Review

Overpressured layer chromatography: From the pressurized ultramicro chamber to BioArena system

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ABSTRACT

The pressurized ultramicro (UM) chamber as a closed adsorbent layer chamber enables the use of a special chromatoplate and a pump to increase and optimize the mobile phase flow velocity through an optional development distance in an adsorbent layer. This chamber is the basic instrument of overpressured-layer chromatography (OPLC), which is a separation technique that combines the advantages of conventional TLC/HPTLC with those of HPLC. The versions of OPLC instrument, the character and achievement of off-line and on-line OPLC systems in analytical and preparative use are described. The development of BioArena as a complex bioautographic system means an exploitation of the unique advantages of planar-layer system for detection, isolation and identification of new antimicrobials, antineoplastics, biopesticides and other biologically active substances as well as for studying fundamental biochemical reactions and mechanisms.

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1. Introductory remarks

Different directions of chromatography from its discovery have undergone innovative developments. Among the technical versions one of the most important is liquid chromatography (LC), which nowadays includes two basic techniques – column LC (CLC) and layer LC (LLC). CLC and LLC techniques – as supplementary techniques due to their similar theoretical basis and technical arrangements – have from the beginnings been progressed similarly.

It is known that layer liquid chromatographic techniques are rather simple and require in general few appliances. Paper chromatography (PC) and its various versions, developed in the 1940s by Martin and his colleagues [1], have to be mentioned first. The technical solutions of PC laid the foundation of modern amino acid and peptide analysis and promoted the discovery of the structure of insulin [1–3]. However, the rapid progress in different fields after 2nd world war demanded the elimination of the limitations of PC (e.g. limited adsorption of paper [4]). Among the innovative developments the discovery of thin-layer chromatography (TLC) by Ismailov and Shraiber [5] as well as Békésy [6], the improvement by Kirchner et al. [7], additionally the standardization and spreading by Stahl et al. [8], opened new horizons in separation science and contributed to the isolation and analysis of many natural and synthetic substances. Today, versions of this classical layer liquid chromatographic technique are indispensable in various fields of scientific research and practice, but the limitations of TLC (e.g. limited efficiency) demanded further development efforts.

It is logic to use an adsorbent layer of fine particle permitting effective separation similar to HPLC technique [9]. High-performance thin-layer chromatography (HPTLC) is based already on the use of chromatoplates coated with fine-particle-size and narrow particle-size distribution adsorbent and instrumentation [10,11]. Comparison of TLC and HPTLC chromatoplates revealed that the latter permitted better separation, higher efficiency, but in a short development distance only, because the migration of the mobile phase was slowed down and therefore, the diffusion of the separated components on the adsorbent layer was increased using a relatively long solvent migration distance, especially if viscous mobile phases were applied. The quadratic development law of classical TLC is also valid in HPTLC [12].

HPTLC is in certain cases considered as the planar layer version of HPLC [13,14]. This is true in part only, since a very important and indispensable step of HPLC is missing in the application of fine particle-size adsorbent layer with a narrow particle-size distribution. Namely, the eluent is dispensed into the system by forced flow (by pressure) in the case of HPLC [9]. That is HPTLC is a half solution to the efficient modern planar layer liquid chromatography.

The steady stream of innovations in layer liquid chromatography has resulted in most diverse technical versions in the last decades. In these modern technical versions the mobile phase migrates through the stationary phase in the layer arrangement under the additional action of forced flow. This can be achieved especially successfully and attractively by application of a pressurized ultramicro (UM) chamber and a pump system in overpressured layer chromatography (OPLC) [15] that is in this system fine particle-size of adsorbent layer can be used with forced flow: it is really a planar layer version of HPLC. The exploitation of the unique potential of the planar adsorbent layer for investigation of the mechanism of action of biologically active compounds and for interactions belongs closely to the innovative development of layer liquid system [16].

This review summarizes the progress direction and the results of the development in OPLC including indirect in vitro biological detection and interactions on adsorbent layers (in vitro studies) and partly in vivo biological investigations as well.

2. Steps to the development of OPLC

2.1. Basic elements of ultramicro (UM) chamber and its importance

Attempts to develop an ultramicro (UM) chamber were already made in the 1960s [17]. In this simple chamber, the adsorbent layer on the chromatoplate is covered by a glass plate such a way that the end of the cover plate is not immersed in the eluent. In this way, the failures caused by capillary effect can be avoided. However, because such a rigid glass plate cover did not provide a secure sealing of the adsorbent layer, thus, a UM chamber as shown in Fig. 1 is used nowadays. The adsorbent layer is covered with a plastic membrane consisting of a plastic sponge wrapped in plastic foil.

2.2. Features of pressurized UM chamber: it is the basic instrument of OPLC

The further possibilities of UM chamber as a closed planar-layer chamber, such as the increase of eluent-flow velocity, the application of optional development distance, and the use of a pump system for optimizing flow velocity, were realized by the development of a pressurized UM chamber [15,18,19].

The essential feature of a pressurized UM chamber system is that the adsorbent layer is completely covered with a flexible membrane under an external pressure so the vapor phase above the adsorbent layer is virtually eliminated. The adsorbent layer is spread onto a flat base plate (preferably in a horizontal position), which is covered by a cushion made from elastic foil or other suitable material, and mounted on the cover plate. The base and cover plates are joined and the cushion is filled, advantageously with water, making an external pressure. Fig. 2 illustrates the main elements of a pressurized circular-type UM chamber [20].

The development of the pressurized UM chamber was an original innovative step in the field of LLC [21] using a pump system [22] or other forcing power (e.g. electroosmosis) [23] for the admission of the eluent into the adsorbent layer. This chamber system was the
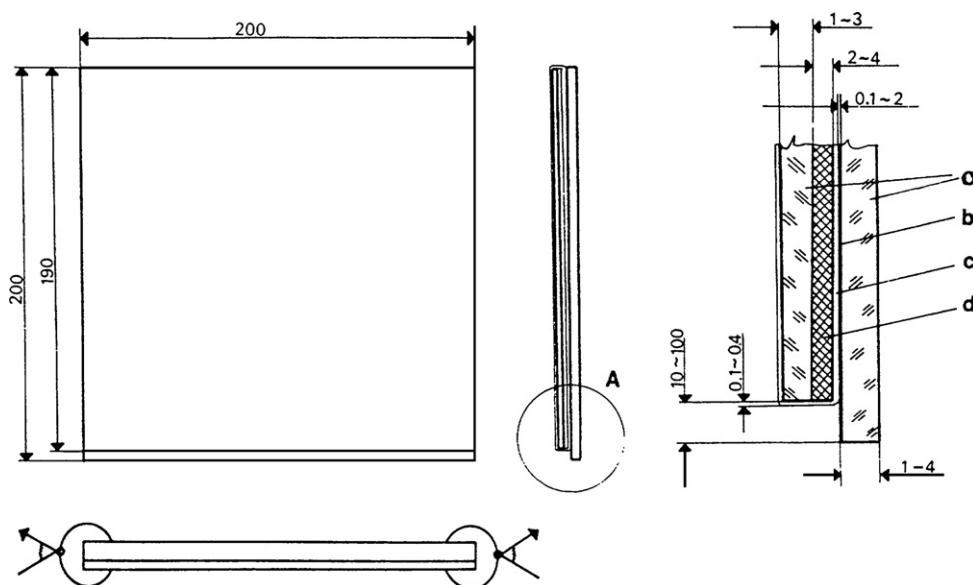
Fig. 1. Schematic drawing of an ultramicro chamber. (a) support plate; (b) sorbent layer; (c) plastic membrane cover; (d) plastic sponge. Distances are in millimetres [20].

first successful step towards a true planar-layer version of HPLC, as well. The pressurized UM chamber can be regarded as a column: the cushion-like half can be controlled with variable external pressure and the other half forms a rigid plastic or metal plate [24]. The ultramicro character of the conventional and pressurized UM chambers originates from the limited space between the surface of the adsorbent layer and the cover plate and from the space between the adsorbent particles [24].

2.3. Development of OPLC instruments and chromatoplates

2.3.1. First commercial OPLC instruments

The essence of OPLC is that the adsorbent layer is closed and pressurized during the separation process and elution can be performed at the highest separation efficiency using the optimum linear velocity generated by a pump. It follows from the principle of OPLC that low (2–5 bar), medium (10–30 bar), and high (50–100 bar

or more) external pressures can be used in this forced flow planar layer liquid chromatographic technique.

On the basis of experience gained with experimental pressurized UM chambers, the first commercial pressurized UM chambers were the Chrompres 10 and Chrompres 25 developed in the nineteen eighties by Labor Instrument Works (Budapest, Hungary). The first commercially available OPLC instrument (Chrompres 10) was a completely off-line system [20]. The second generation instrument (Chrompres 25) was suitable for both off-line and on-line separations [25]. The maximum cushion pressure is 2.5 MPa in this chamber and this higher external pressure on the elastic membrane permitted the use of a superfine-particle-size adsorbent layer, higher viscosity mobile phases. At the same time this system makes possible the increase in the eluent front velocity. The main components of Chrompres 25 are shown in Fig. 3 [22]. This apparatus is suitable for off-line analytical one-directional, 2-D, and continuous separations and
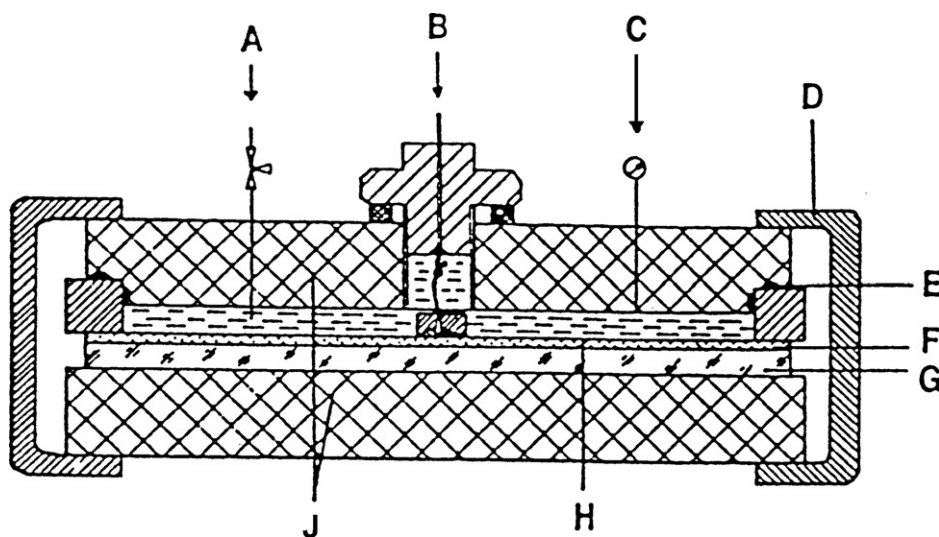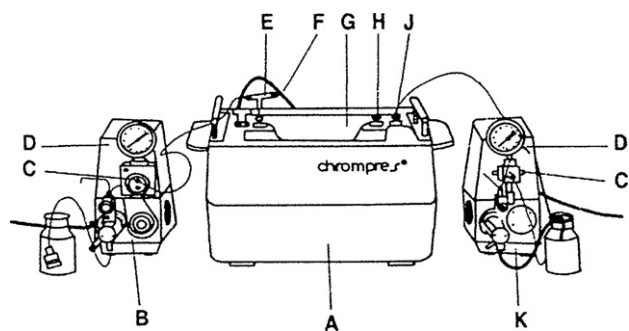

