Two-Dimensional Thin-Layer Chromatography of Selected Coumarins

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Abstract

Polar-bonded stationary phases (CN-silica and Diol-silica) are used with nonaqueous eluents in adsorption mode or with aqueous eluents in partition mode. This enables the application of these systems in two-dimensional separations because of the different selectivity and application to the separation of closely related compounds of similar physicochemical properties and retention behaviour. Similarly, multiphase plates, connected with C18 strips and silica layers, are used with aqueous and nonaqueous eluents. Such layers were applied for the separation of selected coumarins. Thus, differences in separation selectivity are applied for the separation of coumarin fractions from plant extracts of the *Apiaceae* family by two-dimensional thin-layer chromatography.

Introduction

Because of their biological activities, coumarins are very interesting compounds and are widely investigated. They are present in fruits, roots, leaves, and other organs of plants, mainly in the *Apiaceae* and *Rutaceae* families. Furanocoumarins play the role of phytoalexines in plants (1). Some furanocoumarins have pharmacological activity as Ca–channels blockers (2), anticoagulants (3), cytostatics (4), antitumor, anti-inflammator, and antifungal drugs (3). Some substances from this group have photosensibilizing properties and are important drugs in leucodermy therapy (3,4). Psoralene derivatives also have the ability to retard DNA synthesis, which is advantageous in the therapy of psoriasis (3,4). Numerous coumarins have sedative properties, and some of them exhibit toxicity. Some coumarins are also applied as fragrances and cosmetic additives. For this reason, there is a necessity for the analysis of coumarins in various plant material. Coumarins can be also markers for the chemotaxonomic identification of plant species.

Simultaneously, groups of coumarin derivatives, such as simple coumarins, furanocoumarins, or piranocoumarins, have different polarity, and they are relatively easy for group separation, certainly in the extraction step. However, in a particular group, they have similar chemical structures and physicochemical properties and, thus, are difficult to separate in one run. Very often, the separation should be performed in reversed-phase (RP) systems, especially when the molecules differ in the presence or position (or both) of nonpolar fragments (e.g., alkyl group or aromatic ring). When molecules differ in the polar group present or position they frequently require a normal-phase system for separation. This means that in the case of so-called "difficult separations" a multidimensional separation of closely related compounds should be performed.

In planar chromatography, multidimensional separations are relatively easy to perform. Thin-layer chromatography (TLC) gives the possibility of multidimensional separations by the use of the same layer and different eluent systems (5,6) or by the use of multiphase plates (7–9). Both methods can make use of various selectivities to obtain a complete separation in a two-dimensional (2D) process. Polar-bonded stationary phases and multiphase plates can be applied in normal-phase systems with an adsorption mechanism of separation and in RP systems with a partition mechanism of separation. Thus, various separation selectivities can be availed for the separation of structural analogues.

The aim of our work was the optimization of separation of selected coumarins in various chromatographic systems on polar-bonded stationary phases (CN and Diol) on silica- and alkyl-bonded phases. The application of the most selective systems in 2D-TLC separations of standard mixtures and coumarin fractions from fruits of *Archangelica officinalis* Hoffm. and *Heracleum* species is presented. 2D-TLC connected with densitometry or diode-array detection (DAD)-densitometry was found to be an excellent tool for the quantitative analysis of this group of compounds.

Few papers have described fiber optical scanning in TLC. Application of modern fiber optical TLC densitometric scanners with DAD for the identification and quantitative analysis of fenitrothion in apples (10) and flavonol aglycones in extracts of rose leaves (11) has been described.

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Experimental

TLC was performed on 10- × 10-cm glass Diol F_{254} and CN F_{254} high-performance TLC precoated plates (Merck, Darmstadt, Germany). 2D-TLC was also performed on 10- × 10-cm glass-backed dual phase Multi-K CS5 (3-cm zone of C18 parallel to silica layer) purchased from Whatman (Whatman International Ltd., Kent, UK). Plates were developed in horizontal Teflon chambers with an eluent distributor (DS, Chromdes, Lublin, Poland). Plates were conditioned for 15 min in eluent vapours. In some cases, the unidimensional multiple development (UMD) technique, with complete evaporation of the eluent from the plate after each run, was applied (8).

Samples (2 µL) of 2.5% w/v solutions of the solutes in methanol were spotted to the adsorbent layer. Binary mixtures of modifiers, ethyl acetate (AcOEt)–diisopropyl ether (iPr_2O), dichloromethane (DCM) with *n*-heptane (H) or acetonitrile (MeCN), and methanol (MeOH) in water, were used as eluents. In some cases, the addition of formic acid was performed. Solvents were analytical grade from Polish Reagents (POCh, Gliwice, Poland). The location of the spots was determined under UV light (λ = 254 nm). Chromatograms were scanned at λ-254 nm by Camag TLC Scanner 3 assisted by the computer program Cats 4 (Camag, Muttenz, Switzerland). For videoscan, TLC Camag

