This is the final version (unedited) of the paper published in Journal of Chromatography A Volume 1232, 6 April 2012, Pages 3–18
doi:10.1016/j.chroma.2011.11.049
http://www.sciencedirect.com/science/article/pii/S002196731101750X

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

Ernő Tyihák[1], Emil Mincsovics[2] and Ágnes M. Móricz[1]

1) Plant Protection Institute, Hungarian Academy of Sciences, Budapest, Herman O. Str. 15, POB 102, H-1525 Hungary
2) OPLC-NIT Co. Ltd, Budapest, Hungary and Corvinus University, Faculty of Horticultural Sciences, Department of Genetics and Plant Breeding, Budapest, Hungary

Corresponding author: Ernő Tyihák, Tel.: 0036-1-4877-515; Fax: 0036-1-4877-555; e-mail: etyih@nki.hu

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.

28 The development of BioArena as a complex bioautographic system means an exploitation of the
29 unique advantages of planar-layer system for detection, isolation and identification of new
30 antimicrobials, antineoplastics, biopesticides and other biologically active substances as well as
31 for studying fundamental biochemical reactions and mechanisms.
32

33 **1. Introductory remarks**

34

35 Different directions of chromatography from its discovery have undergone innovative
36 developments. Among the technical versions one of the most important is liquid chromatography
37 (LC), which nowadays includes two basic techniques – column LC (CLC) and layer LC (LLC).
38 CLC and LLC techniques – as supplementary techniques due to their similar theoretical basis
39 and technical arrangements – have from the beginnings been progressed similarly.
40 It is known that layer liquid chromatographic techniques are rather simple and require in general
41 few appliances. Paper chromatography (PC) and its various versions, developed in the 1940s by
42 Martin and his colleagues [1], have to be mentioned first. The technical solutions of PC laid the
43 foundation of modern amino acid and peptide analysis and promoted the discovery of the
44 structure of insulin [1-3]. However, the rapid progress in different fields after 2nd world war
45 demanded the elimination of the limitations of PC (e.g. limited adsorption of paper [4]). Among
46 the innovative developments the discovery of thin-layer chromatography (TLC) by Ismailov
47 and Shraiber [5] as well as Békésy [6], the improvement by Kirchner et al. [7], additionally the
48 standardization and spreading by Stahl et al. [8], opened new horizons in separation science and
49 contributed to the isolation and analysis of many natural and synthetic substances. Today,
50 versions of this classical layer liquid chromatographic technique are indispensable in various
51 fields of scientific research and practice, but the limitations of TLC (e.g. limited efficiency)
52 demanded further development efforts.

53 It is logic to use an adsorbent layer of fine particle permitting effective separation similar to
54 HPLC technique [9]. High-performance thin-layer chromatography (HPTLC) is based already on
55 the use of chromatoplates coated with fine-particle-size and narrow particle-size distribution
56 adsorbent and instrumentation [10, 11]. Comparison of TLC and HPTLC chromatoplates
57 revealed that the latter permitted better separation, higher efficiency, but in a short development
58 distance only, because the migration of the mobile phase was slowed down and therefore, the
59 diffusion of the separated components on the adsorbent layer was increased using a relatively
60 long solvent migration distance, especially if viscous mobile phases were applied. The quadratic
61 development law of classical TLC is also valid in HPTLC [12].
62 HPTLC is in certain cases considered as the planar layer version of HPLC [13, 14]. This is true
63 in part only, since a very important and indispensable step of HPLC is missing in the application
64 of fine particle-size adsorbent layer with a narrow particle-size distribution. Namely, the eluent is
65 dispensed into the system by forced flow (by pressure) in the case of HPLC [9]. That is HPTLC
66 is a half solution to the efficient modern planar layer liquid chromatography.
67 The steady stream of innovations in layer liquid chromatography has resulted in most diverse
68 technical versions in the last decades. In these modern technical versions the mobile phase
69 migrates through the stationary phase in the layer arrangement under the additional action of
70 forced flow. This can be achieved especially successfully and attractively by application of a
71 pressurized ultramicro (UM) chamber and a pump system in overpressured layer
72 chromatography (OPLC) [15] that is in this system fine particle-size of adsorbent layer can be
73 used with forced flow: it is really a planar layer version of HPLC. The exploitation of the unique
74 potential of the planar adsorbent layer for investigation of the mechanism of action of
75 biologically active compounds and for interactions belongs closely to the innovative
76 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 Figure 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 vapour 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

102 or other suitable material, and mounted on the cover plate. The base and cover plates are joined
103 and the cushion is filled, advantageously with water, making an external pressure. Figure 2
104 illustrates the main elements of a pressurized circular-type UM chamber [20].
105 The development of the pressurized UM chamber was an original innovative step in the field of
106 LLC [21] using a pump system [22] or other forcing power (e.g. electroosmosis) [23] for the
107 admission of the eluent into the adsorbent layer. This chamber system was the first successful
108 step towards a true planar-layer version of HPLC, as well. The pressurized UM chamber can be
109 regarded as a column: the cushion-like half can be controlled with variable external pressure and
110 the other half forms a rigid plastic or metal plate [24]. The ultramicro character of the
111 conventional and pressurized UM chambers originates from the limited space between the
112 surface of the adsorbent layer and the cover plate and from the space between the adsorbent
113 particles [24].
114

115 **2.3 Development of OPLC instruments and chromatoplates**

116 **2.3.1 First commercial OPLC instruments**

117 The essence of OPLC is that the adsorbent layer is closed and pressurized during the separation
118 process and elution can be performed at the highest separation efficiency using the optimum
119 linear velocity generated by a pump. It follows from the principle of OPLC that low (2-5 bar),
120 medium (10-30 bar), and high (50-100 bar or more) external pressures can be used in this forced
121 flow planar layer liquid chromatographic technique.
122 On the basis of experience gained with experimental pressurized UM chambers, the first
123 commercial pressurized UM chambers were the Chrompres 10 and Chrompres 25 developed in
124 the nineteen eighties by Labor Instrument Works (Budapest, Hungary). The first commercially
125 available OPLC instrument (Chrompres 10) was a completely off-line system [20]. The second
126 generation instrument (Chrompres 25) was suitable for both off-line and on-line separations [25].

127 The maximum cushion pressure is 2.5 MPa in this chamber and this higher external pressure on
128 the elastic membrane permitted the use of a superfine-particle-size adsorbent layer, higher
129 viscosity mobile phases. At the same time this system makes possible the increase in the eluent
130 front velocity. The main components of Chrompres 25 are shown in Figure 3 [22]. This
131 apparatus is suitable for off-line analytical one-directional, 2-D, and continuous separations and
132 on-line analytical and preparative one-directional linear separations.
133 Finally, these conventional OPLC instruments and methods were suitable for demonstration of
134 the advantages of OPLC (the progress) over classical TLC and HPTLC [18, 26-29].
135
136 **2.3.2 Chromatoplates for conventional OPLC**
137
138 Figure 4 shows the various types of the OPLC chromatoplates. In circular OPLC it is not
139 necessary to seal the edges of the chromatoplate and the eluent inlet is placed in the middle of the
140 adsorbent layer. A chromatoplate that is sealed on three sides should be used for one-directional
141 development. If two opposite edges of the chromatoplate are sealed and the eluent inlet is in the
142 middle of the adsorbent layer in a channel, then the system is suitable for a two-directional
143 separation with a large number of samples. For 2-D separation in an off-line system, the four
144 sides of the chromatoplate must be sealed beforehand and the seal opposite the actual inlet must
145 be covered with a strip of filter paper, or an eluent outlet should be used [20, 21, 30-32].
146 The chromatographic plate for conventional on-line OPLC separation contains two-mobile-
147 phase-directing troughs in the adsorbent layer or in the PTFE insert cover plate. The mobile
148 phase inlet trough directs the mobile phase along a linear front and the mobile phase outlet
149 trough collects the mobile phase at the end of the plate and is connected to detector. The
150 combination of several chromatoplates during a single conventional OPLC separation has special
151 advantages (e.g. chromatography of a large number of samples) [20, 21, 33]. The introduction of

152 the mobile phase to parallel-coupled [33] and, especially, serially coupled [28, 34] multi-layer
153 systems is a critical matter. The parallel-coupled is performed by making a perforation in the
154 chromatoplates, of a suitable size and shape, at the mobile phase inlet. For serially coupled multi-
155 layer systems, however, a route for transfer of the effluent mobile phase is also needed [28, 34].
156
157 **2.3.3 Automatic OPLC instrument and other developments**
158
159 On the basis of experience gained with conventional Chrompres chambers, OPLC-NIT Ltd
160 (Budapest, Hungary) developed the automated personal OPLC basic system which includes a
161 separation chamber and a liquid delivery system (Figure 5) [35-37]. The separation chamber
162 generates four main units: holding unit, hydraulic unit, tray-like layer cassette, and attached drain
163 valve. The microprocessor-controlled liquid delivery system includes a two-in-one hydraulic
164 pump and mobile-phase delivery pump. All conditions for single or repeated development can be
165 input and stored in the delivery system software. External pressure (max. 5.0 MPa), mobile phase
166 volume, and mobile phase flow rate can be input, and development time is calculated
167 automatically [36-37].
168 In automatic OPLC system, the parameters can be adjusted for isocratic and stepwise gradient
169 separations: external pressure (P_{ext}), eluent flow rate (FLR), rapid volume (R or Vol. R), eluent
170 volume A (A or Vol. A), eluent volume B (B or Vol. B), and eluent volume C (C or Vol. C).
171 The development of the automated OPLC instrument has required the use of a new technology;
172 however, this is perhaps only the first step in this innovative direction.
173 High-pressure circular OPLC (3.8-4.0 MPa), called high-pressure planar liquid chromatography
174 (HPPLC), was developed by Kaiser and Rieder [38, 39]. HPPLC exploits basically all the
175 advantages of the original circular operating mode and uses the experience gained in the field of
176 circular HPTLC, that is, tries to eliminate the problems of the original HPTLC [10,11].

177 A special version of OPLC was introduced by Witkiewicz et al. [40], who used gas to apply the
178 external pressure; the mobile phase was fed to the adsorbent layer by means of a syringe.
179 OPLC Separation Unit 100 (OSU-100), an experimental instrument, is a chamber with three
180 inlets and three outlets. This built-in hydraulic pump already generates 10 MPa external pressure.
181 This technical solution exploits the advantages of a high external pressure which increases the
182 efficiency on the basis of the theoretical relationship. For on-line sample application and
183 detection one or two injectors and UV-detectors (Liquodet 308, Labor MIM, Budapest, Hungary)
184 were connected [41].
185

186 **2.3.4. Cassette systems for automated OPLC**

187

188 An interesting aspect of automated OPLC that all conventional OPLC operating modes can be
189 performed by use of an appropriate cassette [35, 36]. The cassette containing the chromatoplate
190 of modified adsorbent layer with the samples can be inserted in the pressurized chamber (Figure
191 6). Linear one and two-directional, and two-dimensional off-line and linear one-directional in-
192 line separations can be performed by isocratic as well as two- and three-step gradient
193 development.
194 The introduction of a special double-layer cassette system has enabled the possibility of a
195 relatively long development distance which results in increased spot capacity and better
196 resolution [42].

197 **2.4 Main operating steps in OPLC**

198

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

202

203 **2.4.1. Off-line OPLC**

204

205 In off-line systems, all the principal steps in the chromatographic process such as sample
206 application, separation, quantitative evaluation, and isolation are performed as separate
207 operations [30]. Depending on the application, linear, circular (radial) and triangular operating
208 modes can be performed in off-line OPLC and each has its own particular merits. In the linear
209 developing mode, one- and two-directional, and 2-D developments are possible [43]. In off-line
210 OPLC systems, changes in the composition of the eluent provide good opportunities for special
211 separation modes; that is, isocratic and stepwise-gradient modes. In analytical off-line OPLC,
212 several samples can be processed in parallel. The technique offers further advantages, such as
213 that only the spots and bands of analytical interest need to be assessed, quantitative evaluation
214 can be repeated with various detection parameters. In preparative off-line OPLC after the
215 development the procedures of drying, scraping off the adsorbent layer, elution and
216 crystallization are similar to conventional TLC methods. However, in preparative off-line OPLC,
217 the resolution is considerably increased and thick, fine-particle adsorbent layers can also be used.
218 Fully off-line OPLC has two operations – infusion and transfusion. In infusion mode the mobile
219 phase is introduced into a totally closed adsorbent layer. The air originally present in the
220 adsorbent layer is continuously compressed during the process, helping to fill up the pores of the
221 particles with the eluent. The infusion process is suitable only for off-line developments without
222 overrun and adsorbent layer sealed at four edges has to be used [36]. The transfusion operation
223 mode corresponds to the original OPLC techniques and enables passage of both air and mobile
224 phase [37]. In this system off-line and on-line operation and their combination are possible.

225

226 **2.4.2 On-line OPLC**

227

228 If the eluent outlet of the OPLC chamber is connected to a flow-cell detector, eluted solutes can
229 be detected on-line and fractions can also be collected [25, 44]. The entire chromatographic
230 process can be performed on-line by connecting a loop injector to the eluent inlet and a UV
231 detector to the eluent outlet, in much the same way as in HPLC [30, 45].
232 The hyphenation of TLC/HPTLC with mass spectrometry (MS) via a TLC-MS interface can
233 provide valuable analytical results [46-48].
234 On-line OPLC, a genuine layer chromatographic version of HPLC, is especially suitable for
235 direct coupling to other chromatographic, electrophoretic and/or spectroscopic techniques (e.g.
236 OPLC-FTIR, OPLC-MS, OPLC-MALDI MS) [49-51].
237 Fully on-line hyphenation of a newly developed experimental OPLC separation unit with diode-
238 array detection and mass spectrometry (OPLC-DAD-MS) can be used for analysis of
239 biologically active substances (e.g. xanthine derivatives and green tea leaf extract) [52] and this
240 system can, in future, also be used for metabolite analysis. On-line OPLC separation can also be
241 combined with electrospray mass spectrometric measurement (OPLC 50-ESI-MS) and this
242 combination has been used for detection of glycolipids [53].
243 On-line OPLC-RD (radioactivity detection), and OPLC-DAR (digital autoradiography) and
244 HPLC-RD are rapid, economic and effective separation and detection systems which are highly
245 applicable to animal and human metabolism research (Figure 8) [54].
246

247 3. Some theoretical aspects of OPLC

248

249 The theoretical aspects of OPLC are summarized in different reviews in more details [32, 36, 37,
250 55-58]; therefore, here in this paper we want to demonstrate only some characteristic theoretical
251 aspects of OPLC.

