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Optimization of the high-performance liquid chromatography of coumarins in *Angelica archangelica* with reference to molecular structure

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SUMMARY

Molecular connectivity indices were calculated and compared with measured isocratic and gradient (binary gradient and isoselective multi-solvent gradient) reversed-phase high-performance liquid chromatographic retention data of ten coumarins in *Angelica archangelica*. Retention measurements were performed, using organic solvent–water eluents, containing methanol, ethanol, 1-propanol, tetrahydrofuran, dioxane, acetonitrile and their mixtures, according to the "PRISMA" model in the optimization process. The solvent strength values were obtained experimentally. A baseline separation of the ten coumarins was achieved. The elution order of the coumarins varied according to the solvent. Decreasing the volume fraction in many instances increased the separation factor. The compounds were divided into two groups. The path type of the fourth-order and path/cluster type of the fourth-order valence level indices best described the retention. High correlations were observed between the calculated and measured retentions. The retention could be well predicted for different selectivity points in the "PRISMA" using the molecular connectivity indices.

INTRODUCTION

The retention of compounds in high-performance liquid chromatography (HPLC) analyses can be predicted by using molecular connectivity indices[1–3]. The concept of molecular connectivity was introduced by Randic[4] and further developed by Kier and Hall[5]. The molecular connectivity index terms are numerical values which are fundamental in defining and quantitatively describing the adjacency relationships in the molecular structure. When the nature of the atom is not taken into consideration, the index is referred to as the connectivity level, χ; when it is, the index is described as

the valence level, χ^v. Connectivity indices have been extended to include indices of different orders, in addition to subgraphs composed of paths, clusters and path/clusters, which are described by the subscripts p, c and pc, respectively.

Coumarins usually contain many heteroatoms. The behaviour of heteroatom-containing molecules is sometimes difficult to predict on the basis of molecular connectivity indices. Kier and Hall[5] improved the correlation between the water solubility of oxygen-containing compounds and the corresponding molecular connectivity indices by adding the vertex value, δ, of the oxygen atom to the regression equation for alcohols and ethers. A similar procedure gave a high correlation for boiling points and partition coefficients in the case of primary, secondary and tertiary amines. As shown by Lehtonen[6], the retention of oxygen-containing amines was difficult to predict when analysed together with oxygen-free amines.

The retention behaviour of ten closey related coumarins in *Angelica archangelica* (L.) was studied, using six common solvents in reversed-phase liquid column chromatography. The aim of the study was to compare molecular connectivity indices with the retention measurements. A further aim was to optimize the HPLC separation of ten closely related coumarins by using the "PRISMA" model[7] with reference to molecular connectivity indices describing the molecule structure. The indices were used to study the predictability of the HPLC behaviour and optimization.

EXPERIMENTAL

Apparatus
    A Waters Assoc. Model 6000A liquid chromatograph, equipped with a Pye Unicam PU4020 UV detector and a Hewlett-Packard 3390A integrator, was used.

Chemicals
    The coumarins (Fig. 1) (+)-oxypeucedanin, ostruthol and isoimperatorin were isolated from *Angelica archangelica* (L.) at the Pharmacognosy Division, Department of Pharmacy, University of Helsinki. Angelicin, bergapten, isopimpinellin, umbelliferon, and xanthotoxin were obtained from Roth (Karlsruhe, F.R.G), imperatorin from Serva (Heidelberg, F.R.G.) and scopoletin from Sigma (St. Louis, MO, U.S.A.). The water used was distilled and deionized. 1,4-Dioxane was of HPLC quality from E. Merck (Darmstadt, F.R.G.) and absolute ethanol from Alko (Helsinki, Finland). All other solvents were of HPLC quality from Rathburn Chemicals (Walkerburn, U.K.).

