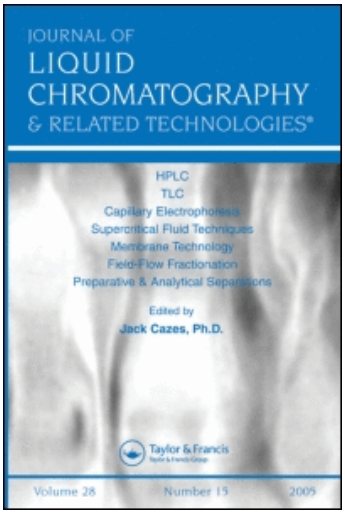

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Overpressured Layer Chromatographic (OPLC) Separation of Closely Related Furocoumarins

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OVERPRESSURED LAYER CHROMATOGRAPHIC (OPLC) SEPARATION OF CLOSELY RELATED FUROCOUMARINS

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ABSTRACT

In this paper, an OPLC method for the analytical separation of eight furocoumarins is described. Of the eight compounds investigated, four are linear furocoumarins (psoralen, bergapten, 8-methoxypsoralen, iso-pimpinellin) and four are angular furocoumarins (angelicin, sphondin, iso-bergapten, pimpinellin). In preliminary experiments, optimization of the mobile phase was made using the "PRISMA" model on TLC plates in unsaturated chambers. Evaluation of the final optimization steps for OPLC separations on HPTLC plates was done densitometrically. With the elaborated OPLC system, seven compounds could be baseline separated, and the resolution between iso-bergapten and angelicin was better than 1.3. Application of the method is demonstrated with the analysis of furocoumarin-containing root extracts from Heracleum sphondylium, H. mantegazzianum and Pastinaca sativa.

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INTRODUCTION

Linear and angular furocoumarins are not only well represented in plants of many genera in the family Umbelliferae [1,2], but also occur in several other plant families [3,4]. Due to the ubiquity of this potently photosensitizing and mutagenic compounds, there is considerable interest in developing reproducible, analytical determination methods.

The four linear furocoumarins examined have been separated by reversed-phase (RP) high-performance liquid chromatography (HPLC) by Beier [5]; all eight compounds investigated were baseline separated with RP-HPLC by Erdelmeier et al. [6]. Glowniak and Bieganowska [7] separated 11 coumarins (including 6 furocoumarins) by RP-thin-layer chromatography (RP-TLC) and RP-HPLC. With stepwise gradient TLC in sandwich chamber, Glowniak et al [8] chromatographed several plant extracts (Pastinaca sativa, Heracleum sphondylium, Sium sisarum, and Libanotis intermedia) containing various coumarins and four furocoumarins, whereby the furocoumarins formed two pairs. For quantitative determinations this method is not appropriate since the resolution, as may be observed on the figures was insufficient.

In addition to the analytical separation of the five main components of Heracleum sphondylium by overpressured layer chromatography (OPLC), the preparative OPLC separation with on-line detection and collection of eluting compounds was demonstrated in [9]. To date the eight investigated furocoumarins have not as yet been separated using planar chromatographic methods.

In this study the OPLC was employed for the separation of the eight isomers. This planar chromatographic method, developed by Tyihák et al [10-12], offers the advantages of a forced flow technique and the feasibility of separations using HPTLC plates over a larger distance than 4.5 cm without loss in resolution. From different optimization methods in liquid chromatography [13], e.g. window diagram technique [14], the "PRISMA" model [15-18] was choosen for the selection of the mobile phase.

EXPERIMENTAL

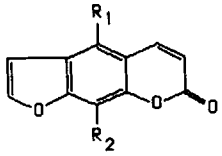
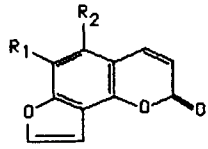
Apparatus : Samples were applied with a Linomat III TLC spotter from Camag (Muttenz, Switzerland) with a 12 mm band width. The OPLC separations were carried out with a Chrompres-10 overpressure layer chromatograph from Labor MIM (Budapest, Hungary). The densitograms were taken with a high speed scanner CS-920 from Shimadzu (Kyoto, Japan) at a wavelength of 295 nm.