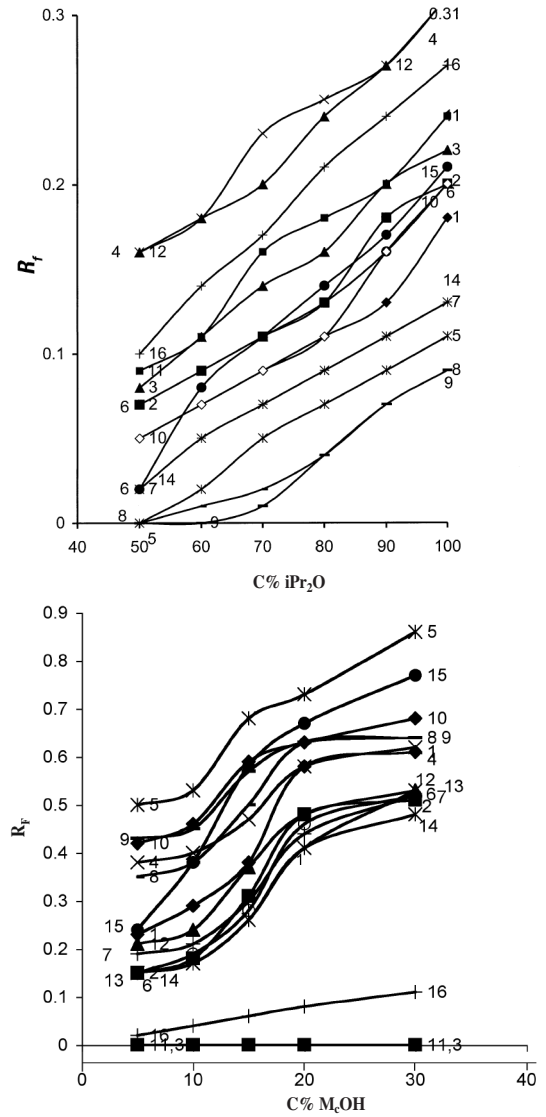
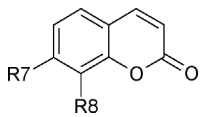
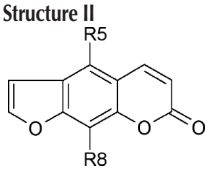
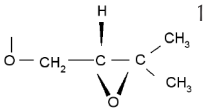
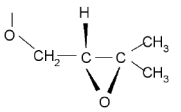
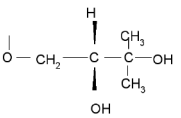
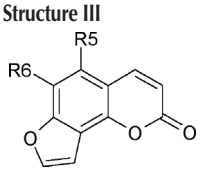


Figure 1. Plots of R_f versus C% (modifier concentration) relationships for investigated compounds on diol layers in eluent systems: diisopropyl ether–*n*-heptane (A) and methanol–water (containing 1% of formic acid) (B). Compound numbers same as Table I.

Table I. Structure of Compounds Investigated

Name of compounds	R5	R6	R7	R8	No.
Structure I 					
Coumarin	–	H	H	–	10
Umbelliferone	–	–	OH	–	4
Aesculetin	–	OH	OH	–	11
Aesculin (6-glicoside aesculetin)	–	OH	OH	–	16
Scopoletin	–	OCH_3	OH	–	7
Isoscopoletin	–	OH	OCH_3	–	15
6-Methylcoumarin	–	CH_3	–	–	20
Fraxidin	–	OCH_3	OCH_3	OH	3
Dimethylfraxetin	–	OCH_3	OCH_3	OCH_3	18
3,4-Dihydrocoumarin	–	–	–	–	17
Structure II 					
Bergapten	OCH_3	–	–	–	12
Xanthotoxin	–	–	–	OCH_3	5
Xanthotoxol	–	–	–	OH	14
Imperatorin	–	–	–	$OCH_2CH=C(CH_3)_2$	6
Phellopterin	OCH_3	–	–	$OCH_2CH=C(CH_3)_2$	19
Heraclenin	H	–	–		13
Byacangelicol	OCH_3	–	–		2
Byacangelicin	OCH_3	–	–		8
Structure III 					
Angelicin	H	H	–	–	9
Isopimpinellin	OCH_3	–	–	OCH_3	1

Reprostar 3 apparatus with a Video store computer program was used (Camag). The TLC-scanner, consisting of a diode-array spectrophotometer (J&M, Aalen, Germany) working in the range λ = 191 to 612 nm, with an average optical resolution better than 2.0 nm (12), was applied to take UV spectra of standards and mixture components. The linear slide system works at a constant speed during reflection measurements (13). The investigated compounds are shown in Figure 1 and listed in Table I.

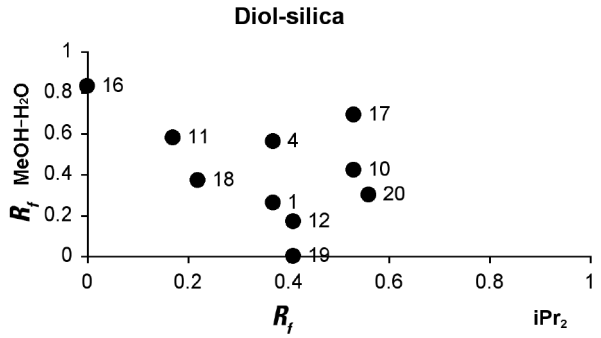


Figure 2. Correlation of R_f parameters for coumarin standards chromatographed on diol-silica in systems: 10% MeOH–water containing 1% formic acid and 100% iPr2O-plate double developed. Compound numbers same as Table I.