Chromatographic conditions
    The column was a 200 × 4 mm I.D. tube, laboratory-packed with Spherisorb S5 ODS-2 (Phase Separations, Queensferry, U.K.), thermostated at 50.0°C. The mobile phase was prepared from acetonitrile, dioxane, ethanol, methanol, 1-propanol and tetrahydrofuran (THF), each diluted with water, and their mixtures (see Results and Discussion). The flow-rate was 1.5 ml/min and detection was effected at 320 nm. The samples were diluted to give the smallest detectable peaks at 320 nm. The dead volume was determined at 250 nm with 0.2 μl of aqueous sodium nitrite.
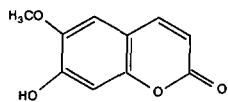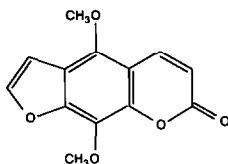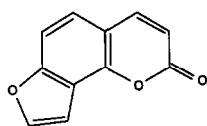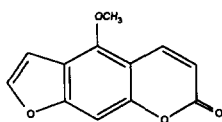
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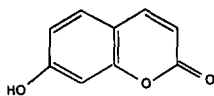
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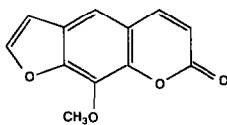
3. Xanthotoxin

4. Isopimpinellin

5. Angelicin

6. Bergapten
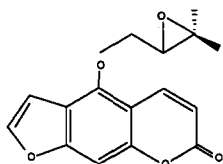
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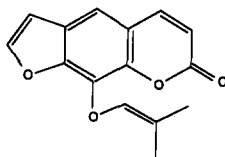
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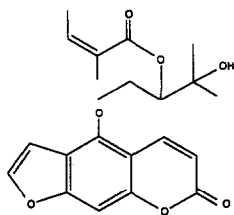
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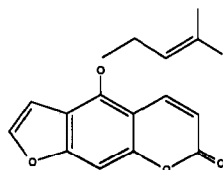
10. Isoimperatorin

Fig. 1. Structures of the coumarins

Calculation of molecular connectivity indices

The molecular connectivity indices for the coumarins were calculated using a BASIC program, as described previously for dansylamides[6]. The following general equation, proposed by Kier and Hall[5], was used for computation of an index of type t and order m:

$$^m\chi_t = \sum_{j=1}^{^m n_s} {^m c_j} = \sum_{j=1}^{^m n_s} \left[\prod_{i=1}^{m} (\delta_i)_j^{-1/2} \right]$$

where $^{m}c_j$ is the subgraph term for mth-order subgraphs, m_{n_s} is the number of mth-order subgraphs and δ is the connectivity level vertex value or the valence level vertex value given to the atoms.

Correlation between retention and molecular connectivity indices

The Macintosh StatView 512+ procedure was used for determining the index best describing retention, using linear regression analysis. The coumarins were divided into two groups according to their retention behaviour with reference to connectivity indices: group I (compounds **3, 6, 8** and **10**; see Fig. 1) and group II (compounds **1, 2, 3, 4, 5, 6, 7** and **9**; see Fig. 1).

RESULTS AND DISCUSSION

Retention measurements were carried out on the closely related coumarins in volume fractions (φ) acetonitrile (54–42%), dioxane (52–42%), ethanol (46–36%), methanol (69–54%), 1-propanol (38–24%) and THF (36–28%), each in six steps, by decreasing the amount of organic solvent in water by 1–4% (v/v).