252

253 ### 3.1. Retention and its influencing factors

254

255 In conventional layer chromatography [TLC, HPTLC and preparative layer chromatography
256 (PLC)], the eluent migrates by means of capillary forces, described by the quadratic equation
257 [15, 18, 19].
258 In OPLC, the eluent can be forced through (or into in the case of infusion operation) the
259 adsorbent bed by means of a pump system with a selected flow rate. Feeding the eluent by
260 constant flow rate onto the chromatoplate, the speed of the α front (Fα) depends on the free
261 cross-sectional area of the adsorbent layer in the direction of the development. Only linear
262 development is able to result in constant linear velocity, chromatoplates with circular and
263 triangular shapes are not. In contrast with linear development circular OPLC yields decreasing
264 velocity of Fα along the radius [59].

265

266 ### 3.2 Relationship between the average theoretical plate height (H) and migration distance
267 ### (L)

268

269 Figure 9 shows clearly that H (HETP) is practically constant in off-line OPLC along the
270 adsorbent layer, is independent on development distance. This is more favourable for
271 conventional fine-particle adsorbent layers than for coarse-particle layers [13, 60]. The use of a
272 superfine-particle-size (e.g. 3 μm) adsorbent layer results in a dramatic increase in the efficiency
273 of the separation; such stationary phases cannot, however, be used in conventional TLC or
274 HPTLC. This arises from the possibility of optimizing the mobile phase velocity in OPLC, by
275 using a pump to direct the mobile phase into the stationary phase. The consequence of the

276 optimization of linear velocity is that two flow profiles also counterbalance each other when
277 superfine-particle adsorbent layers are used [61-63].
278 However, the Figure 9 illustrates clearly the basic difference between the efficiency of TLC,
279 HPTLC, and off-line OPLC and also explains the flow profile of the mobile phase in OPLC.
280
281 **3.3. Relationship between average theoretical plate height (H) and mobile phase velocity (u)**
282 **in OPLC**
283
284 Figure 10 shows the H-u relationship for different OPLC instruments, including the automatic
285 personal OPLC instrument [35]. It is apparent that increasing the external pressure results in
286 increased optimum mobile phase front velocity with an increased optimum velocity range.
287 In OPLC, H value can vary characteristically with linear velocity similarly to HPLC [60, 64, 65].
288 The curves from the plot of H against linear velocity (u) for the different operating modes in
289 OPLC are very similar but H values are different. The lowest H values are obtained for fully off-
290 line OPLC and the highest for fully on-line OPLC. Between these are the two curves of partial
291 off-line (or partial on-line) OPLC. The differences among these systems originate from „extra-
292 column" band broadening; this does not occur in the fully off-line system.
293 The increase of external pressure reduces H in off-line OPLC. Despite of this, the same increase
294 in efficiency was not observed for on-line OPLC [33].
295
296 **4. Multi systems – progress in OPLC**
297
298 For to-day it is clear that the efficiency and attractivity of OPLC technique can be increased
299 dramatically by the use of different multi systems which are coming in this case from the

300 attractivity of the adsorbent layer in multiple mode and from the application of a forced
301 (directed) flow of the eluent in it.
302
303 **4.1 Multilayer systems**
304
305 Parallel solution of overpressured multilayer chromatography (OPMLC) using two or more
306 chromatoplates is very attractive because a large number of samples (50-100 or more) can be
307 separated during one development process [33]. Serial coupled OPMLC (called „long distance"
308 OPLC) can be used for the increase of the theoretical plate number and resolution alike as
309 elaborated by Botz et al. [34]. Several chromatoplates are placed on top of each other to extend
310 the development distance. The potential of these serial connected adsorbent layers can be
311 increased further by use of different (hetero) stationary phases during one development [66]
312 (Figure 11). The end of the first uppermost chromatoplate has a slit-like perforation to enable the
313 mobile phase to flow to a second adsorbent layer, in which the migration continues until the
314 opposite end of the chromatoplate; there the chromatography can be continued onto a subjacent
315 chromatoplate or the eluent is led away.
316
317 **4.2 Multidimensional OPLC**
318
319 For difficult separation problems the application of multidimensional (MD) OPLC is necessary,
320 because the power of one-dimensional chromatography is often inadequate for complete
321 resolution of the components which are present in e.g. complex biological samples [67, 68].
322 According to Giddings [69] the definition of multidimensional chromatography is as follows
323 - The first condition is that the components of a mixture are subjected to two or more
324 separation steps in which their displacements depend on different factors.

325 - The second criterion is that when two components are substantially separated in any
326 single step, they always remain separated until completion of the separation.
327
328 Principle of classical MD TLC (2D-OPLC) development is as follows: chromatographic
329 development in one direction followed by a second development in a direction perpendicular to
330 the first [70]. The method consists of spotting a sample at the corner of a chromatographic plate
331 and enabling migration of the mobile phase in the first direction. After drying, the second
332 development on the chromatoplate, in a direction at right angles to the first, can be started with
333 another mobile phase.
334 There is a proposed theoretical model whereby maximum peak capacity could be achieved by
335 use of 3D TLC (OPLC) separation [71-73]. Because of technical problems, until now this idea
336 could not be realized in practice.
337 Figure 12 - from the different technical efforts - illustrates a special solution proposal in patent
338 level of 2D and 3D separations.
339
340 **4.3 Multichannel OPLC**
341
342 A new general concept has been developed for single-channel and multichannel OPLC
343 separations using a nonsegmented adsorbent layer and a flowing eluent wall (FEW) system for
344 operational segmentation [74]. For FEW system the original hydraulic unit of the OPLC has
345 been changed to a new one which is equipped with two mobile phase inlet connections, one for
346 sample injection and another for the FEW formation, and the outlet can be connected to a flow
347 cell detector and/or a fraction collector (Figure 13). The waste eluent from the FEW formation is
348 collected separated. The experimental four and eight-channel FEW versions are suitable for
349 parallel fully on-line separations [41,74-76].

350 For parallel fully on-line separation the FEW can be used for segmentation of a nonsegmented
351 adsorbent layer, dividing it into active and inactive parts with regard to the separation. Only
352 mobile phase is introduced into the inactive part whereas mobile phase and the sample can be
353 introduced to the active separation part; the unsuitable part of the adsorbent layer is thus
354 excluded from the separation process. The FEW helps eliminate the edge effect of OPLC for
355 single sample injection, and abolishes the band widening in horizontal direction, so the sample
356 mixing effect of neighbouring lanes in multichannel separation processes. The FEW as an
357 innovative technical solution in planar layer liquid chromatography enables real multichannel
358 liquid chromatographic separation on a non-segmented adsorbent layer [74, 76]. Figure 14
359 illustrates an eight-channel FEW version which is an integration of multichannel and
360 multidetection systems.
361
362 **5. Analytical and preparative applications of OPLC**
363
364 OPLC have already been used successfully in different fields. The up-to-date results have been
365 summarized in details [21, 22, 24, 30, 32, 36, 37, 77]. Therefore, in this review we will only
366 demonstrate some interesting trends in the applications of technical versions of OPLC.
367
368 **5.1 Analytical applications**
369
370 OPLC ensures a constant and high flow velocity, even for viscous mobile-phase mixtures with
371 poor adsorbent wetting characteristics (e.g. those used on RP chromatoplates). The development
372 time is shorter than in TLC or HPTLC, and so molecular diffusion is reduced substantially in
373 OPLC, producing compact spots with better resolution than in TLC or HPTLC [78-80].

374 The automated Personal OPLC 50 system with maximum 5 MPa external pressure and tray-like
375 cassette construction has been used for separation of a variety of substances (e.g. ascorbigens,
376 aflatoxins, formaldehyde derivatives etc. [81-85]).
377 Comparison of OPLC with other conventional and new planar layer liquid chromatographic
378 techniques is useful for characterization of the separation potential of these techniques [86-
379 88].OPLC with off-line hyphenation have been used for separation. OPLC has already combined
380 with in- and/or ex-situ spectroscopic and spectrometric detection using different types of
381 instrument (Raman, FAB, MALDI MS and GC-MS etc.) [89-93].
382 The newly developed experimental OPLC Separation Unit 100, generating already 10 MPa
383 external pressure, enables more efficient fully off-line (see Figure 10) and on-line separations
384 than previous generations of OPLC system (Figure 15) [76].
385
386 **5.2 Preparative applications**
387
388 Although, preparative exploitation of OPLC is poor, up-to-date preparative applications of
389 OPLC have been reviewed [36, 94, 95].
390 Similar to analytical OPLC off-line and on-line operating modes can be used in preparative
391 OPLC, too. The on-line OPLC is more effective for preparative applications because the time-
392 consuming scraping and elution are omissible [36, 95, 96]. In on-line preparative OPLC there is
393 a possibility for the isolation of purer substances than in off-line preparative OPLC.
394 On-line sample collection after high-resolution stepwise gradient OPLC separation combined
395 with digital autoradiography (DAR) has been used for isolation of plasma and urine metabolites
396 of ^{3}H or ^{14}C-radiolabeled deramcyclane (a new anxiolytic substance) [97]. This separation
397 process can be followed by different MS techniques for determination of the structure of the
398 minor and major metabolites.

399 OPLC has been used for semipreparative separation-isolation of bound vitamin C from cabbage
400 extract on analytical silica gel layers [98] and for isolation of xanthines from tea leaf extract
401 using 0.5 mm preparative adsorbent layer [35].
402
403 **6. Novel study of biological activity of natural and synthetic compounds**
404
405 **6.1 Unique great potential of adsorbent layer liquid system for biological detection and**
406 **interactions – in-vitro and in-vivo studies**
407
408 It must be noted firstly that the adsorbent bed in the column arrangement is not suitable and so it
409 can not be used for biological detection and interactions because the living cells (e.g. bacterial
410 cells) do not grow there and so their detection, etc. is not possible. This fact increases the
411 importance of planar layer liquid chromatographic techniques because, in the future time,
412 biological detection and interactions of separated substances in-situ in the adsorbent layers will
413 be a crucial and indispensable methodological solution for isolation, identification and
414 characterization of new antimicrobials, antineoplastics, biopesticides and others [99,100], as well
415 as for studying fundamental biochemical reactions and mechanisms [100,101].
416 The well-known direct bioautography, which integrates application of planar layer liquid
417 chromatographic separation and post-chromatographic bioassay, can be regarded as the most
418 efficacious assay for detection of antibiotic-like compounds [102]. Although, direct
419 bioautography is a leading technique in bioautography, nowadays it is already not enough.
420
421 **6.2 In-vitro biological studies – BioArena investigations**
422

423 Although the „antibiotic era" is barely six decades old, nevertheless the mankind is already faced
424 with a global problem of emerging resistance of virtually all pathogens [103]. The tasks are
425 given: among others, such small and large co-factor molecules have to know which play a
426 fundamental role in the antibiotic effect and in the basal and induced disease resistance.
427

428 **6.2.1 Biochemical-chemical background of antibiotic experiments**

429

430 According to recent observations formaldehyde (HCHO) is an endogenous component of all
431 biological systems [104,105], mainly in the form of hydroxymethyl group. Therefore, it can be
432 detected and measured in different biological samples using a HCHO capture molecule (e.g.
433 dimedone) for collecting HCHO molecules from a given biological unit [106].There is a primary
434 HCHO cycle in biological systems in which the formation of S-methyl group of L-methionine
435 from HCHO, originated from natural HCHO generators, and the HCHO-yielding function of S-
436 adenosyl-L-methionine (SAM) are essential components [104, 105].
437 HCHO can be continuously formed by almost all cells both intracellularly and extracellularly.
438 The term „formaldehydome" means the complete set of HCHO-cycle-mediated and non-
439 mediated HCHO pathways of a given biological unit (Figure 16) [107]. The formaldehydome
440 can interlace the big biological units such as genome, proteome etc., so HCHO is a determining
441 endogenous component in all biological systems.
442 Endogenous HCHO can interact with – among others – H_2O_2 as another universal small
443 endogenous molecule. In this interaction $^1O_2$ and excited HCHO (HC*HO) can be formed [108,
444 109]. Figure 17 illustrates the reaction series supposedly [110], taking into account earlier and
445 more recent observations [108-112]. $^1O_2$ can oxidize the H_2O molecules endogenously and H_2O_3
446 can be formed from which, among others, O_3 can be released [111, 112]. This fundamental
447 endogenous reaction can occur in *in vitro* conditions (in chromatographic spots), as well as when

448 using the BioArena system [113, 114]. It is probable that O_3 is generated endogenously from
449 different biochemical reactions in diverse biological systems; however, it seems that HCHO/O_3
450 is a determining pathway in this complicated system.
451 These very reactive small molecules – from HCHO to O_3 – may be the crucial factors of the
452 innate (natural) resistance and form a common bridge between the innate (natural) and adaptive
453 (induced) resistance in plant and animal organisms, alike [77, 110].
454 BioArena system as the first basically further development of the direct bioautography (e.g.
455 coordination of operating steps, using aimed series of endogenous and/or exogenous molecules
456 [16,100]) can be used to exploit the potential of direct biological detection [100]. Figure 18
457 shows the basic elements of the main possibilities of BioArena. The development of BioArena
458 system leads to new possibilities in bioassay-guided detection, fractionation and isolation.
459 BioArena integrates the advantages of layer liquid chromatography (ideally, variants of linear
460 OPLC [15, 22]), the basic direct bioautography [115-117], and visual, spectroscopic as well as
461 spectrometric evaluation of chromatographic spots before and after biological detection. This
462 integration utilizes the possibilities of interactions among (microbial) indicator cells, the
463 separated components and different small and large co-factor molecules in-situ in the adsorbent
464 layer (in the spots). The possibility of the addition (dissolution) of such endogenous and/or
465 exogenous molecules (co-factors) into the culture medium is unlimited. This possibility is
466 illustrated by means of samples in investigation of the role of HCHO and O_3 in the antibiotic
467 effect.
468

469 **6.2.2. Retardation and promotion of antibiotic effect depending on deprivation and**
470 **increase of HCHO and/or O_3 level in the chromatographic spots**
471