Fig. 2. Schematic drawing of the circular type of pressurized ultramicro chamber. A, water inlet, B, developing solvent inlet, C, pressure gauge, D, screw fastener, E, rubber O-ring, F, adsorbent layer, G, support plate, H, plastic foil cushion system, J, polymethacrylate support blocks [20].

Fig. 3. Schematic drawing of Chrompres 25. A, chamber, B, eluent pump, C, safety valve, D, pressure gauge, E, solvent-inlet valve, F, water outlet, G, polymethacrylate cover, H, solvent-outlet pin, J, water inlet, K, water pump.
With permission, from Ref. [22].

on-line analytical and preparative one-directional linear separations.

Finally, these conventional OPLC instruments and methods were suitable for demonstration of the advantages of OPLC (the progress) over classical TLC and HPTLC [18,26–29].

2.3.2. Chromatoplates for conventional OPLC

Fig. 4 shows the various types of the OPLC chromatoplates. In circular OPLC it is not necessary to seal the edges of the chromatoplate and the eluent inlet is placed in the middle of the adsorbent layer. A chromatoplate that is sealed on three sides should be used for one-directional development. If two opposite edges of the chromatoplate are sealed and the eluent inlet is in the middle of the adsorbent layer in a channel, then the system is suitable for a two-directional separation with a large number of samples. For 2-D separation in an off-line system, the four sides of the chromatoplate must be sealed beforehand and the seal opposite the actual inlet must be covered with a strip of filter paper, or an eluent outlet should be used [20,21,30–32].
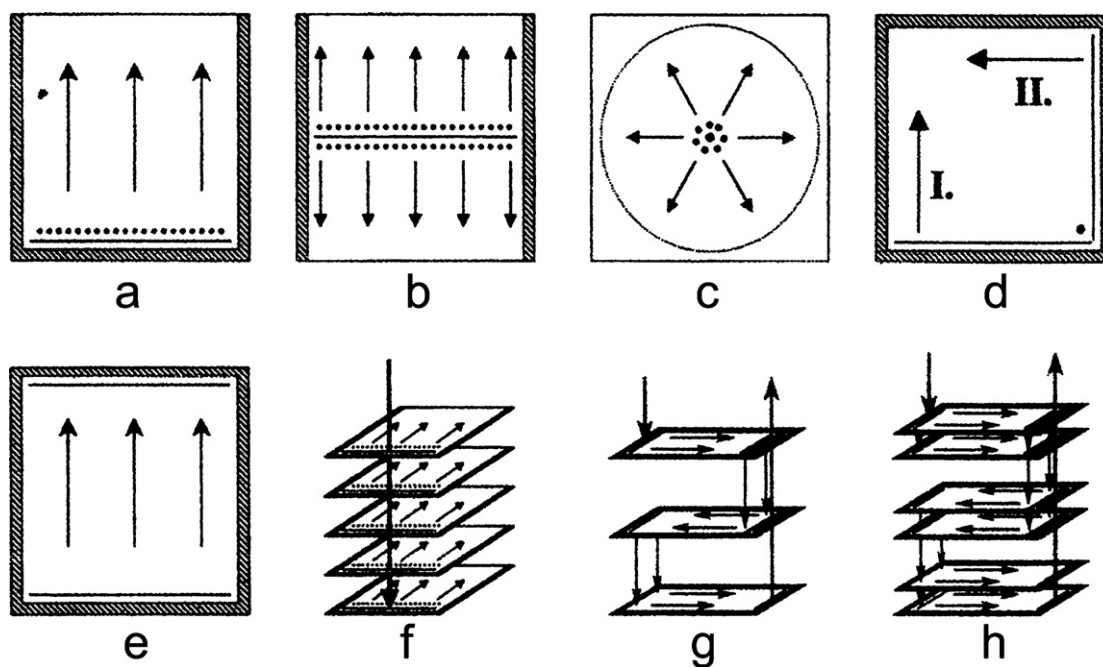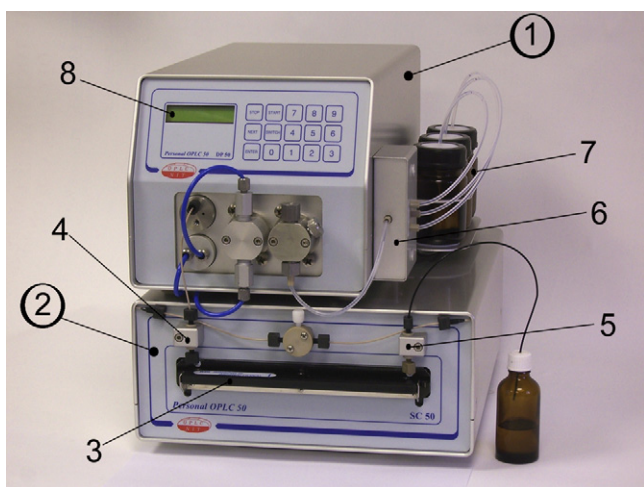
The chromatographic plate for conventional on-line OPLC separation contains two-mobile-phase-directing troughs in the adsorbent layer or in the PTFE insert cover plate. The mobile phase inlet trough directs the mobile phase along a linear front and the mobile phase outlet trough collects the mobile phase at the end of the plate and is connected to detector. The combination of several chromatoplates during a single conventional OPLC separation has special advantages (e.g. chromatography of a large number of samples) [20,21,33]. The introduction of the mobile phase to parallel-coupled [33] and, especially, serially coupled [28,34] multi-layer systems is a critical matter. The parallel-coupled is performed by making a perforation in the chromatoplates, of a suitable size and shape, at the mobile phase inlet. For serially coupled multi-layer systems, however, a route for transfer of the effluent mobile phase is also needed [28,34].

2.3.3. Automatic OPLC instrument and other developments

On the basis of experience gained with conventional Chrompres chambers, OPLC-NIT Ltd (Budapest, Hungary) developed the automated personal OPLC basic system which includes a separation chamber and a liquid delivery system (Fig. 5) [35–37]. The separation chamber generates four main units: holding unit, hydraulic unit, tray-like layer cassette, and attached drain valve. The microprocessor-controlled liquid delivery system includes a two-in-one hydraulic pump and mobile-phase delivery pump. All conditions for single or repeated development can be input and stored in the delivery system software. External pressure (max. 5.0 MPa), mobile phase volume, and mobile phase flow rate can be input, and development time is calculated automatically [36,37].

In automatic OPLC system, the parameters can be adjusted for isocratic and stepwise gradient separations: external pressure (P_{ext}), eluent flow rate (FLR), rapid volume (R or Vol. R), eluent volume A (A or Vol. A), eluent volume B (B or Vol. B), and eluent volume C (C or Vol. C).

The development of the automated OPLC instrument has required the use of a new technology; however, this is perhaps only the first step in this innovative direction.

Fig. 4. Schematic drawing of chromatographic chromatoplates used in conventional OPLC separations; (a) one-directional; (b) two-directional; (c) circular; (d) two-directional; (e) on-line; (f) parallel coupled multilayer; (g) serial coupled multilayer; (h) parallel-serial coupled multilayer.
With permission, from Ref. [36].

Fig. 5. Automated OPLC instrument. 1, liquid-delivery system; 2, separation chamber; 3, cassette; 4, mobile phase inlet; 5 mobile phase outlet; 6, mobile phase switching valve; 7, mobile phase reservoirs; 8, LCD display.

With permission, from Ref. [41].

High-pressure circular OPLC (3.8–4.0 MPa), called high-pressure planar liquid chromatography (HPPLC), was developed by Kaiser and Rieder [38,39]. HPPLC exploits basically all the advantages of the original circular operating mode and uses the experience gained in the field of circular HPTLC, that is, tries to eliminate the problems of the original HPTLC [10,11].

A special version of OPLC was introduced by Witkiewicz et al. [40], who used gas to apply the external pressure; the mobile phase was fed to the adsorbent layer by means of a syringe.

OPLC Separation Unit 100 (OSU-100), an experimental instrument, is a chamber with three inlets and three outlets. This built-in hydraulic pump already generates 10 MPa external pressure. This technical solution exploits the advantages of a high external pressure which increases the efficiency on the basis of the theoretical relationship. For on-line sample application and detection one or two injectors and UV-detectors (Liquodet 308, Labor MIM, Budapest, Hungary) were connected [41].

2.3.4. Cassette systems for automated OPLC

An interesting aspect of automated OPLC that all conventional OPLC operating modes can be performed by use of an appropriate cassette [35,36]. The cassette containing the chromatoplate of modified adsorbent layer with the samples can be inserted in the pressurized chamber (Fig. 6). Linear one and two-directional, and two-dimensional off-line and linear one-directional in-line separations can be performed by isocratic as well as two- and three-step gradient development.

The introduction of a special double-layer cassette system has enabled the possibility of a relatively long development distance which results in increased spot capacity and better resolution [42].

