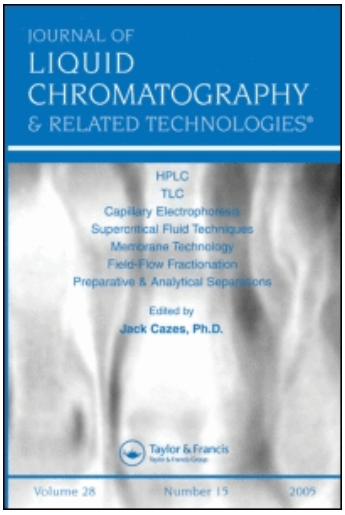

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Separation of Selected Flavonoids by use of RP-HPLC/NP-HPTLC Coupled Methods

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Abstract: Phenolic compounds, such as phenolic acids, flavonoids, flavonoid glycosides, and resveratrol, were chromatographed on thin-layers of silica in various eluent systems. Systems with the highest selectivity were chosen. Also, reversed-phase HPLC systems were optimized for the separation of investigated compounds and most the selective system was applied for the separation of two phenolic extracts – *Polygonum avicularis* and *Polygonum hydropiper*. Partly separated fractions were evaporated and dissolved in methanol. Fractions with partly separated phenolic compounds were spotted onto the silica layer and developed in an appropriate eluent system. After drying, the plate was derivatised with the Naturstoff reagent and videoscanned. Several phenolic compounds were identified, according to their retention coefficients in both systems.

Keywords: RP-HPLC, NP-TLC, Selectivity, *Polygonum* species, Phenolic compounds, Coupled techniques

INTRODUCTION

Phenolic compounds, such as flavonoids and phenolic acids are very important substances occurring in various medicinal plants. Phenolics have also different pharmacological activities and they are useful in therapy of many diseases.

Chromatographic and electrophoretic methods are widely applied in the analysis of phenolic compounds in botannical, chemotaxonomic, and phytochemical research.

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Many papers have been published dealing with capillary electrophoresis[1,2] as well as high performance liquid chromatography[3–8] of phenolics in plant material. Thin-layer chromatography is not so popular, especially in the qualitative analysis of flavonoids, but sometimes this technique is useful[9–13] for these purposes.

Polygonum sp. is the species of herbs which contains phenolic compounds such as flavonoids, phenolic acids, and other components, e.g., resveratrol, which has many pharmacological activities (antioxidant, antimicrobial, anticancer).[14–16] High performance liquid chromatographic methods of analysis of resveratrol in some biological samples, such as vines, peanuts, human plasma, and herbs have been published.[17–28]

Phenolic compounds can be divided into several groups: phenolic acids, coumarins, flavonoids, flavonoid glycosides, and other groups which differ in chemical structure and properties. However, in each of the groups, there are several structural analogues – closely related compounds with similar chromatographic properties, very difficult for separation. When, in plant material, phenolics from various groups occur, the separation is complex. First, group separation of different compounds is possible and, then, separation of structural analogues is performed. In such cases, systems of various properties and separation selectivity should be used. Most often, it should be realized in multidimensional separations. Multidimensionality is difficult to realize by HPLC and can be easy realized by 2D-TLC.

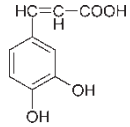
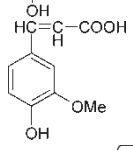
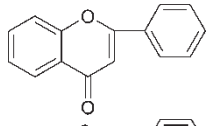
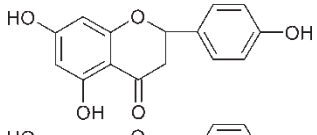
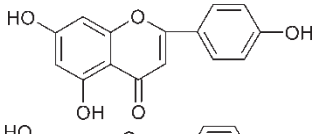
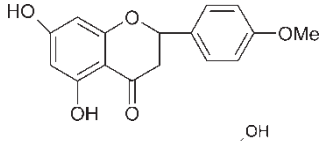
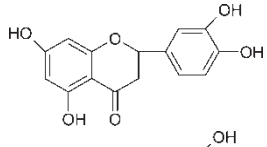
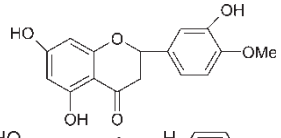
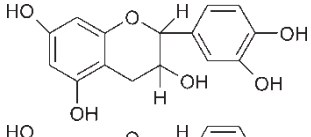
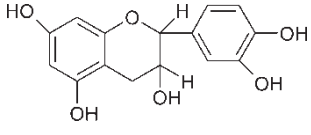
Coupling of HPLC and TLC is an effective method for separation of complex natural mixtures, especially, when RP systems in column chromatography and NP systems in planar chromatography are used. In such cases, various structural differences of groups and structural analogues determine separation of the investigated plant extract components. This method was used in the analysis of coumarins, flavonoids, and phenolic acids in natural materials.[9,11]