The retention orders of the eluates in six different organic solvent–water mixtures are presented in Fig. 2 and Table I. The capacity factors (k') in Table I were calculated from the equation $k' = (t_R - t_0)t_0$, where t_R is the retention time of the compound and t_0 is the dead volume. The volume fraction of each organic solvent was chosen such that k' of the last-eluted peak remained nearly the same, irrespective of the solvent used. The retention order of the compounds varied considerably in different solvents; only compound **10** was consistently the last-eluted compound. Acetonitrile showed great opposing shifts for some compounds, *e.g.*, **4** and **7**. Compounds **1** and **2** were unresolved with ethanol but, when injected separately, **2** was eluted first. The same occurred with acetonitrile. With THF and ethanol **4** and **5** were unresolved, whereas **1, 2** and **3** separated in this order. Compounds **1** and **2**, **4** and **5** and **8** and
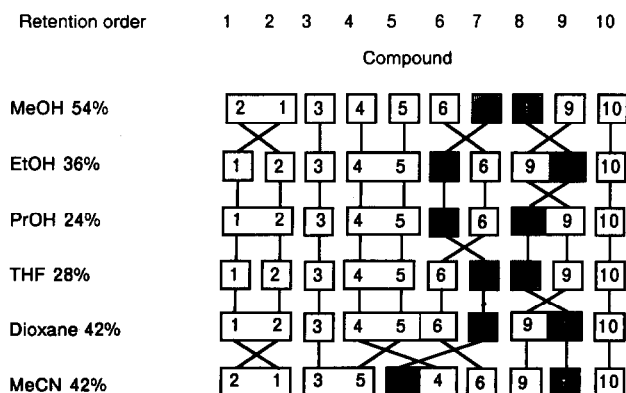
Fig. 2. Retention order of ten coumarins in six organic solvent–water mixtures using isocratic runs with Spherisorb ODS 2 as the stationary phase (compound numbers, see Fig. 1). The concentrations were chosen so that the capacity factor (k') of the last-eluting peak in all mobile phases was *ca.* 25. The compound was regarded to elute in the same peak if the resolution (R_s) was <1.1. MeOH = Methanol; EtOH = ethanol; PrOH = propanol; THF = tetrahydrofuran; MeCN = acetonitrile.

TABLE I

CAPACITY FACTORS, k', OF THE COUMARINS IN SIX ORGANIC SOLVENT WATER MIXTURES

For compounds see Fig. 1.

Compound	k'					
	Methanol (54%)	Ethanol (36%)	1-Propanol (24%)	THF (28%)	Dioxane (42%)	Acetonitrile (42%)
1	0.83	0.51	0.55	0.73	0.44	0.53
2	0.55	0.86	0.60	1.13	0.50	0.47
3	2.44	2.08	1.12	1.95	1.45	2.16
4	2.85	2.69	1.90	2.53	1.66	3.00
5	3.55	2.79	2.28	3.02	1.87	2.23
6	4.30	4.69	3.53	4.77	2.11	4.52
7	5.11	3.99	2.73	8.53	3.40	2.95
8	11.86	11.70	9.22	9.67	7.25	9.87
9	15.61	11.60	9.31	10.94	7.18	8.28
10	24.10	22.05	16.23	17.67	16.48	15.35

9 were unresolved with 1-propanol. THF, 1-propanol and acetonitrile were selected as the most interesting solvents for further studies.

The effect of changing φ on the separation factor (α) was investigated in the six solvents. α-1 was defined as k'_2/k'_1, where k'_2 is the capacity factor for the second-eluting peak and k'_1 that for the first-eluting peak, and similarly α-2 $= k'_3/k'_2$, etc. Fig. 3 shows this effect in THF, 1-propanol and acetonitrile. The change in volume fraction in 1-propanol from 0.24 to 0.27 had the greatest influence on the separation of most of the compounds. However, α-8 and α-5 remained the same or decreased throughout the whole range of φ values. In THF the effect of φ was less than that in 1-propanol, although α-6 and α-5 increased more strongly from 1.4 to 1.7 and from 1.1 to 1.45, respectively. Increasing the amount of acetonitrile improved α to some extent. α-2 remained high throughout the change in φ, i.e., 3.5–4.1.

Table II shows the experimentally obtained solvent strength (S_T) values. S_T values were calculated from observed capacity values using five or six concentrations of the organic solvents (Table I). S_T values were derived from the equation $\log k' = \log k'_w - S_T \varphi$, where k'_w represents the capacity factor of a solute with pure water as mobile phase[8]. The mean S_T values for the solvents varied between 2.8 and 5.3, ethanol having the lowest and THF the highest value. The S_T values obtained here are in good agreement with the values presented elsewhere[6,8–11]. However, the obtained S_T values were strongly dependent on the compounds used in calculating them. The calculated S_T values were used for the solvents when forming the "PRISMA".

As none of the six organic solvents gave a baseline separation in isocratic runs three solvents were selected for further optimization of the mobile phase by the "PRISMA" model[7]. THF, 1-propanol and acetonitrile were chosen on the basis of their effect on the retention order and α for further studies with "PRISMA" (see Fig. 4).

