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Two-dimensional thin-layer chromatography of selected *Polygonum* sp. extracts on polar-bonded stationary phases

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ABSTRACT

Two-dimensional thin-layer chromatographic systems on cyano-bonded polar stationary phases were used for the separation of some phenolic compounds extracted from two species of *Polygonum*: *Polygonum hydropiper* L. and *Polygonum cuspidatum* L. Non-aqueous solvents were used in the first direction and aqueous solvents were used in the second direction on CN silica TLC plates. For the separation of phenolics' standards optimal chromatographic systems were chosen from the retention data collected in one-dimensional TLC experiments by plotting graphs of R_F vs. R_F dependencies. Using above described method the satisfactory results of separations were obtained.

1. Introduction

Plant extracts are usually rich in groups of substances of various physicochemical properties. In the group of phenolics, among others, flavonoid aglycones, flavonoid glycosides, phenolic acids are widely presented in various plant organs. Similar physicochemical properties cause some difficulties in their separation. Separation of these compounds, having similar chromatographic properties, is often impossible in one chromatographic run. For such difficult purposes multidimensional separations by using systems of various selectivities are often applied [1–5]. This needs, however, special equipment and complicated procedures in case of HPLC systems [6–10].

Two-dimensional separations can be much easily performed on one chromatographic plate with two eluents of different selectivities or on two various chromatographic plates (graft TLC).

Polar bonded stationary phases (cyanopropyl, aminopropyl and diol) are the special types of stationary phases which can be used in both normal-phase (NP-TLC) and reversed phase (RP-TLC) systems. In this case, two-dimensional thin layer chromatography can be performed without technical problems in the 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 the use of non-aqueous and perpendicularly aqueous eluents (various properties and selectivities) [11–17].

Polygonum hydropiper L. also known as smartweed has a long history of herbal use, both in Eastern and in Western herbalism. It is not very often used, and it is seen more as a domestic remedy being valued especially for its astringent properties which makes it useful in treating bleeding, skin problems, diarrhoea, etc. The leaves have anti-inflammatory, astringent, carminative, diaphoretic, diuretic, emmenagogue, stimulant, stomachic, and styptic properties. They contain rutin, which helps strengthen fragile capillaries and thus helps prevent bleeding. The seeds are carminative, diuretic and stimulant. The whole plant, either on its own or mixed with other herbs, is decocted and used in the treatment of a wide range of ailments including diarrhoea, dyspepsia, itching skin, excessive menstrual bleeding and haemorrhoids. A poultice of the plant is used in treating swollen and inflamed areas. In Chinese tests, the plant was ranked 20th in a survey of 250 potential antifertility drugs. A homeopathic remedy is made from the leaves. It is used in the treatment of piles, menstrual pains and other menstrual complaints [18].

Polygonum cuspidatum L. (also known as Japanese Knotweed, *Polygonum sieboldii*, *Reynoutria japonica*) has antiphlogistic, bechic, depurative, diuretic, emmenagogue, emollient, febrifuge, stomachic and vulnerary activity. It is also used in the treatment of women's complaints. A decoction is used in the treatment of burn injuries, boils and abscesses, poisonous snakebites, acute hepatitis, appendicitis, traumatic injuries and menstrual irregularities. The leaves can be crushed and applied externally as a poultice to abscesses, cuts, etc., whilst the dried roots can be ground into a powder and applied externally. Extracts of the plant have shown antitumour activity [18,19]. Thin layer chromatographic determination of resveratrol in extract from *P. cuspidatum* was performed by Zhao [20] and Babu et al. [21].

The aim of this paper was the investigation of retention behavior of phenolic compounds in some selected non-aqueous and

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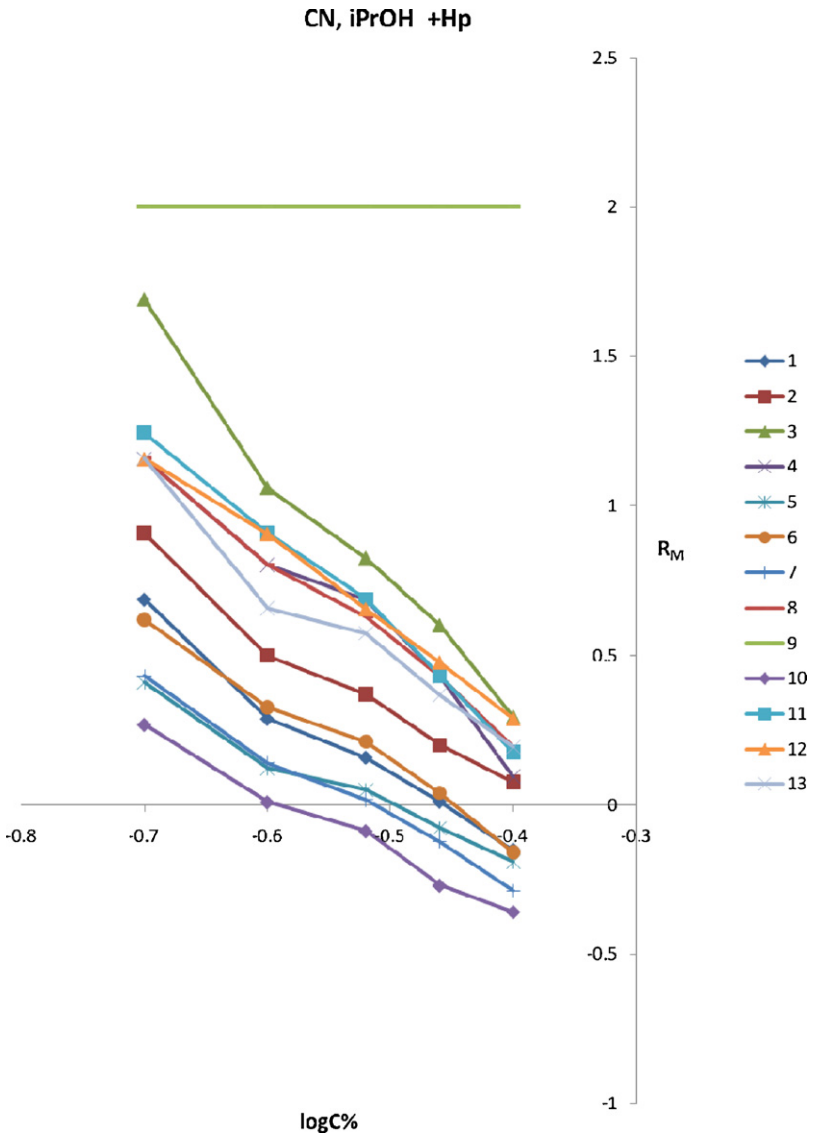