2.4. Main operating steps in OPLC

With present instrumentation, OPLC is suitable for off- and on-line sample applications, separation and detection, a combination of off- and on-line separation and detection, and coupling to other techniques, as it can be seen in Fig. 7.

2.4.1. Off-line OPLC

In off-line systems, all the principal steps in the chromatographic process such as sample application, separation, quantitative evaluation, and isolation are performed as separate
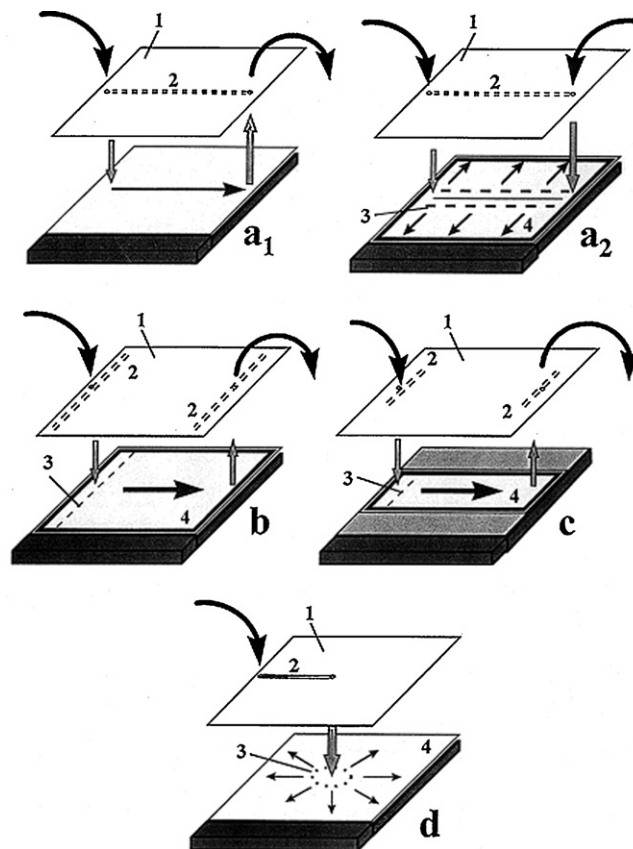
Fig. 6. Cassette solutions to automatic OPLC. (a1) Cassette for system rinsing; (a2) cassette for bidirectional infusion development of 20 cm × 20 cm layer; (b) cassette of 20 cm × 20 cm layer for linear one- and two-dimensional development; (c) cassette of 10 cm × 20 cm glass backed layer for linear one-dimensional development; (d) cassette for circular development. Arrow represents the eluent movement. 1, PTFE coverplate involves eluent directing trough and hole is in floating position; 2, eluent-directing trough; 3, sample; 4, adsorbent layer.

operations [30]. Depending on the application, linear, circular (radial) and triangular operating modes can be performed in off-line OPLC and each has its own particular merits. In the linear developing mode, one- and two-directional, and 2-D developments are possible [43]. In off-line OPLC systems, changes in the composition of the eluent provide good opportunities for special separation modes; that is, isocratic and stepwise-gradient modes. In analytical off-line OPLC, several samples can be processed in parallel. The technique offers further advantages, such as that only the spots and bands of analytical interest need to be assessed, quantitative evaluation can be repeated with various detection parameters. In preparative off-line OPLC after the development the procedures of drying, scraping off the adsorbent layer, elution and crystallization are similar to conventional TLC methods. However, in preparative off-line OPLC, the resolution is considerably increased and thick, fine-particle adsorbent layers can also be used.

Fully off-line OPLC has two operations – infusion and transfusion. In infusion mode the mobile phase is introduced into a totally closed adsorbent layer. The air originally present in the adsorbent layer is continuously compressed during the process, helping to fill up the pores of the particles with the eluent. The infusion process is suitable only for off-line developments without overrun and adsorbent layer sealed at four edges has to be used [36]. The transfusion operation mode corresponds to the original OPLC techniques and enables passage of both air and mobile phase [37]. In this system off-line and on-line operation and their combination are possible.
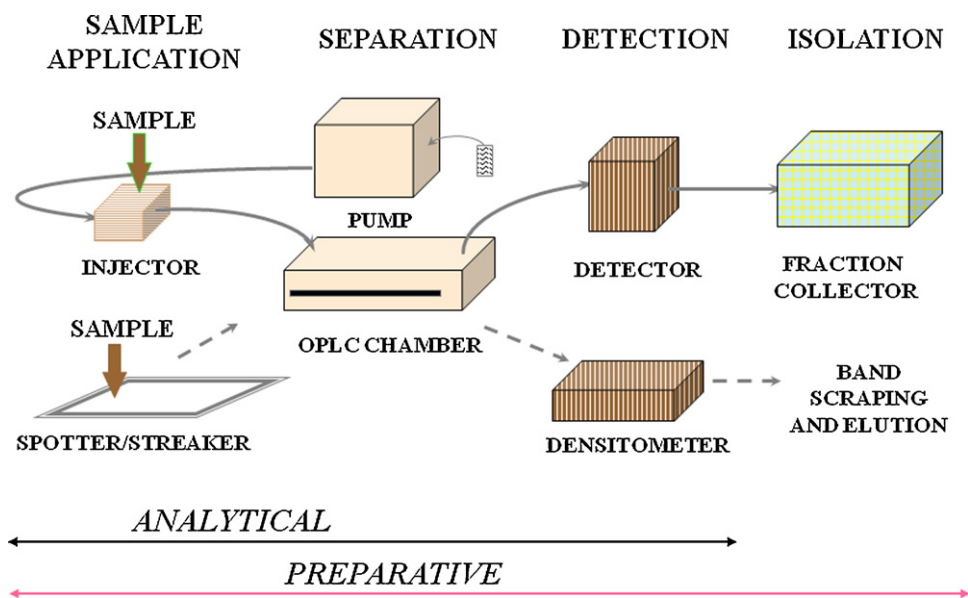
Fig. 7. Schematic diagram of off-line and on-line OPLC. Dashed arrows show the procedure of off-line operating mode.

2.4.2. On-line OPLC

If the eluent outlet of the OPLC chamber is connected to a flow-cell detector, eluted solutes can be detected on-line and fractions can also be collected [25,44]. The entire chromatographic process can be performed on-line by connecting a loop injector to the eluent inlet and a UV detector to the eluent outlet, in much the same way as in HPLC [30,45].

The hyphenation of TLC/HPTLC with mass spectrometry (MS) via a TLC–MS interface can provide valuable analytical results [46–48].

On-line OPLC, a genuine layer chromatographic version of HPLC, is especially suitable for direct coupling to other chromatographic, electrophoretic and/or spectroscopic techniques (e.g. OPLC–FTIR, OPLC–MS, OPLC–MALDI MS) [49–51].

Fully on-line hyphenation of a newly developed experimental OPLC separation unit with diode-array detection and mass spectrometry (OPLC–DAD-MS) can be used for analysis of biologically active substances (e.g. xanthine derivatives and green tea leaf extract) [52] and this system can, in future, also be used for metabolite analysis. On-line OPLC separation can also be combined with electrospray mass spectrometric measurement (OPLC 50-ESI-MS) and this combination has been used for detection of glycolipids [53].

On-line OPLC-RD (radioactivity detection), and OPLC-DAR (digital autoradiography) and HPLC-RD are rapid, economic and effective separation and detection systems which are highly applicable to animal and human metabolism research (Fig. 8) [54].

3. Some theoretical aspects of OPLC

The theoretical aspects of OPLC are summarized in different reviews in more details [32,36,37,55–58]; therefore, here in this paper we want to demonstrate only some characteristic theoretical aspects of OPLC.

3.1. Retention and its influencing factors

In conventional layer chromatography [TLC, HPTLC and preparative layer chromatography (PLC)], the eluent migrates by means of capillary forces, described by the quadratic equation [15,18,19].

In OPLC, the eluent can be forced through (or into in the case of infusion operation) the adsorbent bed by means of a pump system with a selected flow rate. Feeding the eluent by constant flow rate onto the chromatoplate, the speed of the α front (Fα) depends on the free cross-sectional area of the adsorbent layer in the direction of the development. Only linear development is able to result in constant linear velocity, chromatoplates with circular and triangular shapes are not. In contrast with linear development circular OPLC yields decreasing velocity of Fα along the radius [59].

3.2. Relationship between the average theoretical plate height (H) and migration distance (L)

Fig. 9 shows clearly that H (HETP) is practically constant in off-line OPLC along the adsorbent layer, is independent on development distance. This is more favorable for conventional fine-particle adsorbent layers than for coarse-particle layers [13,60]. The use of a superfine-particle-size (e.g. 3 μm) adsorbent layer results in a dramatic increase in the efficiency of the separation; such stationary phases cannot, however, be used in conventional TLC or HPTLC. This arises from the possibility of optimizing the mobile phase velocity in OPLC, by using a pump to direct the mobile phase into the stationary phase. The consequence of the optimization of linear velocity is that two flow profiles also counterbalance each other when superfine-particle adsorbent layers are used [61–63].

However, Fig. 9 illustrates clearly the basic difference between the efficiency of TLC, HPTLC, and off-line OPLC and also explains the flow profile of the mobile phase in OPLC.

3.3. Relationship between average theoretical plate height (H) and mobile phase velocity (u) in OPLC

Fig. 10 shows the H-u relationship for different OPLC instruments, including the automatic personal OPLC instrument [35]. It is apparent that increasing the external pressure results in increased optimum mobile phase front velocity with an increased optimum velocity range.