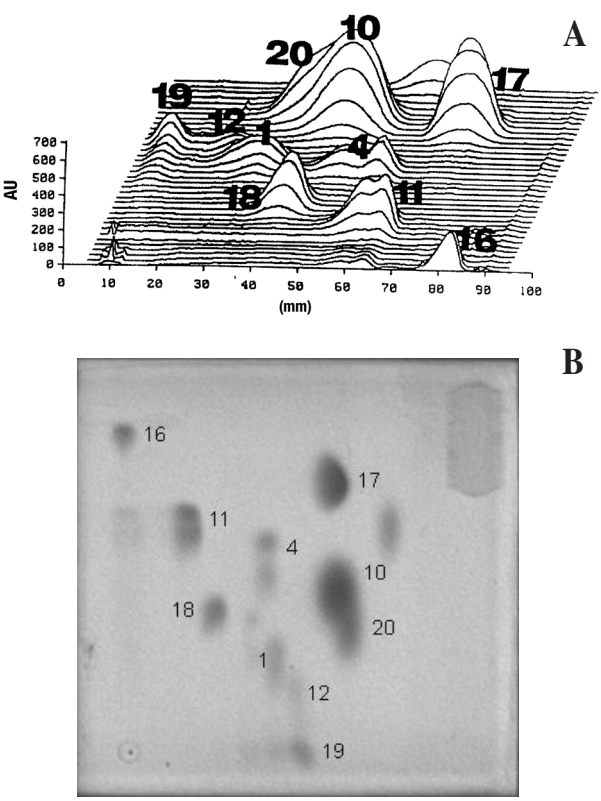


Figure 3. Densitogram (A) and videoscan (B) of two-dimenssional separation of coumarin standards diol-silica in the same systems as Figure 2. Compound numbers same as Table I.

Plant extracts

Plants were collected from the Pharmacognosy Garden of the Medical University of Lublin in the summer of 2003. Fruits of *Heracleum sphondylium*, *Heracleum sibiricum,* and *Archangelica officinalis* and roots of *Heracleum sphondylium* were and dried, ground, and extracted in a Soxhlet apparatus

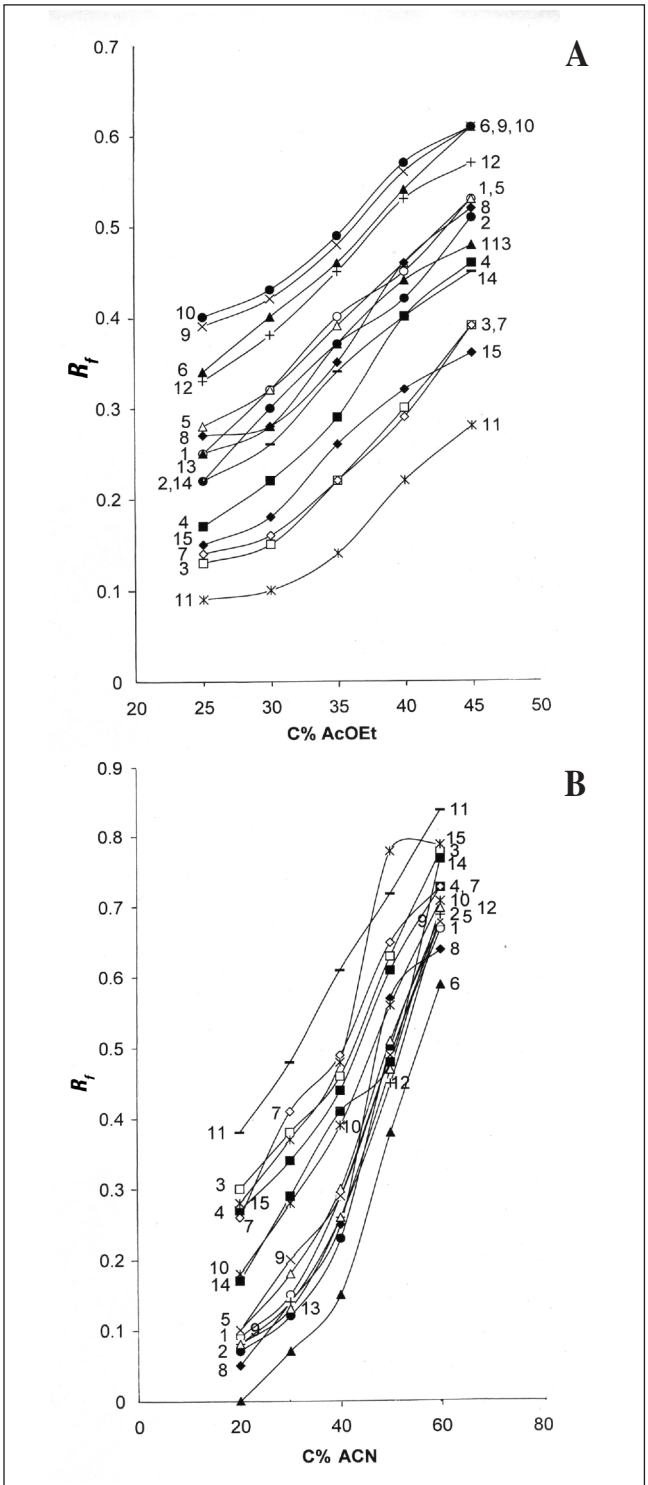


Figure 4. Plots of R_f versus C% (modifier concentration) relationships for investigated compounds on CN-silica layers in eluent systems: ethyl acetate–*n*-heptane (A) and acetonitrile–water (B). Compound numbers same as Table I.

with petroleum ether (20–60°C) for 27 h. Extracts were evaporated to dryness in a vacuum evaporator and dissolved in methanol, filtered, and placed in measured flasks.