472 The effect of Cu(II) ions on the antibiotic effect of trans-resveratrol shows a non-linear
473 relationship. Cu(II) ions in the pure form generate (mobilize) HCHO molecules from the
474 microbial cells and bind them, possibly forming a coordination complex. It seems that the Cu(II)
475 ion is a concentration-dependent HCHO-generating and capturing as well as transporting ion
476 [118]. It has been observed that the use of the Cu(II) ions in culture medium dose-dependently
477 reduces or promotes the antibacterial activity of trans-resveratrol [119]. Dissolving small doses
478 of Cu(II) ions, the mobilized HCHO molecules in the given system are divided between the
479 Cu(II) ions and the trans-resveratrol molecules. High doses of Cu(II) ions mobilize (induce)
480 more HCHO molecules for most of trans-resveratrol molecules in the chromatographic spot. This
481 is a typical non-linear effect [119].
482 Using the aqueous suspension of *Saccharomyces cerevisiae* for the biological detection (Figure
483 19) it can be seen that the HCHO capturer molecules in the culture medium (and so then in the
484 adsorbent layer) can decrease the antiyeast activity of the trans-resveratrol on the silica gel
485 adsorbent layer, while in the presence of Cu(II) ions as HCHO transporting ions the antiyeast
486 activity of trans-resveratrol of the same amount is increased markedly [120].
487 Using the BioArena system, the HCHO molecules could be captured with well-known
488 endogenous HCHO molecules (L-arginine, glutathione) in the spots of paclitaxel (Taxol^{TM}) on
489 the TLC/OPLC adsorbent layer after inoculations (Figure 20) [121]. Capture of HCHO resulted
490 in a dose-dependent decrease in the antibacterial activity of paclitaxel. The antibacterial activity
491 of paclitaxel a well-known antitumor diterpenoid alkaloid in the chromatographic spots can be
492 increased dramatically by dissolving Cu(II) ions as a HCHO mobilizing and carrier ions in the
493 culture medium (Figure 20) [121]. By applying an O_3 scavenger (e.g. Indigo Carmine) this
494 oxidant, as a key reaction product of HCHO, could be detected indirectly in chromatographic
495 spots of paclitaxel. It seems that these small molecules – from HCHO to endogenous O_3 – may

496 be crucial factors of the mechanism of antiproliferative action of paclitaxel including killing of
497 bystander cancer cells also.
498 Figure 21 shows the antibacterial activity of the cinnamic acid. It is supposed that O_3 can be
499 formed in the chromatographic spots of cinnamic acid, which is responsible mainly for its
500 antibacterial effect. Using Indigo Carmine for elimination of O_3 molecules from the
501 chromatographic spots of cinnamic acid it has been established that the antibacterial activity of
502 cinnamic acid decreased really dose-dependently as it can be seen in Figure 21 [113].
503 The main components of thyme (*Thymus vulgaris* L.) essential oil as thymol, carvacrol, and
504 linalool were not appropriately separated by conventional TLC, while OPLC development
505 applying the same stationary and mobile phase gave almost baseline separation (Figure 22,
506 [122]). The densitometric evaluation also confirmed the better resolution between the thymol and
507 carvacrol peaks in the case of OPLC separation. Because of the characteristic functions of
508 mobile phase flow rate, it has to note that the TLC is more time-consuming than OPLC,
509 additionally the use of a longer development distance can improve the resolution only in OPLC
510 separation, where the diffusion does not play so important role like in TLC. The direct
511 bioautographic evaluation showed that each of thymol, carvacrol, and linalool has antibacterial
512 effect against luminescent gene tagged *Pseudomonas syringae* pv. *maculicola*. It can be seen that
513 in TLC separation the inhibition zones (lack of emitted light, dark area) of thymol and carvacrol
514 were not segregated, as it was expected according to their separation efficiency.
515 OPLC with on-line detection and fractionation is an ideal solution for the efficient separation and
516 isolation of different substances, like antimicrobial components of a complex biological matrix.
517 Moreover, it is very simple to couple on-line as well as off-line this system with different
518 spectroscopic, spectrometric techniques for characterisation and identification of the prospected
519 isolated substances. The usually easy adaptation of a TLC method to OPLC makes possible the
520 use of OPLC for isolation of compounds previously found active in a TLC-direct bioautographic

521 study, that is, for bioassay-guided isolation. Figure 23 shows the steps of the bioassay-guided
522 isolation and identification of some antibacterial components of 50% aqueous ethanol
523 chamomile (*Matricaria recutica* L.) flower extract, applying OPLC fractionation and off-line
524 coupled GC-MS identification [123].
525

526 **6.3. New aspects of the characterisation of new ingredients – also *in vivo* investigations**

527

528 BioArena can be used e.g. for different biochemical reaction between cells and small or large
529 molecules. It seems that in the future the BioArena system can be used for the
530 biological/biochemical characterization of the potential biologically active substances separated
531 beside the chemical and physical characterization.
532 According to our preliminary observations the in-vitro observations with the BioArena system
533 can be extended to *in vivo* conditions (e.g. greenhouse experiments). This leads to new, useful
534 utilization of results from planar layer liquid chromatographic separations.
535 On the basis of experiences with the time- and dose-dependent double immune response of
536 plants [110] a logical step was to extend it to the total Avogadro range (i.e. from 10^{-1} to 10^{-23}
537 mol/L^{-1}) in the case there was e.g. four-day interval between pre-treatment and inoculation in the
538 bean-bean pathogen (*Uromyces phaseoli*) relationship. In this system the trans-resveratrol
539 generated a characteristic time- and dose-dependent quadruple immune response of plants to
540 pathogen cells [124] similar to earlier observations with other molecules [77].
541 It has to note that by capturing HCHO as potential endogenous molecule under *in vivo* conditions
542 the immunostimulating activity of inducer (e.g. trans-resveratrol) can be eliminated similar to the
543 BioArena investigations on the adsorbent layer [16].
544

545 **7.0 Conclusions**

546

547 The elimination of the vapour phase above the adsorbent layer in layer liquid chromatography
548 was first realized by development of the ultramicro (UM) chamber. However, further
549 possibilities of this type of closed simple planar-layer chamber, such as increased eluent-flow
550 velocity, optional development distance, and the use of a pump system for optimizing flow
551 velocity, were achieved by the development of a pressurized UM chamber. The essential feature
552 of a pressurized UM chamber is that the adsorbent layer is completely covered by a flexible
553 membrane under an external pressure so the vapour phase above the adsorbent layer is virtually
554 eliminated. The development of a pressurized UM chamber using a pump system for the
555 admission of the eluent was the first successful step towards a true planar-layer version of HPLC.
556 The pressurized UM chamber is the basic instrument in overpressured layer chromatography
557 (OPLC) that is in forced-flow planar layer LC. The characteristic features and development
558 potential of the original technique are the basis of the diversity in the development of
559 experimental and commercial instruments (e.g. the automated personal OPLC basic system 50,
560 or OPLC Separation Unit 100 (OSU-100)). The development of the multi systems as multilayer,
561 multidimensional, multichannel and multidetection systems shows the direction of the progress
562 in OPLC.
563 Biological systems such as microbes or plants contain thousands of constituents and are a
564 valuable source of new biologically active molecules, e.g. antibiotics, antineoplastics and
565 biopesticides. For investigation and isolation of them suitable chemical/biochemical methods and
566 biological assays are important. Model experiments are necessary with complex separation and
567 detection systems at microassay and ultramicroassay levels. It is fact that the adsorbent bed in the
568 column arrangement is not suitable for biological detection because the living cells do not grow
569 there and so their detection etc., is not possible. One solution is the BioArena system, which

570 integrates and exploits the advantages of layer chromatographic separation and direct
571 bioautography.
572 On the basis of up-to-date theoretical and practical biological and biochemical results obtained
573 by the use of the BioArena system, the formaldehyde (HCHO) and ozone (O_3) as characteristic
574 key small molecules play a crucial role in the antibiotic effect of most diverse chemical
575 substances. Therefore, it is especially interesting to know and to understand better the function
576 and place of these molecules in biological world. It seems that these molecules are determining
577 factors in the antibiosis and the resistance, alike. In future investigations with BioArena system,
578 it has to take into account that the *in vitro* results (BioArena studies) can be extended to *in vivo*
579 conditions.
580
581 **Acknowledgements**
582
583 The authors are grateful to *Dr. P.G. Ott* (Plant Protection Institute, Hungarian Academy of
584 Sciences, Budapest, Hungary) for the cooperation in the field of microbiology; also to *Prof. Dr.*
585 *É. Szőke* and *Dr. Sz. Szarka* (Department of Pharmacognosy, Faculty of Pharmacy, Semmelweis
586 University, Budapest, Hungary) for GC-MS analysis.
587 This work was partially supported by OTKA grant No. PD83487.
588
589 **References**
590
591 [1] A.J.P. Martin, R.L.M. Synge, Biochem. J. 35 (1941) 91.
592 [2] A.H. Gordon, A.J.P. Martin, and R.L.M. Synge, Biochem. J. 37 (1943) 86.
593 [3] R. Consden, A.H. Gordon and A.J.P. Martin, Biochem. J. 38 (1944) 224.
594 [4] J.G. Kirchner and G.J. Keller, J. Am. Chem. Soc. 72 (1950) 1867.

595 [5] N.A. Ismailov and M.S. Schraiber, Farmacia (Moscow), 3 (1938) 1.

596 [6] N.v. Békésy, Biochem. J. 312 (1942) 100.

597 [7] J.G. Kirchner, J.M. Miller, G.J. Keller, Anal. Chem. 23 (1951) 420.

598 [8] E. Stahl, G. Schröter, G. Kraft, R. Renz, Pharmazie, 1(1956) 633.

599 [9] Cs. Horváth (Editor) High Performance Liquid Chromatography, Advances and
600 Perspectives, Vol. 1, Academic Press, New York, NY, 1973.

601 [10] R.E. Kaiser (Editor), Einführung in die Hochleistungs-Dünnschicht-
602 Chromatographie, Institute for Chromatography, Bad Dürkheim, 1976.

603 [11] A. Zlatkis and R.E. Kaiser (Eds) HPTLC High Performance Thin-Layer
604 Chromatography, Elsevier, Amsterdam, l977.

605 [12] U.A.Th. Brinkman and G. De Vries, in: W. Bertsch, S. Hara, R.E. Kaiser and A.
606 Zlatkis (Eds)): Instrumental HPTLC, Hüthig, Heidelberg, l980. p. 39.

607 [13] R.E. Kaiser, in: W. Bertsch, S. Hara, R.E. Kaiser and A. Zlatkis (Editors):
608 Instrumental HPTLC, Hüthig, Heidelberg, 1980. p. 165.

609 [14] T. Suzuki, T. Kawada and K. Iwai, J. Chromatogr. 198 (1980) 217.

610 [15] E. Tyihák, E. Mincsovics and H. Kalász, J. Chromatogr. 174 (1979) 75.

611 [16] E. Tyihák, L. Botz, P.G. Ott, J. Nagy, B. Kocsis, Zs. Király-Véghely, E. Mincsovics,
612 Chem. Anal. (Warsaw) 48 (2003) 543.

613 [17] E. Tyihák cited by E. Tyihák and G. Held, in: Progress in TLC and Related
614 Techniques, Vol II, A. Niederwieser and G. Pataki (Eds), Ann. Arbor. Science
615 Publishers, Ann Arbor, MI, 1971. p. 183.

616 [18] E. Mincsovics, E. Tyihák and H. Kalász, J. Chromatogr. 191 (1980) 292.

617 [19] H. Kalász, J. Nagy, E. Tyihák and E. Mincsovics, J. Liquid Chromatogr. 3 (1980)
618 845.

619 [20] E. Tyihák, E. Mincsovics, and F. Körmendi, Hung. Sci. Instrum. 55 (1983) 33.

620 [21] E. Tyihák and E. Mincsovics, LC-GC Int. 4 (1986) 24.
621 [22] E. Tyihák and E. Mincsovics, J. Planar Chromatogr. 1 (1988) 6.
622 [23] D. Nurok, J.M. Koers, A.L. Novotny, M.A. Carmichael, J.J. Kosiba, R.E. Santini,
623 G.L. Hawkins, R.W. Replogle, Anal. Chem. 76 (2004) 1690.
624 [24] K. Ferenczi-Fodor, E. Mincsovics, and E. Tyihák, in: J. Sherma and B. Fried, (Eds)
625 Handbook of Thin Layer Chromatography, Marcell Dekker, Inc. New York, 1991. p.
626 155.
627 [25] E. Mincsovics, E. Tyihák, A.M. Siouffi, in: E. Tyihak (ed.), Proc. Int. Symp. TLC
628 with Special Emphasis on Overpressured Layer Chromatography (OPLC), Labor
629 MIM, Budapest, l986, p. 251.
630 [26] P. Bruno, M. Caselli, and A. Traini, J. Planar Chromatogr. 1 (1988) 299.
631 [27] W.P.N. Fernando and C.F. Poole, J. Planar Chromatogr. 3 (1990) 352.
632 [28] L. Botz, Sz. Nyiredy, and O. Sticher, J. Planar Chromatogr. 4 (1991) 115.
633 [29] Gy. Kátay, E. Mincsovics, Gy. Szókán and E. Tyihák, J. Chromatogr. A 764 (1997)
634 103.
635 [30] E. Mincsovics and E. Tyihák, in: F.A.A. Dallas et al. (eds), Recent Advances in Thin-
636 Layer Chromatography, Plenum Press, New York, l988, pp. 57.
637 [31] E. Tyihák and E. Mincsovics, J. Planar Chromatogr. 23 (2010) 382.
638 [32] E. Mincsovics and E. Tyihak, Nat. Prod. Commun. 6 (2011) 719.
639 [33] E. Tyihák, E. Mincsovics and T.J. Székely, J. Chromatogr. 471 (1989) 375.
640 [34] L. Botz, Sz. Nyiredy and O. Sticher, J. Planar Chromatogr. 3 (1990) 352.
641 [35] E. Mincsovics, M. Garami, L. Kecskés, B. Tapa, Z. Végh, Gy. Kátay, E. Tyihák, J.
642 AOAC Int. 82 (1999) 587.
643 [36] E. Tyihák, E. Mincsovics, in: Sz. Nyiredy (ed.), Planar Chromatography – A
644 Retrospective View for the Third Millennium, Springer, Budapest, 2001, p. 193.