In OPLC, H value can vary characteristically with linear velocity similarly to HPLC [60,64,65]. The curves from the plot of H against linear velocity (u) for the different operating modes in OPLC are very similar but H values are different. The lowest H values are

ISOLATION OF ^{14}C LABELLED METABOLITES BY COMBINED OPLC-HPLC SYSTEM
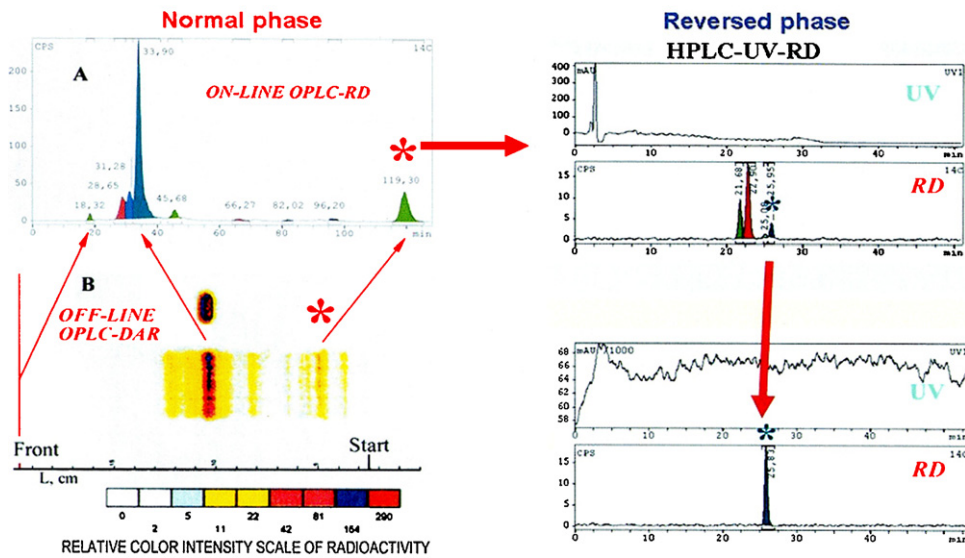

Fig. 8. Isolation of ^{14}C labelled metabolites by combined OPLC-HPLC system. Normal phase OPLC separation (left) reversed phase HPLC separation (right). Peak marked by asterisk has been separated for isolation.

obtained for fully off-line OPLC and the highest for fully on-line OPLC. Between these are the two curves of partial off-line (or partial on-line) OPLC. The differences among these systems originate from "extra-column" band broadening; this does not occur in the fully off-line system.

The increase of external pressure reduces H in off-line OPLC. Despite of this, the same increase in efficiency was not observed for on-line OPLC [33].

## 4. Multi systems – progress in OPLC

For to-day it is clear that the efficiency and attractivity of OPLC technique can be increased dramatically by the use of different multi systems which are coming in this case from the attractivity of the adsorbent layer in multiple mode and from the application of a forced (directed) flow of the eluent in it.
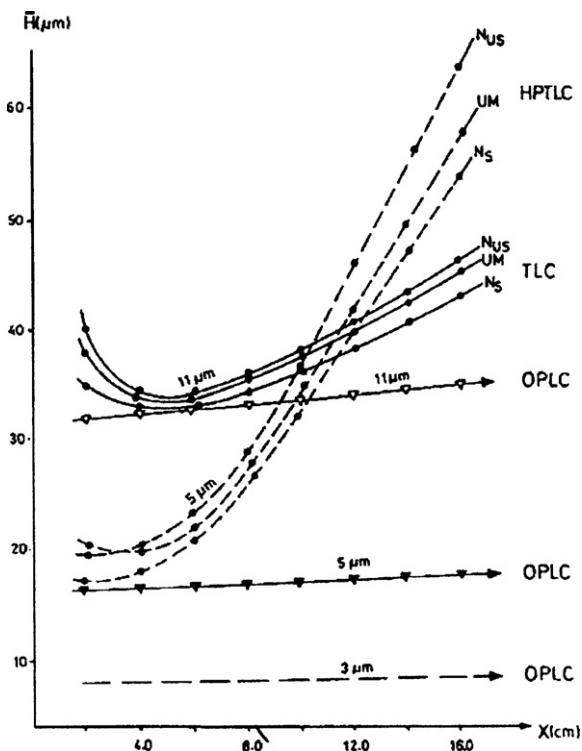
Fig. 9. Correlation between the average theoretical plate height (H) and the distance (X) travelled by the mobile phase on silica gel layers of different particle size and in different chamber systems. Ns, normal saturated chamber; Nus, normal unsaturated chamber; UM, ultramicro chamber (totally closed adsorbent layer, without overpressure); OPLC chamber using 11, 5 and 3 μm particle size, respectively. With permission from Ref. [36].
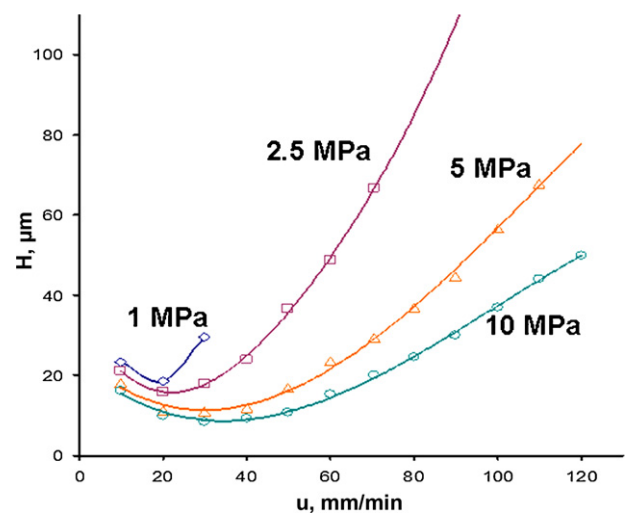
Fig. 10. Effect of linear velocity (u) on theoretical plate height (H) at different external pressures. The results were obtained from the use of the experimental OPLC Separation Unit 100 with the following conditions: 5 μl of a 0.36 mg mL^{-1} PTH-valine solution was applied in 10 mm band, and chromatographed on HPTLC silica gel (Merck) with dichloromethane-ethyl acetate 92:8 (v/v) as mobile phase; the development distance was 170 mm. With permission, from Ref. [31].
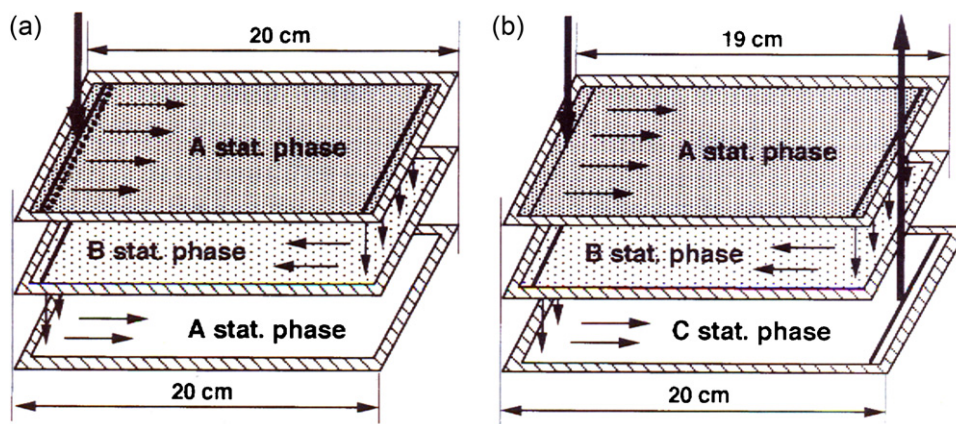
Fig. 11. Schematic diagram of multilayer (ML)-OPLC using different stationary phases of decreasing polarity. (a) Arrangement for analytical off-line separation of eighteen samples or one sample for micropreparative separation. (b) Arrangement for on-line analytical or micropreparative separation. With permission, from Ref. [66].

4.1. Multilayer systems

Parallel solution of overpressured multilayer chromatography (OPMLC) using two or more chromatoplates is very attractive because a large number of samples (50–100 or more) can be separated during one development process [33]. Serial coupled OPMLC (called "long distance" OPLC) can be used for the increase of the theoretical plate number and resolution alike as elaborated by Botz et al. [34]. Several chromatoplates are placed on top of each other to extend the development distance. The potential of these serial connected adsorbent layers can be increased further by use of different (hetero) stationary phases during one development [66] (Fig. 11). The end of the first uppermost chromatoplate has a slit-like perforation to enable the mobile phase to flow to a second adsorbent layer, in which the migration continues until the opposite end of the chromatoplate; there the chromatography can be continued onto a subjacent chromatoplate or the eluent is led away.

4.2. Multidimensional OPLC

For difficult separation problems the application of multidimensional (MD) OPLC is necessary, because the power of one-dimensional chromatography is often inadequate for complete resolution of the components which are present in, e.g. complex biological samples [67,68].

According to Giddings [69] the definition of multidimensional chromatography is as follows

- The first condition is that the components of a mixture are subjected to two or more separation steps in which their displacements depend on different factors.
- The second criterion is that when two components are substantially separated in any single step, they always remain separated until completion of the separation.

Principle of classical MD TLC (2D-OPLC) development is as follows: chromatographic development in one direction followed by a second development in a direction perpendicular to the first [3]. The method consists of spotting a sample at the corner of a chromatographic plate and enabling migration of the mobile phase in the first direction. After drying, the second development on the chromatoplate, in a direction at right angles to the first, can be started with another mobile phase.

There is a proposed theoretical model whereby maximum peak capacity could be achieved by use of 3D TLC (OPLC) separation

[70–72]. Because of technical problems, until now this idea could not be realized in practice.

Fig. 12, from the different technical efforts, illustrates a special solution proposal in patent level of 2D and 3D separations.

4.3. Multichannel OPLC

A new general concept has been developed for single-channel and multichannel OPLC separations using a nonsegmented adsorbent layer and a flowing eluent wall (FEW) system for operational segmentation [73]. For FEW system the original hydraulic unit of the OPLC has been changed to a new one which is equipped with
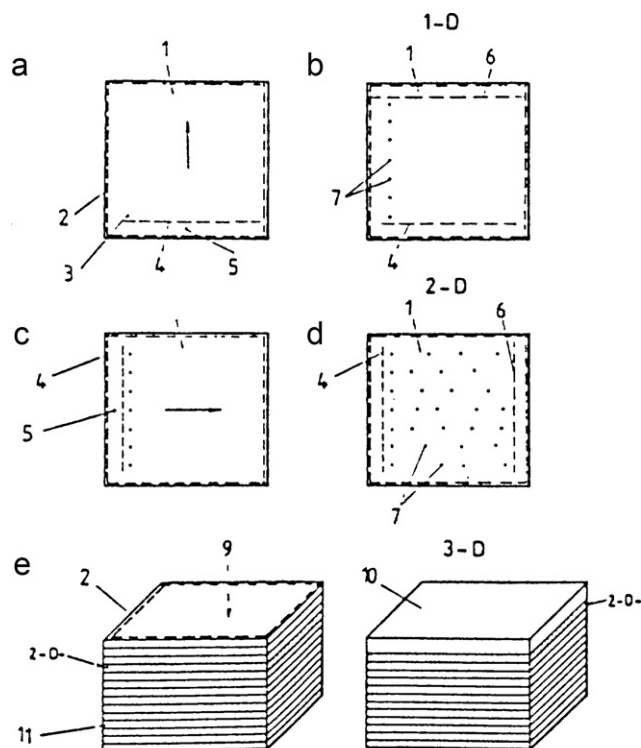
Fig. 12. Schematic drawing of 1-, 2-, and 3-dimensional OPLC separation. 1, adsorbent layer; 2, sealed edge; 3, eluent directing trough; 4, samples; 5, eluent inlet point; 6, eluent front; 7, separated compounds; 9, distributor plate for 3-D; 10, cover plate for 3-D separation; 11, permeable chromatoplates with sealed edges.