Results and Discussion

Coumarin standards were chromatographed in various chromatographic systems on a polar-bonded stationary phases by the use of nonaqueous and aqueous eluents. The examples of retention (eluent composition relationships) are presented in Figures 1A and 1B. Figure 1A shows the retention factor (R_f) versus C% (v/v) of diisopropyl ether–*n*-heptane, whereas in Figure 1B such relationships for methanol–water obtained for the investigated compounds on Diol-silica are presented. It was demonstrated that by changing the modifier concentration, the retention range and selectivity can be controlled. 100% diisopropyl ether and 10% of methanol in water (containing formic acid 1% v/v) was found to be the mobile phases with the best eluent strength and selectivity. In the case of nonaqueous eluent, because of the medium polarity and medium eluent strength of diisopropyl ether, multiple development on the whole distance of the plate with the evaporation of the solvent from the plate after each run was applied. It causes an increase of R_f values and an improvement of the resolution of neighbouring spots (contraction of spots after each run of the mobile phase across the plate) (9). Figure 2 presents a correlation diagram (R_{f1} vs. R_{f2}) simulating separation of investigated standards on the Diol layer. It is seen that diisopropyl ether causes the group separation: coumarin + dihydrocoumarin + 6-methylcoumarin (17 + 10 + 20), isopimpinellin + umbeliferone + bergapten + phelopterin (19 + 12 + 1 + 4), aesculetin + dimethyl fraxetin (18 + 11), and aesculin (16) were partly separated. It should be noted that in the normal-phase system, polar glycoside and coumarins with OH or OCH_3 groups are strongly retained, whereas simple coumarins without any polar substituents are weakly retained. The RP system enabled the complete separation of individual coumarins, availing the differences in their polarity. Furanocoumarins, having a large molecule, are, relatively, strongly retained in this system (compounds 19, 12, and 1), similar to coumarins with methyl or methoxy substituents (compounds 18 and 20). Coumarins with hydroxyl groups, as well as coumarin glycoside, are weakly retained on the diol layer in RP systems. The result of the separation of the coumarin mixture is presented in a videoscan and densitogram (Figure 3A and 3B). This showed that the use of normal-phase and RP systems on the diol layer gives sufficient separation of the selected coumarins [spots (peaks) are dispersed across the whole area of the plate]. Such a 2D separation can be applied for the identification of individual coumarins in natural mixtures.

Similar optimization of separation was performed by use of cyanopropyl-silica and various eluents. Figure 4A shows plots of R_f versus C% of ethyl acetate–*n*-heptane, whereas in Figure 4B, such relationships for acetonitrile–water obtained for investigated compounds on CN-silica are presented. It is seen that by changing the modifier concentration the retention range and selectivity can be controlled. The concentrations, 35% of ethyl acetate in *n*-heptane and 30% of acetonitrile in water, have been found as the mobile phases with sufficient eluent strength and the best selectivity. Thus, coumarins without any substituent

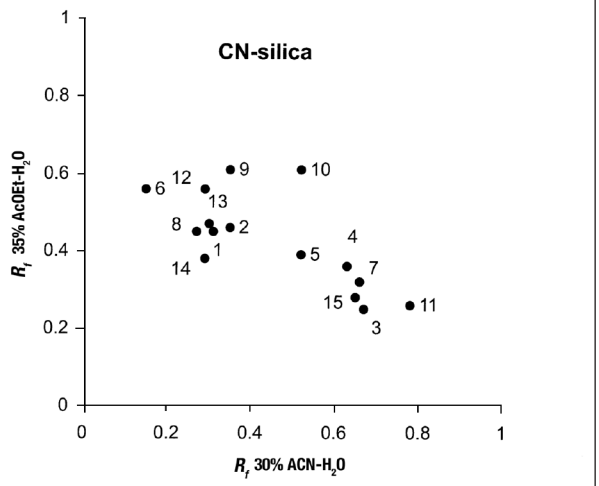


Figure 5. Correlation of R_f parameters for coumarin standards chromatographed on CN-silica in systems with 30% ACN–water, triple developed and 35% AcOEt–*n*-heptane, double developed. Compound numbers same as Table I.

