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SOLVENT SELECTIVITY EFFECTS IN REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF FLAVONOID COMPOUNDS

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SUMMARY

The selectivity properties of methanol, acetonitrile and tetrahydrofuran as organic modifiers in the reversed-phase high-performance liquid chromatographic separation of flavonoid compounds was studied. Conditions for achieving separations between compound classes are described, in particular the possible separation of glycosides from aglycones in acetonitrile and tetrahydrofuran. Eleven retention contributions as $\Delta\log k'$ are reported, and their dependence on mobile-phase composition is described. Solvent strength values and useful gradient elution conditions are given.

INTRODUCTION

Flavonoid compounds are a large group of natural products based on a 2-phenylbenzopyrone structure[1–3]. Reversed-phase high-performance liquid chromatography (RP-HPLC) with isocratic and gradient elution with acid modifiers is a well established method in flavonoid analysis[4,5]. Some work has been carried out on methanol-containing systems[6]. In particular, compound pairs differing in the same substituent group exhibit constant retention differences expressed as $\Delta\log k'$ (= retention group contributions; k' = capacity factor). These retention group contributions are roughly independent of the column, acid modifier and mobile-phase composition[6]. In addition, a detailed study has been made of solvent strength values and their relevance to optimal gradient elution[7,8].

To date, with the exception of methanol, no systematic work has been carried out on the selectivity of organic modifiers, such as tetrahydrofuran (THF) and acetonitrile, which are widely employed in plant extract separations[4,9–12].

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In this work, the chromatographic behaviour of selected flavonoid compounds with these solvents was studied in an extended volume fraction (%,v/v) (φ%) range. This study will be useful for identification purposes and in defining the type and range of experimental variables within which to seek optimal separation conditions in isocratic and gradient elution.

EXPERIMENTAL

Capacity factors (k') for various fixed acetonitrile and THF φ% were determined for standard compounds with a Waters 600 multi-solvent system, equipped with a Rheodyne injection valve (20-μl sample loop), and a Waters 990 photodiode-array detector, coupled with an APC III personal computer (NEC).

All solvents and solutes were of HPLC grade (Rudi-Pont, Hetalab Chemical Corp., Parsippany, NJ, U.S.A.) and analytical-reagent grade, respectively. Acetonitrile, THF and water purified by a Norganic system (Millipore, Bedford, MA, U.S.A.) were the mobile phase solvents. The aqueous phase was buffered at pH 2–3 in 80 m*M* acetic acid–8 m*M* disodium hydrogenphosphate (Carlo Erba, Milan, Italy). Solvent mixtures were filtered through 0.2-μm Millipore filters and degassed with pure helium. The selected flavonoid standards were obtained from Sarsyntex (Merignac, France) and used as received. Standards (in ethanol) had a concentration of 10–100 ppm. The standards selected represent the classes flavones, flavonols, flavanones and glycosides (see Table I). The chromatographic column was a 30 cm × 3.9 mm I.D. 10-μm μBondapak C_{18} column (Waters Assoc., Milford, MA, U.S.A.) and was referred to as W3 in ref. 6. Retention data obtained with methanol taken from ref. 6 and considered here were obtained on a column quoted as Wl in that reference. Between these two columns a mean $\Delta\log k'$ value of 0.3 was observed[6].

RESULTS AND DISCUSSION

The retention data on THF and acetonitrile are presented in Tables I and II, respectively. The dependence of $\log k'$ on φ is nearly linear for THF but markedly deviates from linearity for acetonitrile, as observed previously for these solvents[13–18]. In this respect, THF behaves like methanol[7,8].

THF and methanol retention data were fitted with a linear equation and those for acetonitrile with a parabolic equation. The results of the best fit are reported in Tables III–V. The A data in these tables represent the $\log k'$ value extrapolated to aqueous buffer ($\log k'_w$). The $\log k'_w$ values for the same solute, extrapolated from the three solvents, may differ because they reflect the different molecular environments prevailing in a particular solvent mixture and/or because the extrapolation procedure was imprecise. In Table VI the results of the correlation between $\log k'_w$ values obtained in solvent pairs are presented. It is remarkable that the slope and correlation between the $\log k'_w$ data for methanol and acetonitrile are close to those observed for other compounds in the same solvent systems[17]. For solvent pairs one may observe that linear dependences have intercepts near zero. These findings are good arguments for the coherence of the extrapolation procedure, and hence the mean $\log k'_w$ value for different flavonoid compounds can be taken as a measure of their lipophilicity[17,19–21].