Figure is from a patent application, and lecture in Aix-an Provence, 1992.
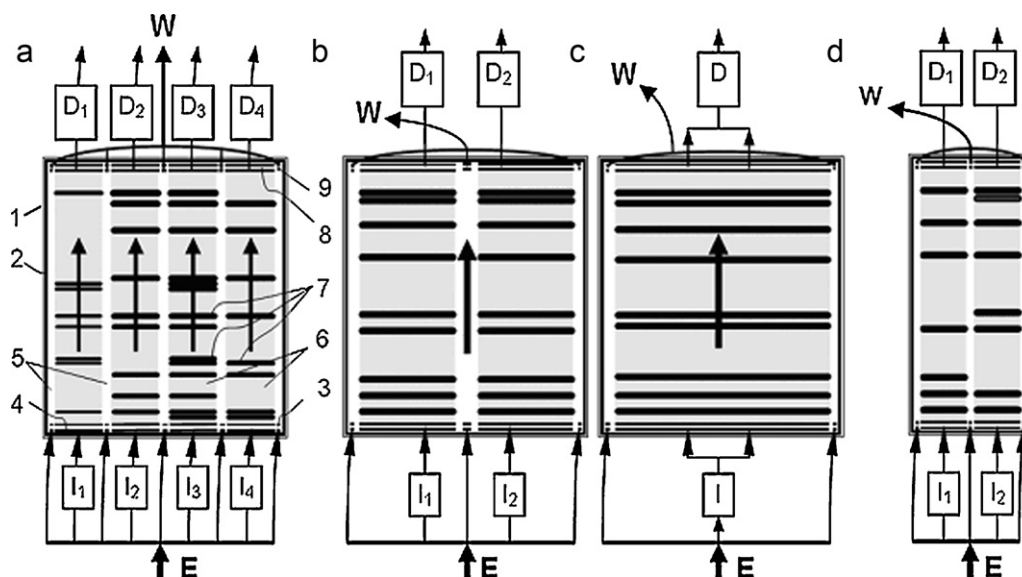
Fig. 13. Schematic diagram of a four-channel OPLC system with the FEW-I/O arrangement dedicated to fully on-line separation of four samples (a), two samples (b), or one sample (c) using 20 cm × 20 cm, and two samples using 10 cm × 20 cm (d) adsorbent layers. 1, non-segmented adsorbent layer; 2, sealed edges; 3, distributor space of mobile phase for FEW formation; 4, distributor space for sample application; 5, FEW part of adsorbent layer; 6, separation part of adsorbent layer; 7, components separated; 8, sample collector space at outlet side; 9, collector space of FEW at outlet side; E, mobile phase; I, injectors; D, detectors; W, waste collected by FEW lines. With permission, from Ref. [75].

two mobile phase inlet connections, one for sample injection and another for the FEW formation, and the outlet can be connected to a flow cell detector and/or a fraction collector (Fig. 13). The waste eluent from the FEW formation is collected separated. The experimental four and eight-channel FEW versions are suitable for parallel fully on-line separations [41,73–75].

For parallel fully on-line separation the FEW can be used for segmentation of a nonsegmented adsorbent layer, dividing it into active and inactive parts with regard to the separation. Only mobile phase is introduced into the inactive part whereas mobile phase and the sample can be introduced to the active separation part; the unsuitable part of the adsorbent layer is thus excluded from the separation process. The FEW helps eliminate the edge effect of OPLC for single sample injection, and abolishes the band widening in horizontal direction, so the sample mixing effect of neighboring lanes in multichannel separation processes. The FEW as an innovative technical solution in planar layer liquid chromatography enables real multichannel liquid chromatographic separation on a non-segmented adsorbent layer [73,75]. Fig. 14 illustrates an eight-channel FEW version which is an integration of multichannel and multidetection systems.

## 5. Analytical and preparative applications of OPLC

OPLC have already been used successfully in different fields. The up-to-date results have been summarized in details [21,22,24,30,32,36,37,31]. Therefore, in this review we will only demonstrate some interesting trends in the applications of technical versions of OPLC.

5.1. Analytical applications

OPLC ensures a constant and high flow velocity, even for viscous mobile-phase mixtures with poor adsorbent wetting characteristics (e.g. those used on RP chromatoplates). The development time is shorter than in TLC or HPTLC, and so molecular diffusion is reduced substantially in OPLC, producing compact spots with better resolution than in TLC or HPTLC [76–78].

The automated Personal OPLC 50 system with maximum 5 MPa external pressure and tray-like cassette construction has been used for separation of a variety of substances (e.g. ascorbigens, aflatoxins, formaldehyde derivatives, etc. [79–83]).

Comparison of OPLC with other conventional and new planar layer liquid chromatographic techniques is useful for characterization of the separation potential of these techniques [84–86]. OPLC with off-line hyphenation have been used for separation. OPLC has already combined with in and/or ex situ spectroscopic
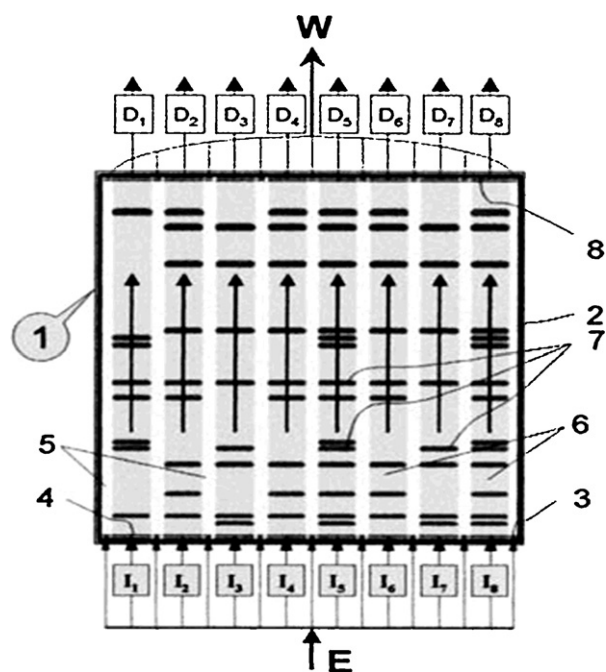
Fig. 14. Schematic diagram of eight-channel OPLC separations using parallel injections and FEW-I/O arrangements. Further conditions are at Fig. 13.
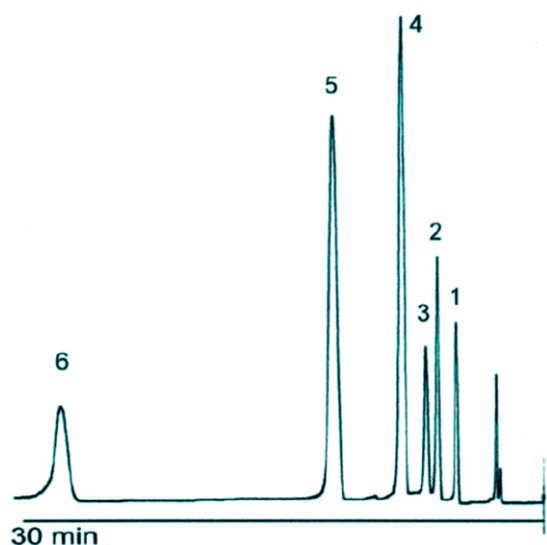
and spectrometric detection using different types of instrument (Raman, FAB, MALDI MS and GC–MS, etc.) [87–91].

The newly developed experimental OPLC Separation Unit 100, generating already 10 MPa external pressure, enables more efficient fully off-line (see Fig. 10) and on-line separations than previous generations of OPLC system (Fig. 15) [75].

5.2. Preparative applications

Although, preparative exploitation of OPLC is poor, up-to-date preparative applications of OPLC have been reviewed [36,92,93].

Similar to analytical OPLC off-line and on-line operating modes can be used in preparative OPLC, too. The on-line OPLC is more effective for preparative applications because the time-consuming scraping and elution are omissible [36,93,94]. In on-line preparative OPLC there is a possibility for the isolation of purer substances than in off-line preparative OPLC.

On-line sample collection after high-resolution stepwise gradient OPLC separation combined with digital autoradiography (DAR) has been used for isolation of plasma and urine metabolites of ^{3}H or ^{14}C-radiolabeled deramcyclane (a new anxiolytic substance) [95]. This separation process can be followed by different MS techniques for determination of the structure of the minor and major metabolites.

OPLC has been used for semipreparative separation–isolation of bound vitamin C from cabbage extract on analytical silica gel layers [96] and for isolation of xanthines from tea leaf extract using 0.5 mm preparative adsorbent layer [35].

