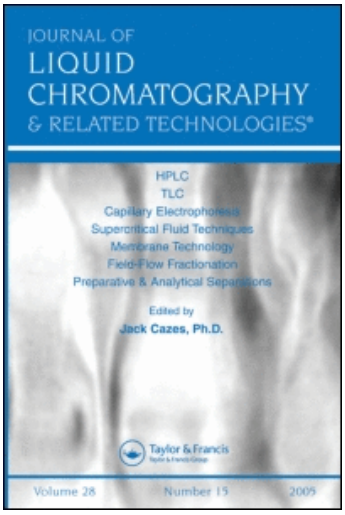

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Mirosław A. Hawrył[a]; Monika Waksmundzka-Hajnos[a]; Tadeusz Inglot[a]
[a] Department of Inorganic Chemistry, Faculty of Pharmacy, Medical University in Lublin, Lublin, Poland

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Retention Behavior of Some Phenolic Compounds in Two-Dimensional Thin Layer Chromatography Systems Using a Diol Bonded Polar Stationary Phase

Mirosław A. Hawrył, Monika Waksmundzka-Hajnos, and Tadeusz Inglot
Department of Inorganic Chemistry, Faculty of Pharmacy, Medical University in Lublin, Lublin, Poland

Abstract: The retention behavior of phenolic compounds was carried out by use of diol precoated plates and non-aqueous solutions (mixtures of dichloromethane and propan-2-ol, methanol and diisopropyl ether, methanol and ethyl acetate) and aqueous solutions (mixtures of methanol and water). The relationships of retention vs. concentration of eluent were presented as plots of R_M vs. logarithm of concentration of a more polar component for non-aqueous solutions and R_M vs. concentration of methanol in aqueous solution. Optimal conditions of separation of investigated compounds were chosen on the basis of correlation plots R_M non-aqueous system vs. R_M aqueous system. On the basis of correlation lines and statistical parameters, optimal two-dimensional thin-layer chromatography conditions were chosen and applied in the separation of investigated plant extracts (*Herba bursae pastoris, Herba polygoni hydropiperis, Herba polygoni avicularis*, and *Flos verbasci*).

Keywords: Two-dimensional separations, Thin-layer chromatography, Diol bonded polar stationary phase, Retention, Selectivity, Flavonoids, Phenolic acids, Plant extracts

Address correspondence to Dr. Monika Waksmundzka-Hajnos, Department of Inorganic Chemistry, Faculty of Pharmacy, Medical University in Lublin, Staszica 6 St., 20-081 Lublin, Poland. E-mail: mwaks@panaceum.am.lublin.pl

INTRODUCTION

Plant extracts are often rich in groups of compounds of various properties. Also, in groups of phenolics, among others, flavonoids, aglycones, flavonoid glycosides, phenolic acids, coumarins are widely presented in plant organs. However, in every group, closely related compounds of similar physicochemical properties occur, which are often difficult to separate. These compounds, having similar chromatographic properties, are impossible to separate in one run. For such difficult purposes multidimensional separations by use of systems of various selectivities are applied.[1–5] This needs, however, special equipment and complicated procedures.[11–15] By use of two-dimensional TLC, separation in simple mode can be performed.

Polar bonded stationary phases (cyanopropyl, aminopropyl, and diol) are the special types of stationary phases which can be used in both systems: normal phase (NP-TLC) and reversed phase (RP-TLC).[6–8] In this case, two dimensional thin layer chromatography can be performed without technical problems of connection of various types of stationary phases (e.g., silica–RP phases). It makes possible the separation of multicomponent natural mixtures on one plate by use of non-aqueous eluent and aqueous eluent (systems of various properties and selectivity).

Two-dimensional thin layer chromatography is the technique in which the development of a chromatographic plate is performed in two directions on one chromatographic plate or on connected plates.[4,9,10] The investigated extract is applied in one corner of the plate and then is developed using the first eluent (e.g., non-aqueous), and after developing and drying is developed in the second direction (perpendicular to the first) using another eluent (e.g., aqueous). After complete development, the chromatogram is dried and visualized (e.g., by a UV lamp). In this way, the more complete separation of investigated plant extracts was achieved, because each direction of development is characterized by various selectivities of separation.

The main aim of this paper was the investigation of retention behavior of some flavonoids and phenolic acids by use of Diol layers in non-aqueous and aqueous eluent systems, and optimization of systems to the 2D separation of *Herba bursae pastoris, Herba polygoni hydropiperis, Herba polygoni avicularis*, and *Flos verbasci.*

EXPERIMENTAL

The compounds investigated are listed in Table 1. All standards were from Sigma (St. Louis, MO, USA) and Indofine (Belle Mead, NJ, USA). *Polygoni avicularis* herb, *Polygoni hydropiperis* herb, *Bursae pastoris* herb, and *Verbasci sp.* flowers were from Herbapol (Lublin, Poland). All solvents: methanol (MeOH), ethyl acetate (AcOEt), propan-2-ol (iPrOH),

Table 1. Compounds investigated

No.	Common name	No.	Common name
1.	Naringenin	13.	Kaempferol 3-glyco-7-rhamnoside
2.	Acacetin	14.	Naringenin 7-glucoside
3.	Flavone	15.	Ferulic acid
4.	Morin	16.	Chlorogenic acid
5.	Hesperetin	17.	Elagic acid
6.	Quercetin	18.	Caffeic acid
7.	Narcizin	19.	p-Coumaric acid
8.	Kaempferol 3,7-dirhamnoside	20.	m-Coumaric acid
9.	Naringin	21.	o-Coumaric acid
10.	Rutin	22.	Gallic acid
11.	Astragalin	23.	Apigenin
12.	Quercitrin		

dichloromethane (DCM), and diisopropyl ether (iPr_2O) were pro analysis grade from Polish Reagents (Gliwice, Poland).

