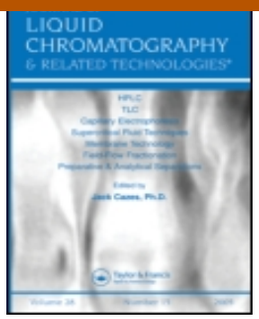

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Novel Results of Two-Dimensional Thin-Layer Chromatography

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Abstract: Various types of two-dimensional thin-layer chromatography are presented. Even using appropriately selective systems, multicomponent mixtures can result in spots around the main diagonal, but they can be spread all over the TLC plate simply by improvements in the mobile phase composition. The use of cyano-silica offers the change of normal-phase to reversed-phase separations in the first and second dimensional developments. Elution type developments can be combined with displacement chromatography; thereby, a unique possibility of different separation mechanisms can be utilized.

Keywords: 2D-TLC, Two-dimensional, Ecdysteroids, Deprenyl, Monomethyl-lysine, Displacement

INTRODUCTION

Recently, there has been increased interest in two-dimensional (2D) separations. In a computer search, the "two-dimensional" key word provided over 500 papers, and 64 of them were published in 2004.[1] There are two basic reasons why 2D-chromatography has recently commanded the interest of chromatographers working in both industry and research. In this way, the peak

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capacity of the separation of complex mixtures is multiplied.[2] In addition, the selectivity of the separation is also definitely increased if the second dimensional chromatography utilizes an essentially different separation mechanism than the first dimension. However, certain methodical problems appear in 2D-chromatography when column (or capillary) techniques are used.

The major problem is the lack of continuous transfer of the effluent from the first to second column. Both separation systems have their special separation mechanisms, so the separation on the second column can generate overlap of the peaks having been satisfactorily separated in the first column. Furthermore, both columns contribute to the increase of the peak width, and this peak-widening is additive. Both shortcomings originate from the fact that in column chromatography the expression "two-dimensional" refers to the conception of the separation mechanisms but not to the geometrical (space) orientation of the mobile phase flow.

The real solution is the use of planar chromatography, where the two-dimensional separations are often called as fingerprints. Observing a wide variety of colorful, well-separated spots on a thin-layer plate is extremely valuable. Detection may generate a third dimension for identification. However, both generation and evaluation of a proper two-dimensional planar chromatogram can hide unforeseen shortcomings. Logically, the major problems are coming from the special circumstances, which differentiate planar chromatography from the column technique, and from duplication of one-dimensional development in real two-dimensional separations.[3]

The nature of planar chromatography offers an easy solution for the gross transfer of the spots separated as outcome of the first dimensional run into the second dimensional chromatography by simply turning the plate by 90°. The problem is, however, how to "turn" the stationary phase to give essentially different separations. Whatman Inc. (Clifton, NJ) offered an evident solution by preparing TLC plates having on one side a track of reversed-phase (RP) material, while the majority of the plates consist of plain silica. The Multi-K C-S5 dual plate has a 3 cm wide C_{18} strip on the 20 × 20 cm plate, and silica covers the remaining 17 × 20 cm field.[4] Further solutions of the problems are the use of two properly selected different mobile phases. This is possible even when only plain silica or RP-silica is used. However, an easier solution is offered by either using plates with cyano-silica coating with aqueous and organic solvents, or elution and displacement chromatography in two-dimensional separations. These arrangements and their results are the subject of the present publication.

EXPERIMENTAL

Solvents and Chemicals

All solvents and chemicals were purchased from commercial sources in the highest purity grade available. L-deprenyl [selegiline hydrochloride;

(−)-N-methyl-N-propynyl(2-phenyl-1-methyl)ethylammonium hydrochloride; (R)-(−)-*N,2*-dimethyl-*N*-2-propynylphenethylamine hydrochloride] was donated by the Chinoin Pharmaceutical and Chemical Works (Budapest, Hungary; a member of the Sanofi-Sintelabo Group). ^{14}C-L-deprenyl [(−)-^{14}C-N-methyl-N-propynyl(2-phenyl-1-methyl)ethylammonium hydrochloride, 98 μCi mg^{-1}] was prepared and provided by the Institute of Isotopes Co., Ltd. (Budapest, Hungary).

Plant Extracts

The roots of *Serratula wolffii* were extracted in the usual way.[5] The extract of *Silene viridifloras* was used without any preliminary purification.[5]

Treatment of Animals

Male Wistar rats (200–250 g) were per os treated with radiolabelled L-deprenyl (5 mg/kg).[6] Urine samples were collected for 6 h.