Fig. 1. R_M vs. log *c*% relationships for tested compounds in the system: cyano bonded stationary phase – propan-2-ol + n-heptane as mobile phase. Numbers in legend as in Table 1.

aqueous mobile phases to compare their separation selectivities and to use optimized most selective systems for the separation of some phenolic compounds occurred in two species of *Polygonum*: *P. hydropiper* and *P. cuspidatum*. This method can also be used as the instrument for comparison of the composition of plant extracts (fingerprints).

2. Experimental

HPTLC CN F254s 10 × 10 cm (Merck, Darmstadt, Germany) plates were used in all experiments. Solvents: propan-2-ol, ethyl acetate, n-heptane and methanol pro analysis grade and were purchased from Polish Reagents (POCh, Gliwice, Poland). Distilled water was mixed with methanol to obtain aqueous phases and n-heptane was mixed with propan-2-ol and ethyl acetate to obtain non-aqueous solvents for 2D-TLC.

All test substances (kaempferol, quercetin, rutin, hyperoside, ferulic acid, gallic acid, caffeic acid, chlorogenic acid, chinic acid, p-coumaric acid, catechin, epicatechin and resveratrol) were acquired from various manufacturers (Sigma, Aldrich, Fluka, Roth). 2,2′-Diphenyl-1-picrylhydrazyl (DPPH) was from Aldrich. 2-(diphenylboryoxy)-ethylamine and PEG4000 – Naturstoff reagent was produced by Merck (Merck, Darmstadt, Germany).

P. hydropiper and *P. cuspidatum* herbs were obtained from Herbapol (Lublin, Poland).

5 g of each herb was closed in the paper case and extracted in Soxhlet apparatus on water bath during 10 h using 350 mL of dichloromethane to get rid of ballast substances (fats, chlorophyll, etc.) and next after drying in air it was extracted by 350 mL of methanol during 12 h. After extraction methanol was evaporated on water bath under reduced pressure and the remnant was washed out with hot water and put in the refrigerator for 12 h. The mixture was filtered using paper filter and the solution was extracted 5 times by the use of 100 mL portions of ethyl acetate. Ethyl acetate extracts were coupled and the solvent was evaporated under low pressure on water bath and the dry residue was dissolved in methanol in 10 mL flask [22]. These extracts were examined in all experiments.

Some mobile phases consisted of propan-2-ol and n-heptane (concentrations: 20%, 25%, 30%, 35% and 50% (v/v)) and ethyl acetate and n-heptane (concentrations: 40%, 50%, 55%, 60% and 70% (v/v)) were prepared to optimize the separation of test substances in non-aqueous systems using CN-bonded chromatographic plates as