Chromatographic plates : The preassays were carried out with TLC plates, while for the OPLC experiments, HPTLC plates with impregnated edges, both with silica gel 60 F_{254} from Merck (Darmstadt, F.R.G.), were used. The impregnation with a special polymer solution from Labor-MIM (Budapest, Hungary) was made so that the plate was compactly sealed all around. Two channels were scraped out of the silica, one at the solvent inlet and the other at the solvent outlet at a distance of 18 cm.

Solvents : All solvents used for the TLC preassays were of reagent grade. The final optimization steps were carried out with solvents of analytical quality (Merck,Darmstadt, F.R.G.). The chloroform used had to be stabilized with 2-methyl-2-butene since ethanol influences the separation.

Samples : The structures of the eight furocoumarins investigated are given in Table 1, where the numbers represent the spot succession in the final OPLC system. Angelicin and psoralen were synthesized [19] and identified with HPLC references from Roth (Karlsruhe, F.R.G.); 8-methoxypsoralen was purchased from Fluka (Buchs, Switzerland). The other five furocoumarins were isolated from Heracleum sphondylium roots [20], which were purchased from Dixa (St. Gallen, Switzerland). Roots of H. mantegazzianum were collected in Switzerland, and those of Pastinaca sativa were collected in Italy.

TABLE 1: Structures of Furocoumarins Investigated

	R_1	R_2	Name	Symbol
	-H	-H	Psoralen	(3)
	$-OCH_3$	-H	Bergapten	(4)
	-H	$-OCH_3$	8-Methoxypsoralen	(7)
	$-OCH_3$	$-OCH_3$	Iso-pimpinellin	(8)
	-H	-H	Angelicin	(2)
	$-OCH_3$	-H	Sphondin	(6)
	-H	$-OCH_3$	Iso-bergapten	(1)
	$-OCH_3$	$-OCH_3$	Pimpinellin	(5)

The dried and pulverized roots (0.5 - 1.0 g of each) were extracted with chloroform. The crude extracts were dissolved in methanol (10 mg/ml) and prepurified over Bond-Elut C_{18} cartridges (Analytichem Intern., Habor City, CA, U.S.A.).

RESULTS AND DISCUSSION

TLC Preassay

Optimization of the mobile phase for the separation of the eight furocoumarins (Table 1) was carried out with the "PRISMA" model [15-18]. For the TLC preassays, they were applied in two groups: on the left side, the five main components of H. sphondylium, and on the right side the other three furocoumarins. Based on their properities as proton acceptors, proton donors, and their dipol interactions, ten selected solvents (Table 2) from the Snyder [21] classification were used as mobile phase for the preliminary experiments on TLC plates in unsaturated chambers (Fig. 1, row A).

TABLE 2 : Selected Solvents for the TLC Preassays

Group	Solvent	Solvent strength value (Si)
-	Hexane	0
I	Diethyl ether	2.8
II	2-Propanol	3.9
	Ethanol	4.3
III	Tetrahydrofuran	4.0
IV	Acetic acid	6.0
V	Dichloromethane	3.1
VI	Dioxane	4.8
	Ethyl acetate	4.4
VII	Benzene	2.7
VIII	Chloroform	4.1