For both isocratic and gradient elution applications the most interesting data

TABLE I

RETENTION (LOG k') OF SELECTED FLAVONOIDS ON A μBONDAPAK C_{18} COLUMN FOR φ(%) TETRAHYDROFURAN IN THE MOBILE PHASE (ACETIC ACID AS ACIDIC MODIFIER)

No.	*Compound*	*φ(%)*						
		20	*25*	*30*	*40*	*45*	*50*	*55*
1	Acacetin	–	–	1.48	0.74	0.58	0.26	0.09
2	Apigenin	–	–	1.20	0.62	0.40	0.14	–0.01
3	Apigenin 7-O-glucoside	1.18	0.80	0.52	–0.03	–0.22	–0.35	–
4	Apiin	1.05	0.67	0.42	–0.14	–0.32	–0.38	–
5	Chrysin	–	–	1.46	0.74	0.60	0.30	0.15
6	Chrysoeriol	–	–	1.18	0.54	0.35	0.10	–0.04
7	Eriodictyol	–	–	1.21	0.62	0.42	0.17	0.03
8	Galangin	–	–	1.69	0.95	0.80	0.46	0.27
9	Luteolin	–	–	1.05	0.48	0.33	0.08	–0.05
10	Luteolin 7-O-glucoside	1.08	0.70	0.43	–0.08	–0.26	–0.34	–
11	Morin	–	–	1.10	0.48	0.29	0.07	–0.10
12	Naringenin	–	–	1.33	0.72	0.51	0.24	0.08
13	Quercetagetin	0.91	0.61	0.32	–0.12	–0.25	–0.37	–
14	Quercetin	–	–	1.23	0.62	0.45	0.18	0.04
15	Quercitrin	1.29	0.92	0.67	0.05	–0.10	–0.25	–
16	Rutin	0.91	0.50	0.29	–0.22	–0.27	–0.34	–

TABLE II

RETENTION (LOG k') OF SELECTED FLAVONOIDS ON A μBONDAPAK C_{18} COLUMN FOR φ(%) ACETONITRILE IN THE MOBILE PHASE (ACETIC ACID AS ACIDIC MODIFIER)

No.	*Compound*	*φ%*						
		15	*20*	*25*	*30*	*40*	*45*	*50*
1	Acetin	–	–	–	1.34	0.85	0.67	0.48
2	Apigenin	–	–	1.08	0.80	0.35	0.21	0.07
3	Apigenin 7-O-glucoside	1.36	0.71	0.17	–0.05	–0.23	–	–
4	Apiin	0.81	0.62	0.06	–0.11	–0.21	–	–
5	Chrysin	–	–	–	1.27	0.80	0.63	0.45
6	Chrysoeriol	–	–	–	0.83	0.38	0.24	0.10
7	Eriodictyol	–	–	–	0.51	0.14	0.03	–0.07
8	Galangin	–	–	–	1.40	0.88	0.72	0.52
9	Luteolin	–	1.26	0.80	0.56	0.13	0.02	–0.10
10	Luteolin 7-O-glucoside	1.12	0.44	–0.03	–0.14	–0.24	–	–
11	Morin	–	–	–	0.45	0.05	–0.05	–0.15
12	Naringenin	–	–	–	0.78	0.36	0.23	0.07
13	Quercetagetin	0.75	0.15	–0.10	–0.21	–0.21	–	–
14	Quercetin	–	1.29	0.82	0.58	0.16	0.05	–0.05
15	Quercitrin	1.37	0.70	0.19	0.00	–0.22	–	–
16	Rutin	1.05	0.32	–0.16	–0.20	–0.22	–	–

TABLE III

LOG k' *VS.* φ LINEAR FITTING ACCORDING TO LOG $k' = A + B\varphi$ WITH METHANOL AS ORGANIC MODIFIER

μBondapak C_{18} column. $\sigma_{y \cdot x}$ = Standard error of regression; R = correlation coefficient.