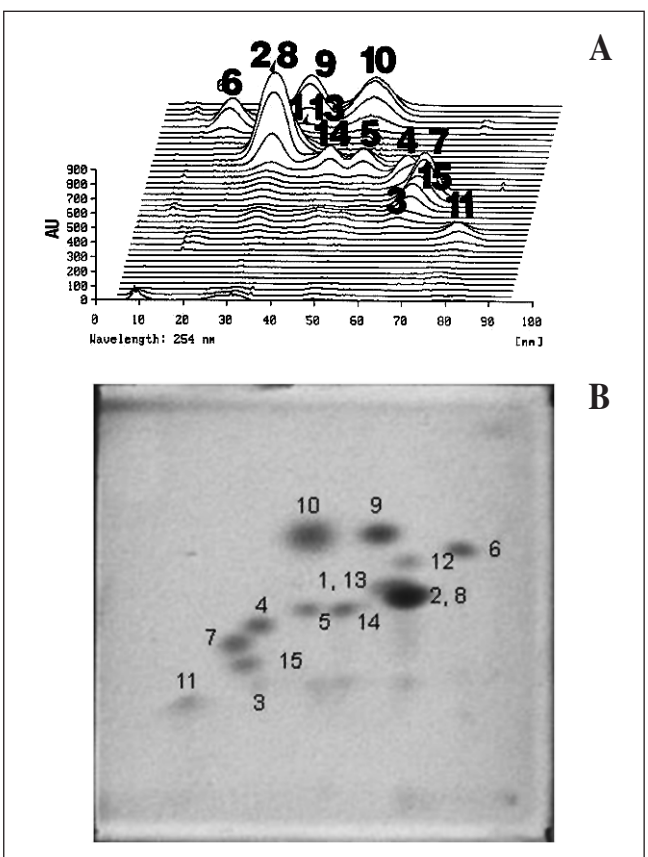


Figure 6. Densitogram (A) and videoscan (B) of two-dimensional separation of coumarin standards on CN-silica plate. Direction eluent: 30% ACN–water, triple developed, I; direction eluent: 35% AcOEt–*n*-heptane, double developed, II. Compound numbers same as Table I.

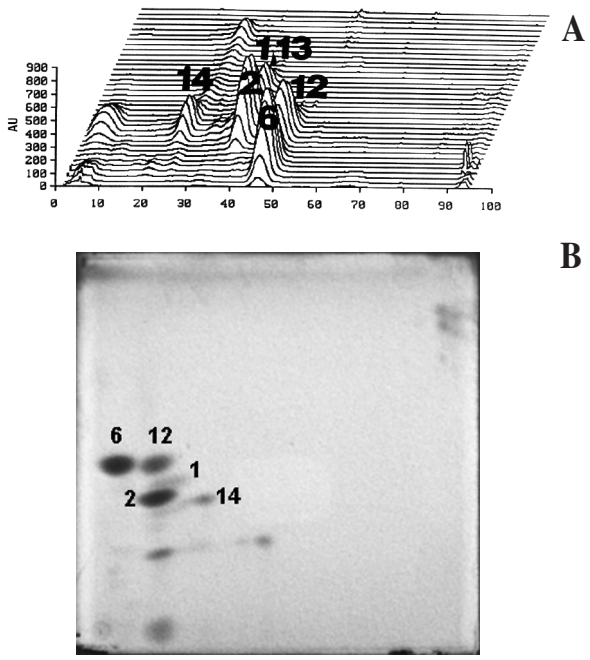


Figure 7. Densitogram (A) and videoscan (B) of two-dimensional separation of *Heracleum sphondylium* fruit extract on CN-silica plate. Same eluents as in Figure 6 and compound numbers same as Table I.

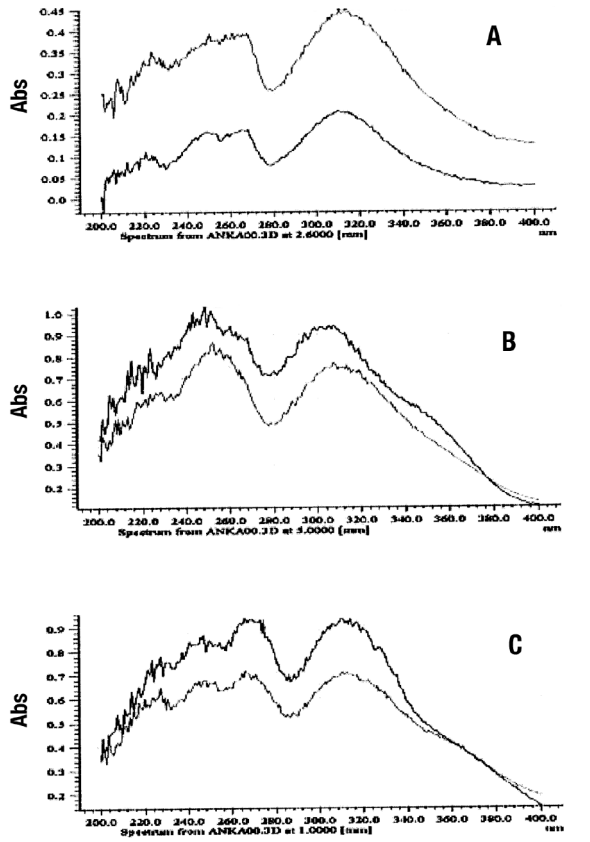


Figure 8. Spectra of some coumarin standards and *H. sphondylium* extract components obtained by DAD densitometer for xanthotoxol (A), imperatorin (B), and byacangelicol (C).

(9 and 10) are weakly retained in a normal-phase system on the CN-silica layer, similar to furanocoumarins with a nonpolar alkyl chain in the 8 position (imperatorin) or with the methoxy group (bergapten). Heraclenin (13), byacangelicin, and byacangelicaol (furanocoumarins with medium polar substituents) were more strongly retained. Simple coumarins with hydroxyl or methoxy groups (or both) (compounds 11, 3, 15, 7, and 4) are more strongly retained on cyanopropyl silica. Different selectivity is obtained for this group in RP systems. In this case, polar coumarins are eluted near the aqueous mobile phase front (compounds 11, 3, 15, 7, and 4). Furanocoumarins are more strongly retained, especially imperatorin with the alkyl chain in the 8 position. The selectivity of separation of the investigated compounds was presented in the correlation diagram of R_f parameters for coumarin standards chromatographed on the CN-silica in systems: 30% acetonitrile–water (v/v) and 35% AcOEt + *n*-heptane (Figure 5). In Figures 6A and 6B, a densitogram and videoscan from the 2D separation of coumarin standards are presented, respectively. By the use of 2D-TLC on CN-silica, the following coumarins can be completely separated: esculetin, 11; fraxidin, 3; isoscopoletin, 15; scopoletin, 7; umbelliferone, 4; xanthotoxin, 5; coumarin, 10; xanthotoxol, 14; byacangelicin + isopimpinellin, 8 + 1; angelicin, 9; byacangelicol + heraclenin, 2 + 13; bergapten, 12; and imperatorin, 6. In Figures 7A and 7B a densitogram and videoscan of the 2D separation of *Heracleum*