All TLC experiments were performed on 10 × 10 cm HPTLC DIOL F_{254s} plates (Merck, Darmstadt, Germany). Before use they were washed with methanol and after drying in air, activated at 100°C for 1 hour. All experiments were repeated three times and final results are their arithmetic average.

Extracts from herbs were prepared by extraction with dichloromethane for 6 hours in a Soxhlet apparatus to remove chlorophyll. After drying in air they were further extracted for 12 hours with methanol. The extract from *Verbascum sp.* flowers was prepared in the same way but without dichloromethane extraction. The methanolic extracts were evaporated on a water bath and the dry residues were dissolved in a mixture of 50% methanol and water (v/v) (extracts purified by SPE on polyamide), or in a mixture of 70% methanol + water (v/v) (extracts purified by SPE on C_8).[16]

Solid phase extraction was performed by use of two methods: 1. on polyamide cartridges—extracts were applied and conditioned during 40 min. After conditioning they were eluted twice by use of 50% methanol + 50% water (v/v). 2. on RP-8 cartridges—extracts were prepared as in part 1 and eluted twice by use of 70% methanol + 30% water (v/v).

C_8 SPE cartridges were from Baker (Bakerbond SPE Octyl (C8)) (J. T. Baker, Deventer, The Netherlands) and polyamide cartridges were home-made (Polyamide from Woelm, Germany).

Extracts, after purification, were collected, dried, and dissolved in 50 mL of methanol.

Methanolic extracts were applied on TLC plates 1 cm from each edge of the plate and developed in the first direction by use of non-aqueous eluent, and after drying in air, by use of aqueous eluent.

After development, the plates were dried in air and derivatized by use of 2-(diphenylboryoxo)-ethylamine (Merck, Darmstadt, Germany) and PEG400 (Merck, Darmstadt, Germany). After derivatization the plates were visualized by Desaga CabUV VIS videoscanner, equipped with Mitsubishi CCD-300E lamp and ProVidoc software (Desaga, Heidelberg, Germany).

RESULTS AND DISCUSSION

Figures 1–4 show R_M vs. log c dependencies for investigated standards. It is seen that decrease of the concentration of a more polar component in binary

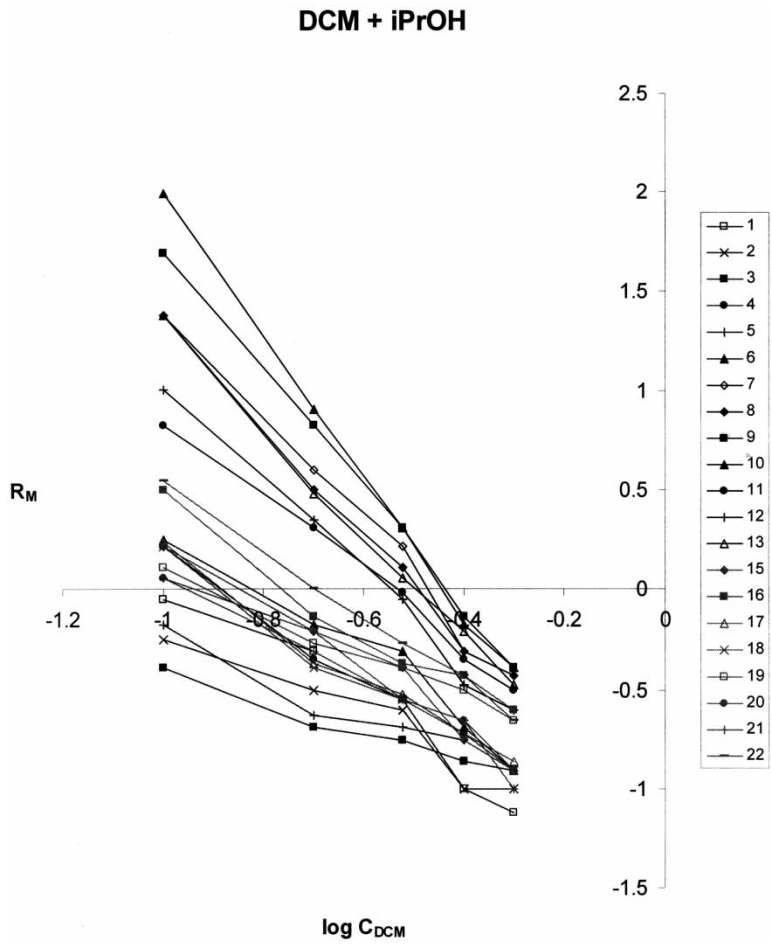

Figure 1. The plot R_M vs. log C_{DCM} for investigated compounds in the system: bonded DIOL phase—dichloromethane + propan-2-ol. Numbers as in Table 1.