The aim of this paper is optimization of reversed-phase systems by use of column chromatography and normal-phase systems on silica layers for multidimensional separation of phenolics which occur in two plants: *Polygonum avicularis* and *Polygonum hydropiper*, by use RP-HPLC on a C_{18} column and re-chromatography of collected fractions by use of an NP-TLC system for full separation of compounds from this group.

EXPERIMENTAL

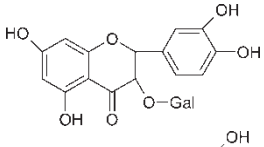
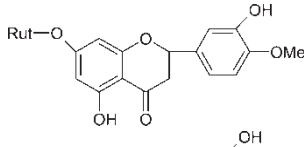
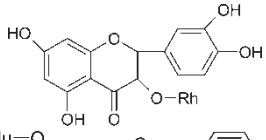
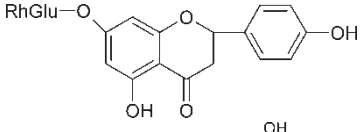
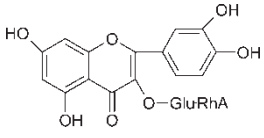
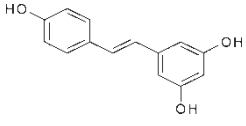
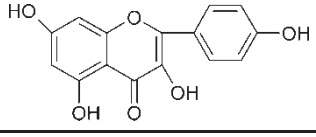
All test substances (Table 1) (flavonoids, phenolic acids, and resveratrol) were from Sigma Chemical, Roth, and Aldrich Chemical. *Polygonum avicularis* herb was from Herbapol (Lublin, Poland) and *Polygonum hydropiper* herb was from Kawon-Hurt (Gostynin Wielkopolski, Poland). Test substances were dissolved in methanol and 0.1% solutions were spotted onto the

Table 1. Compounds investigated

No	Common name	Formula
11	Caffeic acid	
22	Ferulic acid	
33	Flavone	
44	Naringenin	
5	Apigenin	
6	Acacetin	
7	Luteolin	
8	Hesperetin	
9	Catechin	
10	Epicatechin	

(*continued*)

Table 1. Continued

No	Common name	Formula
11	Hyperoside	
12	Hesperidin	
13	Quercitrin	
14	Naringin	
115	Rutin	
116	Resveratrol	
17	Kaempferol	

chromatographic plates and developed using appropriate mobile phases to obtain their retention data.

All TLC solvents (ethyl acetate, propan-2-ol, ethyl-methyl ketone, n-heptane, 1,4-dioxane, acetone, dichloromethane, tetrahydrofuran, acetic acid, and formic acid) were analytical grade and were obtained from the Polish Reagents Co. (POCh, Gliwice, Poland).

The extracts were prepared using a Soxhlet apparatus. 10 g of each material was extracted during 6 hours using dichloromethane to extract chlorophyll and next was re-extracted during 30 minutes using methanol. The methanolic extract was evaporated and the residue was dissolved in hot water. After this, the water extract was cooled in a refrigerator during 24

hours to eliminate ballast substances. Extracts were filtrated through paper filters (No. 388, Spezialpapier-Filtrak GmbH, Germany); the next step was three times re-extraction using ethyl acetate. After evaporating, the dry extract was dissolved in 5 mL of methanol.

All HPLC experiments were performed using a Shimadzu LC-8A pump, Shimadzu SPD-10AVP detector, and a Rheodyne 7520 injection valve equipped with a 20 μL loop. An Agilent Zorbax RX-C18 packed with 5 μm particles chromatographic column (i.d. 4, 6 mm, 15 cm length) was used for HPLC separations. Chromatograms were detected at 254 nm. Methanol LiChrosolv (Merck, Darmstadt, Germany) for chromatography grade in mixtures with bidistilled and deionised water was used.