No.	*Compound*	*A*	*B*	*R*	$\sigma_{y \cdot x}$
1	Acacetin	3.4 ± 0.1	−4.17 ± 0.14	1.00	0.04
2	Apigenin	3.21 ± 0.24	−4.68 ± 0.46	0.99	0.05
3	Apigenin 7-O-glucoside	2.56 ± 0.31	−4.70 ± 0.64	0.98	0.07
4	Apiin	2.13 ± 0.25	−4.06 ± 0.45	0.98	0.09
5	Chrysin	3.28 ± 0.46	−4.20 ± 0.92	0.98	0.07
6	Chrysoeriol	3.0 ± 0.3	−4.1 ± 0.5	0.99	0.04
7	Eriodictyol	2.57 ± 0.25	−4.52 ± 0.43	0.99	0.08
8	Galangin	3.53 ± 0.14	−4.73 ± 0.25	1.00	0.05
9	Luteolin	2.66 ± 0.17	−4.06 ± 0.31	0.99	0.06
10	Luteolin 7-O-glucoside	2.46 ± 0.20	−4.88 ± 0.40	0.99	0.06
11	Morin	2.74 ± 0.11	−4.66 ± 0.22	1.00	0.03
12	Naringenin	2.66 ± 0.26	−4.36 ± 0.50	0.99	0.06
13	Quercetagetin	1.87 ± 0.19	−4.24 ± 0.37	0.99	0.06
14	Quercetin	2.96 ± 0.27	−4.8 ± 0.6	0.99	0.06
15	Quercitrin	2.57 ± 0.14	−4.56 ± 0.26	1.00	0.03
16	Rutin	1.96 ± 0.14	−3.7 ± 0.3	1.00	0.02

TABLE IV

LOG k' *VS.* φ PARABOLIC FITTING ACCORDING TO LOG $k' = A + B\varphi + C\varphi^2$ WITH ACETONITRILE AS ORGANIC MODIFIER

μBondapak C_{18} column. $\varphi_{y \cdot x}$ = Standard error or regression; R = correlation coefficient.

No.	*Compound*	*A*	*B*	*C*	*R*	$\sigma_{y \cdot x}$
1	Acacetin	3.41 ± 0.26	− 8.5 ± 1.4	5.3 ± 1.7	1.00	0.02
2	Apigenin	3.07 ± 0.11	− 9.92 ± 0.64	7.87 ± 0.86	1.00	0.01
3	Apigenin 7-O-glucoside	2.47 ± 0.27	−13.2 ± 1.6	16.0 ± 2.2	1.00	0.02
4	Apiin	2.13 ± 0.48	− 9.7 ± 3.8	8.0 ± 7.0	0.99	0.09
5	Chrysin	3.28 ± 0.26	− 8.3 ± 1.4	5.3 ± 1.7	1.00	0.02
6	Chrysoeriol	3.07 ± 0.28	− 9.8 ± 1.4	7.7 ± 1.8	1.00	0.02
7	Eriodictyol	2.49 ± 0.20	− 8.8 ± 1.0	7.5 ± 1.3	1.00	0.01
8	Galangin	3.68 ± 0.53	− 9.6 ± 2.7	6.5 ± 3.4	1.00	0.03
9	Luteolin	2.68 ± 0.19	− 9.4 ± 1.0	7.8 ± 1.4	1.00	0.02
10	Luteolin 7-O-glucoside	2.52 ± 0.32	−13.9 ± 2.1	17.1 ± 3.2	0.99	0.05
11	Morin	2.7 ± 0.3	−10.2 ± 1.7	9.1 ± 2.1	1.00	0.02
12	Naringenin	2.63 ± 0.41	− 7.8 ± 2.1	5.4 ± 2.7	1.00	0.02
13	Quercetagetin	1.96 ± 0.33	−12.7 ± 2.5	18.0 ± 4.5	1.00	0.02
14	Quercetin	2.78 ± 0.35	−10.0 ± 2.1	8.7 ± 2.9	1.00	0.02
15	Quercitrin	2.53 ± 0.46	−13.3 ± 2.7	16.0 ± 3.8	0.99	0.04
16	Rutin	1.72 ± 0.15	− 8.35 ± 0.97	6.7 ± 1.5	1.00	0.02

TABLE V

LOG k' *VS.* φ LINEAR FITTING ACCORDING TO LOG $k' = A + B\varphi$ WITH TETRAHYDROFURAN AS ORGANIC MODIFIER

μBondapak C_{18} column. $\sigma_{y \cdot x}$ = Standard error of regression; R = correlation coefficient.