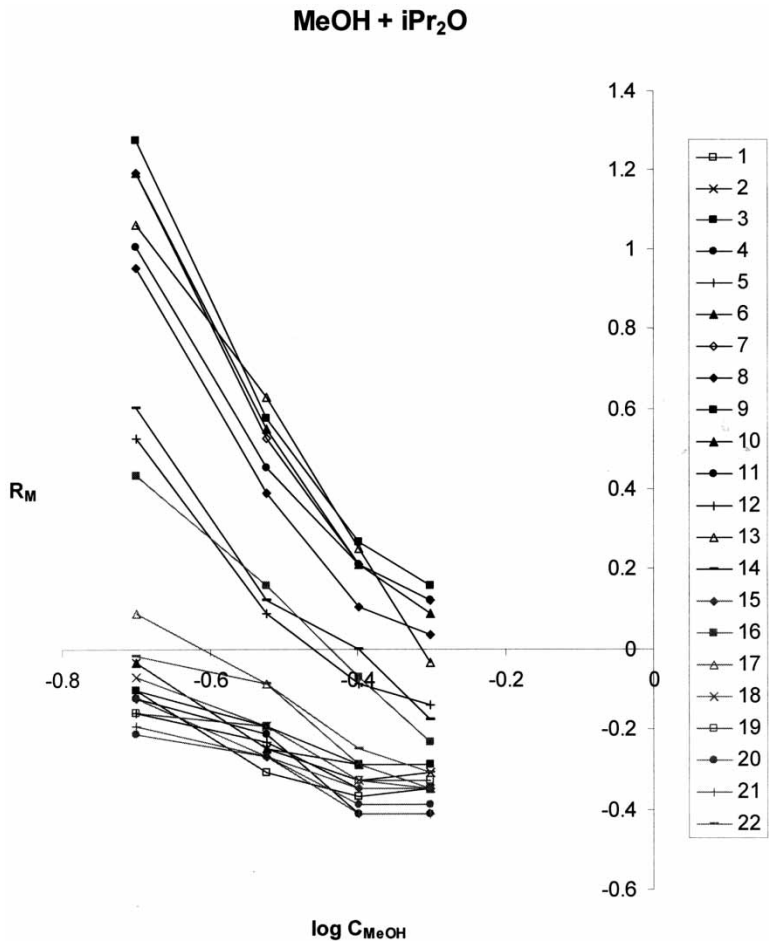

Figure 2. The plot R_M vs. log C_{MeOH} for investigated compounds in the system: bonded DIOL phase—methanol + diisopropyl ether. Numbers as in Table 1.

mobile phase using normal phase systems (bonded polar diol stationary phase + non-aqueous mobile phases, NP-TLC) cause increasing of the systems selectivity and better separations of investigated compounds. Moreover, the investigated group of compounds became separated on two groups: aglycones and phenolic acids in the first group, and flavonoid glycosides in the second one. Such systems can be used (Figures 1–3) to tentatively separate glycosides, aglycones, and phenolic acids mixture on two groups: glycosides (strongly retained) and aglycones + phenolic acids (which are weakly retained) (Figures 1–4).

Besides, the comparison of retention of the investigated compounds shows that the best selectivity is observed in the systems: 10% dichloro-