HPTLC Kieselgel Si F_{254S} 10×10 cm were used in HPLC-TLC coupling. The plates were washed with acetone, dried in air, and activated at 100°C. TLC chromatograms were conditioned during 10 min. to avoid a demixing effect, and were developed in DS-II horizontal TLC chambers (Chromdes, Lublin, Poland) over a distance of 9 cm. Chromatograms were dried in air, derivatised by use of 2-diphenyl(boryoxo)-ethylamine (Merck, Darmstadt, Germany) and PEG400 Merck, Darmstadt, Germany) by use of a Merck TLC sprayer (Merck, Darmstadt, Germany), and was visualized with a Camag Reprostar 3 at 366 nm (Camag, Muttenz, Switzerland).

RESULTS AND DISCUSSION

In the first series of experiments, NP systems were investigated using silica plates and binary mobile phases consisting of non-aqueous solvents mixed with n-heptane. The following mobile phases were studied: ethyl acetate + n-heptane (40%, 50%, 60%, 70%, 80%, and 90%), ethyl-methyl ketone + n-heptane (40%, 50%, 60%, 70%, and 90%), propan-2-ol + n-heptane (20%, 30%, 50%, 70%, and 90%), dioxane + n-heptane (10%, 30%, 50%, 70%, and 90%), tetrahydrofuran + n-hepane (10%, 30%, 50%, 70%, and 90%). Also, ternary mobile phases were investigated, containing formic acid as an ion-supressor (80% ethyl acetate + 10% formic acid + 10% water and 50% propan-2-ol + 5% formic acid + 45% n-heptane).

On the basis of retention data, the relationships R_M vs. concentration of the polar components of the mobile phase were plotted and then the most selective systems were chosen. All retention data are presented in Tables 2–6. The most selective systems were: 60% ethyl-methyl ketone + 40% n-heptane; 50% propan-2-ol + 50% n-heptane; 80% ethyl acetate + 20% n-heptane; 70% dioxane + 30% n-heptane; and 70% tetrahydrofuran + 30% n-heptane. The selectivities of the ternary mobile phases investigated were comparable to the selectivities of the binary systems. Figure 1 shows

Table 2. R_F values for investigated compounds in normal phase TLC system on silica layer and ethyl acetate + n-heptane as the mobile phase

		R_F					
No	Name	40%	50%	60%	70%	80%	90%
1	Caffeic acid	0.11	0.24	0.33	0.42	0.67	0.73
2	Ferulic acid	0.20	0.31	0.36	0.40	0.69	0.75
3	Flavone	0.09	0.16	0.24	0.36	0.38	0.42
4	Naringenin	0.24	0.33	0.40	0.53	0.77	0.80
5	Apigenin	0.13	0.29	0.38	0.49	0.58	0.67
6	Acacetin	0.27	0.36	0.42	0.53	0.57	0.65
7	Luteolin	0.09	0.22	0.31	0.44	0.47	0.49
8	Hesperetin	0.22	0.31	0.38	0.53	0.53	0.64
9	Catechin	0.04	0.20	0.31	0.47	0.68	0.75
10	Epicatechin	0.02	0.27	0.33	0.47	0.68	0.74
11	Hyperoside	0.02	0.10	0.19	0.24	0.30	0.36
12	Hesperidin	0.01	0.01	0.03	0.05	0.06	0.07
13	Quercitrin	0.02	0.04	0.07	0.11	0.49	0.53
14	Naringin	0.01	0.04	0.08	0.13	0.28	0.35
15	Rutin	0.01	0.03	0.04	0.06	0.10	0.14
16	Resveratrol	0.16	0.22	0.36	0.60	0.76	0.81
17	Kaempferol	0.27	0.36	0.47	0.56	0.67	0.74

exemplary plots of R_M vs. C% relationships obtained in the system 80% ethyl acetate + 20% n-heptane.

The main aim of this paper was determination of the possibility of connection of RP-HPLC-NP-TLC chromatographic systems. The optimization of HPLC systems depended on the selection of the most selective system by use of a binary mobile phase in the reversed phase system. Methanol + water mixtures were used for RP separations. The plot of R_M vs. concentration of methanol in the mobile phase is shown in Figure 2. It shows that the best system is 45% methanol + 55% water.

R_M values were correlated for NP-TLC and RP-HPLC systems to check if these optimal systems were chosen correctly (Figure 3).