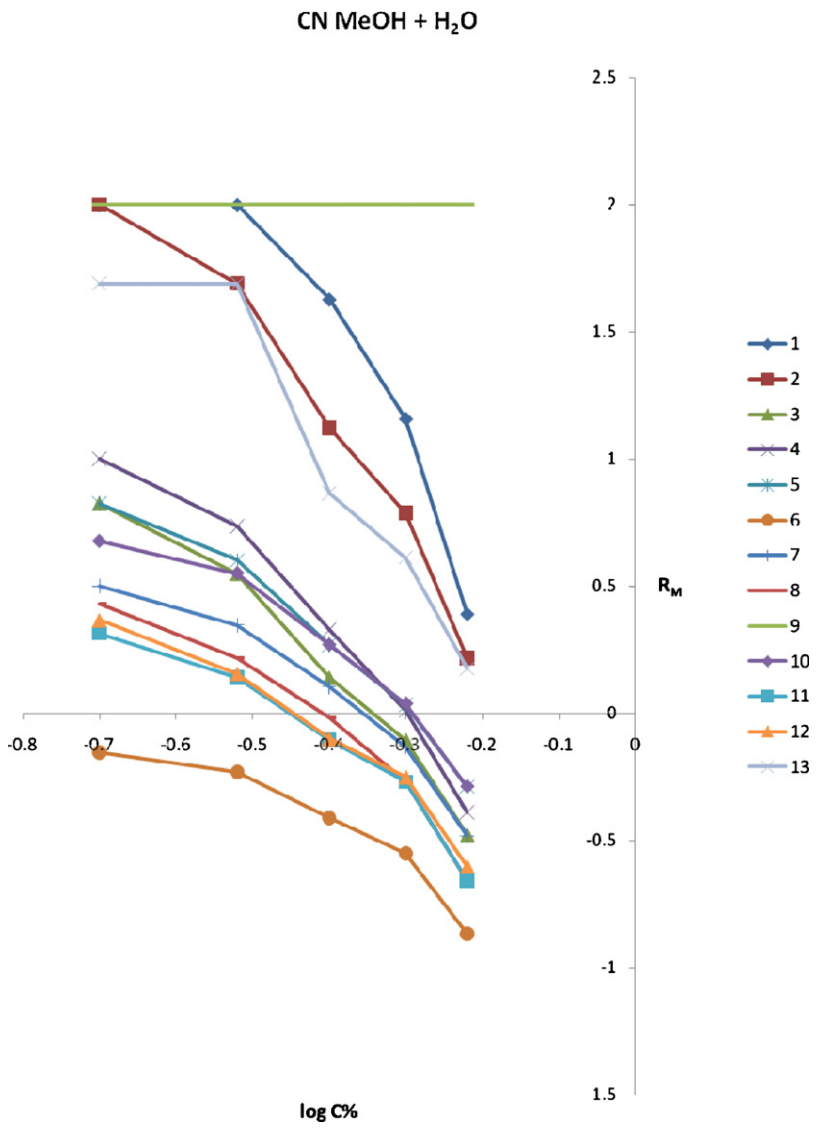


Fig. 2. R_M vs. log *c*% relationship for tested compounds in the system: cyano bonded stationary phase – methanol + water as mobile phase. Numbers in legend as in Table 1.

stationary phase. The mixtures of methanol and water were used for the separation of standards in reversed systems using the same stationary phase. The concentrations of methanol in water were the following: 20%, 30%, 40%, 50% and 60% (v/v). Each mobile phase contained 0.1% (v/v) of formic acid to suppress dissociation of acidic groups.

The 0.1% (v/v) solution of the mixture of test substances and methanolic solution of extracts were spotted by Desaga TLC AS30 applicator (Desaga, Heidelberg, Germany) 1 cm from each edge of the chromatographic plate and developed in two directions by the use of DS-II developing chambers (Chromdes, Lublin, Poland). In the first direction the plate was developed by the use of non-aqueous solvent and after drying in air the same plate was developed by the use of aqueous eluent. In case of non-aqueous solutions each plate was conditioned during 15 min to avoid the demixing effect; in case of RP systems (aqueous eluents) plates were not conditioned. After drying the plates were sprayed by the use of Merck TLC sprayer using 2-(diphenylboryoxy)-ethylamine and PEG4000 (Merck, Darmstadt, Germany) or DPPH and photographed in Camag Cabinet UV lamp at 254 nm and 365 nm by the use of Fuji 8 mpx camera.

Before derivatization the plates were scanned by the use of Desaga CD60 (Desaga, Heidelberg, Germany) densitometer to obtain data for 3D images of chromatographic plates. Parameters of scanning were the following: *X* – 5 mm, start *Y* – 5 mm, end *Y* – 95 mm, meander – 0, number of lanes – 30, distance between lines – 1.0 mm, length of slit – 0.4 mm, height of slit – 0.4 mm, wavelength – 254 nm, resolution – 0.025 mm, smoothing factor – 19. Data were transformed in Excel to text image files and 3D images were generated by ImageJ software.

Examined test substances are listed in Table 1.

3. Results and discussion

Figs. 1 and 2 show R_M vs. log *c* dependencies for investigated test compounds. It is seen that decrease of the concentration of a more polar component (propan-2-ol) in binary mobile phase using normal phase systems (cyano-bonded polar stationary phase + non-aqueous mobile phase, NP-TLC) causes an increase in the system selectivity and improves the separation of investigated compounds. Any chromatographic system applied separately does not allow separation of all tested substances, thus two-dimensional thin layer chromatography was performed to make the separation better. 55 two-dimensional chromatographic systems were tested to find the optimal system for separation of compounds occurred in prepared extracts from *Polygonum* sp. The best results were

Table 1
Test substances examined.

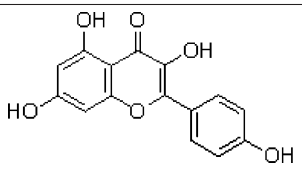
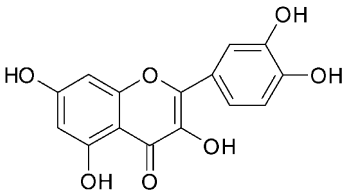
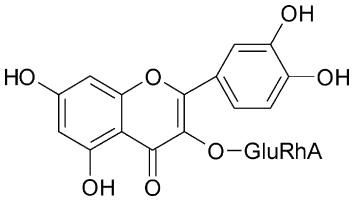
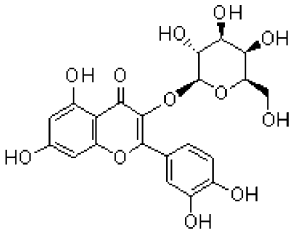
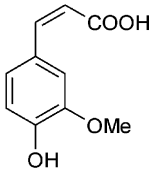
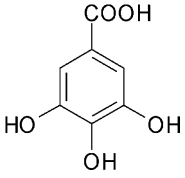
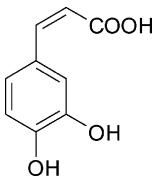
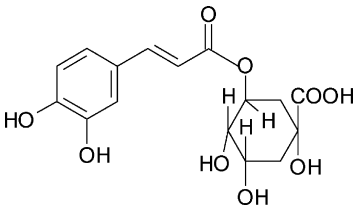
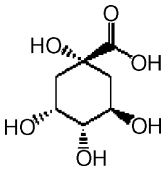
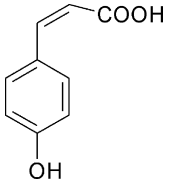
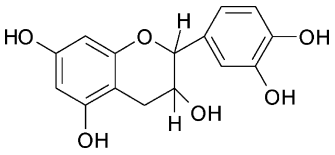
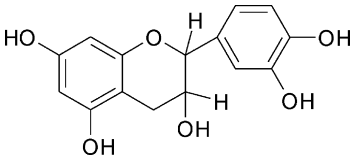
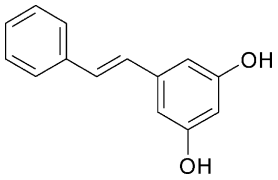
No.	Name	Formula
1.	Kaempferol	
2.	Quercetin	
3.	Rutin	
4.	Hyperoside	
5.	Ferulic acid	
6.	Gallic acid	
7.	Caffeic acid	
8.	Chlorogenic acid	
9.	Quinic acid	