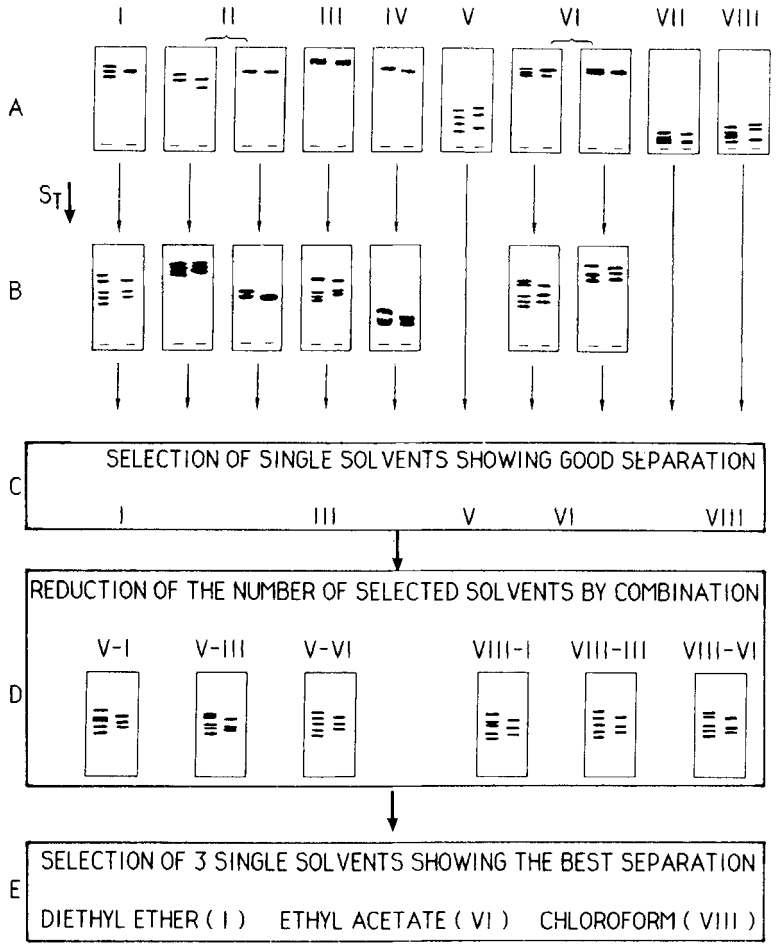


FIGURE 1 : Optimization steps on TLC plates.
Row A: single solvents as mobile phase; Row B : reduction of the solvent strength (S_T) with hexane; Row C : selection of single solvents showing good separation; Row D: combination of dichloromethane (V) and chloroform (VIII) with the solvents giving higher Rf-values; Row E: final selected solvents.

To spread the spots over an Rf range between 0.2 and 0.8, the solvents of group I (diethyl ether), II (2-propanol and ethanol), III (tetrahydrofuran), IV (acetic acid) and VI (ethyl acetate and dioxane) had to be diluted with hexane (Fig. 1, row B). The separation with 2-propanol, ethanol, acetic acid, and dioxane was insufficient. With benzene, the substances rested near the starting point. With regard to further application of the final elaborated system for preparative separations with on-line detection, benzene was inappropriate for the following optimization steps.

Dichloromethane (V), diethyl ether (I) diluted with hexane (1:2) as well as tetrahydrofuran (III) and ethyl acetate (VI) each diluted with hexane (2:1) resulted in good separation. Although the Rf values were lower than 0.4 with chloroform (VIII) good selectivity was observed.

In the next step from the five selected solvents (Fig. 1, row C), dichloromethane and chloroform were combined, each with the solvents giving higher Rf values and with hexane (Fig. 1, row D). With these combinations, tetrahydrofuran (III) gave lower ΔRf values than diethyl ether (I) and ethyl acetate (VI). Chloroform (VIII) showed better selectivity than dichloromethane (V). Using ethyl acetate or diethyl ether as a mobile phase, the succession of the eight compounds was identical, but chloroform showed another spot succession.

Due to these results, ethyl acetate (VI), diethyl ether (I), chloroform (VIII), and hexane were chosen to build the prism (Fig. 1, row E).

The "PRISMA" model, constructed with the three selected solvents (A, B, C) and hexane, allows the following 15 basic combinations (Figure 2):

1 - 3 The three selected single solvents A,B,C, (if necessary diluted with hexane) are represented by the edges of the prism.

4 - 6 Combination of two solvents diluted with hexane; selectivity points along the sides of the regular part of "PRISMA"-model ($S_T < S_C$).

7 Combination of three solvents diluted with hexane within the "PRISMA"-model ($S_T < S_C$).

8 - 10 Combination of two solvents diluted with hexane; selectivity points along the sides of the upper part of the model ($S_T > S_C$).

11 Combination of three solvents diluted with hexane; selectivity point within the upper frustum of the model ($S_T > S_C$.)

12 - 14 Combination of two solvents represented by the edges of the top triangle in the "PRISMA" model.