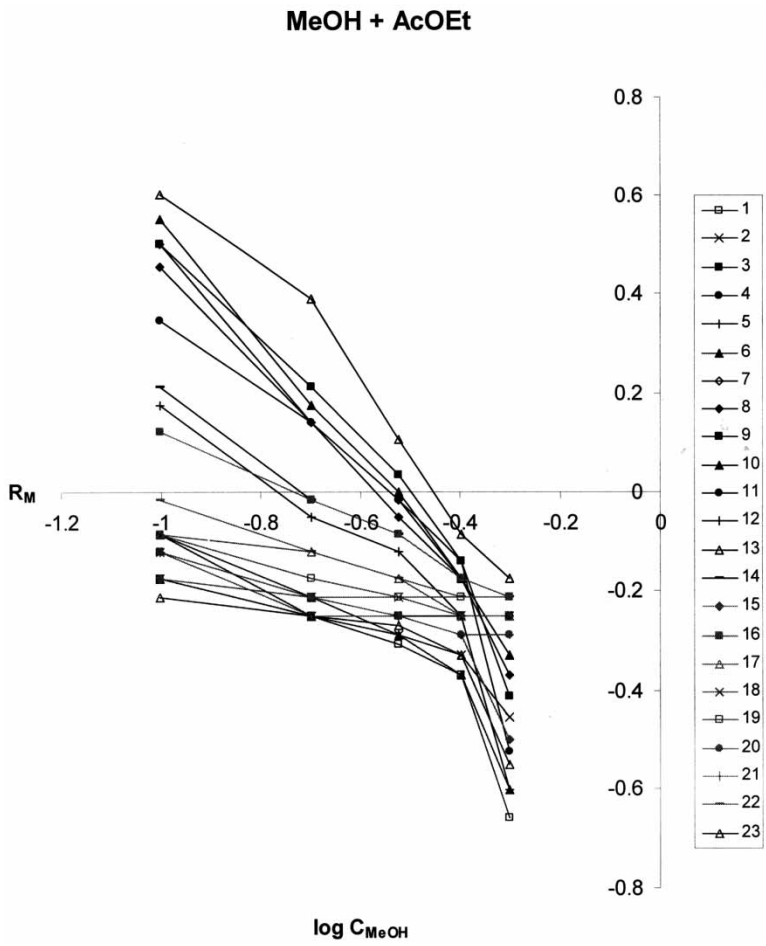

Figure 3. The plot R_M vs. log C_{MeOH} for investigated compounds in the system: bonded DIOL phase—methanol + ethyl acetate. Numbers as in Table 1.

methane + 90% propan-2-ol (v/v), 20% methanol + diisopropyl ether (v/v) and 10% methanol + 90% ethyl acetate (v/v).

The investigated compounds are not separated on two groups in the case of an aqueous system (Figure 4) (bonded polar diol stationary phase + methanol − water as mobile phase); the selectivity of this system is diverse for individual compounds. The best selectivity of separation is observed for the system: bonded diol phase −20% methanol + 80% water (v/v).

The next step was the drawing of correlation diagrams, R_M non-aqueous system vs. R_M aqueous system (Figures 5–7). It is seen that points are strongly dispersed, which is proof of differences in selectivity. On the basis of correlation diagrams and statistical parameters of correlations (Table 2), optimal

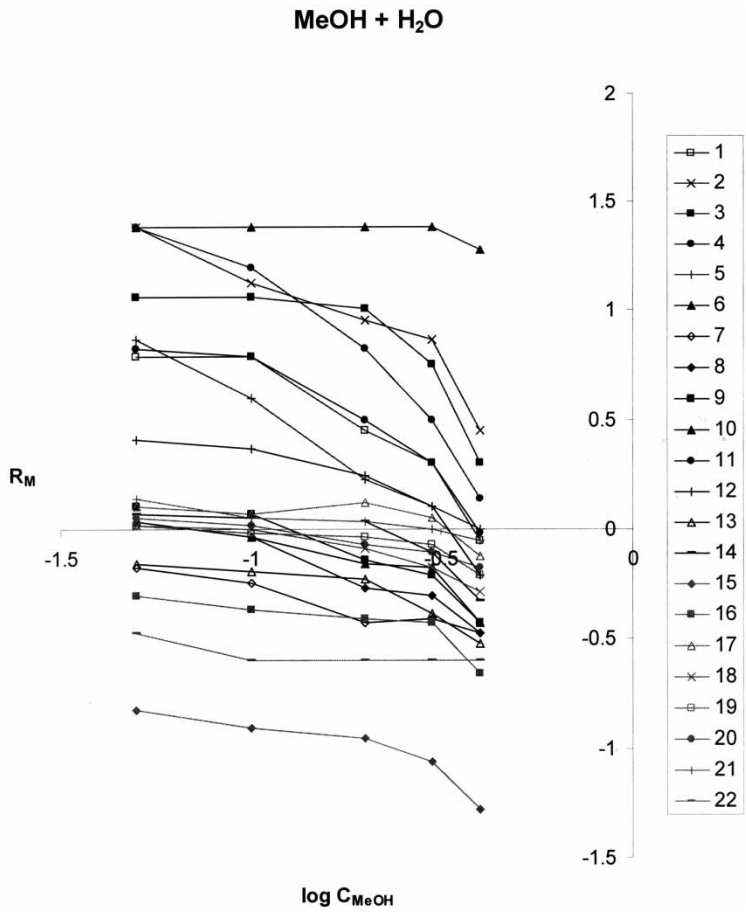

Figure 4. The plot R_M vs. log C_{MeOH} for investigated compounds in the system: bonded DIOL phase—methanol + water. Numbers as in Table 1.

two-dimensional thin-layer chromatography conditions were chosen and applied for separation of investigated plant extracts. The optimal 2D-TLC systems were: 20% dichloromethane + 80% propan-2-ol (v/v) and 20% methanol + 80% water (v/v) (Figure 6), where most of investigated compounds can be sufficiently separated (Figures 5–7).

Extracts were applied onto diol bonded chromatographic plates as spots 1 cm from both edges of chromatographic plate, and developed in the first direction using non-aqueous eluents (10% methanol + 90% ethyl acetate (v/v); 20% dichloromethane + 80% propan-2-ol (v/v) and 20% methanol + 80% diisopropyl ether (v/v)). After the first development the chromatographic plates were air dried and developed in the second direction using aqueous eluents (20% methanol + 80% water (v/v)).