Table 1 (*Continued*)

No.	Name	Formula
10.	p-coumaric acid	
11.	Catechin	
12.	Epicatechin	
13.	Resveratrol	

obtained for systems with propan-2-ol–n-heptane as non-aqueous mobile phase in NP-TLC systems, and methanol–water as aqueous mobile phase in RP-TLC systems. All systems tested are listed in Table 2.

R_F vs. R_F dependencies were plotted to optimize two-dimensional thin layer chromatographic systems. The systems with worse correlation coefficients (orthogonal systems) are the best for two-dimensional separations, but there is not one factor which decides about the separation effect. R_F values of chromatographed compounds should also not be too high and too low. On the basis of these plots optimal 2D-TLC systems were chosen and applied for the separation of examined extracts. Figs. 3 and 4 show the plots of R_F vs. R_F for the systems: 1st direction of development – 30% iPrOH + n-heptane and 2nd direction of development –

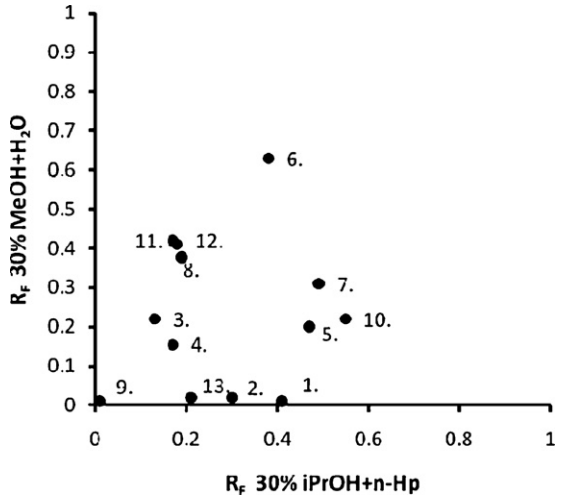


Fig. 3. R_F vs. R_F relationship for 2D-TLC system with cyano bonded stationary phase and: 30% iPrOH + n-heptane in the first direction of development, 30% MeOH + water in the second direction of development. Numbers as in Table 1.

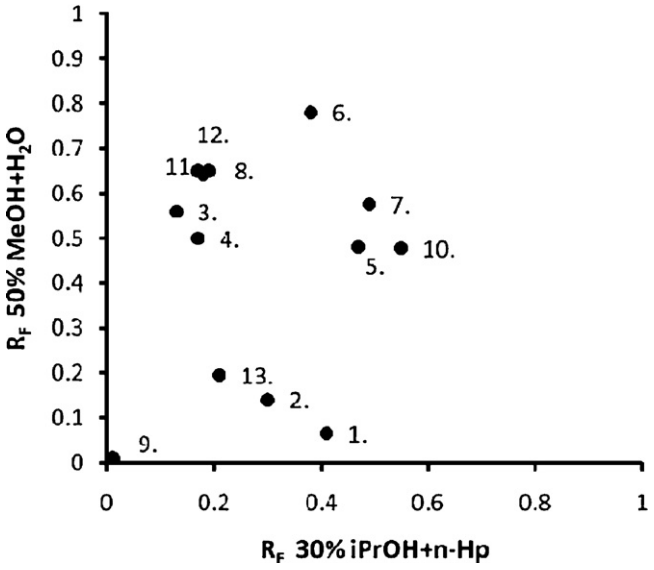


Fig. 4. R_F vs. R_F relationship for 2D-TLC system with cyano bonded stationary phase and: 30% iPrOH + n-heptane in the first direction of development, 50% MeOH + water in the second direction of development. Numbers as in Table 1.

30% MeOH + water and 50% MeOH + water. Simulated separations achieved by the use of these systems were satisfactory and these eluents were applied for the separation of *Polygonum* extracts. Fig. 5 shows the result of the separation of mixture of test compounds and Fig. 6 is the graphical image of the plate before derivatization plotted in ImageJ software on the basis of densitometric scanning data. On the basis of spot location on the plate, the identification of compounds in separated extracts (Figs. 7 and 8) was performed. Identified phenolic compounds in *P. cuspidatum* extract are the following: kaempferol, quercetin, rutin, hyperoside, gallic acid, chlorogenic acid, p-coumaric acid, catechin, epicatechin and resveratrol. Kaempferol, quercetin, rutin, caffeic acid, chlorogenic acid, p-coumaric acid, catechin and epicatechin were identified on plates with the separated *P. hydropiper* extract.

Figs. 5d, 7d and 8d show that separated and identified compounds have antioxidant properties. Identified: kaempferol, quercetin, rutin, gallic acid and p-coumaric acid in Fig. 5d. Chlorogenic acid, catechin and epicatechin are not satisfactorily separated.