No.	*Compound*	*A*	*B*	*R*	$\sigma_{y \cdot x}$
1	Acacetin	3.24 ± 0.23	−6.0 ± 0.6	0.99	0.08
2	Apigenin	3.0 ± 0.04	−6.0 ± 0.1	1.00	0.01
3	Apigenin 7-O-glucoside	2.33 ± 0.09	−5.95 ± 0.32	1.00	0.05
4	Apiin	2.18 ± 0.09	−5.83 ± 0.31	1.00	0.05
5	Chrysin	3.15 ± 0.18	−5.73 ± 0.43	0.99	0.06
6	Chrysoeriol	2.95 ± 0.15	−5.94 ± 0.40	1.00	0.04
7	Eriodictyol	2.6 ± 0.2	−4.73 ± 0.42	0.99	0.08
8	Galangin	3.48 ± 0.17	−6.05 ± 0.41	1.00	0.06
9	Luteolin	2.46 ± 0.15	−4.79 ± 0.36	0.99	0.05
10	Luteolin 7-O-glucoside	2.36 ± 0.16	−6.5 ± 0.6	1.00	0.04
11	Morin	2.61 ± 0.17	−5.15 ± 0.41	0.99	0.06
12	Naringenin	2.8 ± 0.2	−5.04 ± 0.37	0.99	0.07
13	Quercetagetin	1.9 ± 0.1	−5.12 ± 0.30	1.00	0.04
14	Quercetin	2.78 ± 0.20	−5.25 ± 0.49	1.00	0.07
15	Quercitrin	2.48 ± 0.07	−6.1 ± 0.2	1.00	0.04
16	Rutin	1.94 ± 0.14	−5.46 ± 0.48	0.99	0.07

that can be obtained from the observed log k' *vs.* φ dependence are the location and the extent of $\Delta\varphi$ intervals, where the log k' values range between 1 and 0 ($k' = 10$ and $k' = 1$)[13,14]. Table VII reports the $\Delta\varphi$ data together with the mean solvent strength, $\bar{S}$. The $\bar{S}$ values reported in this table are equal to

$$\bar{S} = \Delta \log k'/\Delta\varphi \tag{1}$$

and, as $\Delta \log k' = 1$, they are simply equal to

$$\bar{S} = 1/\Delta\varphi \tag{2}$$

The $\bar{S}$ data for methanol differ from the S values reported in ref. 8. In that case, S was calculated as the slope of the linear portion of the log k' *vs.* φ function in the

TABLE VI

CORRELATION BETWEEN LOG k'_w DATA OBTAINED FROM DIFFERENT ORGANIC MODIFIER PAIRS, LOG $k'_{w,1} = A + B$ LOG $k'_{w,2}$

R = Correlation coefficient.

Modifier pairs (1,2)	*A*	*B*	*R*
Acetonitrile–methanol	−0.15 ± 0.10	1.04 ± 0.05	0.98
Acetonitrile–THF	−0.27 ± 0.12	1.12 ± 0.07	0.97
THF–methanol	0.18 ± 0.10	0.90 ± 0.05	0.98

TABLE VII

USEFUL φ(%) RANGES FOR GRADIENT ELUTION AND MEAN SOLVENT STRENGTHS OF SELECTED FLAVONOID COMPOUNDS IN THE RP C_{18} SYSTEM WITH ACETIC ACID AS THE ACID MODIFIER

