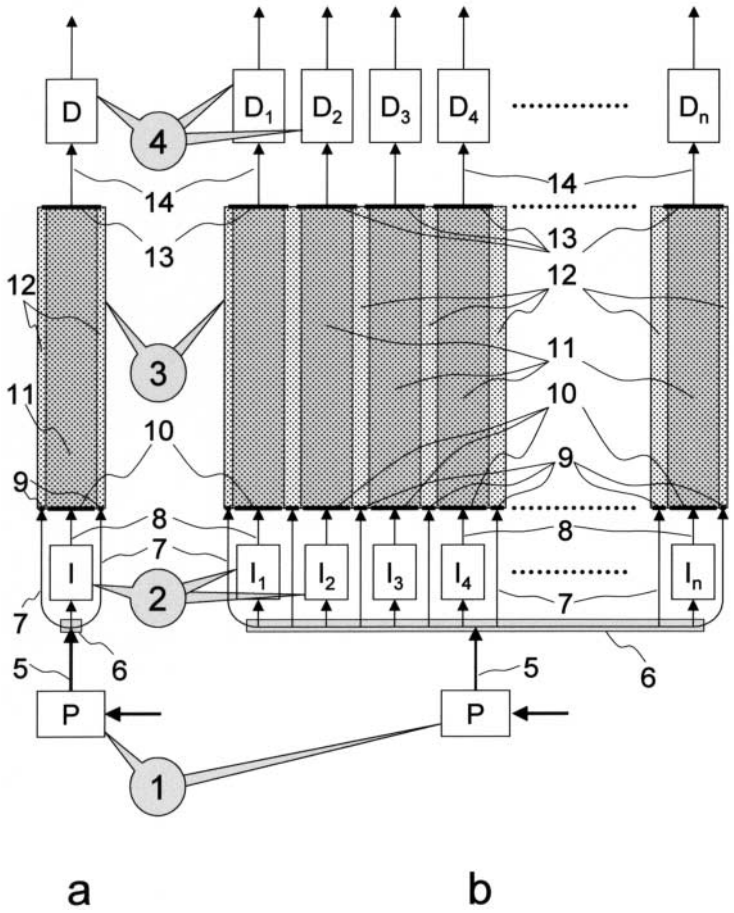

Figure 2. Scheme of one-channel (a) and multi-channel (b) configurations of partially and fully on-line forced-flow separation processes on a non-segmented sorbent bed using an FEW for operating segmentation. *Key:* 1, mobile phase delivery system (pump); 2, injector/multi-channel injector; 3, non-segmented sorbent bed; 4, detector/multi-channel detector; 5, mobile phase stream of inlet side; 6, mobile phase distributor; 7, mobile phase lines of FEW; 8, mobile phase line of sample application (into the stream by injection); 9, distributor space of mobile phase to form FEW; 10, distributor space of sample application; 11, chromatographic separation part of sorbent bed (lane); 12, FEW part of sorbent bed; 13, sample and/or mobile phase collector space at outlet side; 14, stream of sample and/or mobile phase at outlet side.

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space of sample application (item 10) transfer and spread separately the applied liquid connecting to the sorbent bed. These are close to each other and have small volume with a trace of flow resistance perpendicular to the development. The linear velocity of the FEW part (item 12), as well as the separation part (item 11), is roughly the same. The velocity at the sides is distinct from the homogeneous velocity of the bulk. At the outlet side of a non-segmented sorbent bed, the sample and/or mobile phase collector space (item 13) collects and transfers the effluent stream (item 14) to the flowcell detector, which can be connected to a fraction collector for isolation purposes. The collector space at the outlet (item 13) has a small volume and low flow resistance, and it is perpendicular to the mobile phase movement. The shape of the distributor spaces in both the inlet and outlet sides are troughs and can be equipped with filters. In the case of parallel (Fig. 2b) fully on-line separation (i.e., on-line sample application–separation–detection), the FEW can be used for the operating segmentation of non-segmented sorbent bed, detaching it into active and non-active parts regarding separation. Flowing eluent wall works as a spacer during operation. Into the non-active part only mobile phase is introduced, while for the active part eluent and also the sample can be admitted; thus, the unsuitable part of the sorbent bed is excluded from the separation process. Accordingly, FEW can help in the elimination of edge effect of OPLC in the case of single sample injection (Fig. 2a); moreover, it also can eliminate the sample mixing effect of neighboring lanes in the case of multi-channel separation process (Fig. 2b). If both the inlet and outlet connections have a trace of flow resistance, the flow in the sorbent bed is self-regulated.

Figure 3 shows the one-channel FEW system applied for the OPLC Purification Unit. The sample is introduced into the active separation zone where the homogeneous flow exists, and the eluent of the FEW is only introduced into the non-homogeneous zone (compare with Ref.[28]). At the outlet side, the collecting trough directs the eluent from both the active and inactive zones (FEW) as marked by arrows.