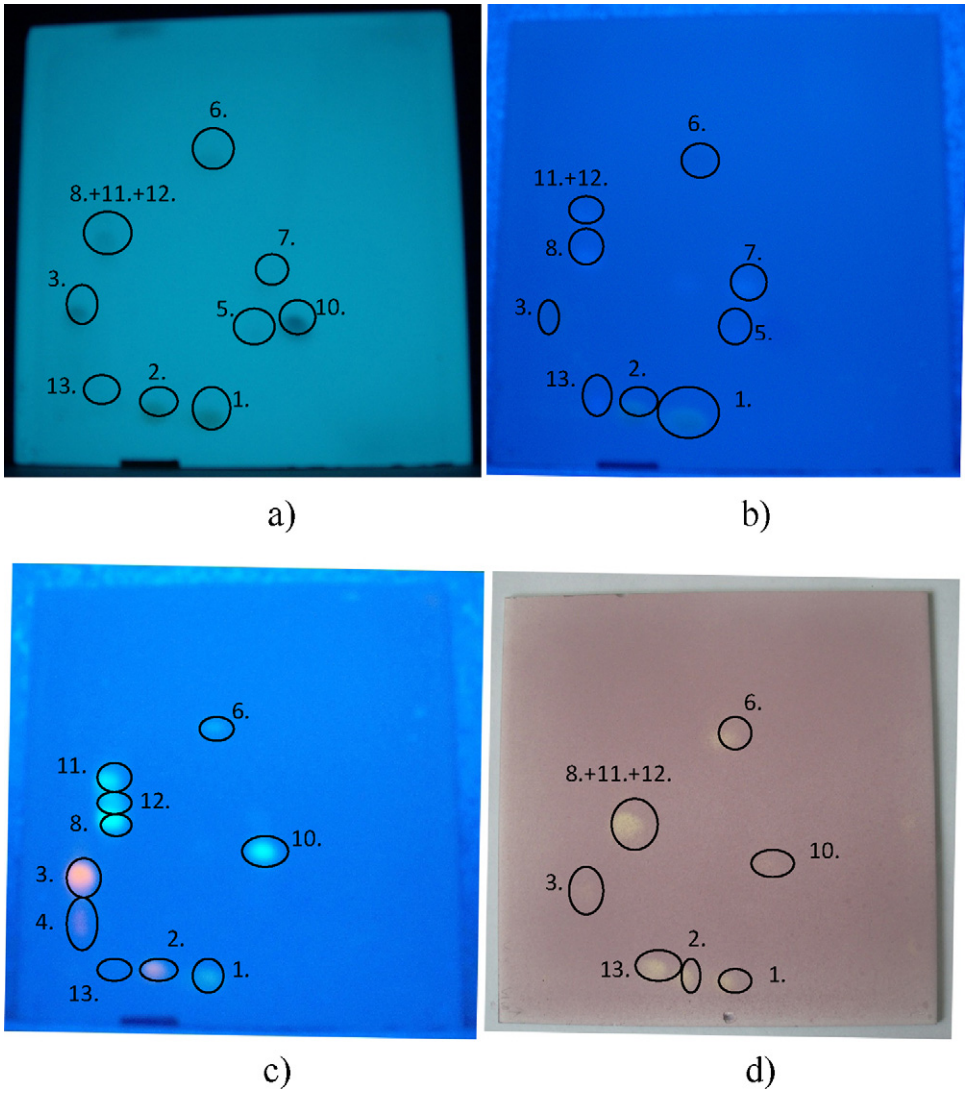


Fig. 5. Photographs of 2D-TLC chromatograms of test compounds using cyano bonded stationary phase and: 30% iPrOH + n-heptane in the first direction of development, 30% MeOH + water in the second direction of development. Visualization: (a) λ = 254 nm, (b) λ = 365 nm, (c) λ = 365 nm after Naturstoff reagent derivatization, (d) after DPPH derivatization. Numbers as in Table 1.

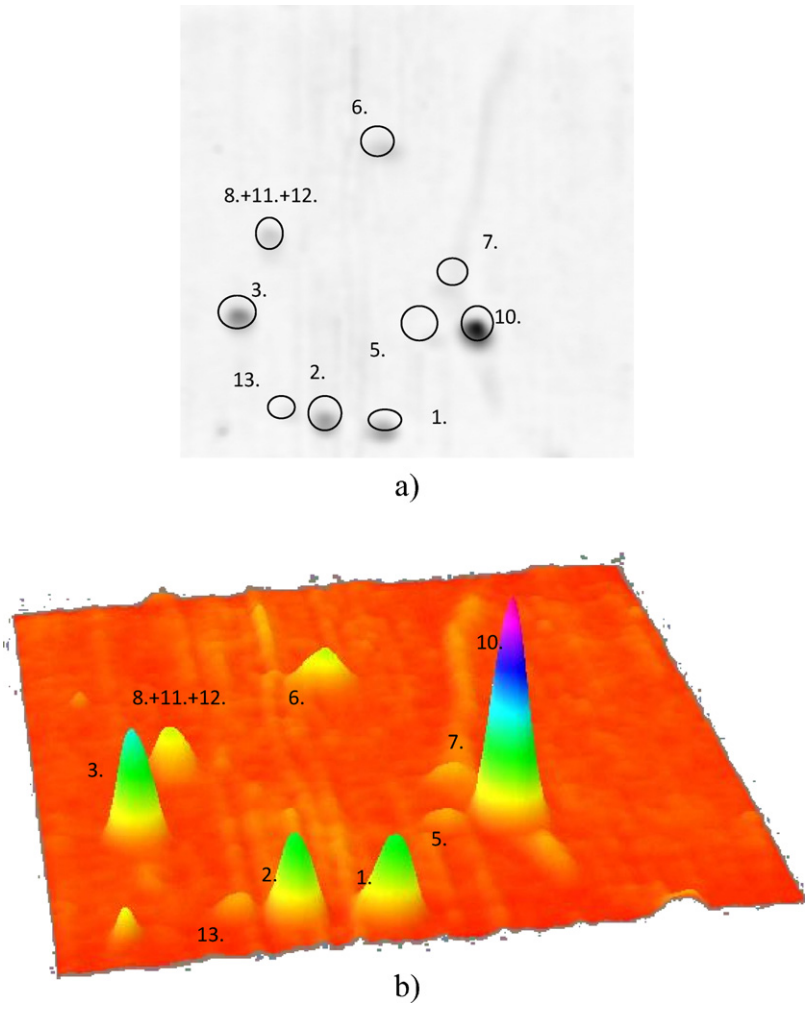


Fig. 6. Image of the plate generated for test compound mixture on the basis of densitometric scanning by ImageJ software. Numbers as in Table 1.