Compound	*Methanol*			*THF*			*Acetonitrile*		
	φ ($k' = 10$)	φ ($k' = 1$)	S	φ ($k' = 10$)	φ ($k' = 1$)	S	φ ($k' = 10$)	φ ($k' = 1$)	S
Eriodictyol	39	55	4.9	34	56	4.5	17	46	3.4
Naringenin	34	61	3.1	35	58	4.3	25	53	3.6
Acacetin	49	82	3.0	33	58	4.0	35	63	3.6
Apigenin	46	74	3.2	34	55	4.8	26	53	3.7
Apigenin 7-O-glucoside	36	54	4.9	23	40	5.9	18	29	9.1
Apiin	37	53	5.4	21	38	5.9	17	27	10.0
Chrysin	56	80	4.2	34	59	4.0	34	63	3.4
Chrysoeriol	45	72	3.7	33	54	4.8	19	53	2.9
Luteolin	43	65	4.2	31	54	4.3	23	46	4.3
Luteolin 7-O-glucoside	33	52	4.5	21	39	5.6	16	25	11.1
Galangin	54	80	3.8	39	62	4.3	36	64	3.6
Morin	36	58	4.2	28	52	4.2	17	43	3.8
Quercetagetin	28	45	4.0	19	37	5.6	13	22	11.1
Quercetin	42	59	4.0	34	57	4.3	23	48	4.0
Quercitrin	38	56	4.9	23	42	5.3	18	30	8.3
Rutin	34	52	4.5	19	36	5.9	15	24	11.1
Common mean and S.D.			4.2 ± 0.7			4.9 ± 0.7			6.1 ± 3.3
Aglycone mean and S.D.			3.8 ± 0.6			4.3 ± 0.3			3.6 ± 0.4
Glycoside mean and S.D.			4.7 ± 0.5			5.7 ± 0.2			10.1 ± 1.2

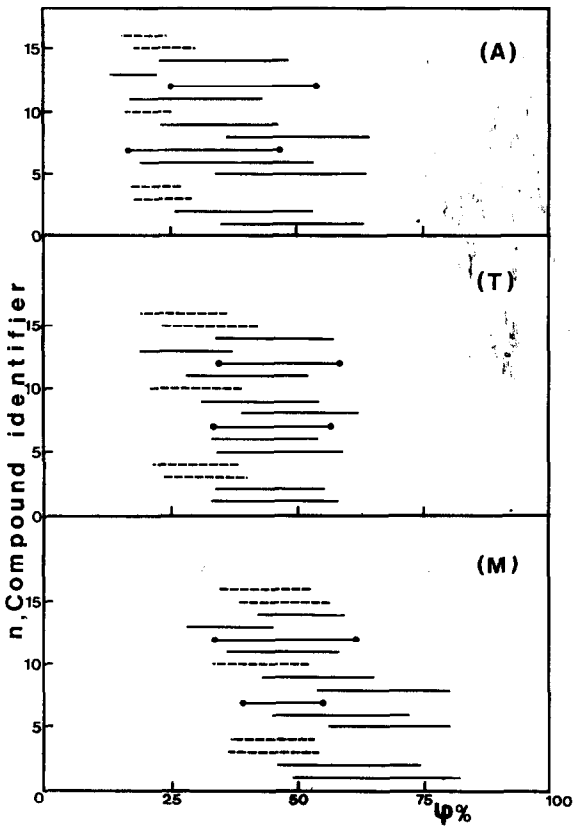

Fig. 1. Useful $\Delta\varphi$ elution range intervals for $1 < k' < 10$. For compound identification, see Table I. - - - = Glycosides; ●—● = flavanones; —— = flavones. A, T and M refer to acetonitrile, THF and methanol respectively.

range log k' = 1–0, whereas here $\bar{S}$ is the mean slope within the same range. This calculation was followed in order to have a common basis when the different solvents are compared.

An overview of the useful $\Delta\varphi$ pattern for gradient and isocratic elution with different solvents is present in Fig. 1. If the minimum φ value giving a k' value lower than 10 is taken as a criterion of elution power, the order is generally acetonitrile > THF > methanol.

Regarding the mean solvent strength, $\bar{S}$, glycosides generally exhibit higher values than aglycones, with the exception of quercitrin, where the extensive hydroxylation is responsible for its anomalous behaviour. Of the three solvents, the effect is more marked for acetonitrile, the most powerful of the series. These high $\bar{S}$ values are peculiar to these compounds and can be explained only by a specific interaction with acetonitrile. Another interesting factor that emerges for the data in Fig. 1 is that it is possible to separate glycosides and aglycones with either THF or acetonitrile. With the former, Snyder's linear solvent strength (LSS) gradient elution theory[13,14] will probably support the selection of a gradient with a nearly constant slope in the range