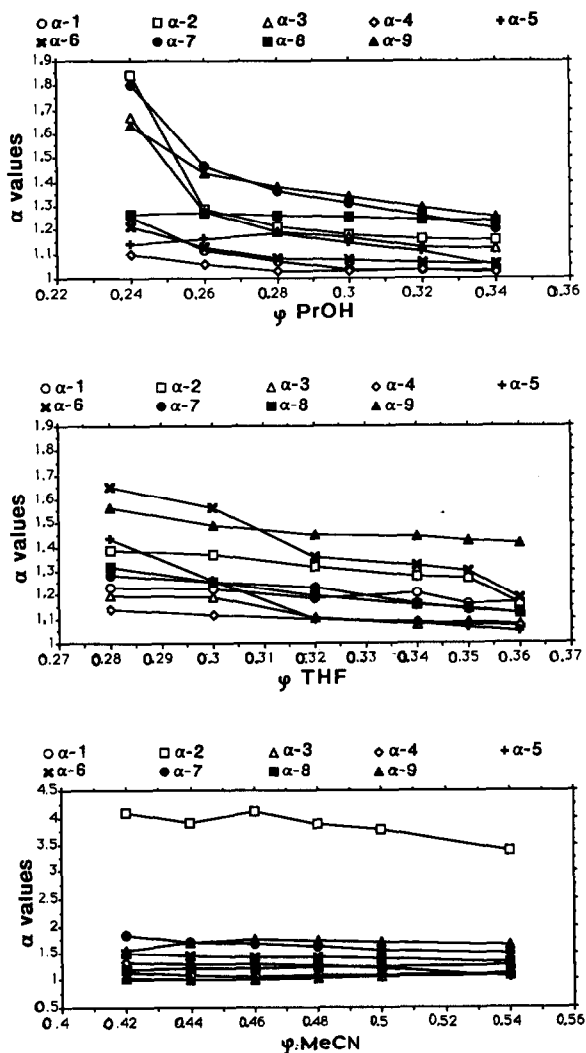Three selectivity points (P_s), 118, 181 and 811, were chosen for further

Fig. 3. Effect of the volume fraction (φ) on the separation factor (α).

optimization of isocratic runs according to "PRISMA" with $S_T = 1.4$. The separation in isocratic runs at any of the P_s was not good. The possibility of carrying out gradient runs was therefore studied. In isocratic runs THF alone proved to be the most promising solvent for obtaining an excellent separation (Fig. 5a). THF was therefore tested in a binary linear gradient run (Fig. 5b). The separation was better than that in the isocratic run, but was still not a baseline separation, especially for **4** and **5** [R_s = 0.82; $R_s = 2(t_2 - t_1)/(w_2 + w_1)$, where t_2 is the retention time for the later eluting compound and t_1 that for the earlier eluting compound, w_2 is the peak width for the later eluting compound and w_1 that for the earlier eluting compound]. Compound **10** was eluted with some impurities that interfered with the separation and made the

TABLE II

SOLVENT STRENGTH (S_T) VALUES CALCULATED FROM OBSERVED k' VALUES USING 5–6 CONCENTRATIONS OF THE ORGANIC SOLVENTS

S_T values were derived from $\log k' = \log k'_w - S_T \varphi$, where k'_w represents the capacity factor of a solute with pure water as mobile phase.