Table 2
2D-TLC systems tested and correlation parameters for R_F vs. R_F relationships.

Lp.	1st direction	2nd direction	*a*	*b*	R^2
1.	20% iPrOH + n-Hp	20% MeOH + H_2O	0.27 ± 0.11	0.13 ± 0.11	0.0330
2.	20% iPrOH + n-Hp	30% MeOH + H_2O	0.24 ± 0.11	0.19 ± 0.12	0.0200
3.	20% iPrOH + n-Hp	40% MeOH + H_2O	0.26 ± 0.22	0.30 ± 0.13	0.0171
4.	20% iPrOH + n-Hp	50% MeOH + H_2O	0.37 ± 0.09	0.39 ± 0.18	0.0266
5.	20% iPrOH + n-Hp	60% MeOH + H_2O	0.47 ± 0.28	0.54 ± 0.24	0.0405
6.	25% iPrOH + n-Hp	20% MeOH + H_2O	0.12 ± 0.08	0.14 ± 0.11	0.0143
7.	25% iPrOH + n-Hp	30% MeOH + H_2O	0.08 ± 0.04	0.21 ± 0.09	0.0049
8.	25% iPrOH + n-Hp	40% MeOH + H_2O	0.08 ± 0.05	0.32 ± 0.16	0.0035
9.	25% iPrOH + n-Hp	50% MeOH + H_2O	0.15 ± 0.09	0.40 ± 0.12	0.0091
10.	25% iPrOH + n-Hp	60% MeOH + H_2O	0.28 ± 0.09	0.54 ± 0.31	0.0280
11.	30% iPrOH + n-Hp	20% MeOH + H_2O	0.17 ± 0.08	0.12 ± 0.09	0.0282
12.	**30% iPrOH + n-Hp**	**30% MeOH + H_2O**	**0.14 ± 0.11**	**0.18 ± 0.09**	**0.0154**
13.	30% iPrOH + n-Hp	40% MeOH + H_2O	0.15 ± 0.07	0.29 ± 0.19	0.0125
14.	**30% iPrOH + n-Hp**	**50% MeOH + H_2O**	**0.22 ± 0.10**	**0.37 ± 0.18**	**0.0213**
15.	30% iPrOH + n-Hp	60% MeOH + H_2O	0.36 ± 0.11	0.50 ± 0.25	0.0519
16.	35% iPrOH + n-Hp	20% MeOH + H_2O	0.18 ± 0.13	0.10 ± 0.09	0.0400
17.	35% iPrOH + n-Hp	30% MeOH + H_2O	0.17 ± 0.09	0.16 ± 0.12	0.0257
18.	35% iPrOH + n-Hp	40% MeOH + H_2O	0.20 ± 0.09	0.27 ± 0.13	0.0250
19.	35% iPrOH + n-Hp	50% MeOH + H_2O	0.27 ± 0.16	0.33 ± 0.19	0.0359
20.	35% iPrOH + n-Hp	60% MeOH + H_2O	0.43 ± 0.21	0.45 ± 0.21	0.0885
21.	40% iPrOH + n-Hp	20% MeOH + H_2O	0.25 ± 0.11	0.06 ± 0.04	0.0757
22.	40% iPrOH + n-Hp	30% MeOH + H_2O	0.27 ± 0.11	0.10 ± 0.12	0.0644
23.	40% iPrOH + n-Hp	40% MeOH + H_2O	0.34 ± 0.21	0.18 ± 0.16	0.0770
24.	40% iPrOH + n-Hp	50% MeOH + H_2O	0.46 ± 0.24	0.22 ± 0.11	0.1125
25.	40% iPrOH + n-Hp	60% MeOH + H_2O	0.65 ± 0.28	0.31 ± 0.13	0.2080
26.	40% AcOEt + n-Hp	20% MeOH + H_2O	−0.13 ± 0.11	0.20 ± 0.10	0.0145
27.	40% AcOEt + n-Hp	30% MeOH + H_2O	−0.23 ± 0.14	0.27 ± 0.12	0.0341
28.	40% AcOEt + n-Hp	40% MeOH + H_2O	−0.32 ± 0.16	0.40 ± 0.17	0.0476
29.	40% AcOEt + n-Hp	50% MeOH + H_2O	−0.32 ± 0.17	0.50 ± 0.23	0.0402
30.	40% AcOEt + n-Hp	60% MeOH + H_2O	−0.17 ± 0.08	0.64 ± 0.27	0.0109
31.	50% AcOEt + n-Hp	20% MeOH + H_2O	−0.01 ± 0.02	0.17 ± 0.12	0.0001

Table 2 (*Continued*)