15 Combination of three solvents, represented by the middle point of the top triangle.

In this case, solvent A is represented by ethyl acetate (S_A=4.4), B by chloroform (S_B=4.1), and C by diethyl ether (S_C=2.8). Of the single solvents tested, diluted with hexane if necessary (see **1-3** in Fig.2), chloroform showed the best selectivity as a mobile phase. With the different combinations of three or four solvents in the regular part of the prism (see **4-7** in Fig. 2), the resolution was not better than with ethyl acetate and hexane or diethyl ether and hexane. Because of these results, the combinations of the upper frustum of the prism (see **8-15** in Fig.2) were tested, except for the solvent combinations **9** and **14** since the solvent strength would be to high.

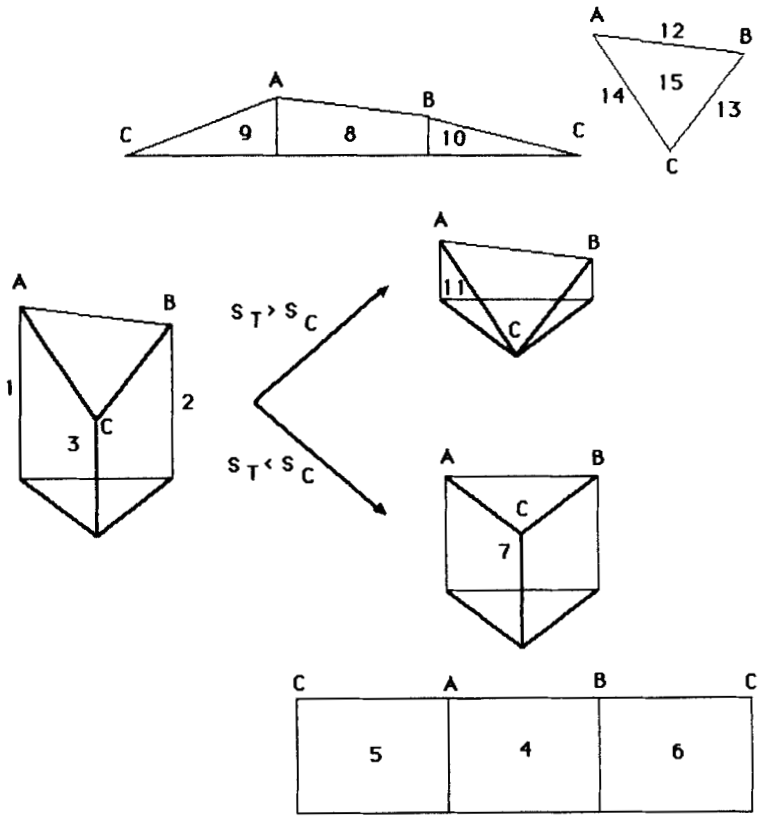


FIGURE 2 : Combination possibilities in the "PRISMA" model

These experiments showed that changes in selectivity and resolution were already highly influenced by low amounts of ethyl acetate and diethyl ether in chloroform. The best separations were obtained with solvent combinations at the top triangle of the prism near the edge of chloroform.

Due to these results, the spot succession was studied by combining chloroform with various amounts of ethyl acetate (Fig. 3) or diethyl