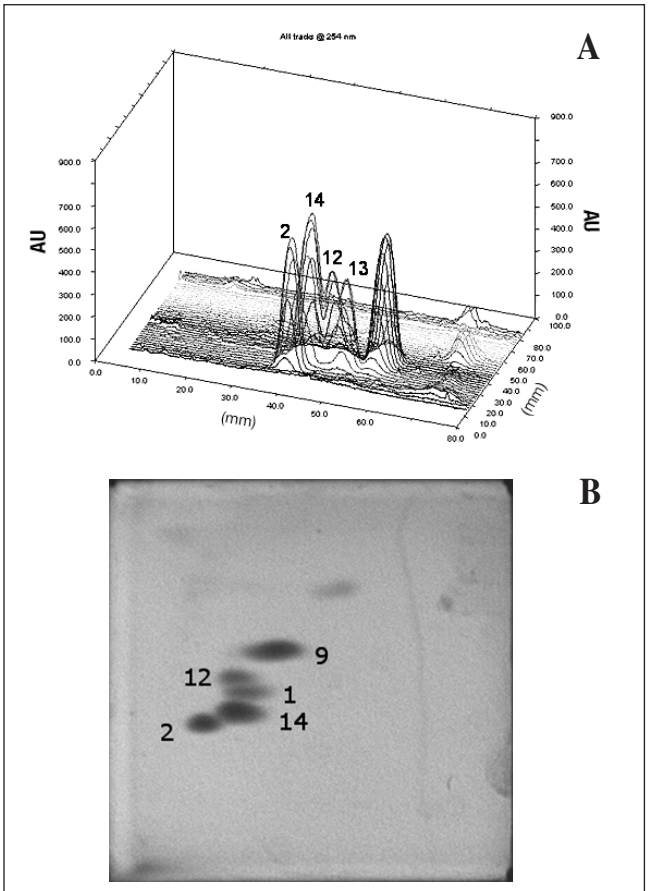


Figure 9. Densitogram (A) and videoscan (B) of two-dimenssional separation of *Heracleum sibiricum* fruit extract on CN-silica plate. Same eluents as in Figure 6.

sphondylium fruit extract in the same systems is presented, respectively. In the chromatogram, the following extract components were identified: isopimpinellin, 1; byacangelicol, 2; imperatorin, 6; bergapten, 12; heraclenin, 13; and xanthotoxol, 14. The identification was also confirmed by the comparison of the UV spectra of the mentioned extract components and the corresponded standards by DAD densitometer (see Figures 8A–8C). TLC–DAD scanner can measure TLC plates simultaneously at different wavelengths without destroying the plate surface and permits parallel recording of chromatograms and in situ UV spectra. Measurements are taken with a set of glass-fibers, which transport light from a deuterium lamp to the surface of the plate.

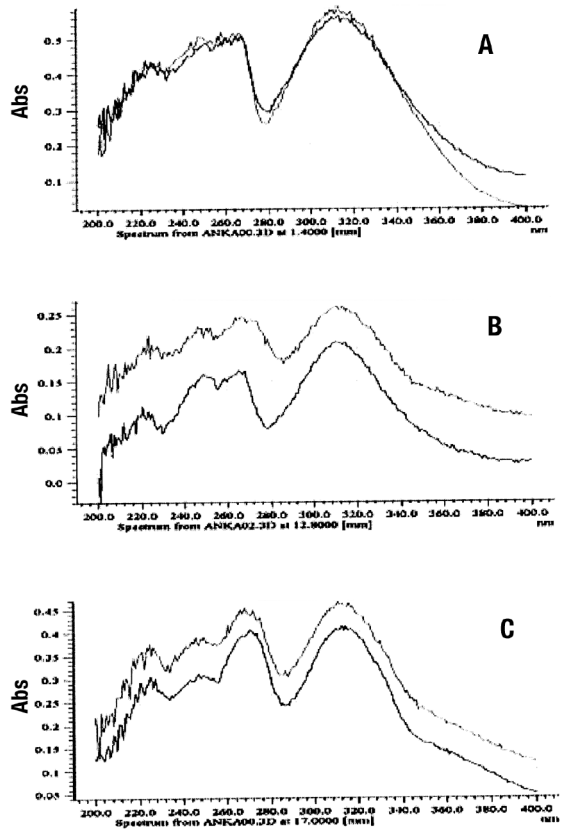


Figure 10. UV spectra of some coumarin standards and *H. sibiricum* extract components obtained by DAD densitometer for bergapten (A); xanthotoxol (B); and isopimpinellin (C). Compound numbers same as Table I.