Compound	S_T					
	Methanol	Ethanol	1-Propanol	THF	Dioxane	Acetonitrile
1	1.18	0.58	1.95	2.25	1.30	2.11
2	2.17	0.41	1.77	3.12	1.32	2.15
3	2.76	2.00	2.31	3.96	2.94	2.76
4	3.09	2.22	3.43	4.70	3.10	3.04
5	2.95	2.25	3.45	5.05	2.96	2.46
6	2.91	3.16	3.38	7.34	2.97	3.20
7	2.95	3.01	4.22	7.96	3.74	3.25
8	4.19	4.27	6.94	6.32	4.46	3.60
9	4.88	4.59	5.52	6.03	4.67	3.82
10	4.96	5.08	7.17	6.25	5.94	3.54
Mean	3.20	2.76	4.01	5.30	3.34	2.99
Standard deviation	1.18	1.58	1.94	1.83	1.44	0.61
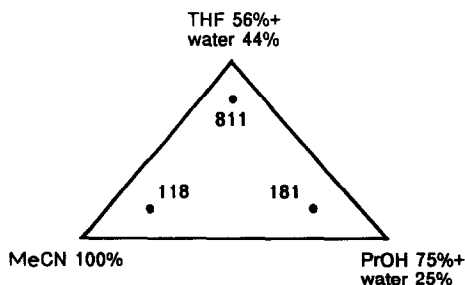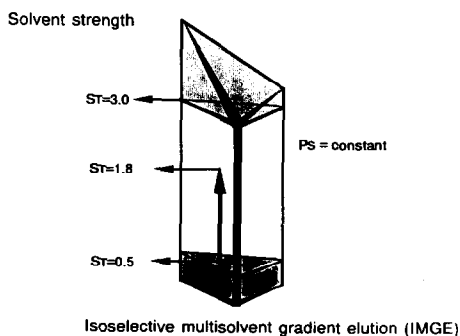
Fig. 4. The "PRISMA" model applied in this study.
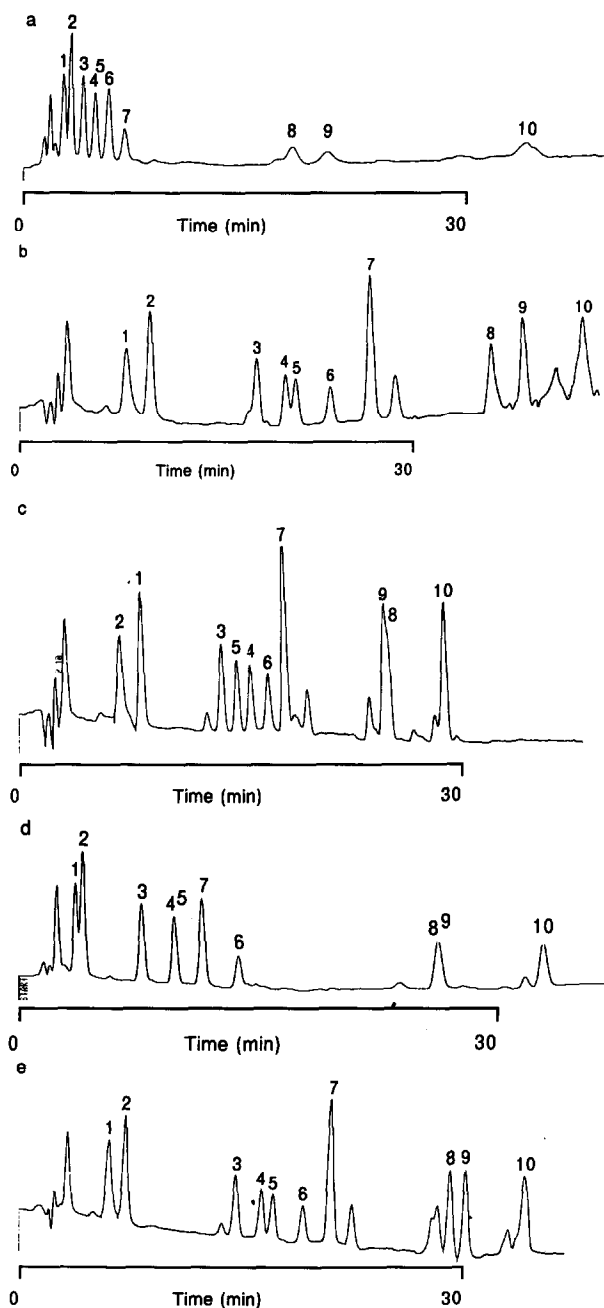
Fig. 5. Separations of the studied coumarins in *A. archangelica*; for compounds, see Fig. 1. Other analytical conditions see Experimental. (a) THF, isocratic run at $S_T = 1.4$; (b) THF, gradient run, S_T from 0.5 to 1.8 in 30 min; (c) IMGE at $P_S = 118$ (THF, 1-propanol, acetonitrile), S_T from 0.5 to 1.8 in 30 min; (d) IMGE at $P_S = 181$, S_T from 0.5 to 1.8 in 30 min; (e) IMGE at $P_S = 811$, S_T from 0.5 to 1.8 in 30 min.