645 [37] E. Mincsovics, K. Ferenczi-Fodor, E. Tyihák, Overpressured Layer Chromatography,
646 in: J. Sherma, B. Fried (eds.), Handbook of Thin-Layer Chromatography, 3rd ed.
647 Marcell Dekker, New York, NY, 2003, p. 171.
648 [38] R.E. Kaiser, R.J. Rieder, in: R.E. Kaiser (ed.) Planar Chromatography, Vol. I, Hüthig,
649 Heidelberg, l986, p. 165.
650 [39] R.E. Kaiser, Einführung in die HPPLC, Hüthig, Heidelberg, l987.
651 [40] Z. Witkiewicz, M. Mazurek, J. Bladek, J. Planar Chromatogr. 6 (1993) 407.
652 [41] E. Mincsovics, J. Planar Chromatogr. 17 (2004) 411.
653 [42] E. Tyihák, Gy. Kátay, Zs. Ostorics, and E. Mincsovics, J. Planar Chromatogr. 11
654 (1998) 5.
655 [43] E. Tyihák, J. Pharm. Biomed. Anal. 5 (1987) 191.
656 [44] Sz. Nyiredy, C.A.J. Erdelmeyer, K. Dallenbach-Toelke, K. Nyiredy-Mikita, and O.
657 Sticher, J. Nat. Products 49 (1986) 885.
658 [45] K.L. Rowlen, K.A. Duell, J.P. Avery, J.W. Birks, Anal. Chem. 63 (1991) 575.
659 [46] G. Morlock, W. Schwack, Trends Anal. Chem. 10 (2010) 1157.
660 [47] G. Morlock, W. Schwack, J. Chromatogr. A 1217 (2010) 6600.
661 [48] M. Sajewicz, L. Wojtal, M. Hajnos, M. Waksmundzka-Hajnos, T. Kowalska, J.
662 Planar Chromatogr. 23 (2010) 270.
663 [49] B. Betti, G. Lodi, C. Bighi, G. Chiorboli and S. Coppi, J. Planar Chromatogr. 7
664 (1994) 301.
665 [50] Sz. Nyiredy and K. Glowniak, in: Sz. Nyiredy (ed.) Planar Chromatography – A
666 Retrospective View for the Third Millennium, Springer, Hungary, 2001, p. 551.
667 [51] E. Mincsovics, G. Kátay, P.G. Ott, Z. Király-Véghely, Á.M. Móricz, E. Tyihák,
668 Chromatographia (Suppl.) 62 (2005) 51.

669 [52] E. Mincsovics, K. Pápai, K. Ludányi, Á.Z. Dávid, M. Budai, I. Antal, I. Klebovich, J.
670 Planar Chromatogr. 21 (2008) 361.
671 [53] W. Chai, C. Leteux, A.M. Lawson, M.S. Stoll, Anal. Chem. 75 (2003) 118.
672 [54] I. Klebovich, Gy. Morovjan, I. Hazai, E. Mincsovics, J. Planar Chromatogr. 15 (2002)
673 404.
674 [55] F. Geiss, Fundamentals of Thin-Layer Chromatography, Hüthig, Heidelberg,
675 Germany, l987.
676 [56] C.F. Poole and S.K. Poole, Chromatography Today, Elsevier, Amsterdam, l991.
677 [57] C.F. Poole, in: Sz. Nyiredy (ed.), Planar Chromatography – A Retrospective View for
678 the Third Millennium, Springer, Budapest, Hungary, 2001, p. 13.
679 [58] G. Guiochon, N. Marchetti, K. Mrizik, R.A. Shalliker, J. Chromatogr. A, 1189 (2008)
680 109.
681 [59] E. Tyihák, E. Mincsovics, H. Kalász, and J. Nagy, J. Chromatogr. 211 (1981) 45.
682 [60] H.E. Hauck and W. Jost, J. Chromatogr. 262 (1983) 113.
683 [61] H. Kalász and M. Báthori, LC-GC Int. 10 (1997) 440.
684 [62] H. Kalász and J. Nagy, J. Liquid Chromatogr. 4 (1981) 985.
685 [63] E. Tyihák, and E. Mincsovics, in: Sz. Nyiredy (ed.) Proc. Planar Chromatogr. 2000,
686 Lillafüred, Hungary, Res. Inst. Med. Plants, Budakalász, 2000, p. 37.
687 [64] J.H. Knox, J. Chem. Soc. (1961) 433.
688 [65] J.C. Giddings, Anal. Chem. 37 (1965) 60.
689 [66] Sz. Nyiredy, In: Sz. Nyiredy (ed.): Planar Chromatography- a Retrospective View for
690 the Third Millennium, Springer, Budapest, Hungary, 2001, p. 103
691 [67] C.F. Poole and S.K. Poole, J. Chromatogr. A 703 (1995) 573.

692 [68] Sz. Nyiredy, in: R.E. Kaiser, W. Günther, H. Gunz and G. Wulff (eds.) Dünnschicht
693 Chromatographie in memoriam Prof. Dr. Hellmut Jork, InCom Sonderband,
694 Düsseldorf, l996, p. 166.
695 [69] J.C. Giddings, in: H.J. Cortes (ed.) Multi-dimensional Chromatography, Marcel
696 Dekker, New York, l990, p. 1.
697 [70] R. Consden, A.H. Gordon and A.J.P. Martin. Biochem. J. 38 (1944) 224.
698 [71] G. Guiochon and A.M. Siouffi, J. Chromatogr. 245 (1982) 1.
699 [72] G, Guiochon, M.F. Gonnord, M. Zakaria, L.A. Beaver and A.M. Siouffi,
700 Chromatographia, 17 (1983) 121.
701 [73] L.A. Beaver and G. Guiochon, USA Patent No. 4469601, l984.
702 [74] E. Mincsovics, M. Manach, L. Kecskés, B. Tapa, D. Papillard, E. Tyihák, J. Liq.
703 Chromatogr. Relat. Technol. 26 (2003) 2611.
704 [75] E. Mincsovics, In: Proc. Int. Symp. On Planar Separations, Planar Chromatography,
705 2005 Milestones in Instrumental TLC , Siófok, Hungary (Ed. Sz. Nyiredy), RIMP,
706 Budakalász, 2005, p. 137.
707 [76] E. Mincsovics, J. Planar. Chromatogr. 21 (2008) 97.
708 [77] E. Tyihák and E. Mincsovics, J. Planar Chromatogr. 23 (2010) 382.
709 [78] E. Tyihák, M. Petro-Turza, K. Kardos, E. Mincsovics, J. Planar Chromatogr. 5 (1992)
710 376.
711 [79] J. Pothier, N. Galand, P. Tvollier, C. Viel, J. Planar Chromatogr. 6 (1993) 220.
712 [80] A. Linard, P. Gueset, G. Durand, J. Planar Chromatogr. 6 (1993) 322.
713 [81] Á. Kovács, L. Simon-Sarkadi, E,. Mincsovics, J. Planar Chromatogr. 11 (1998) 43.
714 [82] A. Ludwiczuk, Sz. Nyiredy, T. Wolski, J. Planar Chromatogr. 18 (2005) 104.
715 [83] E. Tamburini, T. Bernardi, M. Granini, G. Vaccari, J. Planar Chromatogr. 19 (2006)
716 10.

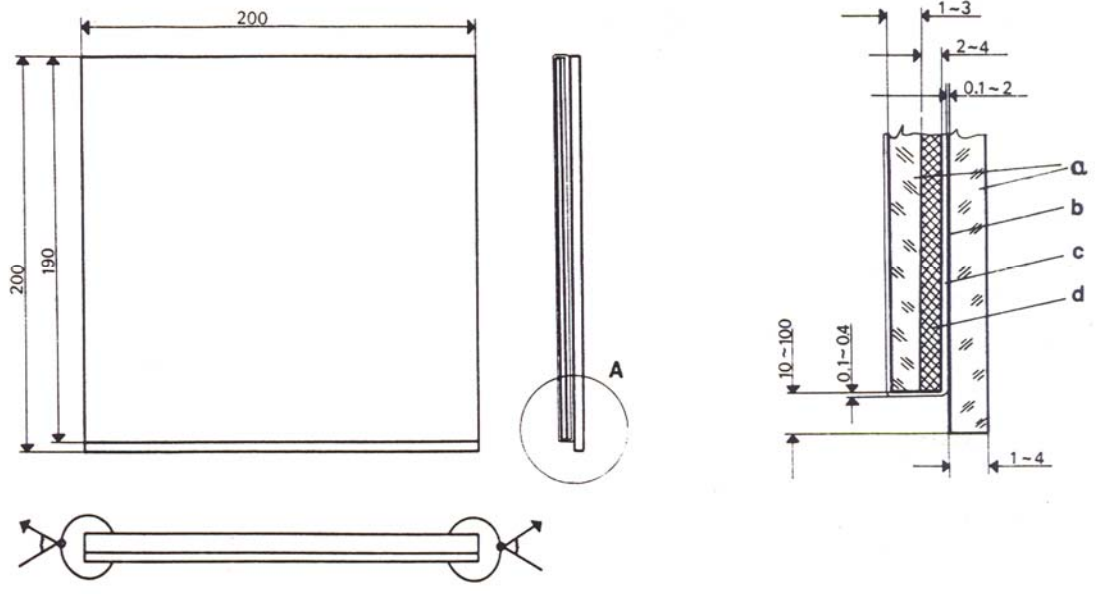
717 [84] Á.M. Móricz, Zs. Fatér, K.H. Otta, E. Tyihák, E. Mincsovics, Microchem. J. 85
718 (2007) 140.
719 [85] A. Pelander, D. Backström, I. Ojanperá, J. Chromatogr. B 857 (2007) 337.
720 [86] J.K. Rozylo, A. Zabinska, J. Matysiek, A. Niewiadomy, J. Chromatogr. Sci. 40
721 (2002) 581.
722 [87] B. Bagocsi, D. Fábián, A. Lauko, S. Maho, Z. Végh, K. Ferenczi-Fodor, J. Planar
723 Chromatogr. 15 (2002) 252.
724 [88] N. Galand, D. Ernouf, F. Montigny, J. Dollet, J. Pothier, J. Chromatogr. Sci. 42
725 (2004) 130.
726 [89] K. Ludányi, K. Vékey, J. Szúnyog, E. Mincsovics, T. Karancsi, K. Újszászy, K.
727 Balogh-Nemes, I. Klebovich, J. AOAC Int. 82 (1999) 231.
728 [90] E. Horváth, Gy. Kátay, E. Tyihák, J. Kristóf, A. Rédey, Chromatographia 51 (2000)
729 297.
730 [91] T.K. Rozylo, R. Siembida, Z.I. Németh, L. Albert, E. Tyihák, Biomed. Chromatogr.
731 14 (2000) 173.
732 [92] A. Pelander, I. Ojhanpera, J. Sistonen, I. Rasanen, E. Vuori, J. Anal. Toxicol. 27
733 (2003) 226.
734 [93] H.S. Yang, M.H. Tang, H.X. Deng, J.L. Yang, Sichuan Da Xue Xue Bao Yi Xue Ban,
735 38 (2007) 1033.
736 [94] Sz. Nyiredy, in: Sz. Nyiredy (ed.) Planar Chromatography – A Retrospective View
737 for the Third Millennium, Springer, Budapest, 2001, p. 177.
738 [95] E. Mincsovics, in: M. Waksmundzka-Hajnos, J. Sherma, T. Kowalska (eds.), Thin-
739 Layer Chromatography in Phytochemistry, CRC Press, Taylor & Frances Group,
740 Boca Raton, 2008, p. 215.
741 [96] Sz. Nyiredy, J. Chromatogr. A, 1000 (2003) 985.

742 [97] I. Klebovich, E. Mincsovics, J. Szunyog, K. Ludányi, T. Karancsi, K. Újszászi, B.
743 Dalmadi Kiss, K. Vékey, J. Planar Chromatogr. 11 (1998) 394.
744 [98] E. Mincsovics, É. Sárdi, I. Velich, Gy. Kátay, E. Tyihák, J. Planar Chromatogr. 15
745 (2002) 280.
746 [99] D.E.Wedge, N.D. Camper, in: H.G. Cutler, S.J. Cutler (eds.), Biologically Active
747 Natural Products: Agrochemicals and Pharmaceuticals, CRC Press, Taylor & Francis
748 Group, Boca Raton, 2000, p. 1.
749 [100] E. Tyihák, Á.M. Móricz, P.G. Ott, in: M. Waksmundzka-Hajnos, J. Sherma, T.
750 Kowalska (eds.), Thin-Layer Chromatography in Phytochemistry, CRC Press, Taylor
751 and Francis Group, Boca Raton, 2008, p. 193.
752 [101] E. Tyihák, P. Ott, Á.M. Móricz, Gy. Kátay, Zs. Király-Véghely, J. Planar
753 Chromatogr. 17 (2004) 84.
754 [102] J.I. Rios, M.C. Recio, A. Villar, J. Ethnopharmacol. 23 (1988) 127.
755 [103] J.N. Eloff, S. Afr. J. Sci. 96 (2000) 116.
756 [104] E.Tyihák, L. Albert, Zs.I. Németh, Gy. Kátay, Zs. Király-Véghely, B. Szende, Acta
757 Biol. Hung. 49 (1998) 225.
758 [105] E. Tyihák, L. Trézl, and B. Szende, Ann. N. Y. Acad. Sci. 851 (1998) 259.
759 [106] Gy. Kátay, M. Adrian-Romero, Zs.I. Németh, G. Blunden, E. Tyihák, L. Albert, J.
760 Planar Chromatogr. 15 (2002) 28.
761 [107] E. Tyihák, E. Mincsovics, Gy. Kátay, Zs. Király-Véghely, Á.M. Móricz, P.G. Ott, J.
762 Planar Chromatogr. 21 (2008) 15.
763 [108] L. Trézl, J. Pipek, J. Mol. Struct. – Theochem. 170 (1988) 213.
764 [109] E. Tyihák, Zs. Rozsnyay, É. Sárdi, G. Gullner, L. Trézl, R. Gáborjányi, Acta Biol.
765 Acad. Sci. Hung. 45 (1994) 3.