6. Novel study of biological activity of natural and synthetic compounds

6.1. Unique great potential of adsorbent layer liquid system for biological detection and interactions – in vitro and in vivo studies

It must be noted firstly that the adsorbent bed in the column arrangement is not suitable and so it cannot be used for biological detection and interactions because the living cells (e.g. bacterial
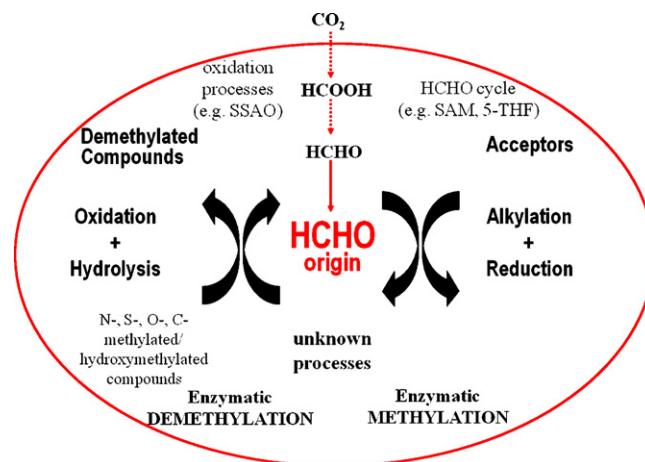
Fig. 16. The main elements of the formaldehydome.
With permission from Ref. [31].

cells) do not grow there and so their detection, etc. is not possible. This fact increases the importance of planar layer liquid chromatographic techniques because, in the future time, biological detection and interactions of separated substances in situ in the adsorbent layers will be a crucial and indispensable methodological solution for isolation, identification and characterization of new antimicrobials, antineoplastics, biopesticides and others [97,98], as well as for studying fundamental biochemical reactions and mechanisms [98,99].

The well-known direct bioautography, which integrates application of planar layer liquid chromatographic separation and post-chromatographic bioassay, can be regarded as the most efficacious assay for detection of antibiotic-like compounds [100]. Although, direct bioautography is a leading technique in bioautography, nowadays it is already not enough.

6.2. In vitro biological studies – BioArena investigations

Although the "antibiotic era" is barely six decades old, nevertheless the mankind is already faced with a global problem of emerging resistance of virtually all pathogens [101]. The tasks are given: among others, such small and large co-factor molecules have to know which play a fundamental role in the antibiotic effect and in the basal and induced disease resistance.

6.2.1. Biochemical–chemical background of antibiotic experiments

According to recent observations formaldehyde (HCHO) is an endogenous component of all biological systems [102,103], mainly in the form of hydroxymethyl group. Therefore, it can be detected and measured in different biological samples using a HCHO capture molecule (e.g. dimedone) for collecting HCHO molecules from a given biological unit [104]. There is a primary HCHO cycle in biological systems in which the formation of S-methyl group of L-methionine from HCHO, originated from natural HCHO generators, and the HCHO-yielding function of S-adenosyl-L-methionine (SAM) are essential components [102,103].

HCHO can be continuously formed by almost all cells both intracellularly and extracellularly. The term "formaldehydome" means the complete set of HCHO-cycle-mediated and non-mediated HCHO pathways of a given biological unit (Fig. 16) [105]. The formaldehydome can interlace the big biological units such as genome, proteome, etc., so HCHO is a determining endogenous component in all biological systems.
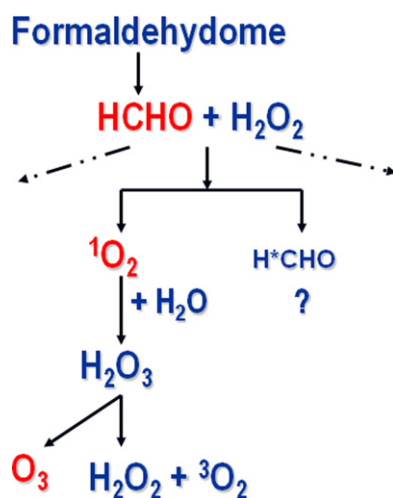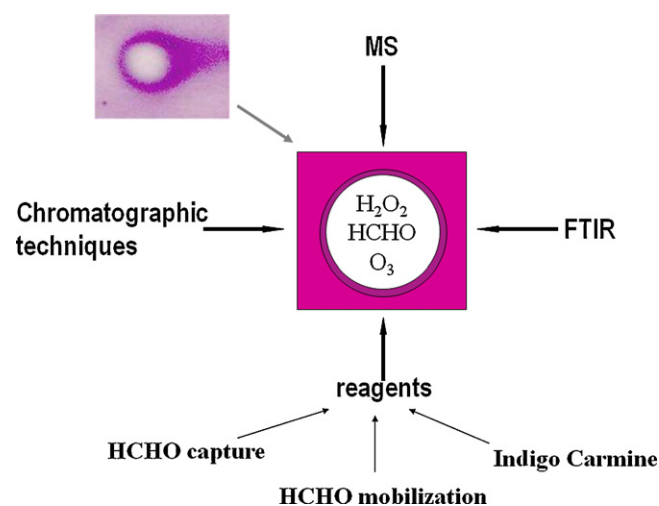
Fig. 17. Possible formation of different reactive oxidants by interaction of HCHO and H_2O_2.
With permission from Ref. [31].

Fig. 18. Main steps in the detection and identification of a typical spot with the BioArena system.
With permission, from Ref. [31].

Endogenous HCHO can interact with – among others – H_2O_2 as another universal small endogenous molecule. In this interaction $^1O_2$ and excited HCHO (HC*HO) can be formed [106,107]. Fig. 17 illustrates the reaction series supposedly [108], taking into account earlier and more recent observations [106–110]. $^1O_2$ can oxidize the H_2O molecules endogenously and H_2O_3 can be formed from which, among others, O_3 can be released [109,110]. This fundamental endogenous reaction can occur in in vitro conditions (in chromatographic spots), as well as when using the BioArena system [111,112]. It is probable that O_3 is generated endogenously from different biochemical reactions in diverse biological systems; however, it seems that $HCHO/O_3$ is a determining pathway in this complicated system.

These very reactive small molecules – from HCHO to O_3 – may be the crucial factors of the innate (natural) resistance and form a common bridge between the innate (natural) and adaptive (induced) resistance in plant and animal organisms, alike [31,108].

BioArena system as the first basically further development of the direct bioautography (e.g. coordination of operating steps, using aimed series of endogenous and/or exogenous molecules [16,98]) can be used to exploit the potential of direct biological detection [98]. Fig. 18 shows the basic elements of the main possibilities of

BioArena. The development of BioArena system leads to new possibilities in bioassay-guided detection, fractionation and isolation. BioArena integrates the advantages of layer liquid chromatography (ideally, variants of linear OPLC [15,22]), the basic direct bioautography [113–115], and visual, spectroscopic as well as spectrometric evaluation of chromatographic spots before and after biological detection. This integration utilizes the possibilities of interactions among (microbial) indicator cells, the separated components and different small and large co-factor molecules in situ in the adsorbent layer (in the spots). The possibility of the addition (dissolution) of such endogenous and/or exogenous molecules (co-factors) into the culture medium is unlimited. This possibility is illustrated by means of samples in investigation of the role of HCHO and O_3 in the antibiotic effect.

6.2.2. Retardation and promotion of antibiotic effect depending on deprivation and increase of HCHO and/or O_3 level in the chromatographic spots

The effect of Cu(II) ions on the antibiotic effect of trans-resveratrol shows a non-linear relationship. Cu(II) ions in the pure form generate (mobilize) HCHO molecules from the microbial cells and bind them, possibly forming a coordination complex. It seems
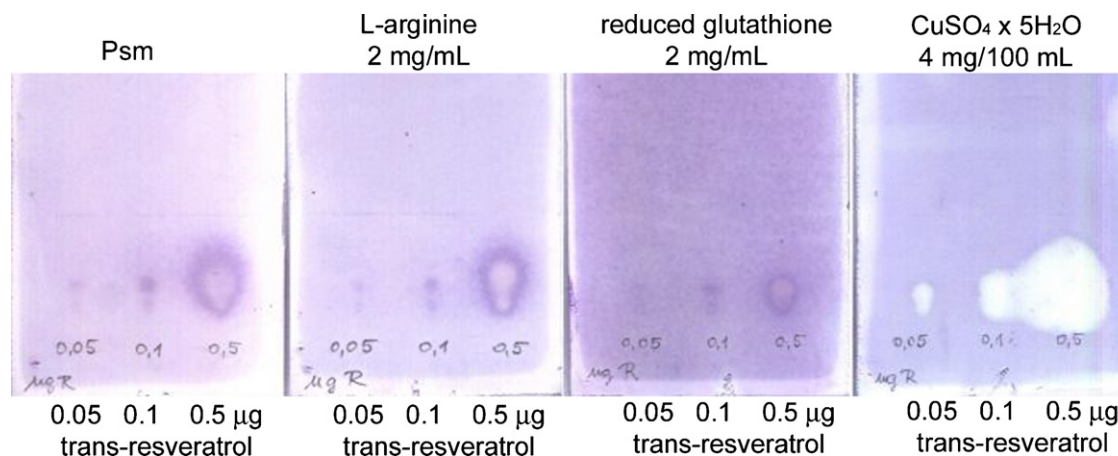
Fig. 19. The influence of endogenous substances on antiyeast (*Saccharomyces cerevisiae*) activity of trans-resveratrol. Chromatographic conditions: silica gel 60 F_{254} (Merck, preconditioning at 120 °C for 3 h), chloroform-methanol 80:4 (v/v). Biological conditions: (a) yeast suspension (3 g yeast in 100 mL distilled water); (b) a + 2 mg L-arginine in 1 mL yeast suspension; (c) a + 2 mg reduced glutathione in 1 mL yeast suspension; (d) a + 4 mg $CuSO_4·5H_2O$ in 100 mL yeast suspension.
With permission, from Ref. [118].
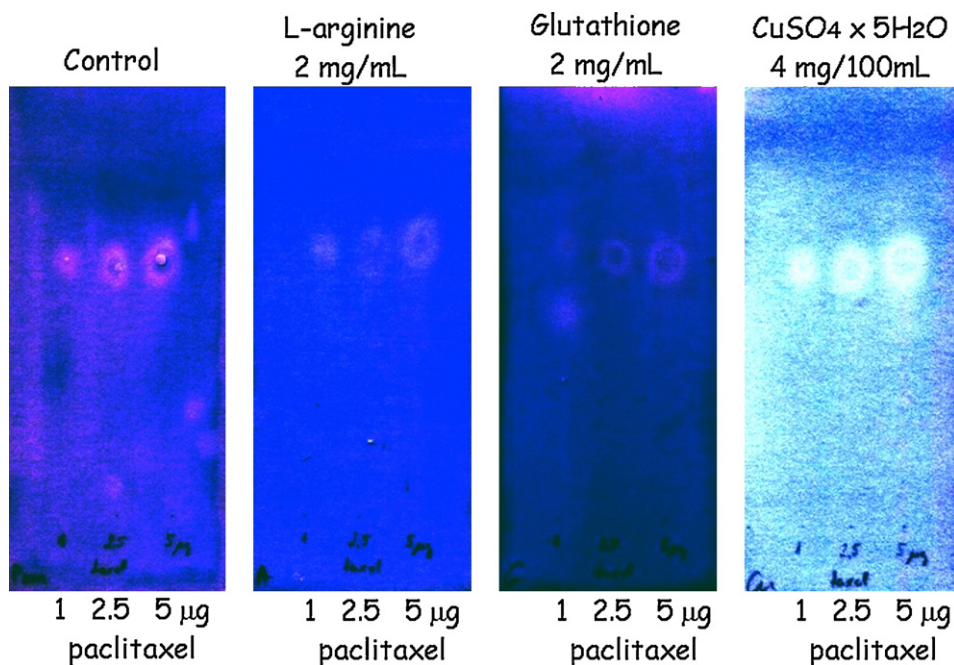
Fig. 20. The influence of endogenous substances on the antibacterial activity of paclitaxel. Chromatographic conditions: silica gel 60 F_{254} (Merck); mobile phase: chloroform-methanol 90:10 (v/v); Desaga glass TLC chamber. Biological conditions: Control: *Pseudomonas* sp., other information is on the chromatograms. Incubation was at the optimum temperature and detection was performed with MTT.