TABLE VIII

COMPARISON BETWEEN ΔLOG k' RETENTION GROUP CONTRIBUTIONS IN DIFFERENT SOLVENT

Group contribution	*Compounds*	*Δlog k' CH_3OH*	*(φ range)**	*Δlog k' CH_3CN*	*(φ range)**	*Δlog k' THF*	*(φ range)**	*Δlog k'_w***
3-OH	8– 5	0.01 ± 0.01	(0.55–0.70)	0.09 ± 0.06	(0.30–0.50)	0.18 ± 0.11	(0.30–0.55)	0.33 ± 0.07
3-OH	14– 9	–0.13 ± 0.15	(0.45–0.60)	0.03 ± 0.03	(0.30–0.50)	0.13 ± 0.09	(0.30–0.59)	0.24 ± 0.12
6-OH	13–14	–0.85 ± 0.06	(0.40–0.50)	–0.80 ± 0.77	(0.20–0.45)	–0.72 ± 0.36	(0.30–0.50)	–0.93 ± 0.14
3'-OH	9– 2	–0.22 ± 0.05	(0.45–0.60)	–0.22 ± 0.11	(0.25–0.50)	–0.09 ± 0.11	(0.30–0.55)	–0.49 ± 0.09
3'-OH	10– 3	–0.19 ± 0.02	(0.30–0.70)	–0.22 ± 0.26	(0.15–0.50)	–0.07 ± 0.07	(0.20–0.50)	–0.01 ± 0.08
3'-OH	7–12	–0.24 ± 0.12	(0.40–0.70)	–0.21 ± 0.13	(0.30–0.50)	–0.09 ± 0.07	(0.30–0.55)	–0.14 ± 0.05
4'-OH	2– 5	–0.29 ± 0.06	(0.45–0.70)	–0.43 ± 0.09	(0.30–0.50)	–0.18 ± 0.12	(0.30–0.55)	–0.14 ± 0.07
3'-OCH_3 (—HO)	6– 2	0.06 ± 0.02	(0.40–0.55)	0.03 ± 0.00	(0.30–0.50)	–0.05 ± 0.05	(0.40–0.55)	–0.09 ± 0.11
4'-OCH_3	1– 5	0.14 ± 0.02	(0.45–0.55)	0.05 ± 0.04	(0.30–0.50)	–0.02 ± 0.06	(0.30–0.55)	0.11 ± 0.02
2,3-Unsaturation	12– 2	–0.44 ± 0.04	(0.40–0.50)	0.01 ± 0.04	(0.30–0.50)	0.11 ± 0.04	(0.30–0.55)	–0.39 ± 0.18
2,3-Unsaturation	7– 9	–0.25 ± 0.07	(0.40–0.55)	0.02 ± 0.08	(0.30–0.50)	0.11 ± 0.08	(0.30–0.55)	–0.05 ± 0.16
3-Glycoside (rhamose)	15–14	–0.25 ± 0.03	(0.40–0.55)	–0.54 ± 0.25	(0.20–0.50)	–0.53 ± 0.12	(0.30–0.50)	–0.31 ± 0.07
3-Glycoside (rutinose)	16–14	–0.44 ± 0.03	(0.40–0.55)	–0.78 ± 0.60	(0.20–0.45)	–0.75 ± 0.42	(0.30–0.50)	–0.97 ± 0.11
7-Glycoside (glucose)	3– 2	–0.68 ± 0.04	(0.45–0.60)	–0.78 ± 0.33	(0.25–0.45)	–0.61 ± 0.19	(0.30–0.50)	–0.64 ± 0.04
7-Glycoside (glucose)	10– 9	–0.65 ± 0.03	(0.40–0.60)	–0.68 ± 0.46	(0.20–0.45)	–0.56 ± 0.16	(0.30–0.50)	–0.16 ± 0.05
7-Glycoside (apiosylglucose)	4– 2	–0.76 ± 0.06	(0.45–0.60)	–0.83 ± 0.46	(0.25–0.45)	–0.69 ± 0.26	(0.30–0.50)	–0.95 ± 0.13

* Mean data with their range values and mobile phase composition ranges are reported for the three organic solvents.

** Mean data with standard deviation are reported for Δlog k'_w with water.

$0.15 < \varphi < 0.60$; for acetonitrile, two slopes are indicated: first low ($0.10 < \varphi < 0.25$) and then three times steeper ($0.25 < \varphi < 0.70$). In addition, the marked positive curvatures of the log k' *vs.* φ plots (see Fig. 2) support convex gradient shapes. This, which requires an optimization procedure, will be the subject of further study.