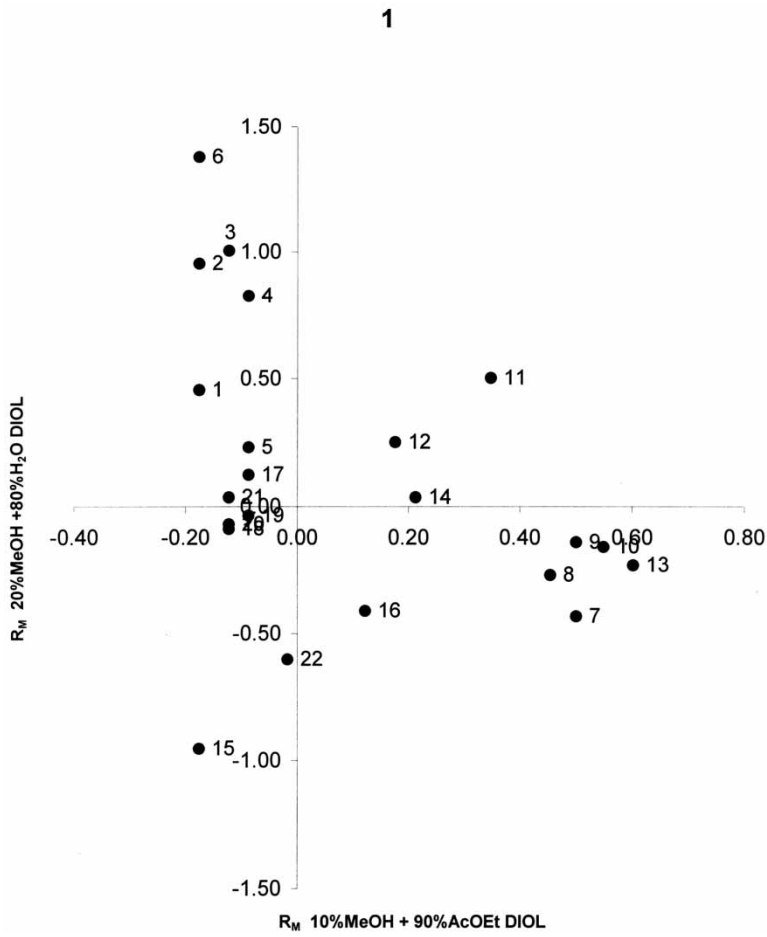

Figure 5. Correlation diagram R_M vs. R_M for the 2D-TLC system: bonded DIOL stationary phase—10% methanol + 90% ethyl acetate and 20% methanol + 80% water. Numbers as in Table 1.

After development and air drying, the plates were derivatized by 2-diphenyl-boryoxoethylamine and PEG400, and photographed by a Desaga video scanner at $\lambda = 365$ nm. Figures 8–14 show photographs of two-dimensional thin layer chromatograms of investigated extracts purified by various SPE methods (polyamide or C_8 cartridges).

Figure 8 shows the two-dimensional thin layer chromatogram of extract from *Capsella bursa pastoris*, purified by the SPE method using a polyamide cartridge. Some compounds can be identified: narcizin, kaempferol 3,7-dirhamnoside, rutin, kaempferol 3-glyco-7-rhamnoside, o-coumaric acid, and hesperidin.

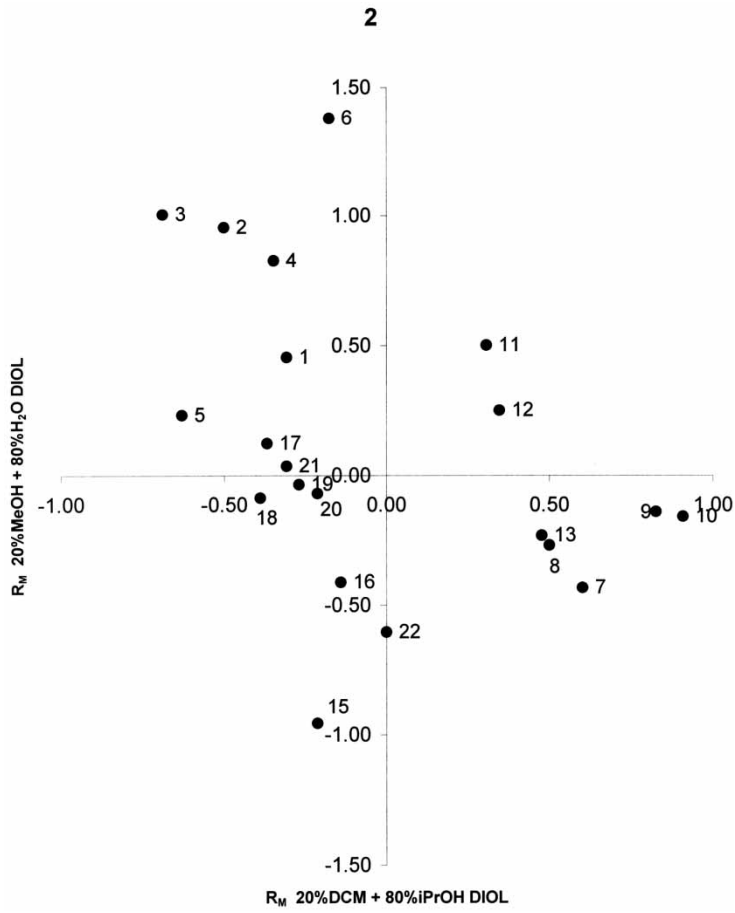

Figure 6. Correlation diagram R_M vs. R_M for the 2D-TLC system: bonded DIOL stationary phase—20% dichloromethane + 80% propan-2-ol and 20% methanol + 80% water. Numbers as in Table 1.