The coupling of RP-HPLC and NP-TLC method was realized, in this way, that the eluate fractions from the RP column containing partially separated analytes were spotted onto the silica plate and, after mobile phase evaporation, were developed in a normal phase system which was optimized previously. The extracts from two *Polygonum* species were applied onto the reversed phase chromatographic column and, after the preliminary separation on four peak groups (in case of *Polygonum aviculare* extract) and on five fractions (in case of *Polygonum hydropiper* extract) (Figures 4, 5), the mobile phase from all fractions were evaporated. The dry residues were dissolved in 25 μL of methanol and spotted onto the silica chromatographic plate. One

Table 3. R_F values for investigated compounds in normal phase TLC system on silica layer and ethyl-methyl ketone + n-heptane as the mobile phase

		R_F				
No	Name	40%	50%	60%	70%	90%
1	Caffeic acid	0.13	0.22	0.38	0.40	0.58
2	Ferulic acid	0.20	0.29	0.40	0.42	0.60
3	Flavone	0.38	0.40	0.51	0.53	0.62
4	Naringenin	0.24	0.33	0.49	0.51	0.67
5	Apigenin	0.20	0.31	0.44	0.47	0.67
6	Acacetin	0.31	0.36	0.53	0.53	0.67
7	Luteolin	0.13	0.22	0.40	0.40	0.64
8	Hesperetin	0.20	0.31	0.51	0.47	0.67
9	Catechin	0.04	0.09	0.29	0.27	0.62
10	Epicatechin	0.02	0.13	0.24	0.33	0.60
11	Hyperoside	0.01	0.01	0.01	0.01	0.18
12	Hesperidin	0.01	0.01	0.01	0.02	0.09
13	Quercitrin	0.01	0.04	0.07	0.09	0.36
14	Naringin	0.01	0.01	0.01	0.09	0.11
15	Rutin	0.01	0.01	0.01	0.01	0.07
16	Resveratrol	0.18	0.29	0.42	0.51	0.67
17	Kaempferol	0.27	0.36	0.47	0.56	0.69

of the optimized TLC systems was applied for normal phase separation – 80% ethyl acetate + 20% n-heptane. Before development, the plate was saturated during 10 minutes to avoid a demixing effect. After drying in air, the plate was photographed at the wavelength 366 nm (Figure 6). Some of the phenolic compounds were identified.

Several polar compounds are eluted in one fraction from a C_{18} column: phenolic acids, resveratrol, and flavonoid glycosides such as rutin, quercitrin, and naringin contained in the *Polygonum avicularis* extract. These compounds are well separated on silica layers – there is high resolution between diglycosides and monoglycosides. The positions of polar groups or sugar molecules strongly influence retention in normal phase systems. In the extract of *Polygonum hydropiper*, several polar compounds are also eluted in one fraction from a C_{18} column. There are resveratrol, phenolic acids, catechin, epicatechin, and flavonoid glycosides (rutin, quercitrin and naringin), which have been well separated on the silica layer. The positions of the polar groups or sugar molecules strongly influence retention in normal phase systems.

In *Polygonum avicularis*, the following compounds are identified: rutin, naringin, quercitrin, caffeic acid, ferulic acid, resveratrol, hyperoside, and kemferol. In *Polygonum hydropiper*: rutin, naringin, quercitrin, ferulic acid, resveratrol, catechin + epicatechin, naringenin, and luteolin are identified.

Table 4. R_F values for investigated compounds in normal phase TLC system on silica layer and propan-2-ol + n-heptane as the mobile phase

		R_F				
No	Name	20%	30%	50%	70%	90%
1	Caffeic acid	0.20	0.33	0.53	0.64	0.71
2	Ferulic acid	0.20	0.33	0.51	0.60	0.69
3	Flavone	0.40	0.44	0.56	0.67	0.69
4	Naringenin	0.20	0.38	0.56	0.69	0.73
5	Apigenin	0.20	0.38	0.58	0.71	0.76
6	Acacetin	0.29	0.40	0.56	0.67	0.69
7	Luteolin	0.18	0.36	0.53	0.67	0.76
8	Hesperetin	0.16	0.31	0.51	0.64	0.71
9	Catechin	0.07	0.22	0.49	0.62	0.73
10	Epicatechin	0.09	0.24	0.60	0.62	0.64
11	Hyperoside	0.02	0.02	0.18	0.33	0.44
12	Hesperidin	0.04	0.09	0.38	0.22	0.60
13	Quercitrin	0.04	0.11	0.36	0.53	0.60
14	Naringin	0.01	0.01	0.11	0.27	0.38
15	Rutin	0.04	0.02	0.09	0.24	0.38
16	Resveratrol	0.24	0.36	0.67	0.71	0.69