The scheme of a four channel FEW model can be seen in Fig. 4. If the sorbent bed is regarded as a homogeneous medium, the flow resistance for the whole active bed is the same, and the effect of irregularity on linear velocity at the sides is negligible regarding the active part of the bed. Based on the above-mentioned facts, we concluded that for a mobile phase stream that previously distributed to several parts and was introduced through tubes to mobile phase distributor spaces contacting separately with the sorbent bed surface (both should have same and low flow resistances), the resulting linear velocity should homogeneously be the same in the whole bed except at the sides. As can be seen in Fig. 4 using a flat bed (sorbent layer), the number of separation channels ($n = 1, 2, 3, \ldots, n$; injection as well as detection) should be tailored to the number of FEWs ($n + 1$).

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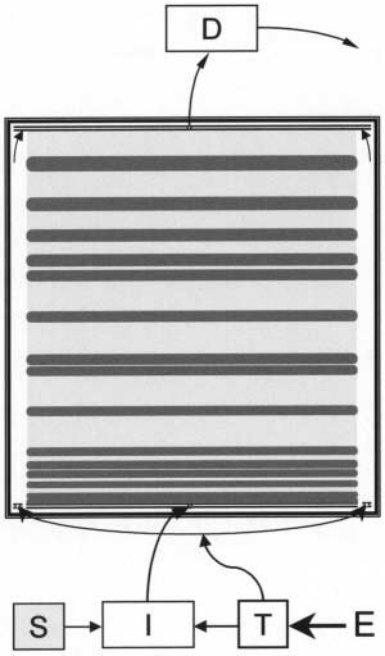

Figure 3. Scheme of one-channel OPLC FEW separation. *Key:* E, eluent to form FEW; S, sample injected into the stream of eluent; T, tee for distributing the eluent stream to the injector and FEW; I, sample injector; D, flowcell detector.

Figure 5 shows a one channel FEW sample application–separation of dyes using 50 μL (a) and 250 μL (b) injections. Both chromatograms show the separation of quite linear sample bands, and the disturbance of the edge effect is eliminated. Of course the 250 μL injected volume yields broader bands than 50 μL. This one channel version is well suited to rapid isolation in case of "dirty samples." Because of the openable sorbent bed, after several injections the layer can be washed by a strong eluent and the purity of sorbent layer can be checked by densitometry or visually using a UV lamp. Generally, 2–5 mg of sample can be injected onto the 20 × 20 cm analytical layer, and the loading can be linearly increased with the thickness.[22–24]

Figure 6 shows the difficulties of parallel sample application without the use of an FEW in the case of the four channel version. The chromatogram of the parallel sample injection–separation clearly shows the side effect and the effect of sample mixing; moreover, the sample partly moves into the back side of the trough. If the inlet troughs are placed close to the bottom seal, the back

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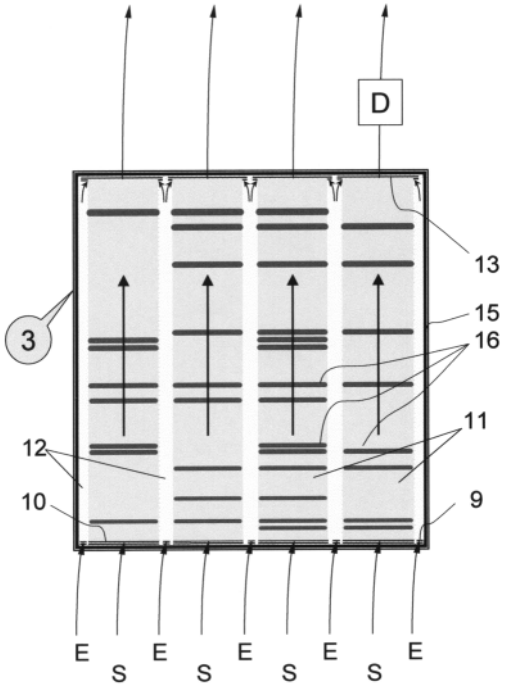

Figure 4. Scheme of four-channel version OPLC separation using FEW and multi-injection suitable for on-line and/or off-line detection/fraction collection. *Key:* 3, non-segmented sorbent bed; 9, distributor space of mobile phase to form flowing eluent wall; 10, distributor space of sample application; 11, chromatographic separation part of sorbent bed (lane); 12, FEW part of sorbent bed; 13, sample and/or mobile phase collector space at outlet side; 15, sealed edge of sorbent layer; 16, components separated; E, eluent to form FEW; S, samples injected into the stream of eluent.

side effect can be reduced. This solution is not feasible without care. Figure 7 shows the chromatogram of a four parallel on-line injection–separation using an FEW configuration. As can be seen, all of the three disturbing effects mentioned above have been eliminated, and all the components in every channel move with same retention. It should be noted that all of the layer and trough inhomogeneity are copied to the sample bands, thus both the layer and the PTFE cover sheet should carefully be used.