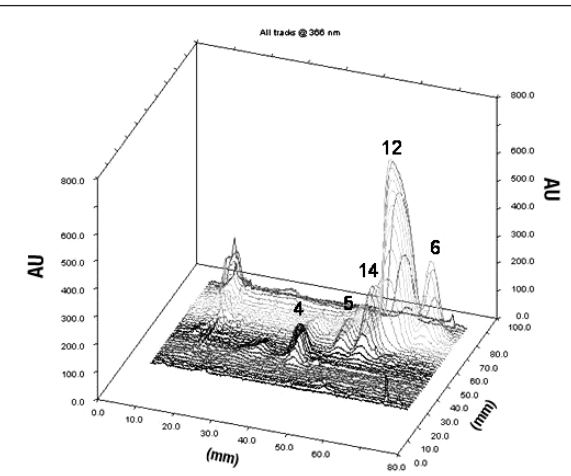


Figure 11. Densitogram of two-dimensional separation of *Archangelica officinalis* fruit extract on CN-silica plate.

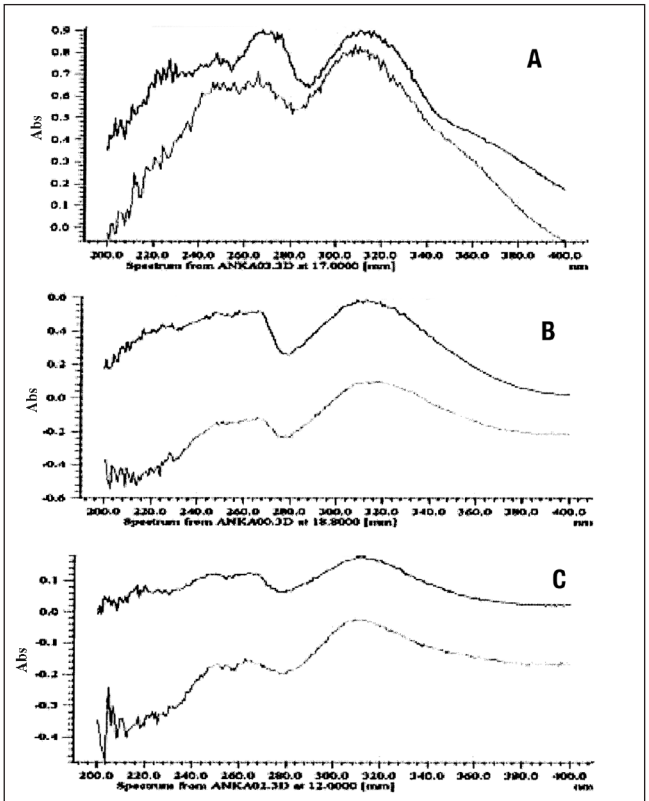


Figure 12. UV spectra of some coumarin standards and Archangelica off. extract components obtained by DAD densitometer. Same eluents as in Figure 6 for phellopterin (A); bergapten (B); and xanthotoxol (C). Compound numbers same as Table I.

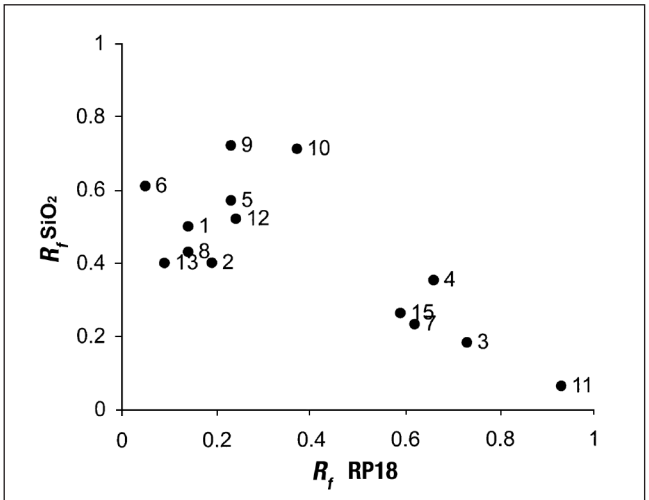


Figure 13. Correlation of R_f parameters for coumarin standards chromatographed in systems: C18 W/55% MeOH-water; silica/35% AcOEt–*n*-heptane, double developed. Compound numbers same as Table I.

The scattered light is reflected by the surface, and the light carries the relevant information. Another set of glass-fibers transports the remission light to the diode-array detector (14,15). Quantitative evalution of thin-layer chromatograms by the optical method is based on differential measurement of light emerging from analyte-free and analyte-containing zones on the plate (16).

According to its position on 2D-TLC CN-layer (see Figures 9A and 9B) and UV spectra (Figure 10), the following compounds were identified in *Heracleum sibiricum* fruit extract: byacangelicol, 2; bergapten, 12; isopimpinelin, 1; and xanthotoxol, 14. The following compounds were also identified from *Archangelica officinalis* fruit extract from a similar chromatogram: umbelliferone, 4; xanthotoxin, 5; xanthotoxol, 14; bergapten, 12; and imperatorin, 6 were identified (see Figure 11A, 11B, and 12). Moreover, based on similar 2D-TLC separations in *Heracleum sphondylium* roots, imperatorin, byacangelicol, and xanthotoxol were identified.