The general pattern of structure retention relationships in these two solvents was compared with results obtained previously with methanol[6] by calculating the group contribution to the retention as $\Delta\log k'$ for various substitutions in the benzopyran ring. Only log k' data roughly in the range 1–0 were employed. The dependence on φ is presented in Figs. 2 and 3 for selected groups in the three solvents. A marked dependence of $\Delta\log k'$ on φ is often observed in THF and acetonitrile. The general independence of $\Delta\log k'$ from φ and also from other variables (*e.g.*, the chain length of the bonded phase and the type of acid modifier), as previously observed for methanol[6], must be considered peculiar to methanol. Another distinct feature of THF and acetonitrile is their levelling effect on the $\Delta\log k'$ contributions: negative $\Delta\log k'$ values increase with an increase in the organic content of the mobile phase, whereas positive values decrease. In Table VIII the mean $\Delta\log k'$ values are reported together with their ranges. It can be seen that both the $\Delta\log k'$ values and their ranges for glycosides are always higher for acetonitrile and THF than for methanol. Hence the fact that glycosides are eluted early by THF and especially by acetonitrile must be ascribed to specific, strong polar and/or hydrogen-bonding acceptor properties of these two solvents with respect to methanol. The same kind of strong specific interaction is observed for the 6-OH group in quercetagetin, thus explaining why this compound is eluted with the glycosides. Another specific behaviour strongly dependent on solvent type is the unsaturation contribution, which distinguishes flavones from

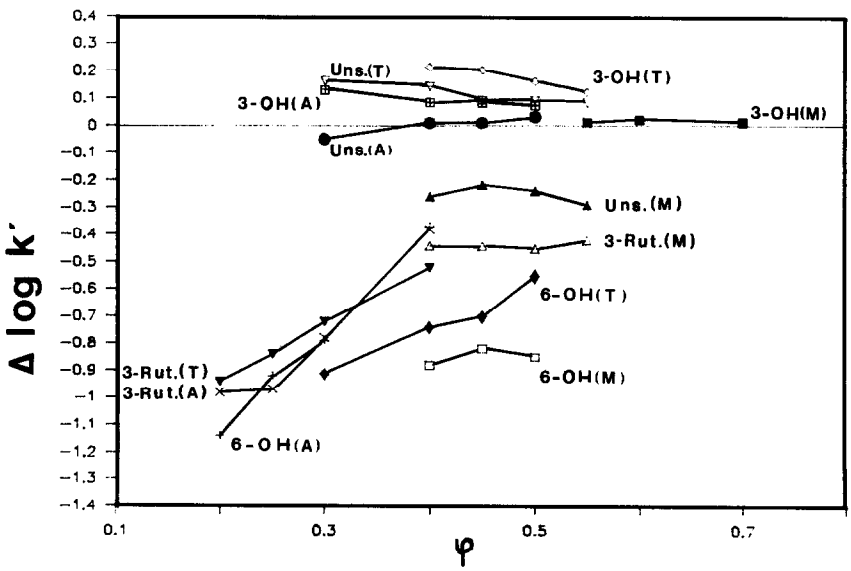

Fig. 2. Dependence of $\Delta\log k'$ group contributions on mobile phase composition. 3-OH refers to the pair of compounds 8 and 5; Uns. refers to the pair of compounds 7 and 9; 3-Rut refers to the pair of compounds 16 and 14; 6-OH refers to the pair of compounds 13 and 14 (see Table VIII). A, T and M as in Fig. 1.

flavanones. Despite the fact that there were differences in molecular structure, the flavones being planar and the flavanones partially planar, only in methanol is there a marked difference with a significant negative group contribution and early elution among glycoside compounds. This effect is lower for acetonitrile and reversed for THF (see Fig. 2). It is not easy to explain this behaviour, which is probably a combined effect of solvation enthalpy and entropy. The ordered solvent structure of the aqueous methanol system implies a larger solvation entropy for the planar flavones than for the non-planar flavanones[22]. This effect must be reversed in THF or other solvation processes must be operative.

The $\Delta\log k'$ data now discussed can be compared with the $\Delta\log k'_w$ data, which can be calculated by using $\log k_w'$ data obtained by extrapolation procedures (see Table VIII). No systematic differences are observed between the extrapolated values with water and those observed with various solvent mixtures as mobile phases. However, a significant negative $\Delta\log k'_w$ value for unsaturation, as in methanol, is observed. In addition, the decreased polarity effect of the 3-OH group, giving rise to an intramolecular hydrogen bond, appears strongest with water.