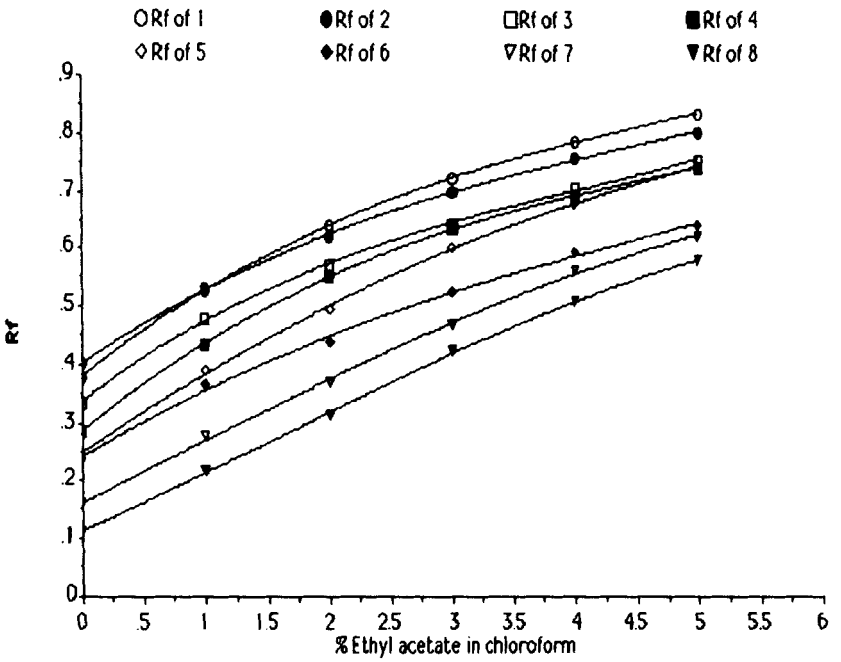


FIGURE 3 : Rf values for furocoumarins as a function of % ethyl acetate in chloroform.

ether (Fig. 4). The densitometric evaluation of this experiment is shown in Figures 3 and 4.

The curves in Figure 3 show that with chloroform as the mobile phase the separation of pimpinellin (5) and sphondin (6) was insufficient. With 1 % ethyl acetate, iso-bergapten (1), and angelicin (2) changed their place and with 3 % or more the separation of psoralen (3), bergapten (4), and pimpinellin (5) was insufficient.

As shown in Figure 4, iso-bergapten (1) and angelicin (2) changed their succession with 1,5 % diethyl ether in chloroform, and the ΔRf

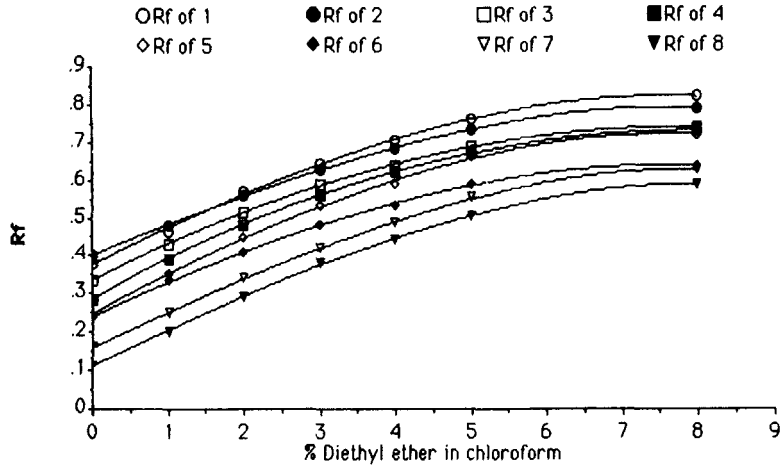


FIGURE 4 : Rf values for furocoumarins as a function of % diethyl ether in chloroform.

values between psoralen (3), bergapten (4), and pimpinellin (5) are decreased with more than 4 % diethyl ether in chloroform.

The best separations in the TLC preassays were obtained with 2 % ethyl acetate or with 3 % diethyl ether in chloroform.

Final Optimization Steps in OPLC

For the OPLC separation of these eight furocoumarin isomers three single solvents or (if necessary) the three solvents diluted in hexane were tested (Figure 5). In addition, various mixtures close to the best solvent combinations determined in the TLC preassays were examined.