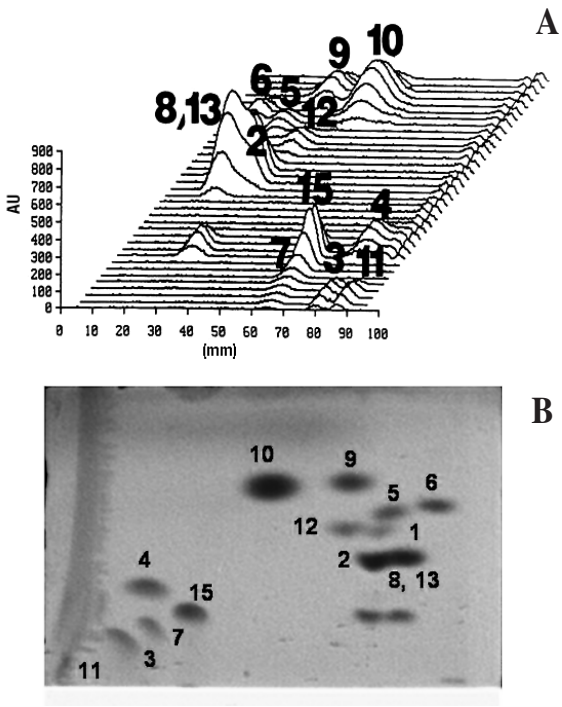


Figure 14. Densitogram (A) and videoscan (B) of two-dimensional separation of coumarin standards on a multiphase plate in systems: C18 W/55% MeOH–water; silica/35% AcOEt–*n*-heptane, double developed. Compound numbers same as Table I.

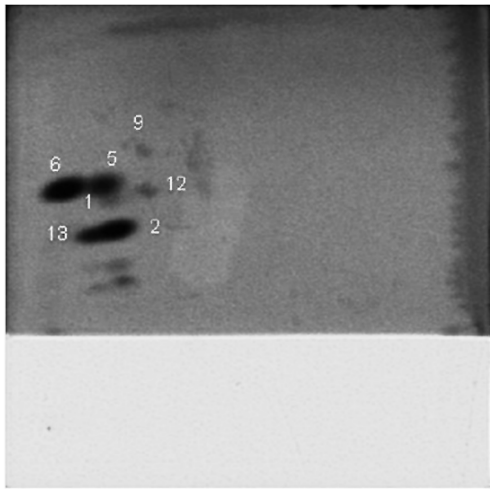


Figure 15. Videoscan of two-dimensional separation of *Heracleum sphondylium* fruit extract on multiphase plate in systems, same as in Figure 13. Compound numbers same as Table I.

Figure 13 presents a correlation diagram of R_F parameters for coumarin standards chromatographed on C18 in an eluent system of 55% MeOH–water and on silica in an eluent system 35% AcOEt–*n*-heptane. This showed that RP-18-TLC enables the group separation of: imperatorin, 6; haraclenin, 13; isopimpinellin, 1; byacangelicin, 8; and byacangelicol, 2. Furanocoumarins with an alkyl chain in the 8 position are the most strongly retained; bergapten, 9; xanthotoxin, 5; and angelicin, 12, while furanocoumarins without any hydroxyl group, also strongly interact with C-18 ligands. Coumarin, without any hydroxyl substituent, is among the simple coumarins most strongly retained on the C18 layer, whereas coumarins with one hydroxyl group (umbeliferone, 4; isoscopoletin, 15; and scopoletin, 7) eluted relatively high in this system. Aesculetin, 11, possessing two hydroxyl groups in the molecule, is eluted most strongly. In a normal-phase system, the molar volume of the molecule does not have a strong effect on retention. The polarity of the molecule has the greatest influence of its retention behaviour. Aesculetin (11), with two hydroxyl groups, was the most strongly retained when compared with other simple coumarins possessing hydroxyl and methoxy groups (fraxidin, scopoletin, and isoscopoletin). The highest R_f values have coumarin and angelicin without any substituent. These differences in retention behaviour enable the separation of an investigated group on two-phase plates connected from a strip of RP sorbent and the silica layer. In Figure 14A and 14B the densitogram and videoscan from the 2D separation of coumarin standards are presented. By the use of 2D-TLC on a multiphase plate, the following coumarins can be completely separated: esculetin, 11; fraxidin, 3; scopoletin, 7; isoscopoletin, 15; umbelliferone, 4; coumarin, 10; bergapten, 12; angelicin, 9; byacangelicol, 2; heraclenin, 13; byacangelicin, 8; isopimpinellin, 1; xanthotoxin, 5; and imperatorin, 6. Figure 15 shows a densitogram and videoscan of a 2D separation of *Heracleum sphondylium* fruit extract in the same systems. The following coumarins were identified: isopimpinellin, 1; byacanglicol, 2; heraclenin, 13; xanthotoxin, 5; imperatorin, 6; angelicin, 9; and bergapten, 12.

Conclusion

Polar-bonded statoionary phases (CN-silica and diol-silica) enable the application of aqueous and nonaqueous eluents, which can be use in 2D-TLC separations. It can be applied in difficult separations of compounds with closely related structures and properties, for example, coumarins.

2D separations can be realized on multiphase plates connected

with the C18 strip, and the silica layer can also be applied for the separation of a coumarin mixture.

2D separations in optimized eluent systems connected with densitometry and DAD-densitometry can be applied for the qualitative analysis of coumarins and furanocoumarins present in *Archangelica officinalis* and *Heracleum species* fruits and roots.

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