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Journal of Chromatography A, 791 (1997) 289–297

JOURNAL OF CHROMATOGRAPHY A

Separation of hydroxylated and methoxylated flavonoids by micellar electrokinetic capillary chromatography Determination of analyte partition coefficients between aqueous and sodium dodecyl sulfate micellar phases

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Received 14 October 1996; received in revised form 3 July 1997; accepted 7 July 1997

Abstract

The capacity factors (k'_{corr}) of ionized flavonoids have been determined after correction from the influence of buffer pH and their dependence versus sodium dodecyl sulfate (SDS) micelle concentration was found to be linear in micellar electrokinetic capillary chromatography (MEKC). The partition coefficients between aqueous and SDS micellar phases of several flavonoids have been deduced from these linear curves and their values interpreted from the structural features of these compounds. Additionally, the critical micelle concentration (CMC) value of SDS surfactant was determined. Finally, a mixture of seven hydroxylated and methoxylated flavonoids has been resolved by MEKC using 20 m*M* $Na_2B_4O_7$–25 m*M* SDS, 20% CH_3OH electrolyte (pH 8). © 1997 Elsevier Science B.V.

Keywords: Partition coefficients; Critical micelle concentration; Flavonoids

1. Introduction

Flavonoids constitute one of the largest groups of naturally occurring phenols and are widespread components in all parts of plants. These compounds have structures based on 2-phenyl benzopyrone and differ in the pattern of hydroxylation, degree of saturation and type and position of sugar links [1].

The most common methods for the analysis of plant flavonoids are thin-layer chromatography (TLC) and liquid chromatography (LC) but neither of these methods has the resolution power of capillary electrophoresis (CE). Several papers have reported separation and analysis of flavonoids by CE. Both capillary zone electrophoresis (CZE) [2] and isotachophoresis (ITP) [3] have been used to resolve pharmaceutically important flavonols. CE has also been mentioned for the separation and quantitative analysis of sugarcane-flavones [4]. Recent reports [5,6] dealing with the separation of flavonoid-glycosides by free solution CE have focused on the role of borate complexation in the separation of flavonol glycosides. The influence of flavonoid structure on CZE electrophoretic mobility has shown that the dominant factors are the molecular size and the position and number of free phenolic hydroxyl

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0021-9673/97/$17.00
PII S0021-9673(97)00774-7

groups remaining on the flavonol [7,8]. A recent paper reviews the possibilities and applications of CE as an analytical technique for plant secondary metabolites [9]. Other authors have used micellar electrokinetic capillary chromatography (MEKC) to separate flavonoids [10–13].

This paper deals with the determination of the partition coefficient between aqueous and sodium dodecyl sulfate (SDS) micellar phases of ionizable flavonoids and of the SDS critical micelle concentration (CMC) value by CE. Finally, the separation of seven hydroxylated and methoxylated flavonoids by CZE and MEKC is reported.

2. Experimental

2.1. Instrumental

All open-tube CZE and MEKC separations were performed on a SpectraPhysics (San Jose, CA, USA) Spectrophoresis 1000 instrument using a silica capillary tube (70 cm×50 μm I.D.×375 μm O.D.). Data were processed on an IBM PS/2 Model 70386 computer. Software operating under IBM OS/2 was supplied by Spectra Physics. The instrument contains a programmable, high-speed scanning mode, multi-wavelength UV detector. Using the fast scanning mode, we were able to record on-column UV spectra of these compounds from 200 to 360 nm in 5 nm increments.

Separations were carried out at 30°C and +20 kV (electrical field strength 286 V/cm). Analytes were injected in the hydrodynamic mode using a 0.75 p.s.i vacuum for 1 s (1 p.s.i.=6894.76 Pa). Each capillary was conditioned daily by washing first with 0.1 *M* sodium hydroxide (5 min) at 40°C, water at 40°C (5 min) and the electrophoretic buffer (10 min) at 30°C. Between two consecutive analyses, the capillary tubing was flushed with water (5 min) and electrophoretic buffer (2 min) in order to improve repeatability and reproducibility of the migration time, peak shape and capacity factor. This conditioning step is important to ensure that the capillary silica surface is uniformly charged. It is sometimes necessary to clean the capillary with methanol.