Elution-Displacement TLC

TLC silica gel 60 F_{254} plates (Merck, Darmstadt, Germany) were used.[7] The mobile phases were chloroform–methanol–water (7 : 5 : 1) and dichloromethane–triethanolamine (19 : 1) for elution and displacement, that is for the first and second dimensional developments, respectively.

Derivatization

Pre-TLC derivatization of formaldehyde with dimedon resulting in formaldemedon had been detailed in our previous publications.[6,8]

Detection

Ecdysteroids were detected using a "triple-detection"[5,9] involving (1) observation under ultraviolet light at 254 nm, (2) heating after use of vanillin-sulfuric acid spray reagent and observing the plates in day-light, and (3) observing the fluorescence of the ecdysteroids under the light at 366 nm (after using the spray reagent and heating). The separated bands of deprenyl metabolites were detected using an x-ray film, with an exposure time of 120 h. Details were given in an earlier paper.[6,8]

Elution 2-D TLC

In both dimensions in normal-phase elution TLC silica gel 60 F_{254} plates from Merck KGaA were used.[7]

In normal phase vs. reversed-phase elution, the stationary phase was LiChrospher® CN (Merck), 10 μm spherical silica particles with γ-cyanopropyl function, having 10 nm pore size, 1.25 mL/g pore volume, and 350 m^2/g specific surface area; the carbon coverage was 6.6%.[7]

The following mobile phases were used:

Mobile phase No. 1.: acetone–ethanol (96%)–ammonia (25%) (140 : 3 : 9)

Mobile phase No. 2.: ethyl acetate–ethanol (96%)–water (16 : 2 : 1)

Mobile phase No. 3.: toluene–acetone–ethanol (96%)–ammonia (25%) (100 : 140 : 32 : 9)

Mobile phase No. 4.: water–acetonitrile (4 : 1)

Mobile phase No. 5.: n-hexane–acetone (3 : 2)

RESULTS

Extracts of the root of *Serratula wolffi* were subjected to the usual cleanup. The ecdysteroids (black spots) and the remaining flavonoids were analyzed using thin-layer chromatography on silica stationary phase. The mobile phases contained acetone–ethanol–ammonia and ethyl acetate–ethanol–water in the first and second dimensional runs, respectively. Figure 1 shows separation resulting in spots located mainly in the main diagonal of the TLC plate. TLC silica stationary phase was used. The same extract (side section, inner track) and an artificial mixture of the appropriate ecdysteroids standards (side section, outer track) were also separated.

Figure 2 shows separation where the spots are spread around the TLC plate. The proper mobile phase combination (Mobile phases Nos. 3 and 2) was selected here; also, the sample contained a wide spectrum of solutes, including four earlier identified ecdysteroids, and also several flavonoids.

Cyano-silica stationary phase was used to generate normal phase versus reversed phase separations on the same plate by simply changing the mobile phase composition. The ecdysteroid-containing extract was subjected to 2-D separation using a mobile phase providing straight phase separation [*n*-hexane–acetone (6 : 4)] as well as a mobile phase [water–acetonitrile (4 : 1)] adequate for reversed-phase separation on cyano-silica. The spots were detected by the so-called triple detection method that is for ecdysteroids. The 2-D TLC method resulted in adequate separation of ecdysteroids, and it can routinely be used for obtaining reliable information on the ecdysteroid spectrum of plants, and also to monitor ecdysteroid purification from plant extracts.