Lp.	1st direction	2nd direction	*a*	*b*	R^2
32.	50% AcOEt + n-Hp	30% MeOH + H_2O	−0.07 ± 0.05	0.25 ± 0.11	0.0063
33.	50% AcOEt + n-Hp	40% MeOH + H_2O	−0.14 ± 0.11	0.38 ± 0.21	0.0168
34.	50% AcOEt + n-Hp	50% MeOH + H_2O	−0.16 ± 0.13	0.48 ± 0.22	0.0165
35.	50% AcOEt + n-Hp	60% MeOH + H_2O	−0.03 ± 0.02	0.61 ± 0.23	0.0006
36.	55% AcOEt + n-Hp	20% MeOH + H_2O	−0.01 ± 0.02	0.17 ± 0.11	2×10^{-5}
37.	55% AcOEt + n-Hp	30% MeOH + H_2O	−0.07 ± 0.04	0.25 ± 0.15	0.0059
38.	55% AcOEt + n-Hp	40% MeOH + H_2O	−0.14 ± 0.09	0.38 ± 0.21	0.0162
39.	55% AcOEt + n-Hp	50% MeOH + H_2O	−0.16 ± 0.09	0.49 ± 0.23	0.0168
40.	55% AcOEt + n-Hp	60% MeOH + H_2O	−0.02 ± 0.01	0.61 ± 0.34	0.0004
41.	60% AcOEt + n-Hp	20% MeOH + H_2O	0.06 ± 0.08	0.15 ± 0.11	0.0064
42.	60% AcOEt + n-Hp	30% MeOH + H_2O	0.01 ± 0.05	0.28 ± 0.12	0.0003
43.	60% AcOEt + n-Hp	40% MeOH + H_2O	−0.03 ± 0.04	0.35 ± 0.22	0.0009
44.	60% AcOEt + n-Hp	50% MeOH + H_2O	−0.03 ± 0.03	0.45 ± 0.26	0.0010
45.	60% AcOEt + n-Hp	60% MeOH + H_2O	0.09 ± 0.06	0.56 ± 0.26	0.0070
46.	70% AcOEt + n-Hp	20% MeOH + H_2O	0.11 ± 0.08	0.12 ± 0.07	0.0229
47.	70% AcOEt + n-Hp	30% MeOH + H_2O	0.08 ± 0.06	0.19 ± 0.11	0.0100
48.	70% AcOEt + n-Hp	40% MeOH + H_2O	0.05 ± 0.06	0.31 ± 0.22	0.0031
49.	70% AcOEt + n-Hp	50% MeOH + H_2O	0.05 ± 0.04	0.41 ± 0.26	0.0036
50.	70% AcOEt + n-Hp	60% MeOH + H_2O	0.20 ± 0.11	0.51 ± 0.27	0.0304
51.	50% iPrOH + n-Hp	20% MeOH + H_2O	0.26 ± 0.18	0.03 ± 0.02	0.0763
52.	50% iPrOH + n-Hp	30% MeOH + H_2O	0.31 ± 0.19	0.06 ± 0.04	0.0779
53.	50% iPrOH + n-Hp	40% MeOH + H_2O	0.44 ± 0.23	0.10 ± 0.06	0.1160
54.	50% iPrOH + n-Hp	50% MeOH + H_2O	0.59 ± 0.21	0.12 ± 0.11	0.1674
55.	50% iPrOH + n-Hp	60% MeOH + H_2O	0.85 ± 026	0.15 ± 0.12	0.3230

Abbreviations: CN – cyano bonded polar stationary phase, iPrOH – propan-2-ol, Hp – n-heptane, AcOEt – ethyl acetate, MeOH – methanol.