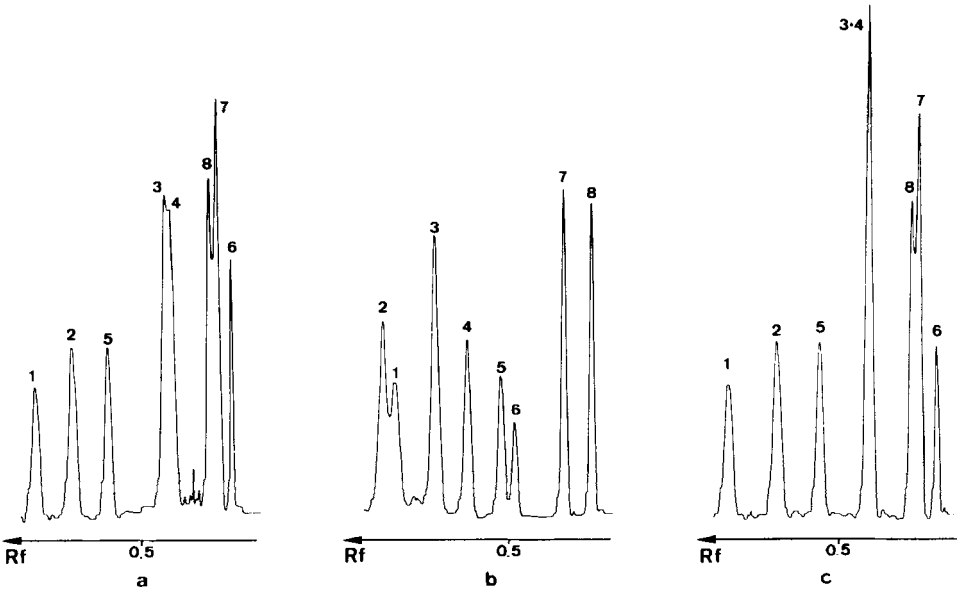


FIGURE 5: Densitograms of OPLC separations with single solvents. a) ethyl acetate-hexane (10:90); b) chloroform; c) diethyl ether-hexane (10:60).

To avoid the disturbing zone [22], a prerun with hexane was tested, but in this case it had a negative influence on separation in the higher Rf range. The disturbing zone was, therefore, eliminated by increasing the inlet pressure (to 1.5 bar) and by reducing the solvent strength of the mobile phase by diluting with hexane. Due to this modification, the separation was complete in 40 minutes. Using this method to eliminate the disturbing zone, the separations were further optimized.

The densitograms of the separations with the single solvents (see case 1, 2 and 3 in Fig. 2) are given in Figure 5. With ethyl

acetate and diethyl ether, each diluted with hexane, psoralen (3) and bergapten (4), iso-pimpinellin (8) and 8-methoxypsoralen (7) could not be separated. Chloroform as a mobile phase resulted in good separation except for angelicin (2) and iso-bergapten (1).

The spots of angelicin (2) and psoralen (3) were placed exactly between the spots of iso-bergapten (1) and bergapten (4) by choosing new mobile phase combinations near the selectivity points found in the previous TLC preassays.

For the combination of chloroform with ethyl acetate (see 12 in Fig. 2) the best separation was obtained with a proportion of 97.6 to 2.4 diluted with 65 % hexane. With this mobile phase, the resolution between psoralen (3) and bergapten (4) and between sphondin (6) and 8-methoxypsoralen (7) was better than 1.25 with all other compounds baseline separated.

From the combination of chloroform with diethyl ether (see case 13 in Fig. 2), the best result was obtained with the same relation of 97 to 3 as found in the TLC preassays. This combination was diluted with 70 % hexane. The resolution between iso-bergapten (1) and angelicin (2) was better than 1.25 and better than 1.1 between peak 3 (psoralen) and peak 4 (bergapten). For all other compounds baseline separations could be achieved.

No other three solvent combination resulted in better resolution for all eight furocoumarins. The optimal OPLC separation was achieved with a four solvent combination of ethyl acetate, chloroform, and diethyl ether (1.0:97.5:1.5) (see case 7 in Fig. 2), diluted for OPLC with 65 % hexane. The densitometric evaluation of this separation is presented in Figure 6. Except for iso-bergapten and angelicin, which exhibited a resolution better than 1.3, all compounds were baseline separated.