In order to check the homogeneous elution at outlet side, the eluent is applied continuously onto the layer, and the effluents at the four outlets are collected separately after parallel injection using a moving chromatographic paper at constant speed. Figure 8 shows the parallel sample bands eluted.

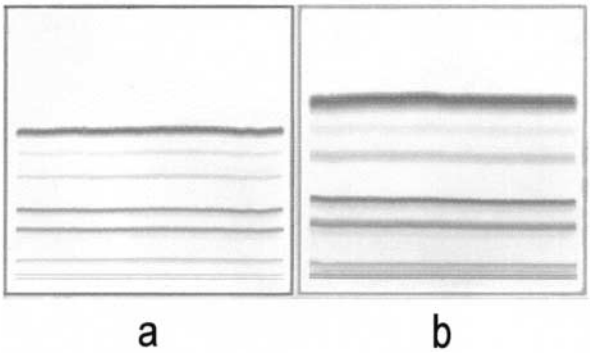

Figure 5. Chromatogram of one-channel OPLC separation of dyes using FEW and 50 μL (a) and 250 μL (b) injection volumes. External pressure, 5 MPa; flow rate, 1 mL/min; eluent, toluene.

Of course this detection mode is far from the ideal one, however, the elution time of parallel bands can be compared. Because of the diverse elution characteristics of components regarding the paper, the shapes of bands are different. To check the sample mixing effect at outlet side, the UV detector was connected to the second outlet channel, and the sample was injected into three channels in parallel and only eluent was introduced into the fourth one (second) in much the same way as in case of the FEW. No peak was found in the non-loaded channel at a sensitivity of 0.01 AUFS (at 280 nm) using hexane–ethyl acetate (90 : 10) with a flowrate of 2 mL/min and dye mixture dissolved in the eluent.

In fully off-line OPLC, where the separation starts with dry sorbent bed, the eluent constituents move according to the principles of frontal analysis while the separation of sample components happens by means of elution chromatography. In the case of an apolar–polar mixture, the polar constituent sorbed strongly to the sorbent sites causing secondary fronts and zones with different solvent strengths. In a zone, the solvent strength is practically the same. At a secondary front (between neighboring zones), the dramatic change of eluent strength can disturb the separation by collecting the slowly moving components in the preceding zone, as well as the small amount of impurities previously collected from the air by the sorbent (see Fig. 1). After the last secondary front leaves the layer, the eluent composition becomes practically constant, but the balance between the stationary and mobile phases is not complete yet. In a lucky case, when the applied eluent is isohydric, the sorbent activity, as well as, the retention of sample components become constant. As is known, the above mentioned transition period is not used in column liquid

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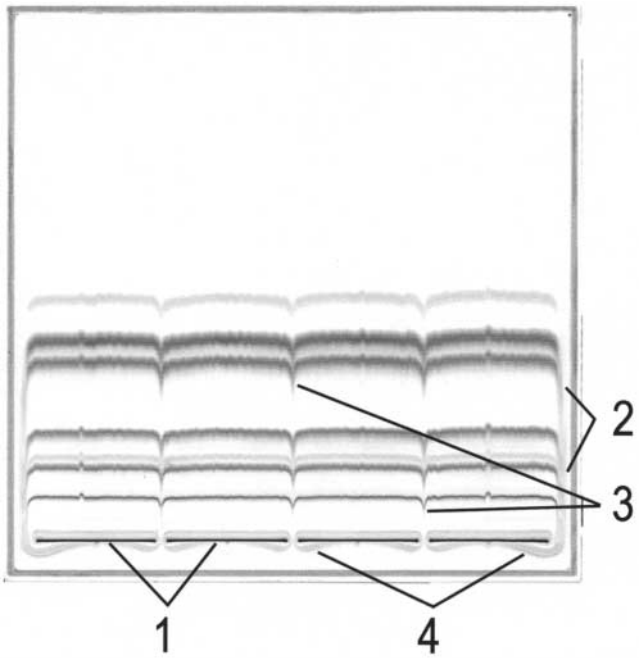

Figure 6. Chromatogram of four-channel OPLC separation of dyes without FEW using 50 μL injection volume for four parallel channels. External pressure, 5 MPa; flow rate, 1 mL/min; eluent, toluene. *Key:* 1, parallel injection troughs; 2, edge effect; 3, sample mixing effect of neighboring sample lanes; 4, back side effect.