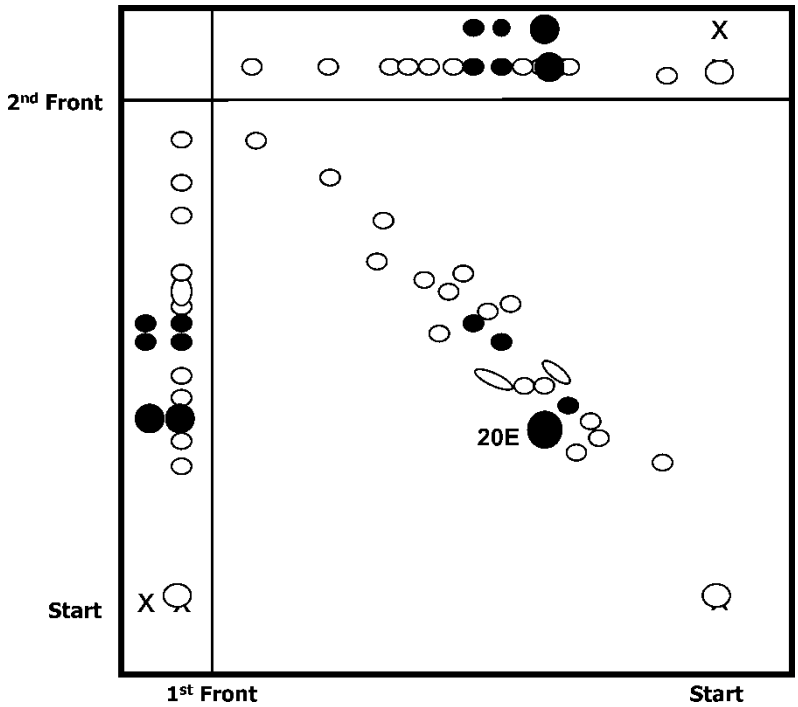

Figure 1. 2D-TLC of an extract of *Silene viridiflora*s. The stationary phase was TLC silica gel F_{254}. The mobile phases were acetone–ethanol (96%)–ammonia (25%) (140 : 3 : 90) and ethyl acetate–ethanol (96%)–water (16 : 2 : 1) in the first and second dimensional runs, respectively. The spot of 20-hydroxyecdysone is marked as 20E. The same extract was loaded on both inner side tracks, and three ecdysteroids were spotted on the outer side tracks.

Figures 1, 2, and 3 are the graphical reproduction of the chromatograms. The triple-detection approach is specific for the ecdysteroids (given as dark spots). The contaminating flavonoids were detected under UV light at 254 nm, they are shown as open circles.

Elution-displacement 2D-TLC is operating under essentially different mechanisms in the first and second dimensional separations. Figure 4 presents the 2-D separation of (−)-deprenyl metabolites, including the parent drug; the sample also contained (−)-nordeprenyl, (−)-methamphetamine, (−)-amphetamine, as well as formaldemedon.

DISCUSSION

Thin-layer chromatography is carried out using a disposable stationary phase. The sample cleanup can be restricted to the removal of contaminants that

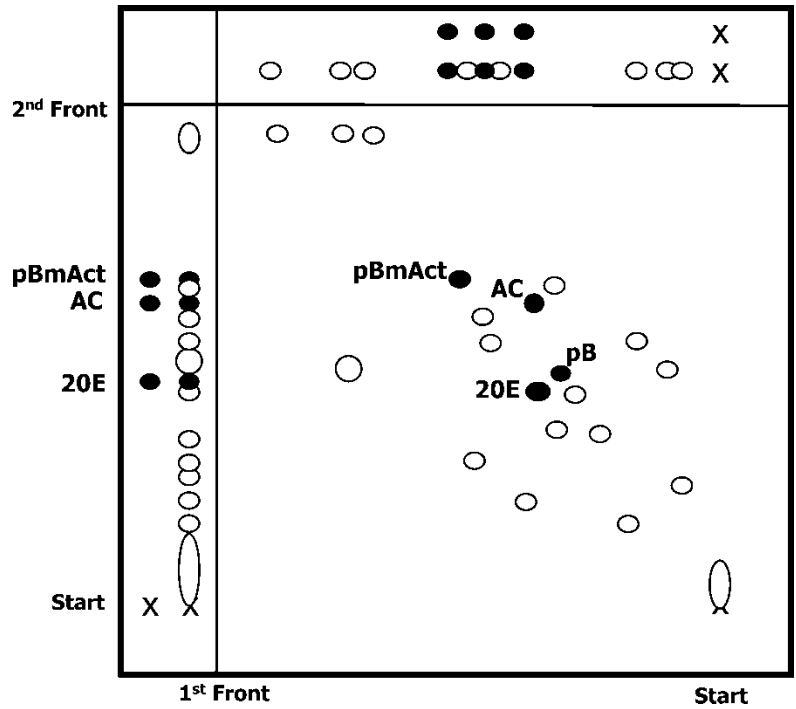

Figure 2. 2D-TLC of a root extract of *Serratula wolffii*. The stationary phase was TLC silica gel F_{254}. The mobile phases were toluene–acetone–ethanol (96%)–ammonia (25%) (100 : 140 : 32 : 9) and ethyl acetate–ethanol (96%)–water (16 : 2 : 1) in the first and second dimensional runs, respectively. The spots of 20-hydroxyecdysone, polypodine B, Ajugasterone C, and polypodine B monoacetonide are marked with 20E, pB, AC, and pBmAct, respectively. The same extract was loaded on both inner side tracks, and three ecdysteroids were spotted on the outer side tracks.

disturb the separation of the solutes to be determined. In situ cleanup is also possible using sesqui-dimensional development.[10] The first dimensional development serves to remove the major amount of contaminants, while the second directional development improved the separation of the important solutes.