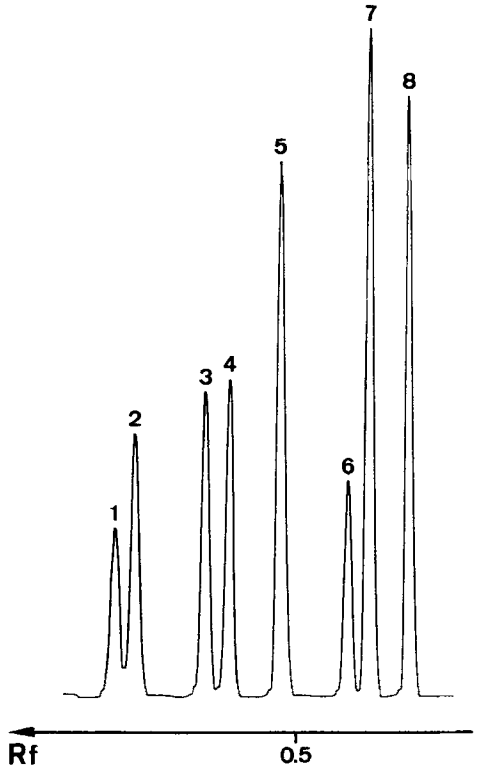


FIGURE 6 : Densitogram of eight investigated furocoumarins separated by OPLC; Separation on HPTLC plate; distance 18 cm; time 34 min; cushion pressure 10 bar; starting pressure 1.5 bar.

With the final elaborated OPLC system, the prepurified root extracts of Heracleum sphondylium, H. mantegazzianum and Pastinaca sativa were analyzed (Figure 7) [23]. The extract of H. mantegazzianum contained all eight furocoumarin isomers of interest. In the extract of H. sphondylium (except for psoralen) all investigated furocoumarins were detected in different concentrations. The main components in the extract of Pastinaca sativa were angelicin, psoralen and 8-methoxypsoralen.

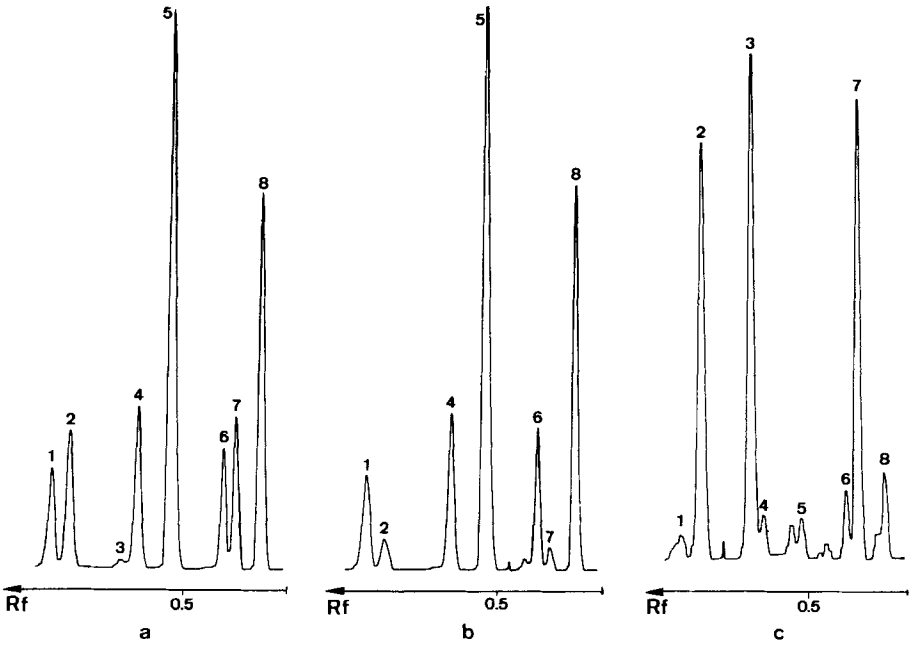


FIGURE 7 : Densitograms of furocoumarins in root extracts
a) Heracleum mantegazzianum; b) H. sphondylium and c) Pastinaca sativa.

In comparison with the earlier published HPLC system [8], the presented planar chromatographic method needs no gradient and allows analysis of between 10 and 17 samples in parallel with good resolution. With a densitometer, the spots may be detected independently of the chromatographic process, allows selection of the most appropriate wavelength.

Due to the ubiquitous distribution of the investigated furocoumarins, they could serve as chemotaxonomic markers, and the elaborated OPLC system may be applied for such systematic investigations.

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