quantitative determination inaccurate. This is the reason why the previously mentioned three P_s values were tested in linear gradient runs (Fig. 5c, d and e). The gradient used here was so-called isoselective multi-gradient elution (IMGE), where the selectivity point remains the same in the gradient run but the solvent strength is changed (Fig. 4)[12]. As can be seen, IMGE at P_s 811 and S_T from 0.5 to 1.8 in 30 min gave an excellent baseline separation of the compounds (Fig. 5e). IMGE was necessary owing to the difference in the polarities of the compounds.

The correlation between indices describing the molecular structure and retention in isocratic and gradient runs was studied. The retention was correlated with the molecular connectivity level (χ_p) and valence level (χ_p^v) indices for the path type through to the sixth order. The connectivity and valence level indices for the cluster (χ_c, χ_c^v) and path/cluster (χ_{pc}, χ_{pc}^v) types were calculated to the fourth order. The compounds were divided into two groups on the basis of their different behaviour in HPLC (see Experimental for groups I and II).

The indices best describing the retention in isocratic runs are presented in Table III. The index best describing the retention for group I was $^4\chi_p$ (Table IV). Group II was best described in most of the eluents by a valence level index i.e., $^4\chi_{pc}^v$ (Table IV). In THF $^2\chi^v$ and in acetonitrile $^3\chi_p$ were selected as best describing the retention (Table IV). The correlation for the solvents showed r values ranging from 0.93 to 0.98. The regression equations are given in Table IV as $k' = A\chi + B$, where A is the slope and B the intercept of the regression curve. A correlation coefficient of 0.95 ($2p < 0.0001$) was achieved (Fig. 6) when correlating the experimental k' values obtained from the isocratic runs with pure solvents with calculated k' values obtained from the equations in Table IV and indices from Tabe III. In isocratic runs the retention can be well predicted. This has also been shown by Vuorela and Lehtonen[13] for eight furo-coumarins.

The correlation between retention and the molecular connectivity indices in gradient runs has not been studied very much. It was investigated here for three solvents, THF, acetonitrile and 1-propanol, with S_T changing from 0.5 to 1.8. The observed k' values are shown in Table V. In all three solvents the index $^4\chi_{pc}^v$ showed the

TABLE III

MOLECULAR CONNECTIVITY INDICES BEST DESCRIBING THE RETENTION OF THE CLOSELY RELATED COUMARINS

Compound	$^3\chi_p$	$^4\chi_p$	$^2\chi^v$	$^4\chi_p^v$	$^4\chi_{p_c}^v$
1	5.01	4.02	2.82	1.25	0.67
2	4.14	3.36	2.78	1.27	0.43
3	6.20	5.54	3.42	1.79	0.76
4	6.98	6.40	3.73	2.08	0.85
5	5.49	4.79	3.07	1.57	0.67
6	6.20	5.59	3.41	1.83	0.82
7	6.92	6.08	5.32	2.09	1.17
8	7.05	6.12	4.81	2.03	0.98
9	10.07	7.80	7.04	2.65	2.04
10	7.22	6.29	4.80	2.06	1.01