With permission from Ref. [119].

that the Cu(II) ion is a concentration-dependent HCHO-generating and capturing as well as transporting ion [116]. It has been observed that the use of the Cu(II) ions in culture medium dose-dependently reduces or promotes the antibacterial activity of trans-resveratrol [117]. Dissolving small doses of Cu(II) ions, the mobilized HCHO molecules in the given system are divided between the Cu(II) ions and the trans-resveratrol molecules. High doses of Cu(II) ions mobilize (induce) more HCHO molecules for most of trans-resveratrol molecules in the chromatographic spot. This is a typical non-linear effect [117].

Using the aqueous suspension of *Saccharomyces cerevisiae* for the biological detection (Fig. 19) it can be seen that the HCHO capturer molecules in the culture medium (and so then in the adsorbent layer) can decrease the antiyeast activity of the

trans-resveratrol on the silica gel adsorbent layer, while in the presence of Cu(II) ions as HCHO transporting ions the antiyeast activity of trans-resveratrol of the same amount is increased markedly [118].

Using the BioArena system, the HCHO molecules could be captured with well-known endogenous HCHO molecules (L-arginine, glutathione) in the spots of paclitaxel (Taxol^{TM}) on the TLC/OPLC adsorbent layer after inoculations (Fig. 20) [119]. Capture of HCHO resulted in a dose-dependent decrease in the antibacterial activity of paclitaxel. The antibacterial activity of paclitaxel a well-known antitumor diterpenoid alkaloid in the chromatographic spots can be increased dramatically by dissolving Cu(II) ions as a HCHO mobilizing and carrier ions in the culture medium (Fig. 20) [119]. By applying an O_3 scavenger (e.g. Indigo Carmine) this oxidant, as a
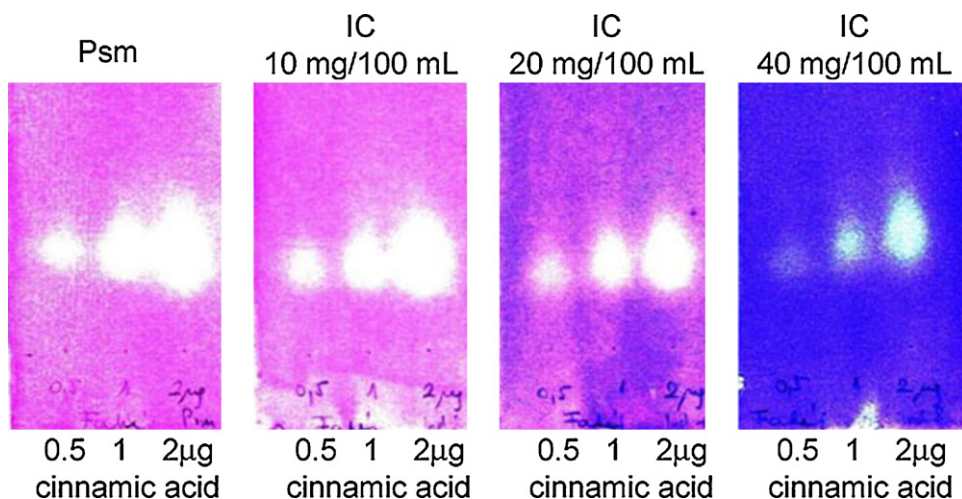
Fig. 21. Effect of Indigo Carmine on the antibacterial activity of cinnamic acid. Chromatographic conditions: silica gel 60 F_{254} (Merck); mobile phase: chloroform-methanol 80:8 (v/v); Desaga glass TLC chamber. Biological conditions: (a) 100 mL *Pseudomonas savastanoi* suspension, (Psm); (b) a + 10 mg Indigo Carmine; (c) a + 20 mg Indigo Carmine; (d) a + 40 mg Indigo Carmine.
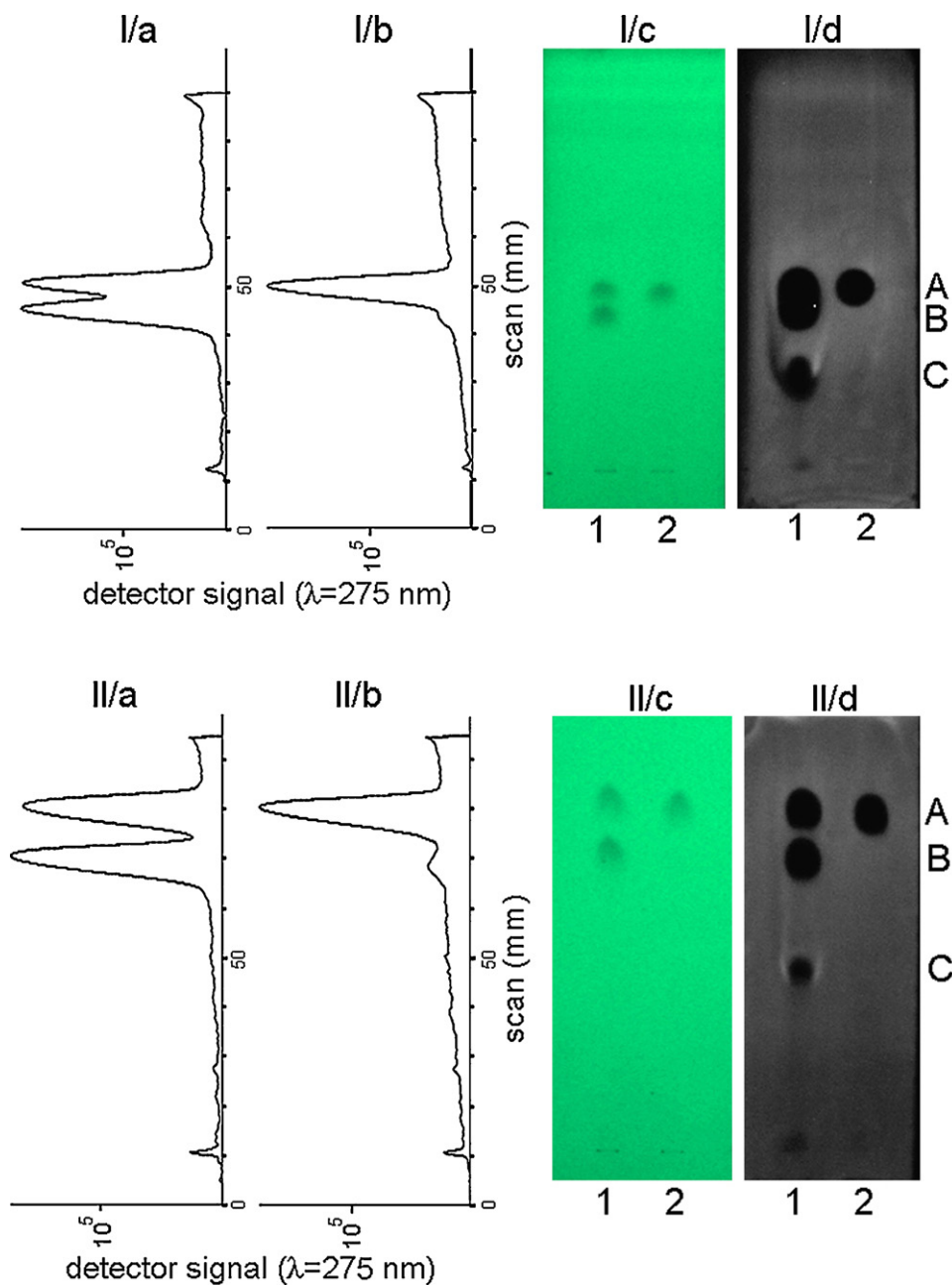
Fig. 22. Detection of the main components of *Thymus vulgaris* L. essential oil separated by TLC (I) and OPLC (II). Track 1, standards, 10 μg each of thymol (A), carvacrol (B), and linalool (C). Track 2, *Thymus vulgaris* L. oil, 25 μg. The components were detected before biological detection by densitometry (a – track 1, b – track 2) and under UV light (254 nm) (c); The bioautograms (d) were obtained by use of luminescent *Pseudomonas syringae* pv. *maculicola*.
With permission, from Ref. [120].

key reaction product of HCHO, could be detected indirectly in chromatographic spots of paclitaxel. It seems that these small molecules – from HCHO to endogenous O_3 – may be crucial factors of the mechanism of antiproliferative action of paclitaxel including killing of bystander cancer cells also.

Fig. 21 shows the antibacterial activity of the cinnamic acid. It is supposed that O_3 can be formed in the chromatographic spots of cinnamic acid, which is responsible mainly for its antibacterial effect. Using Indigo Carmine for elimination of O_3 molecules from the chromatographic spots of cinnamic acid it has been established that the antibacterial activity of cinnamic acid decreased really dose-dependently as it can be seen in Fig. 21 [111].