766 [110] E. Tyihák, in: J.A. Teixeira da Silva (ed.) Foliculture, Oriental and Plant
767 Biotechnology, Advances and Topical Issues Vol 3, Global Science Books, London,
768 UK, 2006, p.380.
769 [111] A.D. Wentworth, I.H. Jones, P. Wentworth, K.D. Janda, R.A. Lerner, Proc. Natl.
770 Acad. Sci. USA 97 (2000) 10930.
771 [112] P. Wentworth Jr., A.D. Wentworth, X.Y. Zhu, L.A. Wilson, K.D. Janda, A.
772 Eschenmoser, R.A. Lerner, Proc. Natl. Acad. Sci. USA 100 (2003) 1490.
773 [113] E. Tyihák, Á.M. Móricz, P.G. Ott, J. Planar Chromatogr. 21 (2008) 77.
774 [114] Á.M. Móricz, N. Adányi, E. Horváth, P.G. Ott, E. Tyihák, J. Planar Chromatogr. 21
775 (2008) 417.
776 [115] R.R. Goodall and A.A. Levi, Nature 158 (1946) 675.
777 [116] Á.M. Móricz, P.G. Ott, F. Billes, K.-H. Otta, E. Tyihák, J. Appl. Microbiol. 103
778 (2007) 2525.
779 [117] I.M. Choma, E.M. Grzelak, J. Chromatogr. A, 1218 (2011) 2684.
780 [118] E. Tyihák, A. Takátsy, Á.M. Móricz, P.G. Ott, R. Ohmacht, in: M. Szilágyi and K.
781 Szentmihályi (eds), Proc. Trace Elements in the Food Chain, Vol.3, Budapest,
782 Hungary, 2009, p. 292.
783 [119] E. Tyihák, Á.M. Móricz, P.G. Ott, Gy. Kátay, and Zs. Király-Véghely, J. Planar
784 Chromatogr. 18 (2005) 67.
785 [120] E. Tyihák, Gy. Kátay, Á.M. Móricz, P.G. Ott, Zs. Király-Véghely, Acta Pharm.
786 Hung. 77 (2007) 53.
787 [121] E. Tyihák, Á.M. Móricz, P.G. Ott, M.L. Hajnos, and K. Glowniak, J. Planar
788 Chromatogr. 21 (2008) 331.
789 [122] Á.M. Móricz, Gy. Horváth, P. Molnár, B. Kocsis, A. Böszörményi, É. Lemberkovics,
790 P.G. Ott, J. Planar Chromatogr. 23 (2010) 406.

791 [123] Á.M. Móricz, Sz. Szarka, P.G. Ott, É.B. Héthelyi, É. Szőke, E. Tyihák, Med.
792 Chem.(in Press)
793 [124] E. Tyihák, Zs. Király-Véghely, and Á.M. Móricz, Nat. Prod. Commun. 6 (2011) 631
794
795

796 **Captions to figures**

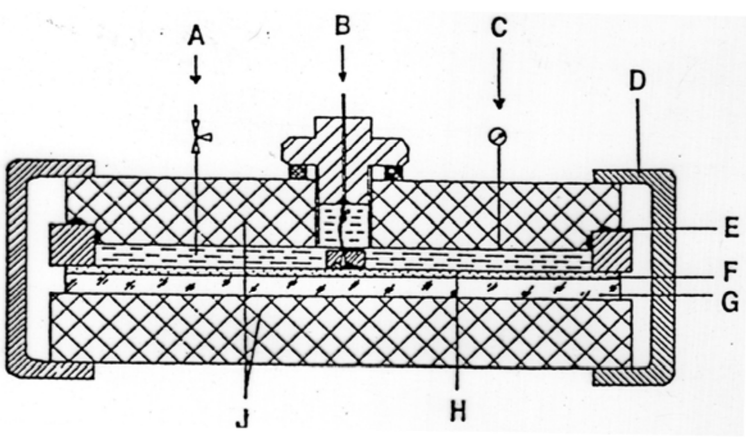


797

798 Figure 1

799 Schematic drawing of an ultramicro chamber

800 Distances are in millimetres [20]



801

802 Figure 2

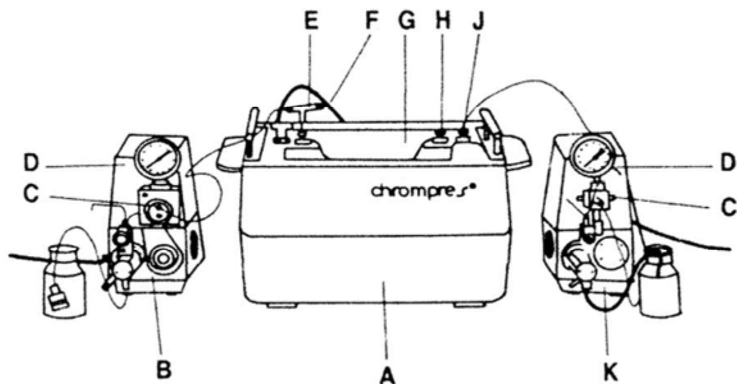
803 Schematic drawing of the circular type of pressurized ultramicro chamber

804 A, water inlet, B, developing solvent inlet, C, pressure gauge, D, screw fastener, E, rubber O-

805 ring, F, adsorbent layer, G, support plate, H, plastic foil cushion system, J, polymethacrylate

806 support blocks [20]

807



808 Figure 3

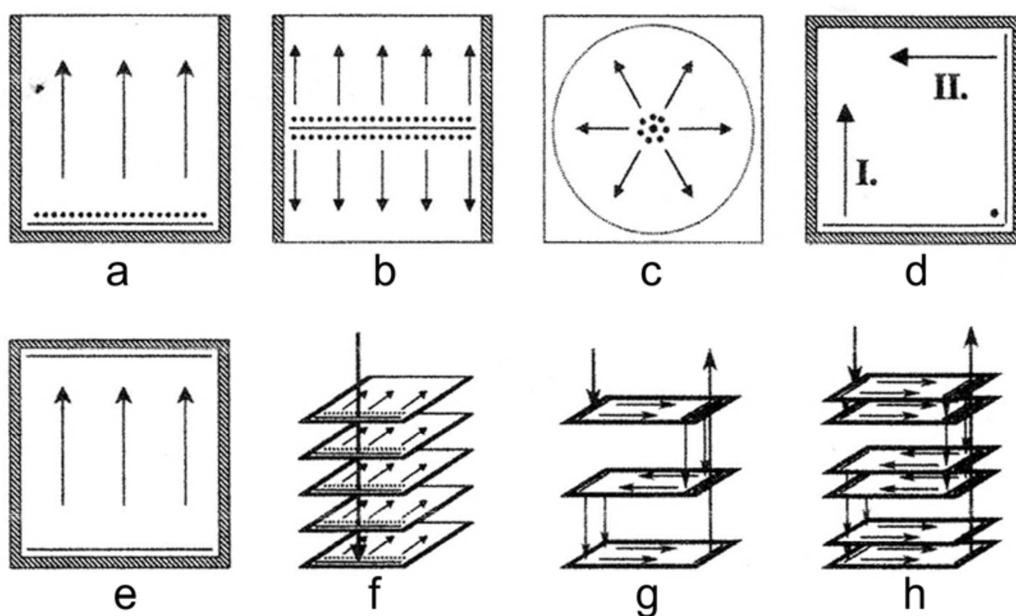
809 Schematic drawing of Chrompres 25

810 A, chamber, B, eluent pump, C, safety valve, D, pressure gauge, E, solvent-inlet valve, F, water

811 outlet, G, polymethacrylate cover, H, solvent-outlet pin, J, water inlet, K, water pump (with

812 permission, from Ref. [22]).

813



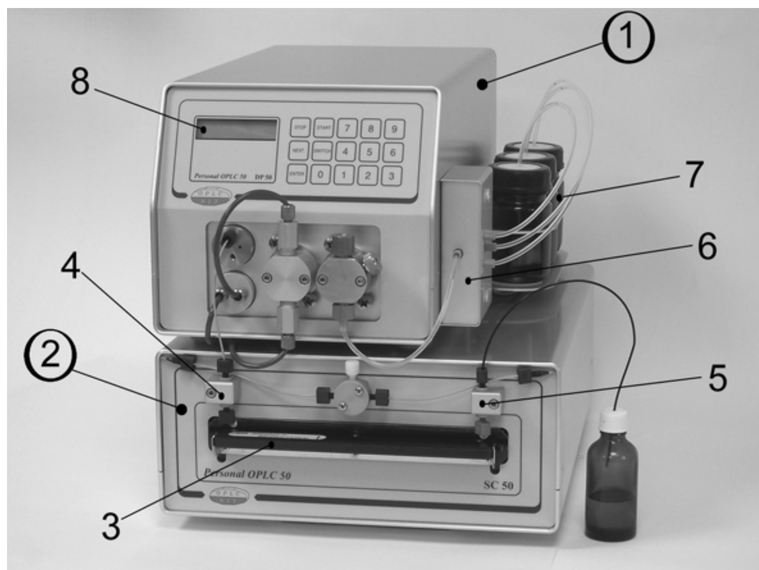
814 Figure 4

815 Schematic drawing of chromatographic chromatoplates used in conventional OPLC separations;

816 a, one-directional; b, two-directional; c, circular; d, two-directional; c, on-line; f, parallel

817 coupled multilayer; g, serial coupled multilayer; h, parallel-serial coupled multilayer. (With

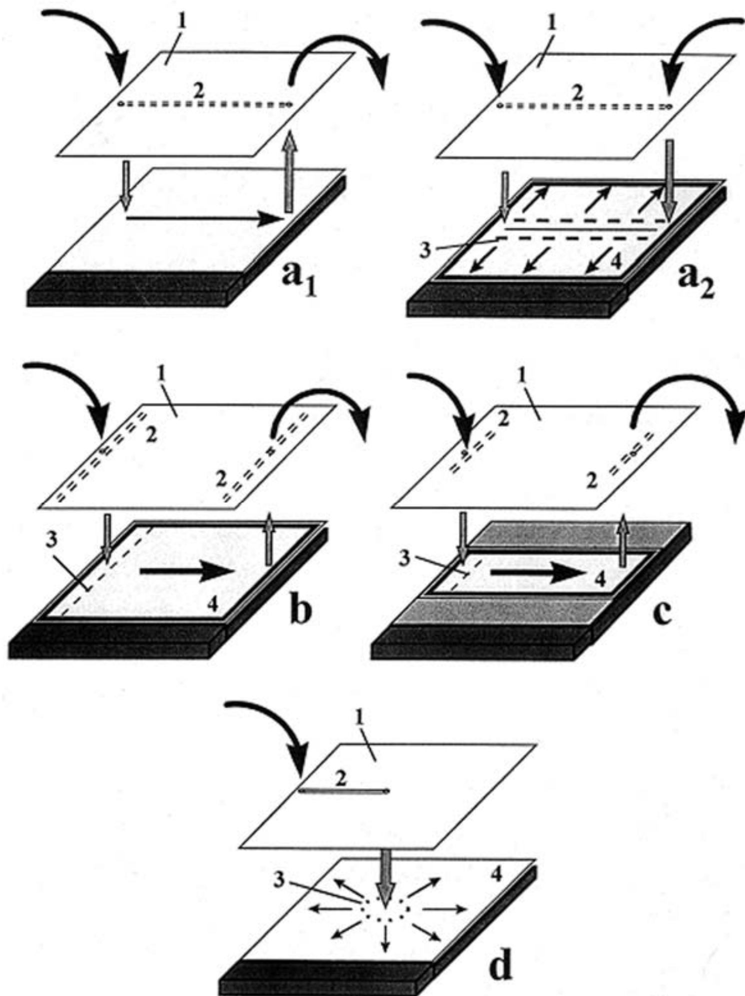
818 permission, from Ref. [36].)

819 

820 Figure 5

821 Automated OPLC instrument

822 1, liquid-delivery system; 2, separation chamber; 3, cassette; 4, mobile phase inlet; 5 mobile
823 phase outlet; 6, mobile phase switching valve; 7, mobile phase reservoirs; 8, LCD display. (With
824 permission, from Ref. [41]).

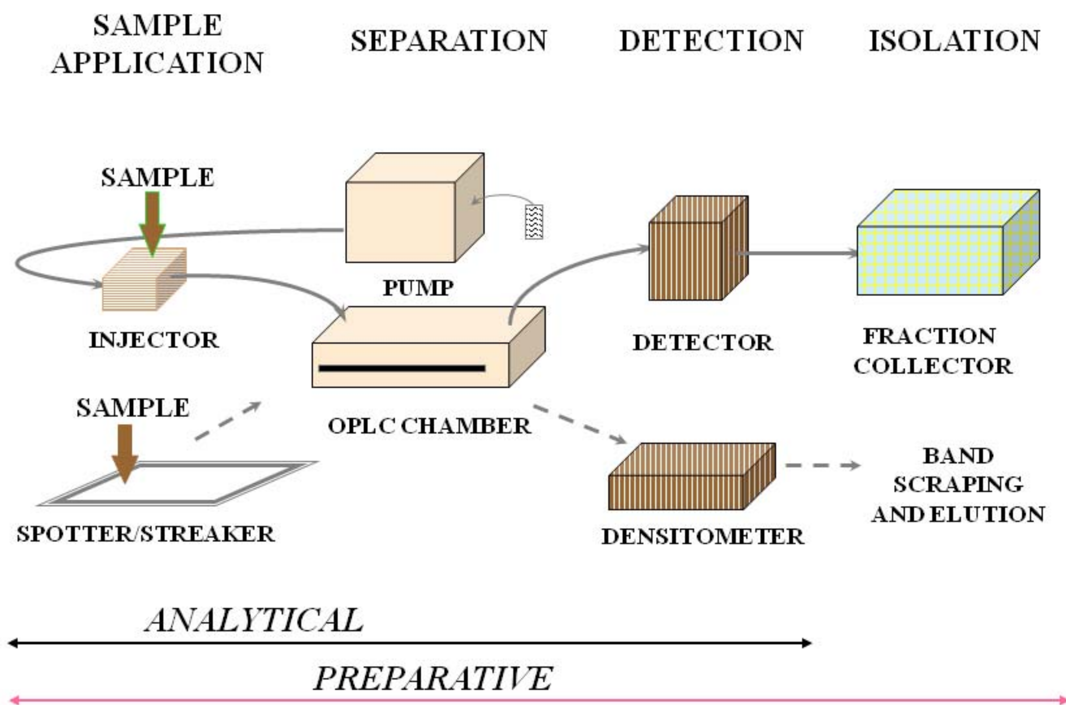


825

826 Figure 6

827 Cassette solutions to automatic OPLC

828 a1, cassette for system rinsing
829 a2, cassette for bidirectional infusion development of 20x20 cm layer
830 b, cassette of 20x20 cm layer for linear one- and two-dimensional development
831 c, cassette of 10x20 cm glass backed layer for linear one-dimensional development;
832 d, cassette for circular development.
833 Arrow represents the eluent movement.
834 1, PTFE coverplate involves eluent directing trough and hole is in floating position; 2, eluent-
835 directing trough; 3, sample; 4, adsorbent layer
836