Figures 9 and 10 show the photograph of the plate with the separated extract from *Polygonum hydropiper* purified by SPE on polyamide (Figure 9) and C_8 column (Figure 10). In the extract the following compounds can be identified: quercetin, kaempferol 3,7-dirhamnoside, naringin, rutin, quercitrin, naringenin 7-glucoside, caffeic acid, m-coumaric acid, hesperidin, and astragalin; in Figure 10 the following substances can be identified: acacetin, quercetin, kaempferol 3,7-dirhamnoside, naringin, rutin, quercitrin, naringenin, 7-glucoside, caffeic acid, p-coumaric acid, apigenin, astragalin, and hesperidin. Probably the lightest spot is hyperoside.

Part of the flavonoids are strongly retained on polyamide, however, they can be eluted from RP8 columns. The extracts purified on polyamide columns

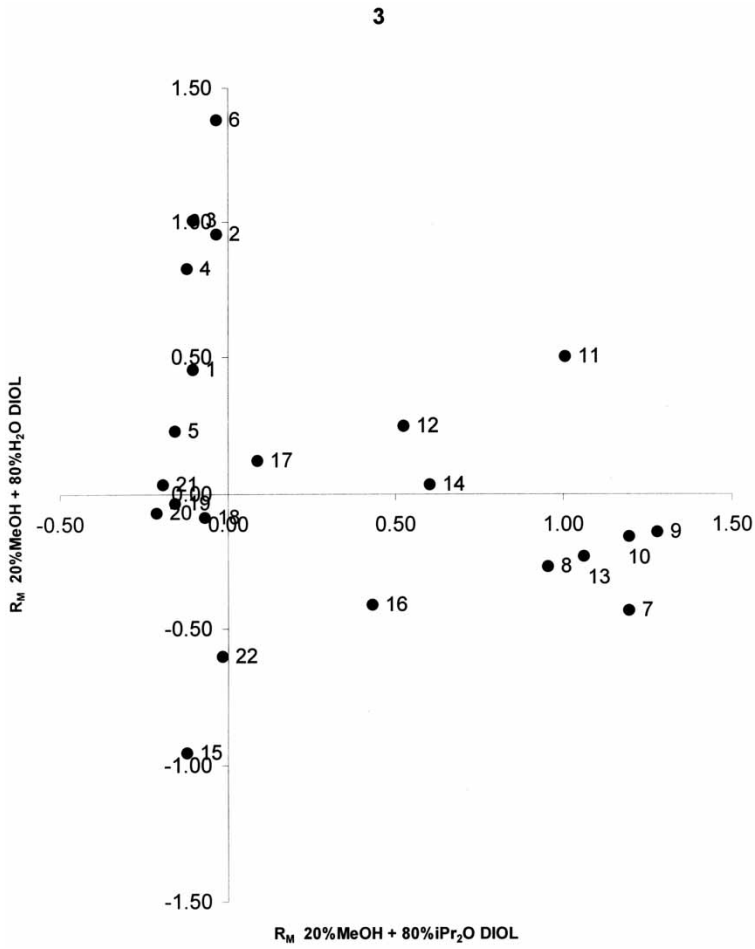

Figure 7. Correlation diagram R_M vs. R_M for the 2D-TLC system: bonded DIOL stationary phase—20% methanol + 80% diisopropyl ether and 20% methanol + 80% water. Numbers as in Table 1.

do not contain, e.g., apigenin and acacetin, which are present in extracts purified by use of a RP8 column (Figures 9 and 10).

Figures 11 and 12 depict two-dimensional TLC chromatograms obtained from *Polygonum aviculare* extracts purified by SPE methods using polyamide (Figure 11) and RP8 (Figure 12). The following phenolics can be identified in Figure 11: rutin, quercitrin, naringenin 7-glycoside, chlorogenic acid, caffeic acid, p-coumaric acid, m-coumaric acid, o-coumaric acid, hesperidin, and astragalin, and in Figure 12 the following compounds can be identified: naringenin, acacetin, flavone, kaempferol 3,7-dirhamnoside, rutin, quercitrin, naringenin 7-glycoside, caffeic acid, o-, m- and p-coumaric acids, apigenin,

Table 2. Correlation parameters for R_{MI} vs. R_{MII} relationships

1.	DIOL 10%MeOH + 90%AcOEt − 20%MeOH + 80%H_2O	−0.73 ± 0.42	0.17 ± 0.12	0.3606	2.989	0.0993	0.54	22
2.	DIOL 20%DCM + 80%iPrOH − 20%MeOH + 80%H_2O	−0.49 ± 0.26	0.10 ± 0.12	0.3998	3.615	0.0725	0.54	21
3.	DIOL 20%MeOH + 80%iPr_2O − 20%MeOH + 80%H_2O	0.30 ± 0.22	0.20 ± 0.14	0.3340	2.849	0.1290	0.25	22

hesperidin, and astragalin. Similarly, in this case, part of the compounds were strongly adsorbed on polyamide (apigenin, acacetin, naringenin, flavone).