The main components of thyme (*Thymus vulgaris* L.) essential oil as thymol, carvacrol, and linalool were not appropriately separated by conventional TLC, while OPLC development applying the same stationary and mobile phase gave almost baseline separation (Fig. 22, [120]). The densitometric evaluation also confirmed the better resolution between the thymol and carvacrol peaks in the case of OPLC separation. Because of the characteristic functions of mobile phase flow rate, it has to note that the TLC is more time-consuming than OPLC, additionally the use of a longer development distance can improve the resolution only in OPLC separation, where the diffusion does not play so important role like in TLC. The direct bioautographic evaluation showed that each of thymol, carvacrol, and linalool has antibacterial effect
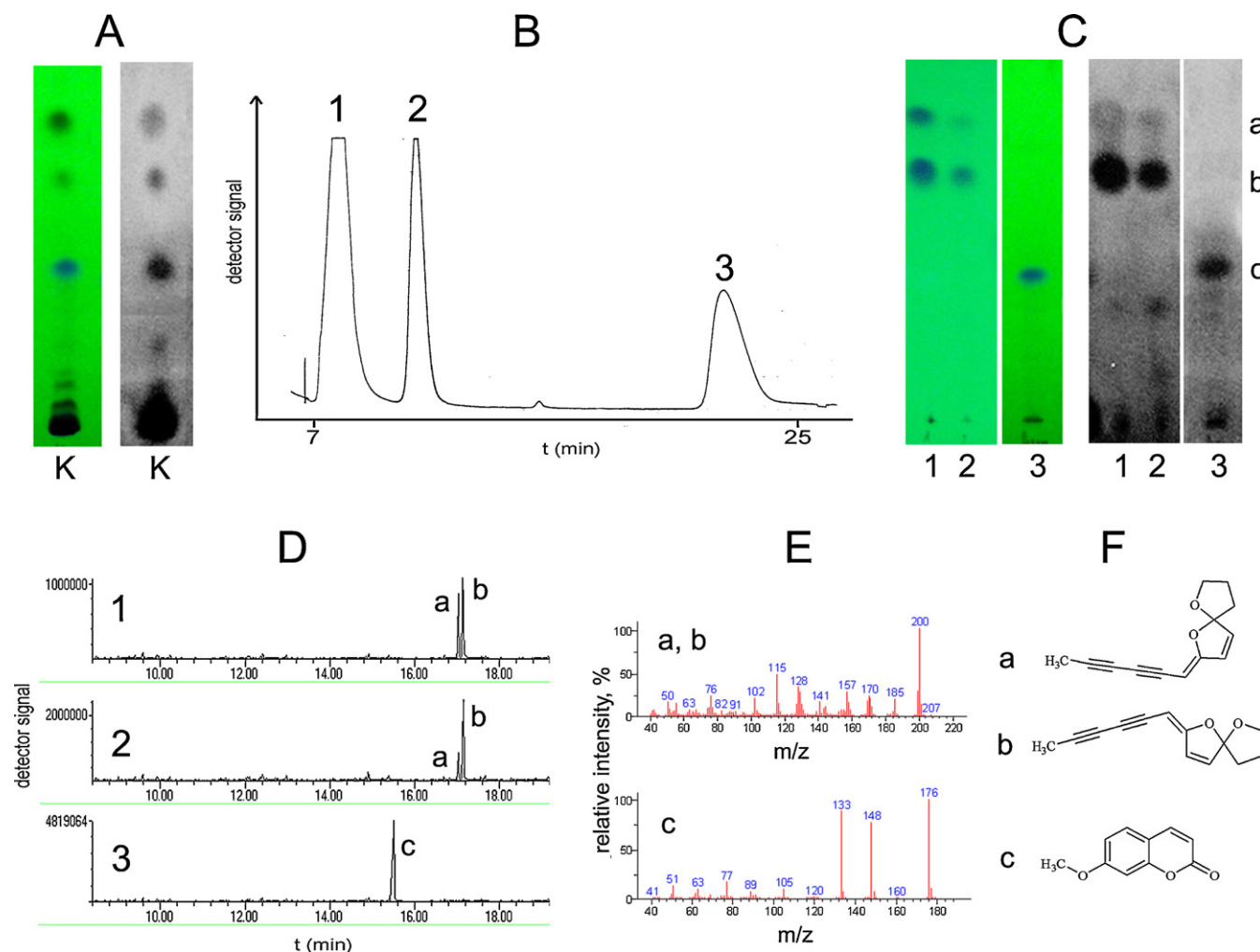
Fig. 23. Bioassay-guided isolation and identification of antibacterial components of 50% aqueous ethanol extract of chamomile flower (150 mg in 1 mL); (A) The active components, separated on TLC layer (Merck, #5554) with chloroform-acetone 99:1 (v/v), were visualized under UV (254 nm) (on the left) and with direct bioautography using luminescent gene tagged *Pseudomonas savastanoi* pv. *maculicola* plant pathogen bacteria (on the right); (B) OPLC separation of 0.5 mL chamomile flower extract, performed by a Personal OPLC BS50 system (OPLC-NIT, Budapest, Hungary), and the collected fractions (1–3, the 3 peaks); OPLC conditions were as follows: sealed 20 cm × 20 cm normal particle size silica gel layer (Merck, #5554), sample application in a 16 cm wide band at 3 cm from the edge, 50 bar external pressure, 450 μL rapid mobile-phase flush, 1 mL min^{-1} mobile phase flow rate; the mobile-phase was chloroform, the on-line UV detection was achieved at 350 nm; (C) the TLC re-chromatography of the collected fractions (chloroform-acetone 99:1 (v/v)); the active components were visualized under UV (254 nm) (on the left) and with direct bioautography using luminescent gene tagged *Pseudomonas savastanoi* pv. *maculicola* plant pathogen bacteria (on the right); (D) GC–MS analyses of the 3 fractions (TIC chromatograms); (E) the MS spectra of the main components of the chamomile flower fractions; (F) chemical structures of identified components: a – *cis*-spiroether, b – *trans*-spiroether, c – herniarin [121].

against luminescent gene tagged *Pseudomonas syringae* pv. *maculicola*. It can be seen that in TLC separation the inhibition zones (lack of emitted light, dark area) of thymol and carvacrol were not segregated, as it was expected according to their separation efficiency.

OPLC with on-line detection and fractionation is an ideal solution for the efficient separation and isolation of different substances, like antimicrobial components of a complex biological matrix. Moreover, it is very simple to couple on-line as well as off-line this system with different spectroscopic, spectrometric techniques for characterization and identification of the prospected isolated substances. The usually easy adaptation of a TLC method to OPLC makes possible the use of OPLC for isolation of compounds previously found active in a TLC-direct bioautographic study, that is, for bioassay-guided isolation. Fig. 23 shows the steps of the bioassay-guided isolation and identification of some antibacterial components of 50% aqueous ethanol chamomile (*Matricaria recutica* L.) flower extract, applying OPLC fractionation and off-line coupled GC–MS identification [121].

6.3. New aspects of the characterization of new ingredients – also in vivo investigations

BioArena can be used, e.g. for different biochemical reaction between cells and small or large molecules. It seems that in the future the BioArena system can be used for the biological/biochemical characterization of the potential biologically active substances separated beside the chemical and physical characterization.

According to our preliminary observations the in vitro observations with the BioArena system can be extended to in vivo conditions (e.g. greenhouse experiments). This leads to new, useful utilization of results from planar layer liquid chromatographic separations.

On the basis of experiences with the time- and dose-dependent double immune response of plants [108] a logical step was to extend it to the total Avogadro range (i.e. from 10^{-1} to 10^{-23} mol L^{-1}) in the case there was, e.g. four-day interval between pre-treatment and inoculation in the bean-bean

pathogen (*Uromyces phaseoli*) relationship. In this system the trans-resveratrol generated a characteristic time- and dose-dependent quadruple immune response of plants to pathogen cells [122] similar to earlier observations with other molecules [31].

It has to note that by capturing HCHO as potential endogenous molecule under in vivo conditions the immunostimulating activity of inducer (e.g. trans-resveratrol) can be eliminated similar to the BioArena investigations on the adsorbent layer [16].

7. Conclusions

The elimination of the vapor phase above the adsorbent layer in layer liquid chromatography was first realized by development of the ultramicro (UM) chamber. However, further possibilities of this type of closed simple planar-layer chamber, such as increased eluent-flow velocity, optional development distance, and the use of a pump system for optimizing flow velocity, were achieved by the development of a pressurized UM chamber. The essential feature of a pressurized UM chamber is that the adsorbent layer is completely covered by a flexible membrane under an external pressure so the vapor phase above the adsorbent layer is virtually eliminated. The development of a pressurized UM chamber using a pump system for the admission of the eluent was the first successful step towards a true planar-layer version of HPLC. The pressurized UM chamber is the basic instrument in overpressured layer chromatography (OPLC) that is in forced-flow planar layer LC. The characteristic features and development potential of the original technique are the basis of the diversity in the development of experimental and commercial instruments (e.g. the automated personal OPLC basic system 50, or OPLC Separation Unit 100 (OSU-100)). The development of the multi systems as multilayer, multidimensional, multichannel and multidetection systems shows the direction of the progress in OPLC.

Biological systems such as microbes or plants contain thousands of constituents and are a valuable source of new biologically active molecules, e.g. antibiotics, antineoplastics and biopesticides. For investigation and isolation of them suitable chemical/biochemical methods and biological assays are important. Model experiments are necessary with complex separation and detection systems at microassay and ultramicroassay levels. It is fact that the adsorbent bed in the column arrangement is not suitable for biological detection because the living cells do not grow there and so their detection, etc., is not possible. One solution is the BioArena system, which integrates and exploits the advantages of layer chromatographic separation and direct bioautography.

On the basis of up-to-date theoretical and practical biological and biochemical results obtained by the use of the BioArena system, the formaldehyde (HCHO) and ozone (O_3) as characteristic key small molecules play a crucial role in the antibiotic effect of most diverse chemical substances. Therefore, it is especially interesting to know and to understand better the function and place of these molecules in biological world. It seems that these molecules are determining factors in the antibiosis and the resistance, alike. In future investigations with BioArena system, it has to take into account that the in vitro results (BioArena studies) can be extended to in vivo conditions.

Acknowledgements

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