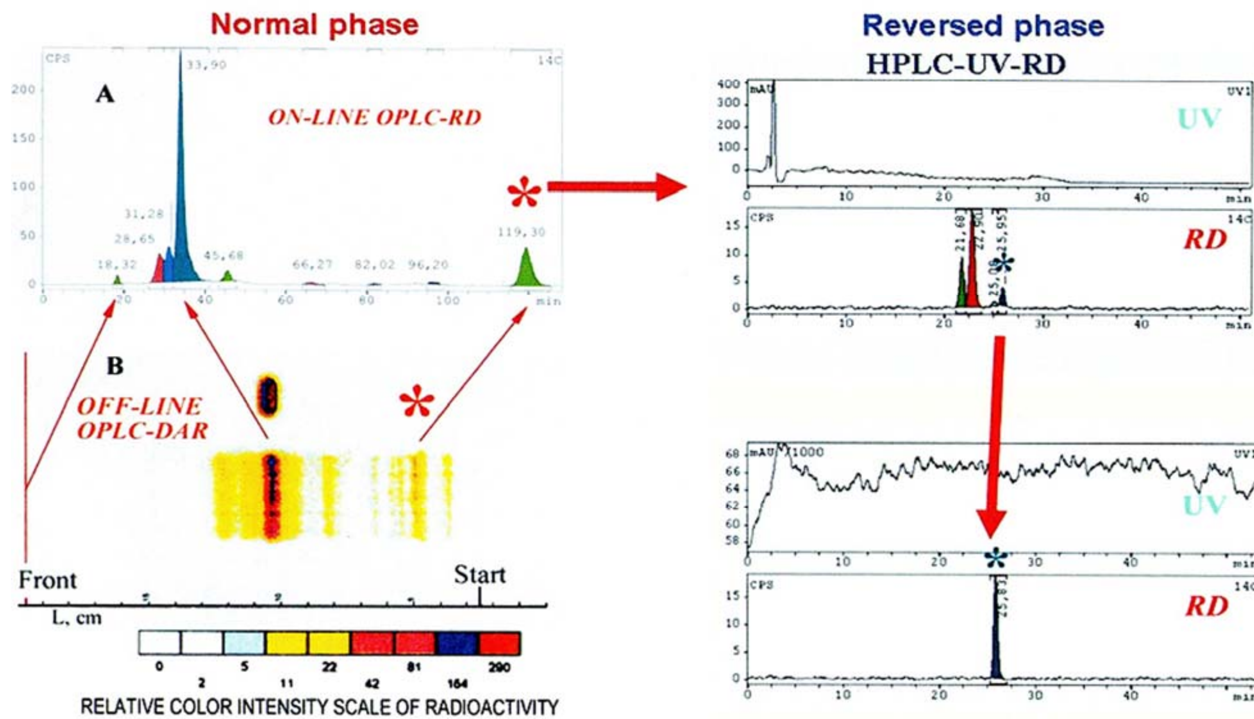


837

838 Figure 7

839 Schematic diagram of off-line and on-line OPLC. Dashed arrows show the procedure of off-line

840 operating mode.



841

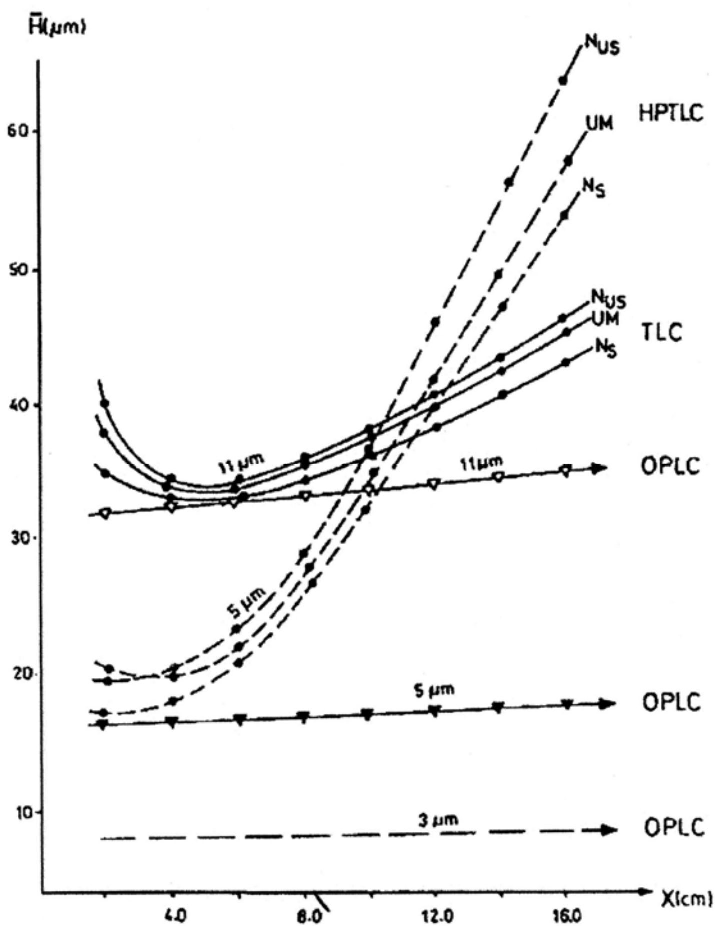
842 Figure 8

843 Isolation of ^{14}C labelled metabolites by combined OPLC-HPLC system

844 Normal phase OPLC separation (left) reversed phase HPLC separation (right).

845 Peak marked by asterisk has been separated for isolation.

846



847 Figure 9

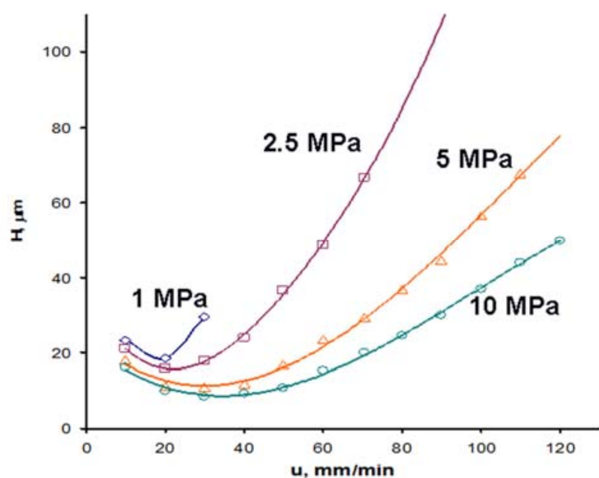
848 Correlation between the average theoretical plate height (H) and the distance (X) travelled by the

849 mobile phase on silica gel layers of different particle size and in different chamber systems.

850 Ns, normal saturated chamber; Nus, normal unsaturated chamber; UM, ultramicro chamber

851 (totally closed adsorbent layer, without overpressure); OPLC chamber using 11, 5 and 3 μm

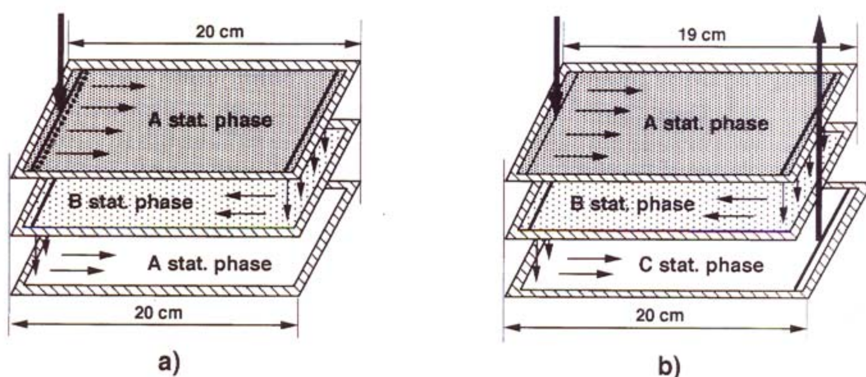
852 particle size, respectively (with permission from Ref. [36]).



853

854 Figure 10

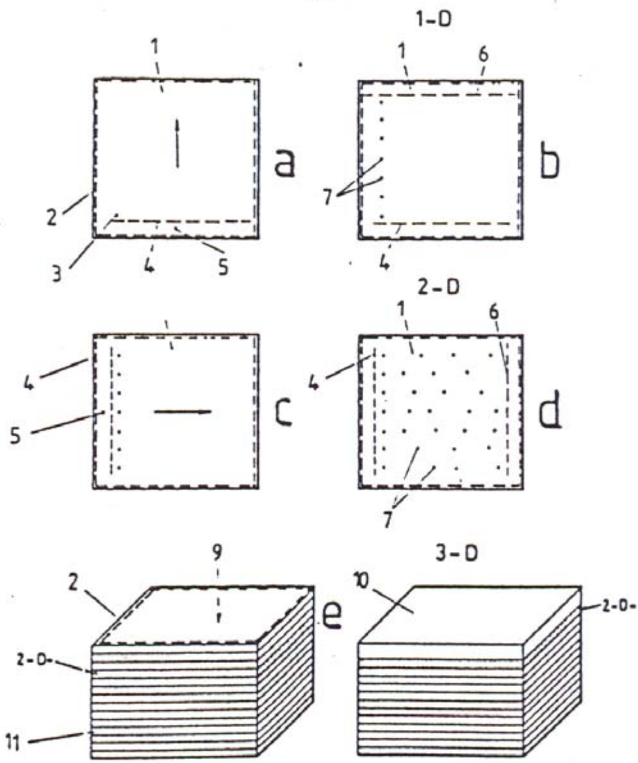
855 Effect of linear velocity (u) on theoretical plate height (H) at different external pressures. The
856 results were obtained from the use of the experimental OPLC Separation Unit 100 with the
857 following conditions:
858 5 µl of a 0.36 mg mL^{-1} PTH-valine solution was applied in 10 mm band, and chromatographed
859 on HPTLC silica gel (Merck) with dichloromethane-ethyl acetate 92:8 (v/v) as mobile phase; the
860 development distance was 170 mm. (With permission, from Ref. [77]).



861

862 Figure 11

863 Schematic diagram of multilayer (ML)-OPLC using different stationary phases of decreasing
864 polarity. (a) Arrangement for analytical off-line separation of eighteen samples or one sample for
865 micropreparative separation. (b) Arrangement for on–line analytical or micropreparative
866 separation.

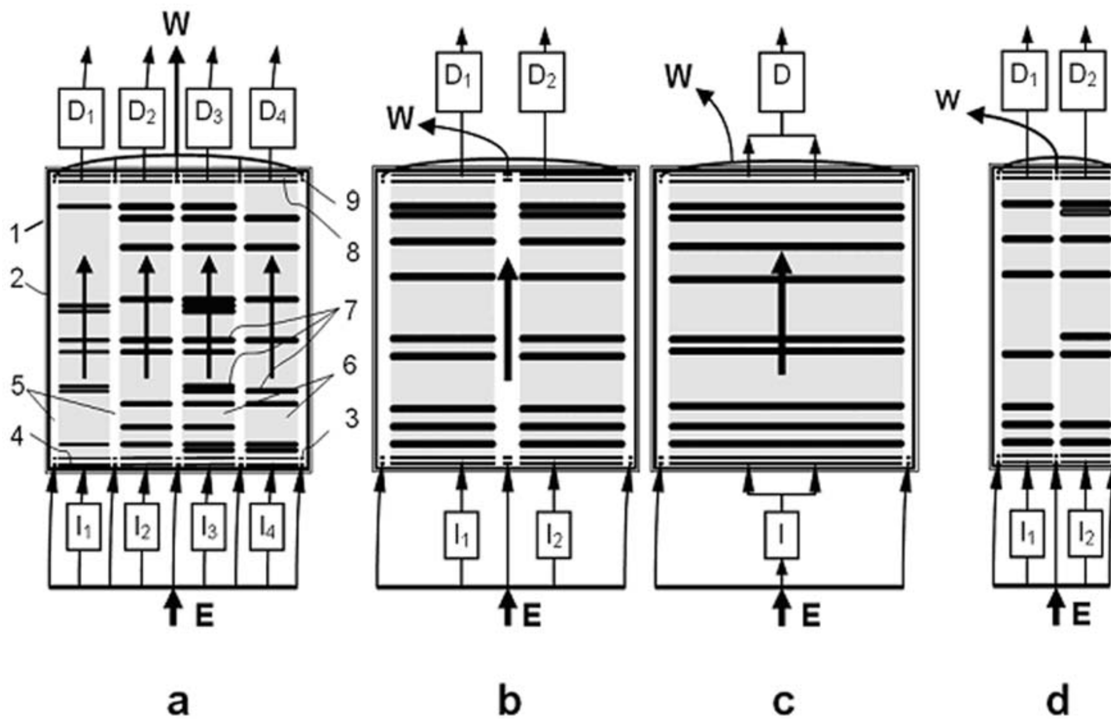
867 

868 Figure 12

869 Schematic drawing of 1-, 2-, and 3-dimensional OPLC separation.

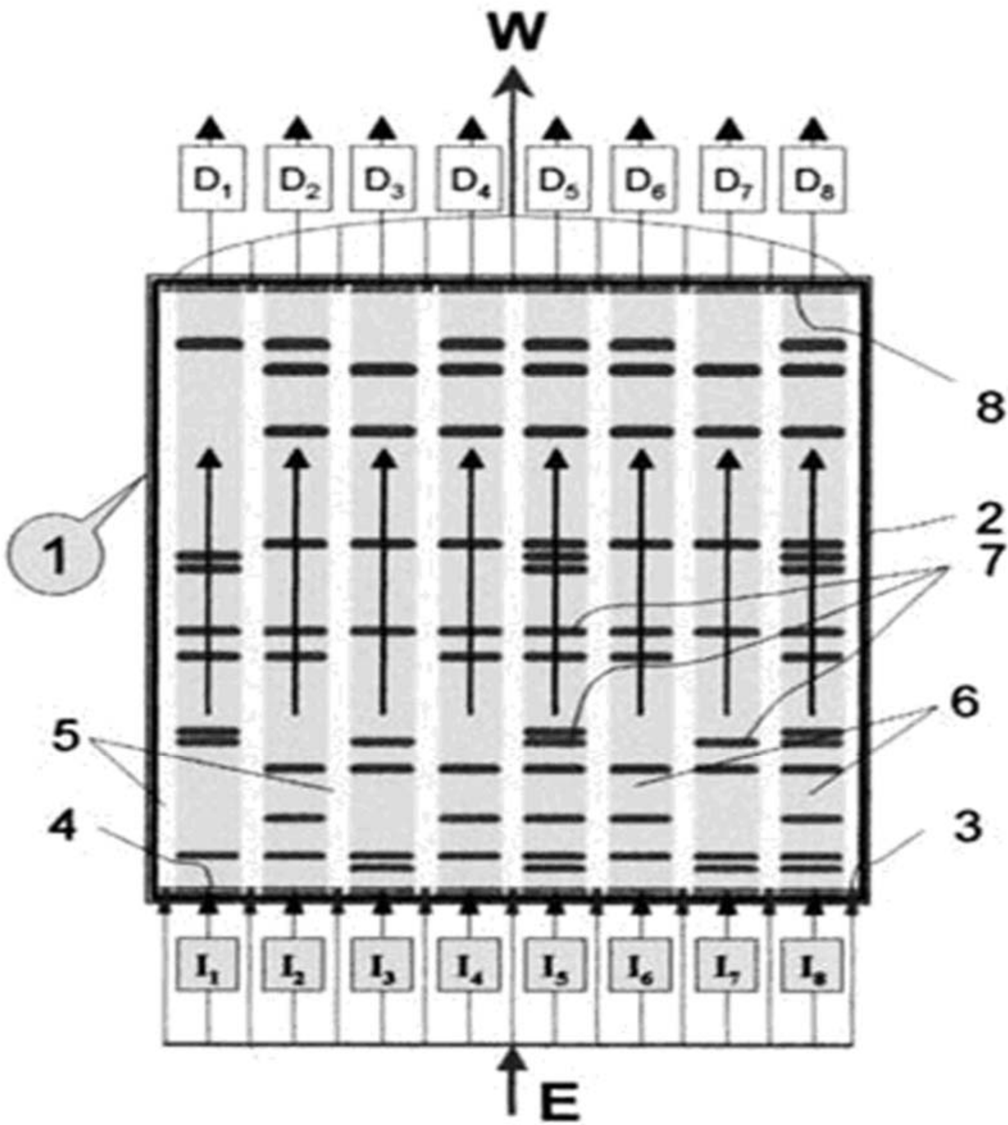
870 (Figure is from a patent application, and lecture in Aix-an Provence, 1992.)