Figures 13 and 14 show 2D-TLC chromatograms of *Verbascum sp.* extracts purified by SPE methods on polyamide (Figure 13) and RP8

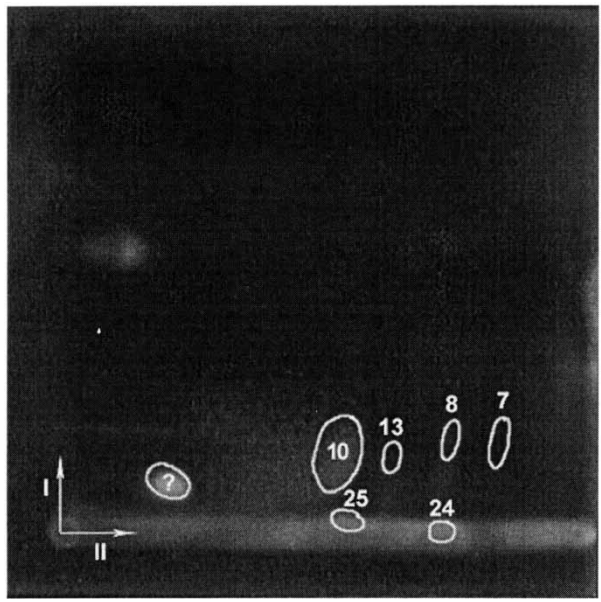

Figure 8. The photography of 2D-TLC chromatogram of the *Capsella bursae pastoris* extract at $\lambda = 365$ nm.

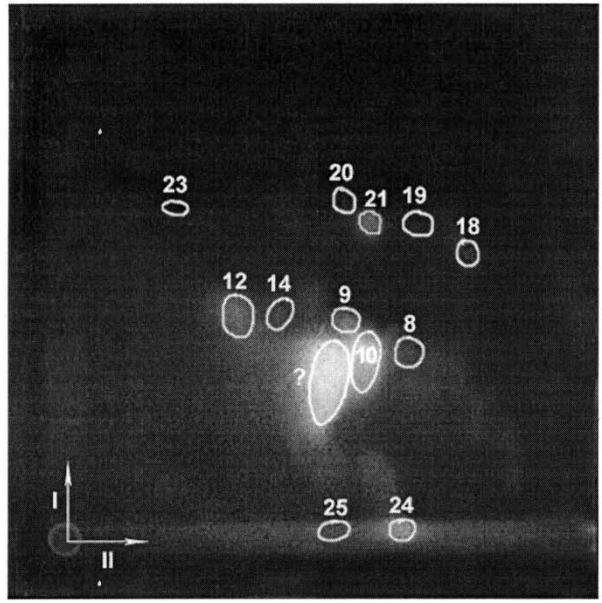

Figure 9. The photography of 2D-TLC chromatogram of the *Polygoni hydropiperis* extract purified by SPE on polyamide cartridges at $\lambda = 365$ nm.

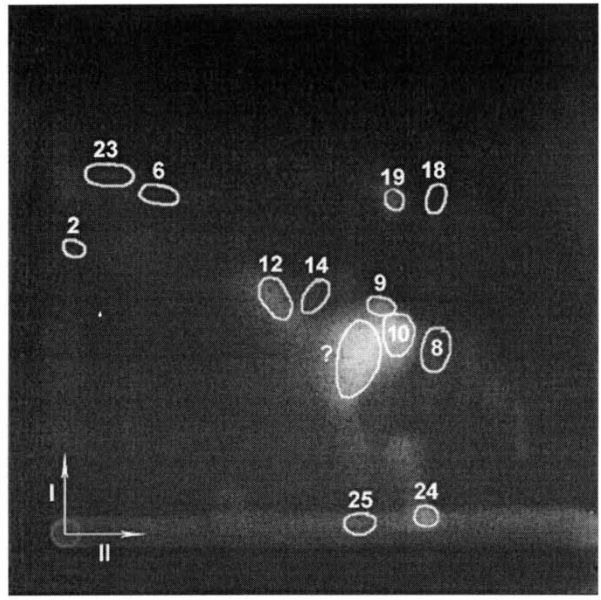

Figure 10. The photography of 2D-TLC chromatogram of the *Polygoni hydropiperis* extract purified by SPE on C8 cartridges at $\lambda = 365$ nm.