TABLE IV

REGRESSION COEFFICIENTS FOR EQUATIONS OF THE TYPE $k' = A\chi + B$ IN SIX ORGANIC SOLVENTS

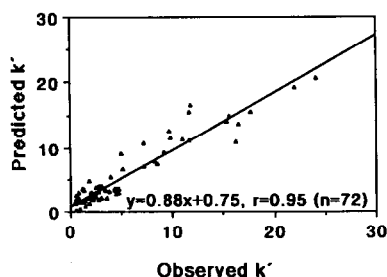| Coefficient	Methanol (54%)		Ethanol (36%)		1-Propanol (24%)		THF (28%)		Dioxane (42%)		Acetonitrile (42%)	
	I^a	II^a	I	II	I	II	I	II	I	II	I	II
A	24.40	9.35	22.40	6.69	17.10	5.38	17.19	2.36	16.96	4.28	15.10	1.32
B	−132.93	−4.26	−121.72	−2.55	−93.14	−2.23	−92.64	−5.13	−93.03	−1.64	−80.94	−5.39
r	0.94	0.97	0.95	0.95	0.96	0.94	0.95	0.95	0.93	0.98	0.97	0.93
Index χ	$^4\chi_p$	$^4\chi_{pc}^v$	$^4\chi_p$	$^4\chi_{pc}^v$	$^4\chi_p$	$^4\chi_{pc}^v$	$^4\chi_p$	$^2\chi^v$	$^4\chi_p$	$^4\chi_{pc}^v$	$^4\chi_p$	$^3\chi_p$

^{a} Groups I and II (see text).

Fig. 6. Plot of the observed and predicted capacity factors (k') for isocratic runs. The predicted values were calculated from equations of the type $k' = A\chi + B$ (see Table IV).

best correlation for group I (Table VI). The indices of the type $^4\chi_p$ best correlated with retention in acetonitrile and 1-propanol for compounds in group II, whereas in THF the $^4\chi_p^v$ indices best described the retention. The correlation for the solvents showed r values from 0.91 to 0.99. The regression equations are given in Table VI as

TABLE V

CAPACITY FACTORS, k', OF THE COUMARINS IN THREE ORGANIC SOLVENT–WATER MIXTURES AND SELECTIVITY POINTS IN GRADIENT RUNS

Compound	k'					
	THF	1-Propanol	Acetonitrile	$P_s = 811$	$P_s = 181$	$P_s = 118$
1	5.14	0.81	2.87	3.58	1.72	5.87
2	6.56	1.20	2.69	4.51	2.05	4.12
3	12.65	5.64	9.46	10.07	4.82	9.31
4	14.42	7.08	12.17	11.40	6.37	10.79
5	14.88	7.08	9.46	11.98	6.37	10.12
6	16.88	9.00	13.82	13.66	9.43	10.67
7	19.31	7.98	12.17	15.11	7.69	12.53
8	26.19	22.68	17.80	21.14	18.91	17.93
9	23.08	22.68	17.08	22.05	18.91	17.70
10	31.53	25.34	20.74	25.18	23.92	20.80

TABLE VI

REGRESSION COEFFICIENTS FOR EQUATIONS OF THE TYPE $k' = A\chi + B$ IN THE THREE ELUENTS FOR GRADIENT RUNS

Coefficient	THF		1-Propanol		Acetonitrile	
	I^a	II^a	I	II	I	II
A	56.61	12.27	31.64	2.92	39.01	3.40
B	−32.72	−10.47	−15.32	−7.38	−19.16	−8.48
r	0.99	0.94	0.91	0.91	0.97	0.93
Index χ	$^4\chi_{pc}^v$	$^4\chi_p^v$	$^4\chi_{pc}^v$	$^4\chi_p$	$^4\chi_{pc}^v$	$^4\chi_p$

^{a} Groups I and II (see text).

TABLE VII

REGRESSION COEFFICIENTS FOR EQUATIONS OF THE TYPE $k' = \Sigma\Pi(A\chi + B)$ AT THREE DIFFERENT SELECTIVITY POINTS IN IMGE RUNS

These equations are calculated on the basis of the equations in Table VI.

Coefficient	$P_s = 118$		$P_s = 181$		$P_s = 811$	
	I^a	II^a	I	II	I	II
A	40.03	10.21	34.87	9.23	52.35	11.70
B	−20.13	− 8.49	−17.44	−7.75	−29.62	− 9.95
r	1.00	1.00	1.00	1.00	1.00	1.00
Index χ	$^4\chi^v_{pc}$	$^4\chi^v_p$	$^4\chi^v_{pc}$	$^4\chi^v_p$	$^4\chi^v_{pc}$	$^4\chi^v_p$

^{a} Groups I and II (see text).