871



872 Figure 13

873 Schematic diagram of a four-channel OPLC system with the FEW-I/O arrangement dedicated to
874 fully on-line separation of four samples (a), two samples (b), or one sample (c) using 20 x 20 cm,
875 and two samples using 10 x 20 cm (d) adsorbent layers. 1, non-segmented adsorbent layer; 2,
876 sealed edges; 3, distributor space of mobile phase for FEW formation; 4, distributor space for
877 sample application; 5, FEW part of adsorbent layer; 6, separation part of adsorbent layer; 7,
878 components separated; 8, sample collector space at outlet side; 9, collector space of FEW at
879 outlet side; E, mobile phase; I, injectors; D, detectors; W, waste collected by FEW lines (with
880 permission, from Ref. [76).



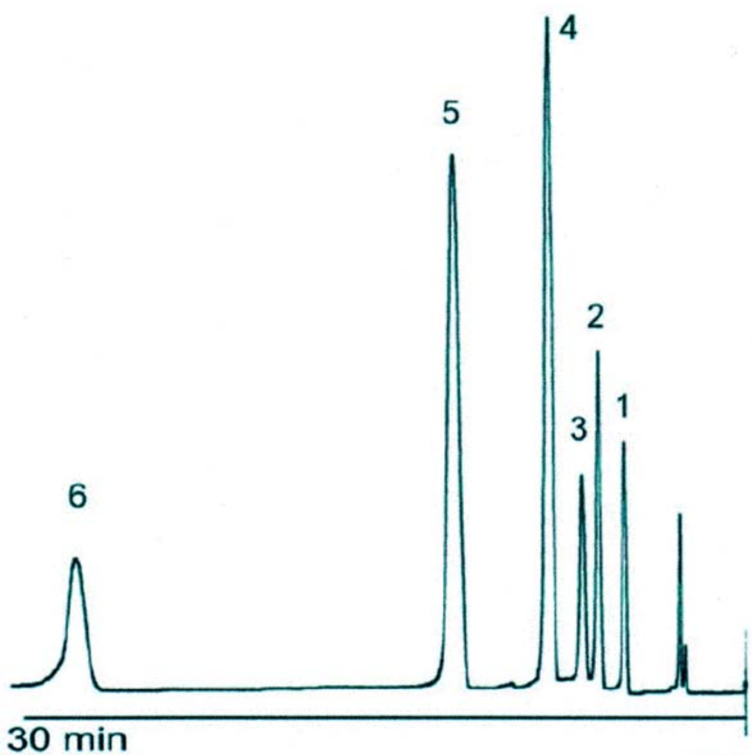
881

882 Figure 14

883 Schematic diagram of eight-channel OPLC separations using parallel injections and FEW-I/O

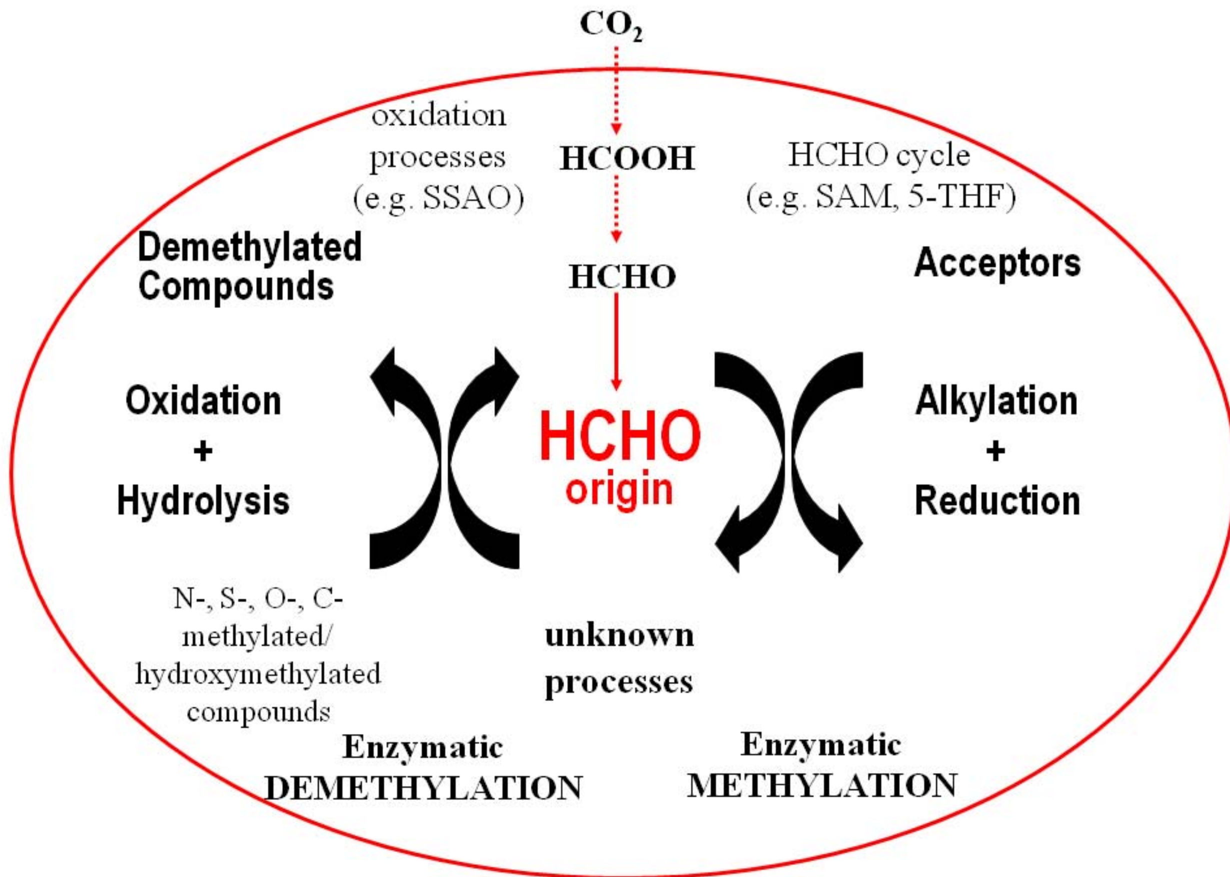
884 arrangements. Further conditions are at Figure 13.

885



886 Figure 15

887 One-channel fully on-line FEW-OPLC separation of some PTH-amino acids using the
888 experimental OPLC Separation Unit 100. The acids were separated on a 5 cm x 20 cm fine-
889 particle silica gel layer with chloroform-ethyl acetate 9:1 (v/v) as mobile phase at a flow rate of
890 0.3 mL min^{-1}; injection volume, 0.5 µl; cell volume , 1 µl; detection at 275 nm; AUFS 0.05/0.5V.
891 1, PTH, proline; 2, PTH-leucine; 3, PTH-isoleucine; 4, PTH-valine; 5, PTH-methionine; 6, PTH-
892 glycine (with permission, from Ref. [41]).

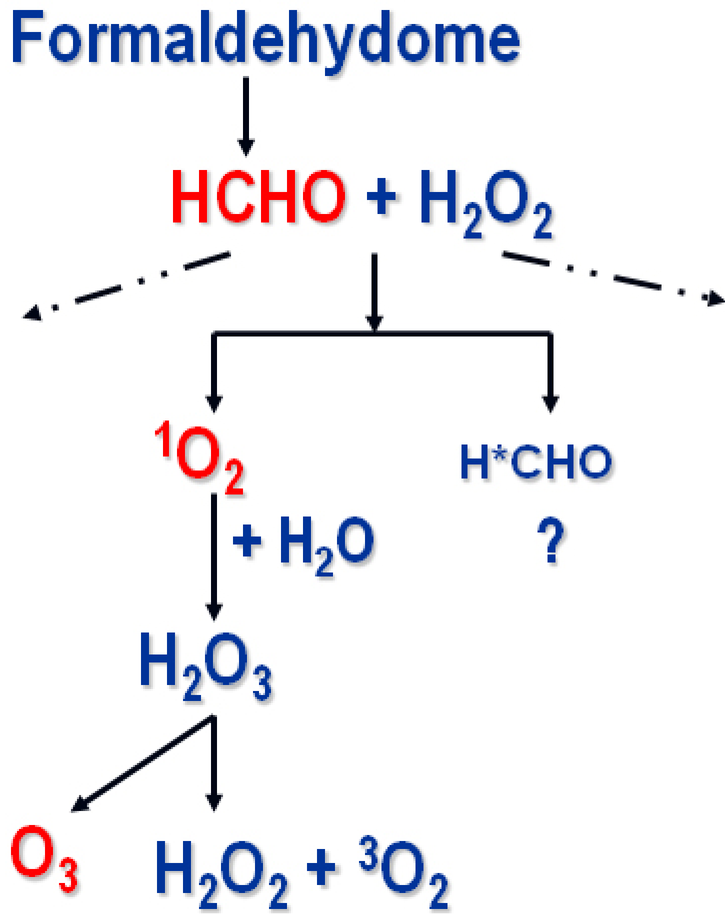


893

894 Figure 16

895 The main elements of the formaldehydome (with permission, from Ref. [77]).

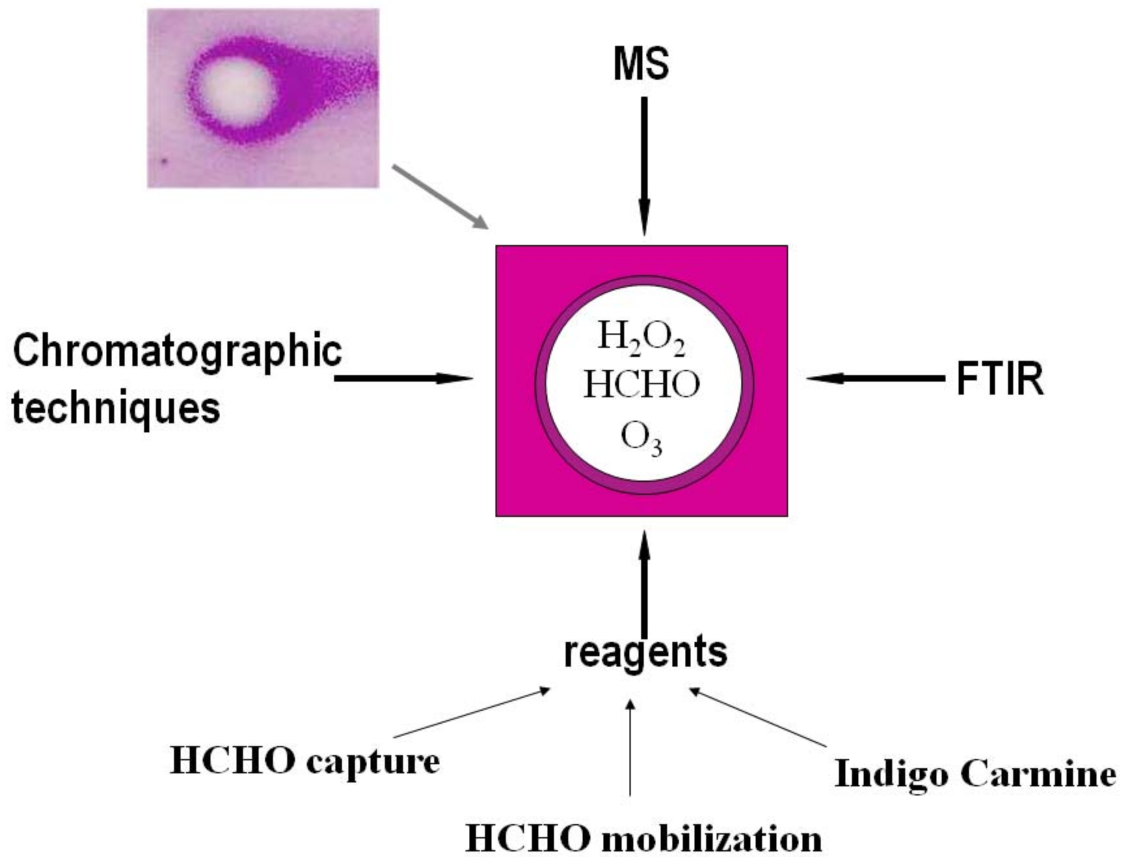
896



897 Figure 17

898 Possible formation of different reactive oxidants by interaction of HCHO and H_2O_2 (with

899 permission, from Ref. [77]).