The 2-D chromatogram is an outcome of the individual one-dimensional development. In general, the R_F value of each spot on the 2-D chromatogram has to correspond to the same solute on the side tracks. However, this is only a general rule. There are several exceptions explained by the special circumstances that differentiate the chromatographic processes in the side track from that on the 2-D TLC field. For example, 2-D-elution-displacement TLC, 2-D-reaction TLC, and sesqui-dimensional TLC belong to these exceptions.

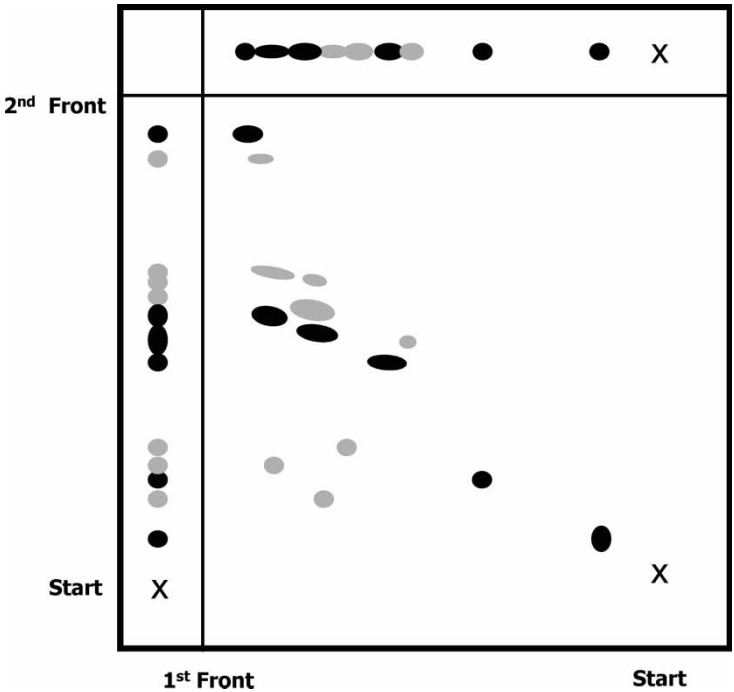

Figure 3. 2D-TLC of the extract of *Serratula wolffii.* The stationary phase was TLC cyano-silica gel F_{254}. The mobile phases were water–acetonitrile (4 : 1) and *n*-hexane–acetone (3 : 2) in the first and second dimensional runs, respectively. The spots were visualized by spraying with vanillin–sulfuric acid and observing under 365 nm UV light. The same extract was loaded on the side tracks.

The result shown in Figure 1 is an example when adequate separations were reached in both dimensional developments. However, the selectivity of the two separation systems was similar with respect to the stationary phase–mobile phase–solute combination; therefore the spots were arranged around the main diagonal of the 2-D chromatogram.

The TLC picture in Figure 2 shows a situation in which interactions among the stationary phase, mobile phases, and solutes resulted in different selectivities in the first and second dimensional developments. Therefore, the spread of the spots covers a wide portion of the 2-D chromatogram.

TLC on cyano-silica is utilizing diverging mechanisms if the first dimensional run is using a water-containing mobile phase and a water-free mobile phase is used for the second dimensional development. [*n*-Hexane–acetone (6 : 4)] generates normal-phase separation, while reversed-phase chromatography results from using water–acetonitrile (4 : 1). It is suggested that if the beneficial results are confirmed by trial-and-error, then the diverging