2.2. Reagents

All chemicals were of analytical-reagent grade. Sodium tetraborate, acetic acid and surfactant SDS (Fluka, Buchs, Switzerland) and methanol (Merck, Darmstadt, Germany) were used as received. Water used for dilution or as buffer solution was of HPLC grade (Carlo Erba, Milan, Italy).

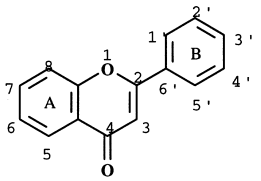
For free solution CE, the running buffer was 20 m*M* $Na_2B_4O_7$ (pH adjusted with 1 *M* acetic acid) while the MEKC buffer was 20 m*M* $Na_2B_4O_7$ (pH adjusted with 1 *M* acetic acid), SDS and sometimes methanol (20% v/v). Methanol and anthracene were injected to determine the retention time of neutral unretained solute and the migration time of the micelles, respectively. All buffers were freshly prepared and filtered through a Whatman (Maidstone, UK) polypropylene filter (0.2 μm pore size, 25 mm diameter).

The studied flavonoids were synthesised by Prof. Gaydou (University of Aix, Laboratory of Phytochemistry, Marseille III, France). A methanolic solution of each flavonoid was prepared at a concentration of ca. 15 ppm.

3. Results and discussion

In two recent papers [5,6], we reported the CE separation of a mixture of flavonoid-O-glycosides having either different sugar or aglycone moieties; in the first case, the electrolyte required the addition of borate anion to the alkaline buffer to promote the complexation of *cis*-1-2 hydroxyl groups in the glycoside structure, and in the second analysis, the use of SDS to provide the hydrophobic interaction useful for the differentiation of these aglycones. The MEKC technique has also been employed to resolve a flavonoid mixture containing both glycosylated flavonoids and aglycones [10].

The purpose of the present work was to resolve – using CE – a mixture of seven flavonoids differing in the number, the position and the nature of their functional groups (methoxy and hydroxy) upon rings A and B of the molecule and to assess the capability of MEKC to determine the partition coefficient of these analytes between aqueous and micellar phases.



Number	Flavone	4'	7
1	7-OCH_3		OCH_3
2	4'-OCH_3	OCH_3	
3	7,4'-$(OCH_3)_2$	OCH_3	OCH_3
4	4'-OH	OH	
5	7-OH,4'-OCH_3	OH	OCH_3
6	7-OH		OH
7	7,4'-$(OH)_2$	OH	OH

Fig. 1. Structure of studied methoxy- and hydroxylated flavonoids.

The formulae of the flavonoids studied are shown in Fig. 1.

3.1. Free solution CE

The CZE separation mechanism is based on differences in the charge-to-mass ratio of solutes. The investigated mono- and dihydroxylated flavones have ionizable phenolic groups at different positions on the flavone skeleton. Phenolic ionization is one of the main determinants of electrophoretic mobility in CZE for this family. Wolfbeis et al. [14] have previously determined the phenolic group pK_a data for some mono- and dihydroxyflavonoids; for instance, 7.39 for 7-OH, 8.28 for 4′-OH and 11.6 for 5-OH. Unfortunately, the pK_a values of 7,4′-$(OH)_2$ and 7-OH,4′-OCH_3 are not mentioned; consequently, we employed the Pallas software (CompuDrug Chemistry, Budapest, Hungary) to make pK_a predictions based on the structural formulae of these flavonoids (Table 1). These solutes are charged at high pH values and the molecules with greater negative charge (smaller pK_a value) will be species with larger migration times. CZE can also be achieved with complexing buffer by the addition of borate anion which can form a negatively charged complex with hydroxy groups of flavonoids. In alkaline conditions, the resolution of the analysis generally increases with increasing concentration of boric acid [6,7] but only hydroxyl groups in vicinal position with a *cis* configuration can form stable complexes.