Let us now consider some practical applications for the selectivity properties of these three organic modifiers. It may be recalled that the $\Delta\log k'$ data reported in Table VIII and Figs. 2 and 3 are also the logarithms of the relative retentions. A difficult resolution, unobtainable with a particular organic modifier, may be obtained by simply changing it or by modifying the volume fraction. For example, the flavonol galangin (compound 8), which cannot be separated from the corresponding flavone chrysin (compound 5) in methanol, can be resolved with acetonitrile or even better with THF (see Fig. 2). Another, similar pair (quercetin *vs.* luteolin) is better separated in either THF or methanol (see Table VIII). It is relevant that the minor differences,

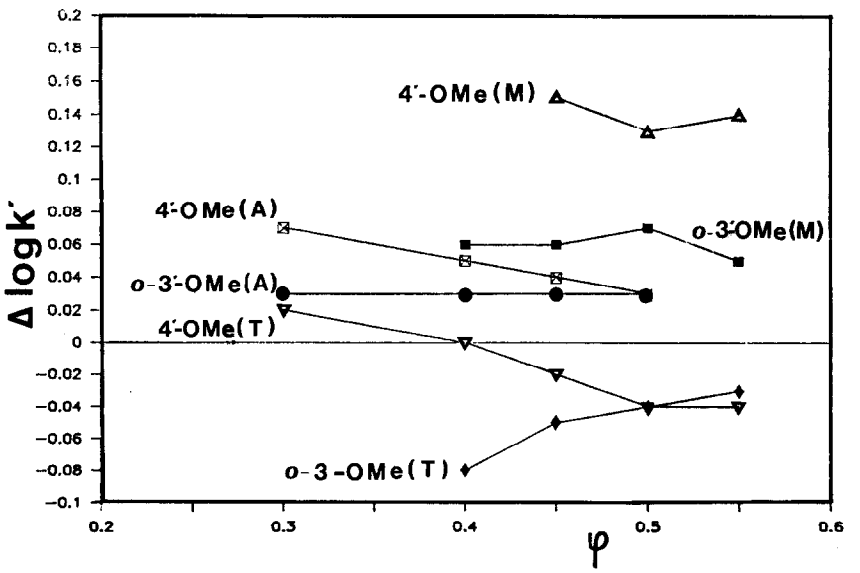

Fig. 3. Dependence of $\Delta\log k'$ group contributions on mobile phase composition (see Table VI). Me = Methyl. A, T and M as in Fig. 1.

from the same 3-OH group contribution, imply different chromatographic conditions. Other particularly difficult separation problems are compound pairs that differ in the occurrence of a OCH_3 group. An isolated OCH_3 [the 4'-OCH_3 group in the pair of compounds 1 and 5] clearly enhances the lipophilicity with a corresponding increase in retention (see Fig. 3). The latter is most pronounced for methanol and less so for acetonitrile, and inversion effects take place in THF. When an OH group is close to OCH_3 (*e.g.*, in the pair of compounds 6 and 2), the total lipophilicity of the molecule is lowered through the *ortho* effect, and separation can be achieved with both THF and methanol (see Fig. 3). Note that a $\Delta \log k'$ value of 0.05 means a retention difference of 12%. Finally, in acetonitrile and THF the general dependence of $\Delta \log k'$ on φ makes the mobile phase composition change a useful parameter in solving particularly difficult separation or identification problems.

CONCLUSION

The three organic modifiers considered exhibit significant selectivity peculiarities toward flavonoid compounds in RP-HPLC. The overall effect, derived by a careful analysis of group contributions to retention, is complex and probably the result of the many simultaneously acting factors (different specific and non-specific interactions, together with different solvophobic effects)[20–25]. For methanol[6], the group contributions are largely independent of solvent composition, which probably helps in understanding gradient elution behaviour. Nonetheless, as organic modifiers, THF and acetonitrile can be useful in facilitating the selective elution of different classes of compounds, such as glycosides and the parent aglycone, and in achieving particular resolutions and identifications. Minor changes in $\Delta \log k'$ values for the same substituent group likewise have important practical relevance, and a more extended study of different flavonoids may lead to additional insights into the secondary effects which may, nonetheless, play an important rôle in certain separations.

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