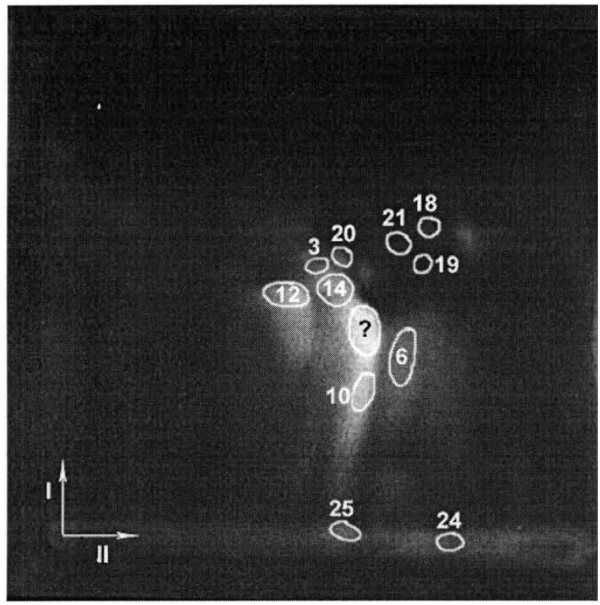

Figure 11. The photography of 2D-TLC chromatogram of the *Polygoni avicularis* extract purified by SPE on C_8 cartridges at $\lambda = 365$ nm.

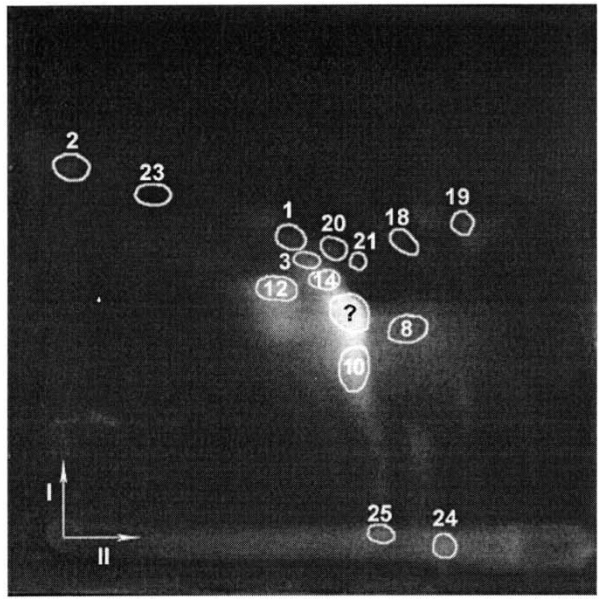

Figure 12. The photography of 2D-TLC chromatogram of the *Polygoni avicularis* extract purified by SPE on polyamide cartridges at $\lambda = 365$ nm.

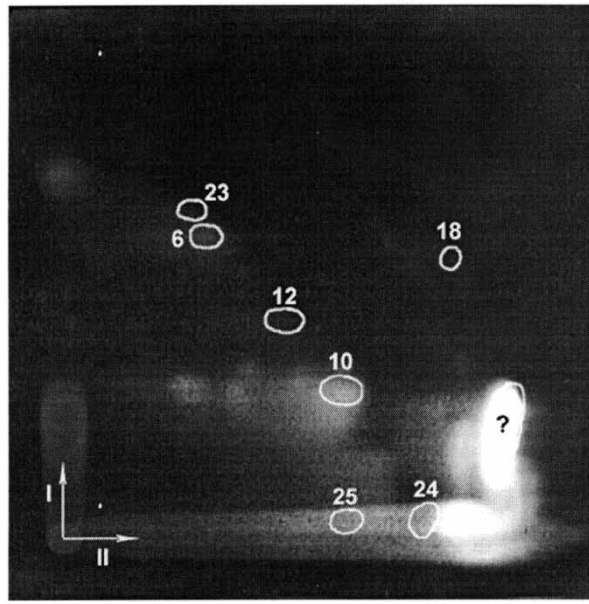

Figure 13. The photography of 2D-TLC chromatogram of the *Verbascum* extract purified by SPE on polyamide cartridges at $\lambda = 365$ nm.

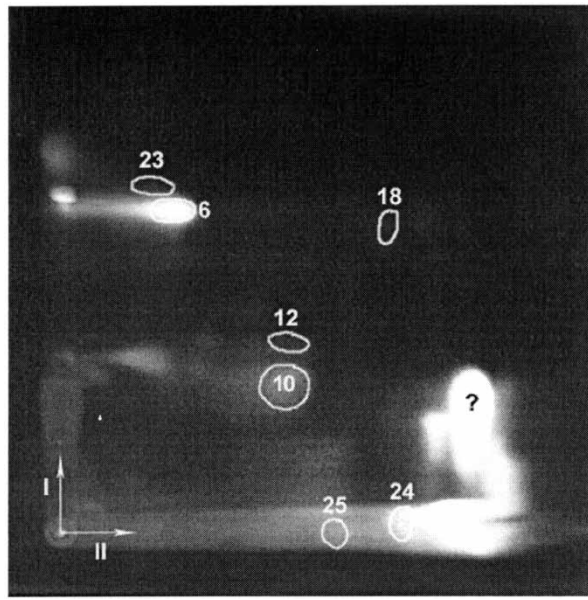

Figure 14. The photography of 2D-TLC chromatogram of the *Verbascum* extract purified by SPE on C_8 cartridges at $\lambda = 365$ nm.

(Figure 14). Quercetin, rutin, quercitrin, caffeic acid, apigenin, hesperidin, and astragalin were identified in both chromatograms.

Two-dimensional chromatograms can be used in chemotaxonomic investigations, in qualitative investigations of plant material, and to rapidly check the procedure of sample preparation.

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