$k' = A\chi + B$. A correlation coefficient of 0.91 ($2p < 0.0001$) was achieved when correlating the experimental k' values obtained from the gradient runs with pure solvents (THF, 1-propanol and acetonitrile) with calculated k' values obtained from the equations in Table IV. In the gradient runs the retention could be well predicted. This is in good agreement with results obtained by Lehtonen[14] for amines.

The retention of the coumarins was estimated for the IMGE run at the selectivity points (P_s) 118, 181 and 811 using regressions obtained with pure solvents. The capacity factors were calculated from the regression curves of the type $k' = A\chi + B$ shown in Table VI and it follows at the different selectivity points of $k' = \Sigma\Pi(A\chi + B)$, where Π is the fraction of the solvent used in forming the "PRISMA", when assuming that a linear correlation for retention between different selectivity points exists (Table VII). Fig. 7 demonstrates the efficiency of the method for calculating the k' values at different selectivity points within the "PRISMA" based on the retention information at the "PRISMA" edges, *i.e.*, gradient runs with THF, 1-propanol and acetonitrile only. The correlation between the calculated and measured k' values (Table V) at $P_s = 811$ is highly significant ($2p < 0.0001$) with a correlation coefficient of 0.97 in regression analysis (Fig. 8). The existence of the same relationship at other selectivity points was also tested using factor analysis. Table VIII shows the orthogonal transformation solution varimax with two factors. It shows clearly that when the measured retention of the compounds correlates with the measured retention at
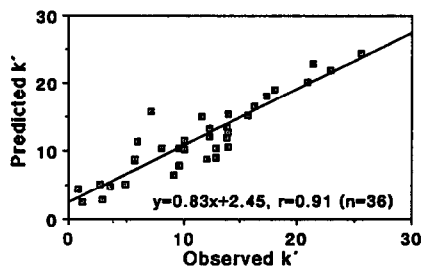

Fig. 7. Plot of the observed and predicted capacity factors (k') for gradient runs. The predicted values were calculated from equations of the type $k' = A\chi + B$ (see Table VI).

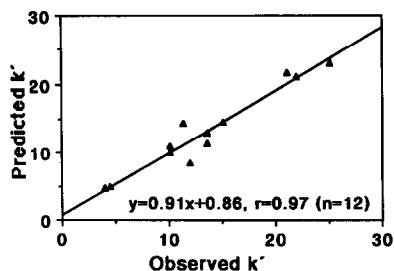Fig. 8. Plot of the observed and predicted capacity factors (k') for the IMGE runs at $P_S = 811$ and $S_T = 0.5-1.8$ in 30 min. The predicted values were calculated from equations of the type $k' = \Sigma \Pi (A\chi + B)$ (see Table VII).

TABLE VIII

DEPENDENCE BETWEEN OBSERVED AND CALCULATED k' VALUES AT THE SELECTIVITY POINTS TESTED WITH FACTOR ANALYSIS USING THE ORTHOGONAL TRANSFORMA-TION SOLUTION VARIMAX WITH TWO FACTORS

Value[a]	Factor 1	Factor 2
Obs ($P_S = 118$)	0.985	0.131
Obs ($P_S = 181$)	0.964	0.078
Obs ($P_S = 811$)	0.039	0.996
Calc ($P_S = 118$)	0.968	0.180
Calc ($P_S = 181$)	0.963	0.205
Calc ($P_S = 811$)	0.240	0.967

[a] Obs = Observed k' values, Calc = calculated k' values at the indicated P_S.

different P_S, a correlation also exists between the calculated values at different P_S. When the retention at a certain P_S is not correlated with that at different P_S, then there is only the tie between the measured and calculated retention at a certain P_S.

CONCLUSIONS

A baseline separation between the ten coumarins was achieved using the "PRISMA" model in the optimization of the mobile phase. The retention of coumarins under different HPLC conditions correlated well with the indices describing molecular structure. The retention could be well predicted for the different selectivity points in the "PRISMA" using the molecular connectivity indices.

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