chromatography and fully on-line OPLC because the equilibrium is not perfect (see Ref.[29]). Figure 9 demonstrates the relationship between off-line and on-line sample application followed with on-line separation–detection regarding retention and efficiency. Here, the four channel FEW configuration was applied according to Fig. 4. In the case of off-line sample application (a), parallel streaking was applied onto the dry layer, which was followed by on-line elution and only one channel detection. When the alpha front leaves the layer, the eluent appears in the detector, resulting in a high signal (item 1). This period causes detection problems because the effluent initially contains a mixture of air and eluent. (In contrast, the on-line radioactivity detector is not sensitive to air bubbles.[30]) Contrary to the prewashed layer, the secondary fronts (beta and gamma) yield remarkable signals that were not found in the case of the fully on-line process (b). This indicates an improper prewashing procedure of the layer. Notwithstanding that the injection happened after 20

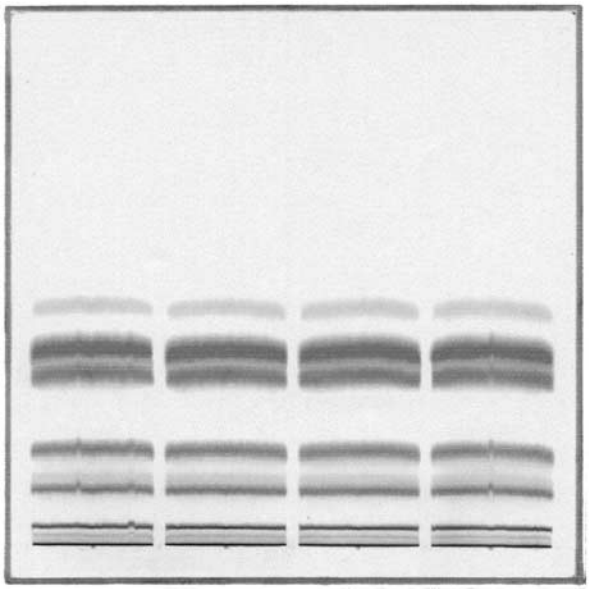

Figure 7. Chromatogram of four-channel OPLC separation of dyes using FEW for segmentation and 250 μL injection volume for four parallel channels. External pressure, 5 MPa; flow rate, 1 mL/min; eluent, toluene.

total volumes of the layer, the ascorbigen retention of the off-line (a) and on-line (b) processes has quite the same values (a lucky case). Owing to reduced extra column band broadening, off-line sample application and on-line separation–detection results in more efficient separation (HETP, 46 μm) than the fully on-line method (HETP, 65 μm). The use of the fully on-line OPLC

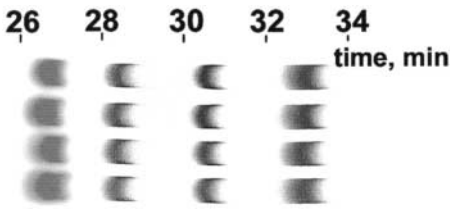

Figure 8. Four-channel fully on-line OPLC separation of dyes collected in parallel by chromatographic paper at outlets using FEW for segmentation. External pressure, 5 MPa; flow rate, 500 μL/min; eluent, methylene chloride; 250 μL injection for four parallel channels; speed of chromatographic paper, 50 mm/min.

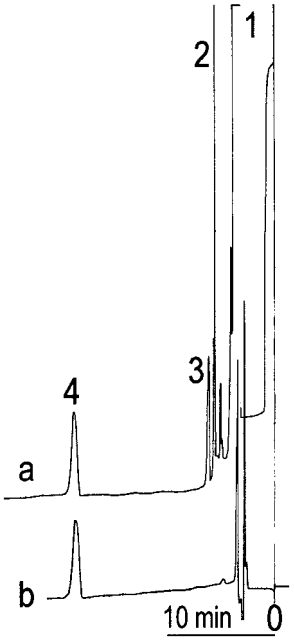

Figure 9. Comparison of off-line sample application and on-line separation–detection (a) with fully on-line process using four channel FEW configuration of OPLC system with one channel on-line detection (b). External pressure, 5 MPa; eluent chloroform–methanol–acetic acid (90 : 10 : 1); flow rate, 1.5 mL/min; sample, cabbage extract in ethyl acetate. *Key:* a, four times 25 μL/40 mm for four separate bands; b, 100 μL for four parallel channels; 1, alpha front and t_0; 2, beta front; 3, gamma front; 4, ascorbigen; absorbance at 285 nm, AUFS 0.2.

process in parallel (4, 8, or 12 channels) with an FEW configuration will be favorable for fields requiring higher throughput for both analysis and isolation (e.g., combinatorial chemistry, medicinal plant research). Because OPLC is a bridge between TLC and HPLC, both eluents can easily be adapted; moreover, combined on-line/off-line detection can be fully exploited. We believe that the multi-channel FEW solution can be extended to column liquid chromatography, also.

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