Table 1
Electrophoretic mobilities of some hydroxylated flavonoids at pH 8

OH position	pK_a	Electrophoretic mobility $\cdot 10^5$ ($cm^2/V/s$)
4′-OH	9.13[a] (8.28[b])	5.0
7-OH, 4′-OCH_3	7.81[a]	17.9
7-OH	7.81[a] (7.39[b])	19.5
7,4′-$(OH)_2$	7.78[a]; 9.27[a]	22.9

Fused-silica capillary: 70 cm×50 μm I.D; applied voltage: +20 kV; temperature: 30°C; UV detection at 250 nm; electrolyte: 20 m*M* $Na_2B_4O_7$, pH 8 (adjusted with 1 *M* CH_3COOH); hydrodynamic injection time: 1 s; solute concentration: 15 μg/ml.
[a] pK_a values predicted by Pallas software.
[b] Ref. [14].

Fig. 2 shows the CZE separation of a standard mixture of seven methoxylated and hydroxylated flavonoids by using the 20 m*M* $Na_2B_4O_7$ (pH 8) running buffer. Under these alkaline conditions, anionic species moved towards the detector electrode (cathode) with an effective mobility equal to the sum of the electroosmotic flow (EOF) mobility and the electrophoretic mobility. Neutral sample molecules, such as methoxylated flavonoids (Nos. 1–3) migrated at the same velocity as that of EOF and were not resolved. In contrast, the four hydroxylated flavonoids (Nos. 4–7) were well resolved with peak efficiencies close to 250 000 theoretical plates; their respective mobilities are given in Table 1.

The greater electrophoretic mobility of the 7,4′-$(OH)_2$ flavonoid (solute No. 7) compared to that of monohydroxyflavonoids (solute Nos. 4–6) at pH 8 comes from two hydroxyl groups in positions 7 and 4′ which increase the apparent negative charge of the molecule at pH 8 [−0.72 for 7,4′-$(OH)_2$ flavonoid compared to −0.64 for 7-OH flavonoid]. The 4′-hydroxyflavonoid (solute No. 4) has a smaller electrophoretic mobility than 7-hydroxyflavone (solute No. 6); this difference in electrophoretic mobility does not depend in this case on the molecular size, but is due to the degree of molecular ionisation. Indeed, the increased acidity of the hydroxyl group