Table 5. R_F values for investigated compounds in normal phase TLC system on silica layer and dioxane + n-heptane as the mobile phase

		R_F				
No	Name	10%	30%	50%	70%	90%
1	Caffeic acid	0.01	0.02	0.24	0.53	0.69
2	Ferulic acid	0.01	0.07	0.31	0.60	0.71
3	Flavone	0.01	0.29	0.49	0.69	0.78
4	Naringenin	0.07	0.04	0.31	0.60	0.76
5	Apigenin	0.01	0.04	0.24	0.53	0.71
6	Acacetin	0.01	0.09	0.38	0.64	0.80
7	Luteolin	0.01	0.02	0.18	0.47	0.69
8	Hesperetin	0.01	0.04	0.29	0.60	0.78
9	Catechin	0.01	0.01	0.31	0.58	0.80
10	Epicatechin	0.01	0.01	0.01	0.53	0.69
11	Hyperoside	0.01	0.01	0.01	0.20	0.42
12	Hesperidin	0.01	0.01	0.01	0.18	0.47
13	Quercitrin	0.01	0.01	0.01	0.31	0.58
14	Naringin	0.01	0.01	0.01	0.16	0.44
15	Rutin	0.01	0.01	0.01	0.09	0.38
16	Resveratrol	0.01	0.02	0.01	0.64	0.80

Table 6. R_F values for investigated compounds in normal phase TLC system on silica layer and tetrahydrofuran + n-heptane as the mobile phase

		R_F				
No	Name	10%	30%	50%	70%	90%
1	Caffeic acid	0.01	0.07	0.38	0.49	0.69
2	Ferulic acid	0.01	0.16	0.40	0.51	0.69
3	Flavone	0.09	0.29	0.51	0.60	0.73
4	Naringenin	0.01	0.13	0.42	0.56	0.71
5	Apigenin	0.01	0.09	0.40	0.51	0.71
6	Acacetin	0.02	0.16	0.44	0.56	0.71
7	Luteolin	0.01	0.07	0.38	0.49	0.71
8	Hesperetin	0.01	0.11	0.47	0.56	0.69
9	Catechin	0.01	0.01	0.40	0.42	0.73
10	Epicatechin	0.01	0.01	0.18	0.36	0.60
11	Hyperoside	0.01	0.01	0.04	0.07	0.29
12	Hesperidin	0.01	0.01	0.01	0.07	0.27
13	Quercitrin	0.02	0.01	0.07	0.20	0.44
14	Naringin	0.01	0.01	0.01	0.07	0.24
15	Rutin	0.01	0.01	0.01	0.04	0.20
16	Resveratrol	0.01	0.09	0.36	0.49	0.64

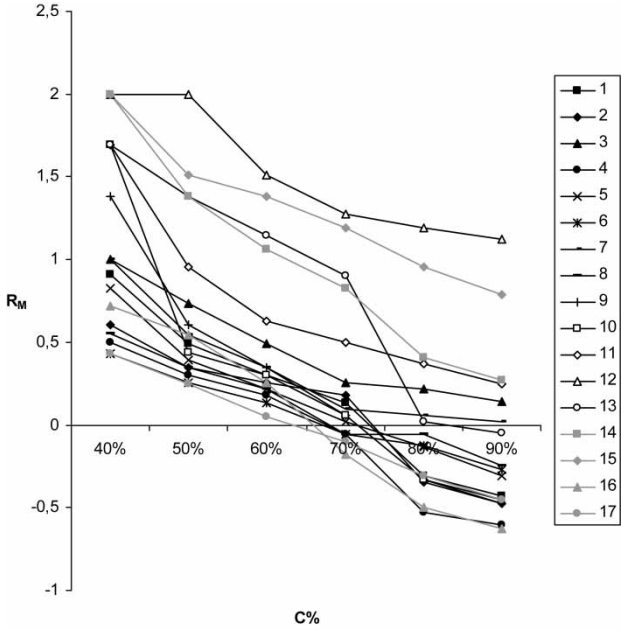


Figure 1. R_M vs. C% plot for the NP-TLC system with silica as stationary phase and ethyl acetate + n-heptane as mobile phase. Numbers of compounds as in Table 1.