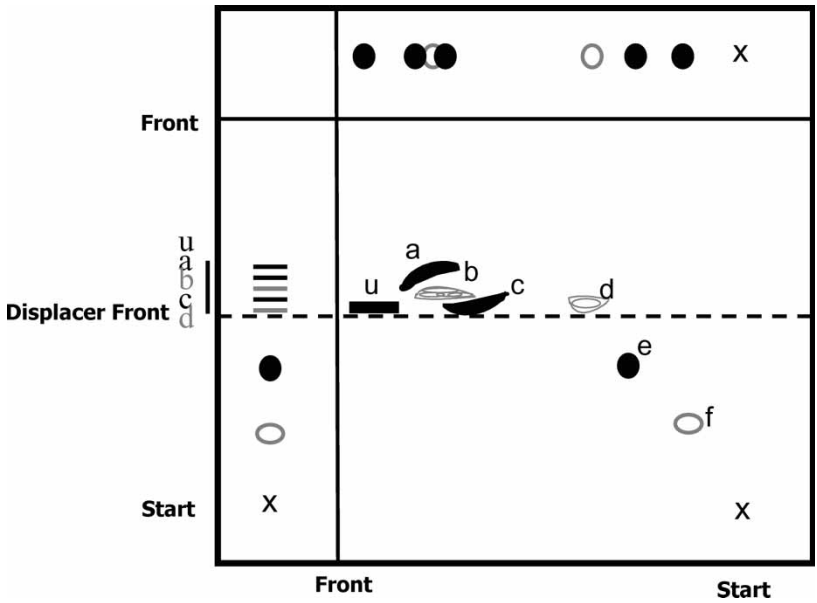

Figure 4. 2D-elution-displacement chromatography of (−)-deprenyl metabolites on TLC silica F_{254} stationary phase. The mobile phases were dichloromethane–methanol–water (7 : 5 : 1) and triethanolamine–chloroform (5 : 95) in the first and second dimensional developments, respectively. The sample components **d** (L-amphetamine) and **u** (formaldemedon = dimedon derivative of formaldehyde) were taken out from the displacement train, where the other components **a** (L-deprenyl), **b** (L-nordeprenyl), and **c** (L-methamphetamine) while subjected to a shoulder-to-shoulder displacement. Two spots, as e (para-hydroxy-L-methamphetamine) and f (para-hydroxy-L-amphetamine) were not displaced. Dark spots indicate radioactivity (and UV absorbance at 254 nm), open circles gave UV absorbance only.

separation mechanisms can even be transferred to the HPLC separation of multicomponent mixtures.

Two-dimensional displacement TLC can also be carried out using elution-type development in the first dimensional run, followed by displacement type development in the second dimension. Two distinct displacement trains have to be considered if the elution type TLC separates at least one component from the group of solutes to be displaced. The phenomenon of two discrete displacement trains is presented in Figure 4. This is the reason that only the elution development (ED) is monitored on the side track of 2D-ED-TLC.[11]

Two-dimensional reaction TLC is the case when certain solutes are chemically modified on-site between the first and second dimensional developments. Derivatization reaction does not take place unconditionally for each solute, therefore, the 2D-TLC separation cannot be derived from the one-dimensional parallel procedures at the side tracks.

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REFERENCES

1. http://www.sciencedirect.com.
2. Gilar, M.; Dali, A.M.; Kele, M.; Neue, U.D.; Gebler, J.C. Implication of column peak capacity on the separation of complex peptide mixtures in single- and two-dimensional high performance liquid chromatography. J. Chromatogr. A **2004**, *1061*, 183–192.
3. Ettre, L.S.; Kalász, H. The story of thin-layer chromatography. LC-GC NA **2001**, *19*, 712–721.
4. www.whatman.com.
5. Báthori, M. Phytoecdysteroids effects on mammalian, isolation and analysis. Mini-Rev. Med. Chem. **2002**, *2*, 285–293.
6. Kalász, H.; Lengyel, J.; Szarvas, T.; Morovján, G.; Klebovich, I. Investigation of metabolism using TLC-DAR and reaction-displacement TLC. J. Planar Chromatogr.—Mod. TLC **2003**, *18*, 383–387.
7. www.merck.hu.
8. Kalász, H.; Szarvas, T.; Szarkáné-Bolehovszky, A.; ALengyel, J. TLC analysis of formaldehyde produced by metabolic *N*-demethylation. J. Liq. Chromatogr. & Relat. Technol. **2002**, *25*, 1589–1598.
9. Báthori, M. Purification and characterization of plant ecdysteroids of *Silene otites.* Trends Anal. Chem. **1998**, *17*, 372–383.
10. Kalász, H.; Benkő, A.; Szücs, Z.; Szilágyi, Á; Szarvas, T.; Lengyel, J. HPLC analysis of urinary N^{ε}-monomethyl-lysine. J. Chromatogr. Sci. **2005**, 43, 165–168.
11. Kalász, H.; Báthori, M.; Ettre, L.S.; Polyák, B. Displacement thin-layer chromatography of some plant ecdysteroids with forced-flow development. J. Planar Chromatogr.-Mod. TLC **1993**, *6*, 481–486.

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