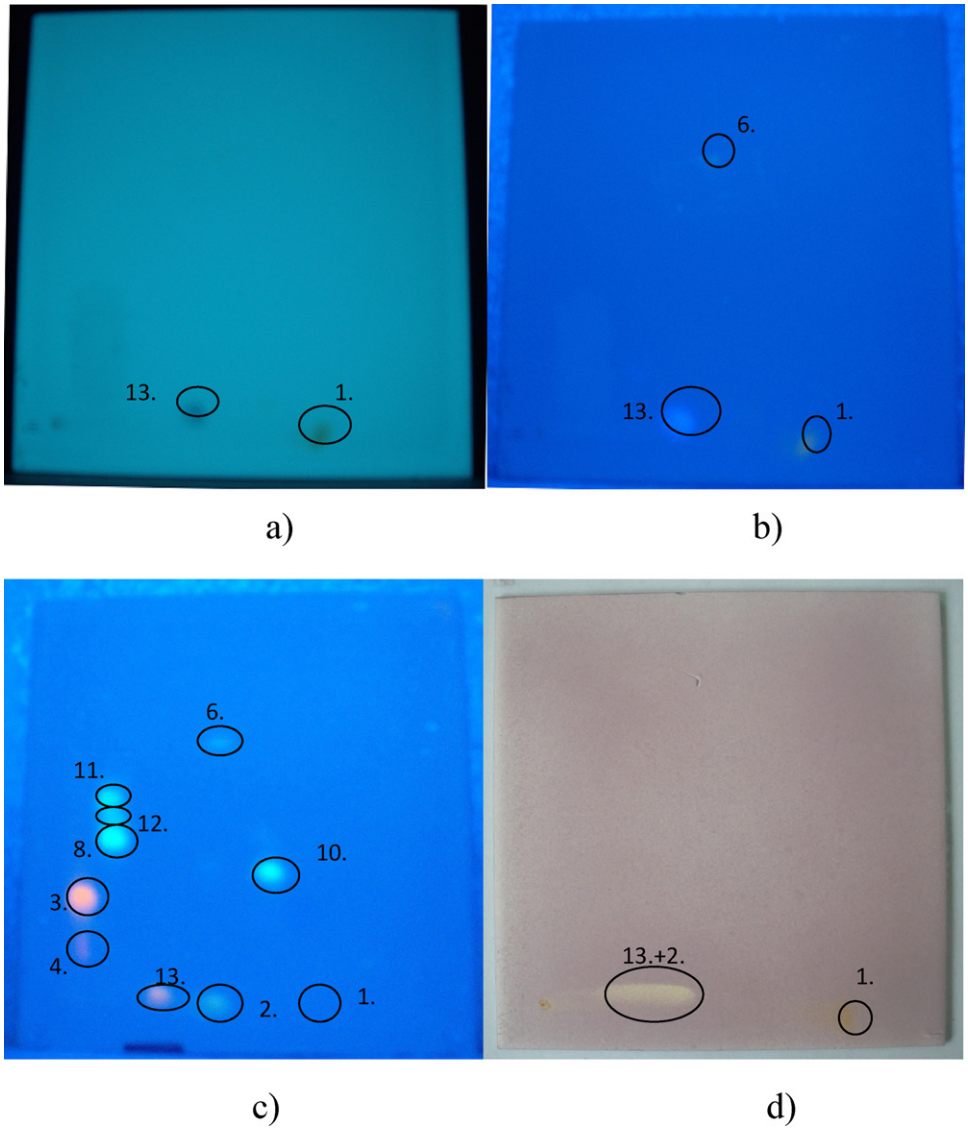


Fig. 7. Photographs of 2D-TLC chromatograms of *Polygonum cuspidatum* extract using cyano bonded stationary phase and: 30% iPrOH + n-heptane in the first direction of development, 30% MeOH + water in the second direction of development. Visualization: (a) λ = 254 nm, (b) λ = 365 nm, (c) λ = 365 nm after Naturstoff reagent derivatization, (d) after DPPH derivatization. Numbers as in Table 1.

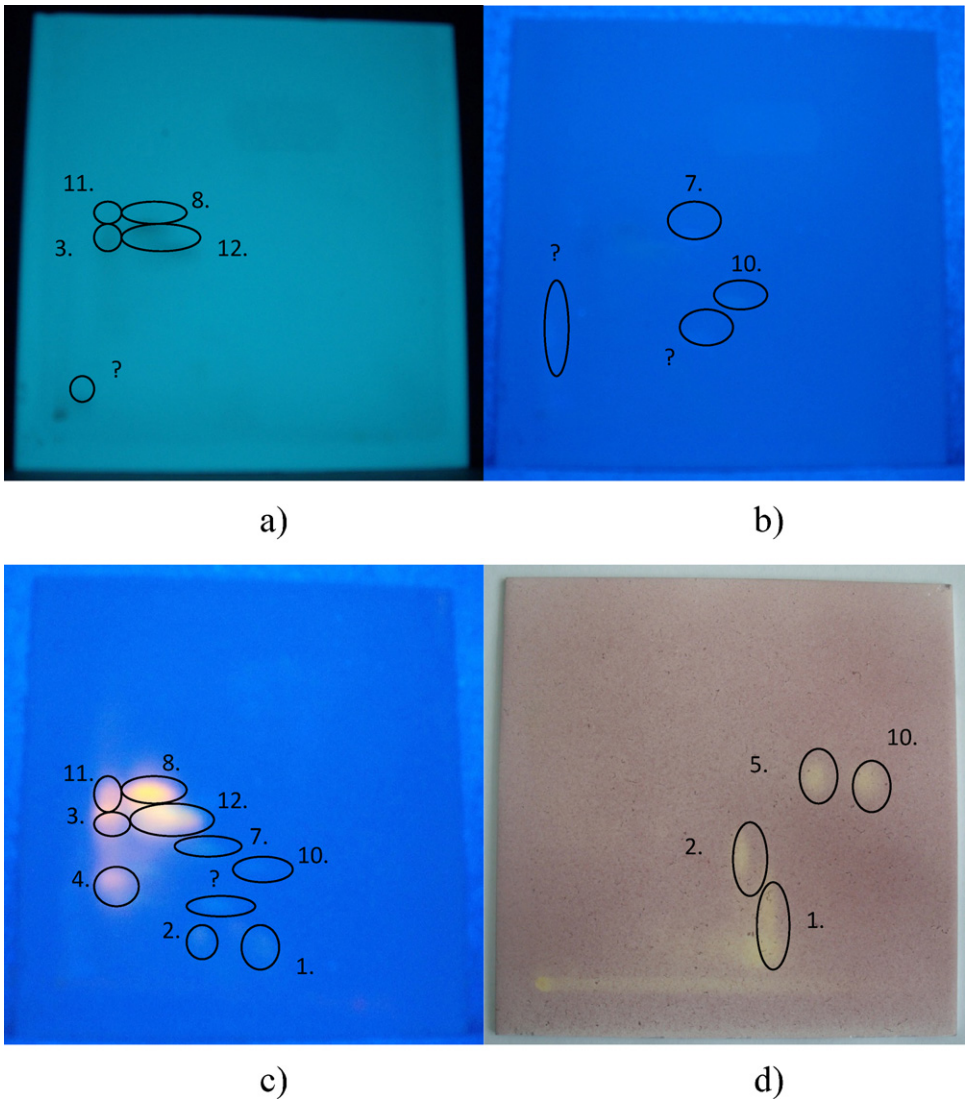


Fig. 8. Photographs of 2D-TLC chromatograms of *Polygonum hydropiper* extract using cyano bonded stationary phase and: 30% iPrOH + n-heptane in the first direction of development, 50% MeOH + water in the second direction of development. Visualization: (a) λ = 254 nm, (b) λ = 365 nm, (c) λ = 365 nm after Naturstoff reagent derivatization, (d) after DPPH derivatization. Numbers as in Table 1.

4. Conclusions

2D-TLC technique by the use of CN silica plates enables separation of phenolic fraction from *P. cuspidatum* and *P. hydropiper* plant extracts.

R_F vs. R_F dependencies are one of the best modes for the optimization of 2D-TLC separations of complex mixtures e.g. plant extracts.

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