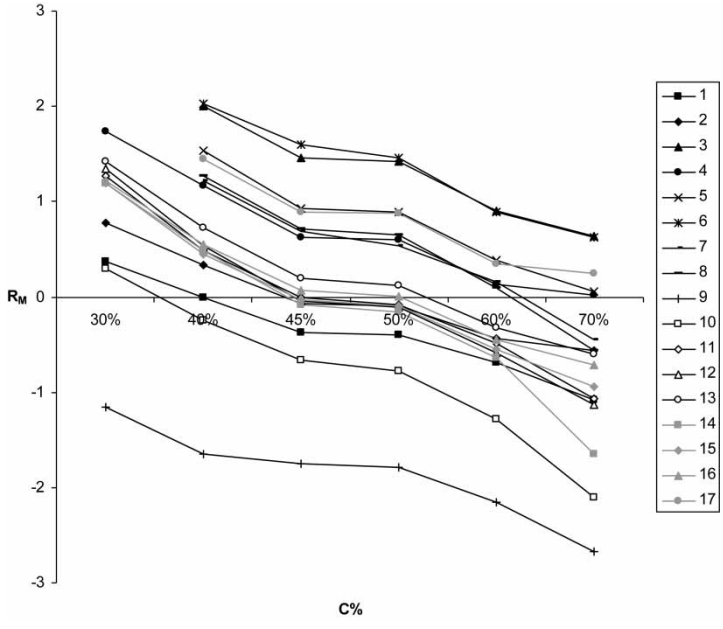


Figure 2. R_M vs. C% plot for the RP-HPLC system with RP18 as stationary phase and methanol + water as mobile phase. Numbers of compounds as in Table 1.

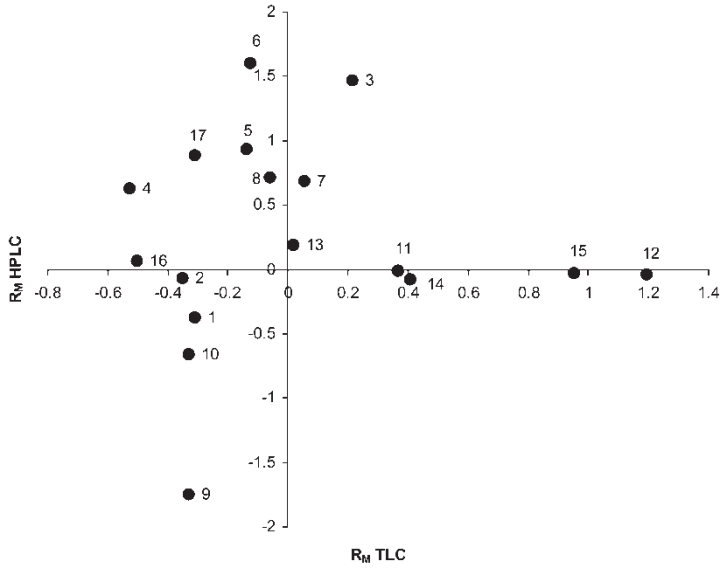


Figure 3. Correlation graph $R_{M\ TLC}$ vs. $R_{M\ HPLC}$. Numbers of compounds as in Table 1.