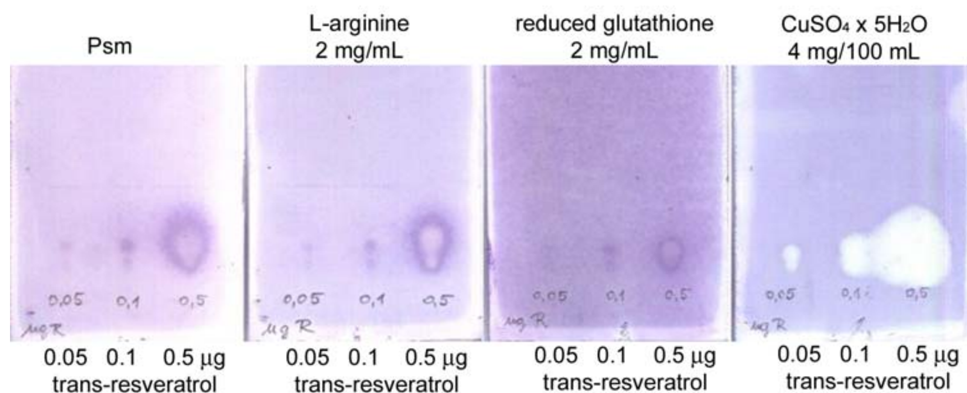


900

901 Figure 18

902 Main steps in the detection and identification of a typical spot with the BioArena system (with

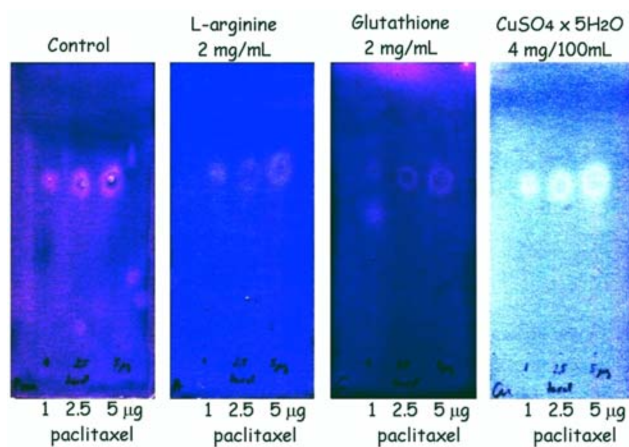
903 permission, from Ref. [77]).



904

905 Figure 19

906 The influence of endogenous substances on antiyeast (*Saccharomyces cerevisiae*) activity of
907 trans-resveratrol. Chromatographic conditions: silica gel 60 F_{254} (Merck, preconditioning at 120
908 ^{o}C for 3 h), chloroform-methanol 80:4 (v/v). Biological conditions: (A) yeast suspension (3 g
909 yeast in 100 mL distilled water); (B) A + 2 mg L-arginine in 1 mL yeast suspension; (C) A + 2
910 mg reduced glutathione in 1 mL yeast suspension; (D) A + 4 mg $CuSO_4$ x 5 H_2O in 100 mL
911 yeast suspension. (With permission, from Ref. [120]).

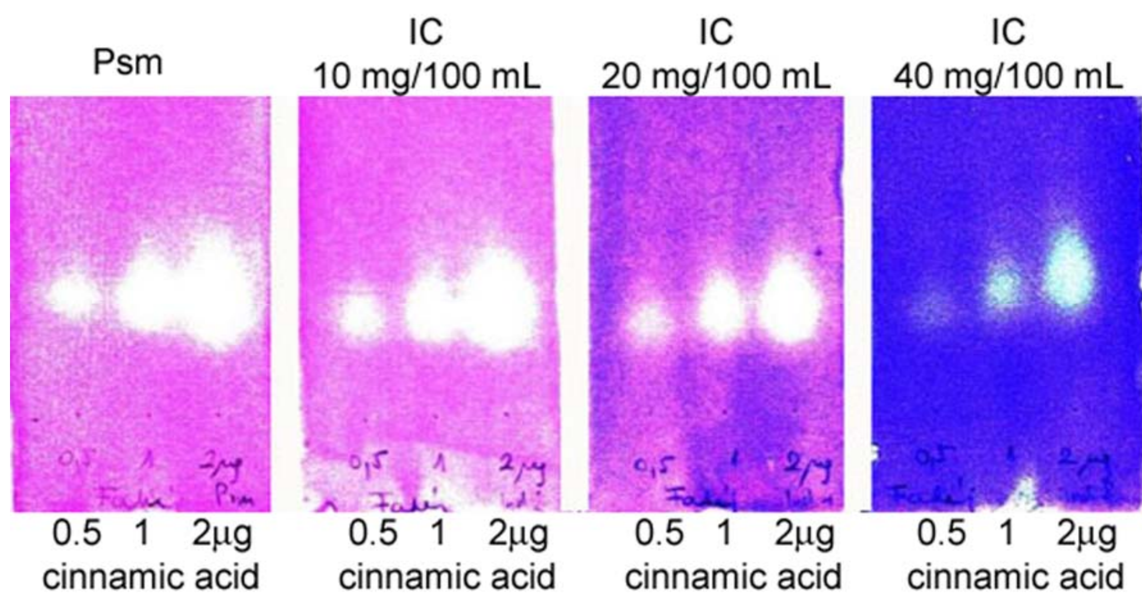


912

913 Figure 20

914 The influence of endogenous substances on the antibacterial activity of paclitaxel.
915 Chromatographic conditions: silica gel 60 F_{254} (Merck); mobile phase: chloroform-methanol
916 90:10 (v/v); Desaga glass TLC chamber. Biological conditions: Control: *Pseudomonas* sp., other
917 information is on the chromatograms. Incubation was at the optimum temperature and detection
918 was performed with MTT. (With permission, from Ref. [121].)

919



920 Figure 21

921 Effect of Indigo Carmine on the antibacterial activity of cinnamic acid. Chromatographic

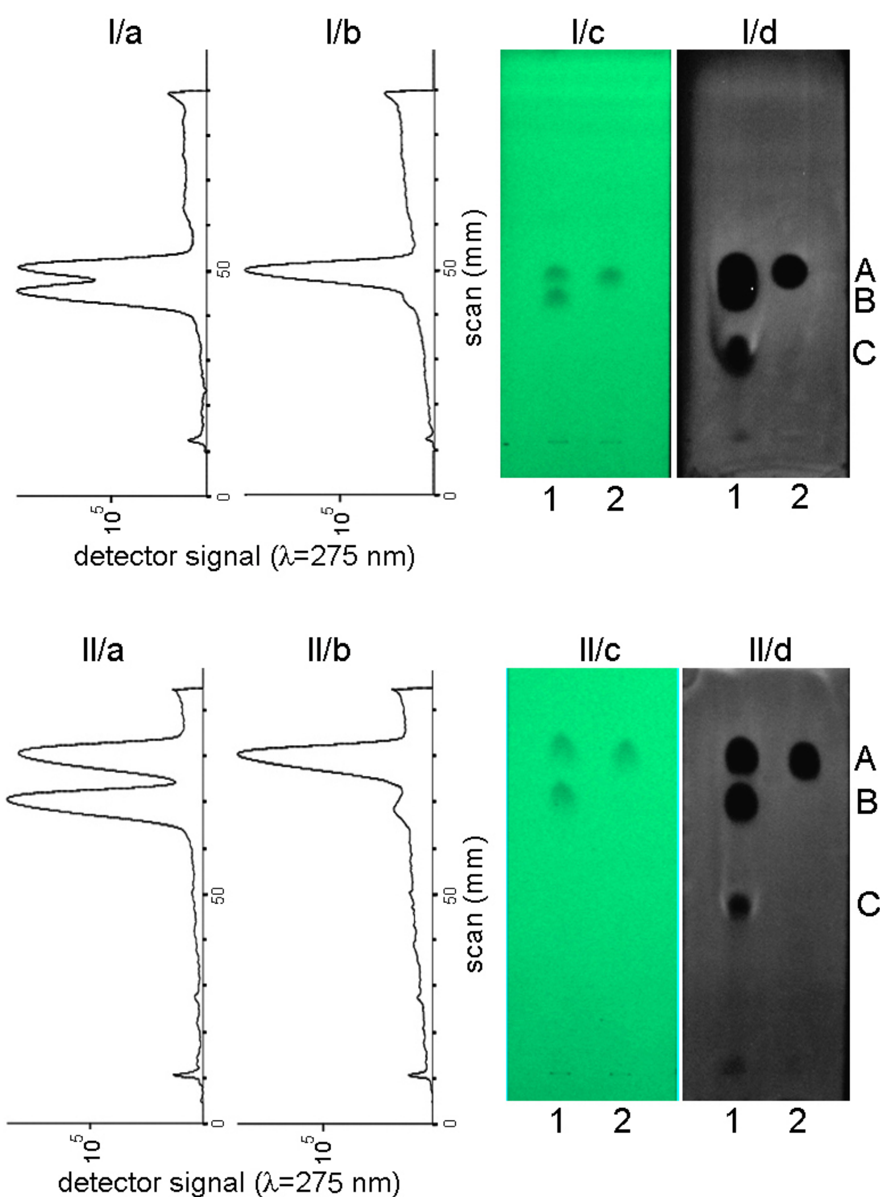
922 conditions: silica gel 60 F_{254} (Merck); mobile phase: chloroform-methanol 80:8 (v/v); Desaga

923 glass TLC chamber. Biological conditions: (A) 100 mL *Pseudomonas savastanoi* suspension,

924 (Psm); (B) A + 10 mg Indigo Carmine; (C) A + 20 mg Indigo Carmine; (D) A + 40 mg Indigo

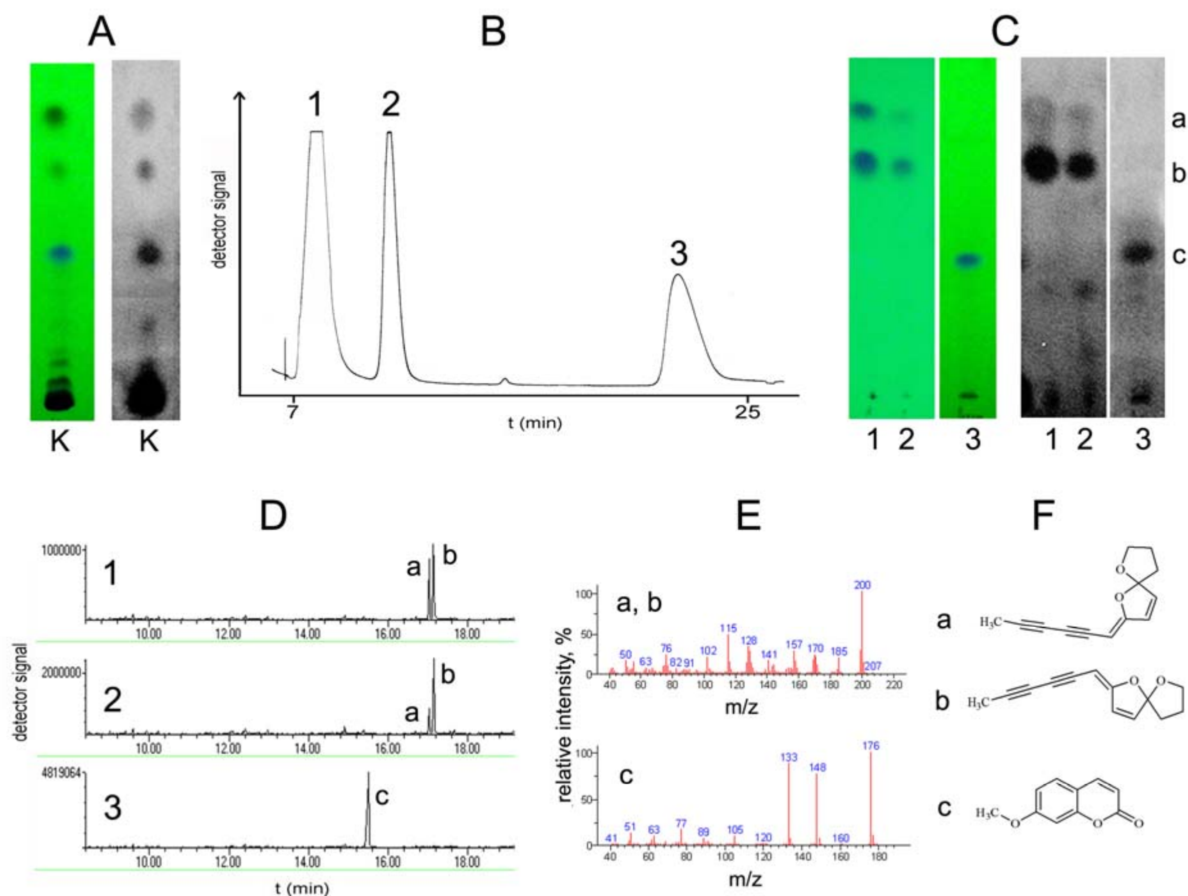
925 Carmine.

926



927 Figure 22

928 Detection of the main components of *Thymus vulgaris* L. essential oil separated by TLC (I) and
929 OPLC (II). Track 1, standards, 10 µg each of thymol (A), carvacrol (B), and linalool (C). Track
930 2, *Thymus vulgaris* L. oil, 25 µg. The components were detected before biological detection by
931 densitometry (a – track 1, b – track 2) and under UV light (254 nm) (c); The bioautograms (d)
932 were obtained by use of luminescent *Pseudomonas syringae* pv. *maculicola*. (With permission,
933 from Ref. [122].)



934

935 Figure 23

936 Bioassay-guided isolation and identification of antibacterial components of 50% aqueous ethanol

937 extract of chamomile flower (150 mg in 1 mL);

938 A – The active components, separated on TLC layer (Merck, #5554) with chloroform-acetone

939 99:1 (v/v), were visualized under UV (254 nm) (on the left) and with direct bioautography using

940 luminescent gene tagged *Pseudomonas savastanoi* pv. *maculicola* plant pathogen bacteria (on

941 the right);

942 B – OPLC separation of 0.5 mL chamomile flower extract, performed by a Personal OPLC BS50

943 system (OPLC-NIT, Budapest, Hungary), and the collected fractions (1-3, the 3 peaks); OPLC

944 conditions were as follows: sealed 20x20 cm normal particle size silica gel layer (Merck, #5554),

945 sample application in a 16 cm wide band at 3 cm from the edge, 50 bar external pressure, 450 µL

946 rapid mobile-phase flush, 1 mL min^{-1} mobile phase flow rate; the mobile-phase was chloroform,

947 the on-line UV detection was achieved at 350 nm;

948 C – The TLC re-chromatography of the collected fractions (chloroform-acetone 99:1 (v/v)); the

949 active components were visualized under UV (254 nm) (on the left) and with direct

950 bioautography using luminescent gene tagged *Pseudomonas savastanoi* pv. *maculicola* plant

951 pathogen bacteria (on the right);

952 D – GC-MS analyses of the 3 fractions (TIC chromatograms);

953 E – The MS spectra of the main components of the chamomile flower fractions;

954 F – Chemical structures of identified components: a – *cis*-spiroether, b – *trans*-spiroether, c –

955 herniarin [123].

956

957