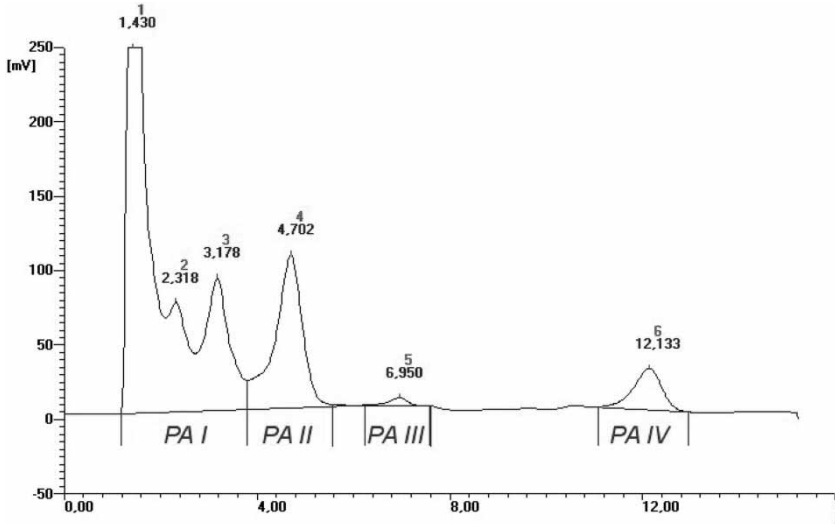


Figure 4. RP-HPLC chromatogram for *Polygonum aviculare* extract. PAI, PAII, PAIII, PAIV – numbers of fractions collected from the chromatographic column during the elution. Arabic numbers – peaks numbers and their retention times calculated by POL-LAB program.

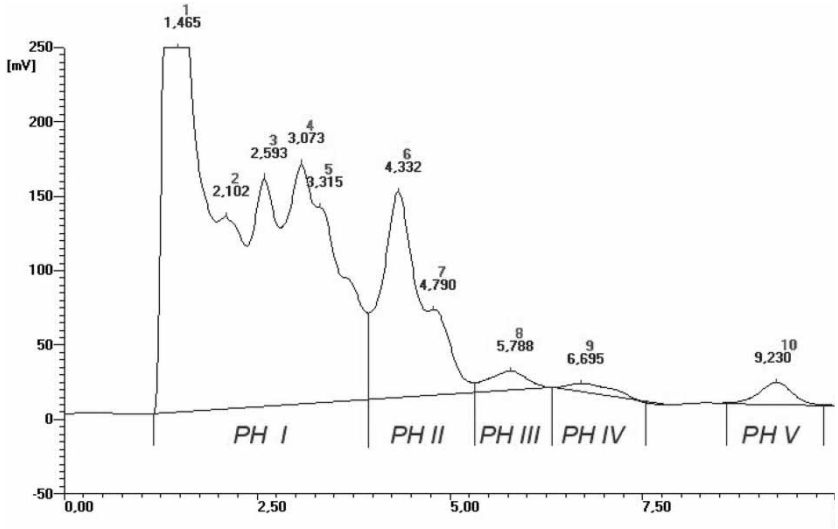


Figure 5. RP-HPLC chromatogram for *Polygonum hydropiper* extract. PHI, PHII, PHIII, PHIV, PHV – numbers of fractions collected from the chromatographic column during the elution. Arabic numbers – peaks numbers and their retention times calculated by POL-LAB program.

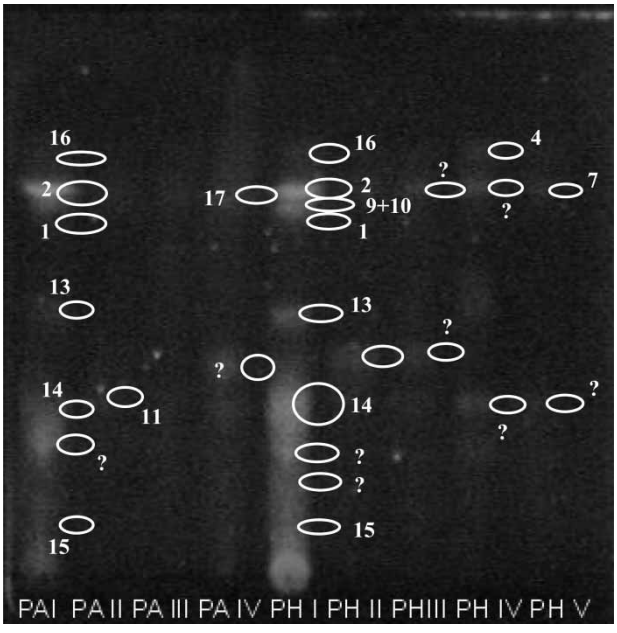


Figure 6. The photograph of silica chromatographic plate scanned at 366 nm by Reprostar 3 after development of collected HPLC fractions. Numbers as in Table 1. Other abbreviations as in Figs. 4 and 5.

CONCLUSIONS

Normal phase and reversed phase systems have different selectivities; they are most selective for separation of investigated groups of compounds. The coupling of TLC and HPLC methods is more suitable for the separation of complicated mixtures (especially herbal) than the application of each of them separately.

The off-line coupling of RP and NP systems allowed separation of investigated extracts; however, not all compounds were identified.

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