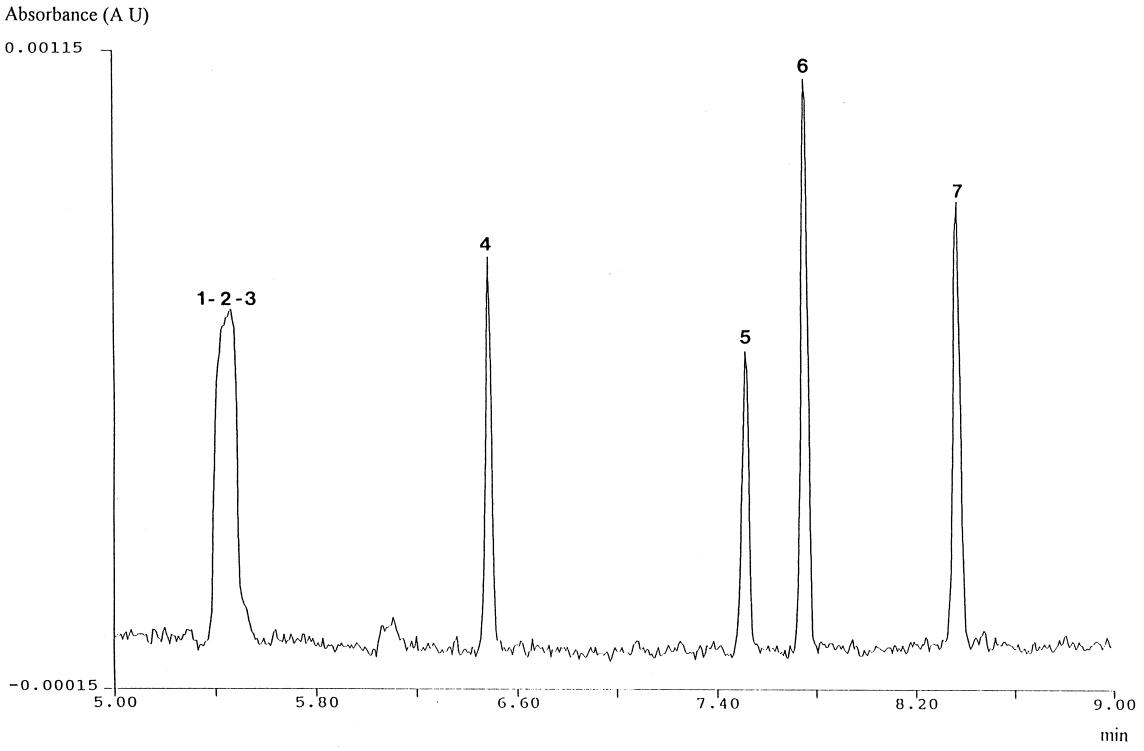


Fig. 2. Separation of a standard mixture of seven methoxylated and hydroxylated flavonoids by CZE. Fused-silica capillary: 70 cm×50 μm I.D; applied voltage: +20 kV; temperature: 30°C; UV detection at 250 nm; electrolyte: 20 m*M* $Na_2B_4O_7$, pH 8 (adjusted with 1 *M* CH_3COOH); hydrodynamic injection time: 1 s; solute concentration: 15 μg/ml. Solute numbers referred to are given in Fig. 1.

in position 7 (pK_a=7.81) compared to the one in position 4′ (pK_a=9.13) affects the apparent negative charge and consequently the electrophoretic mobility of the hydroxylated flavonoids. Lastly, the difference observed between the electrophoretic mobilities of 7-OH,4′-OCH_3 flavone (solute No. 5) and 7-OH flavone (solute No. 6) comes from the additional methoxy on ring B (solute No. 5) and its increased molecular size.

Thus, CZE is clearly a key method to determine the pK_a values of hydroxyl groups on flavonoid cycles, and to compare the hydroxyl acidity between the positions on the A and B rings.

While hydroxylated flavonoids may be resolved by CZE using an alkaline buffer (with or without complexing agents), the separation of a methoxy- and hydroxyl-flavonoid requires additional separation mechanism based on the differences in hydrophobicity between solutes.

3.2. Micellar electrokinetic capillary chromatography

This technique, first described by Terabe et al. [15] and dedicated to the separation of nonionic or neutral molecules [16–18] is often used to improve the separation of ionic compounds. The most used micelles are those formed with the anionic surfactant SDS, which are negatively charged in the common pH range. In MEKC, the selective migration is totally dependent on the selective interaction with the micelles. For charged analytes, both native mobility and partitioning are involved.

In MEKC, the capacity factor is defined as the amount of material in the pseudo-stationary phase divided by the amount in the mobile phase. For charged analytes, the apparent capacity factor (k'_{app}) has been determined from the measurement of the migration times of the analyte (t_m), of the neutral

marker (t_0) and of the micelle marker (t_{mc}) and calculated according to the following expression:

$$k'_{app} = \frac{t_m - t_0}{t_0\left(1 - \frac{t_m}{t_{mc}}\right)} \quad (1)$$

Methanol and anthracene have been selected as neutral and micelle markers, respectively. Due to the low available amount of these flavonoids, the repeatability test has been evaluated in MEKC by carrying out six consecutive injections of a methanolic solution of quercetin (25 μg/ml) as analyte and of anthracene (25 μg/ml) as micelle marker. For the reproducibility evaluation, injections have been done over three days. The selected MEKC buffer was 20 m*M* $Na_2B_4O_7$, 50 m*M* SDS (pH 8) and the separation conditions were 25°C and +15 kV. Statistical analyses of t_0 and t_{mc} values are reported in Table 2.

For the three migration times [t_0, t_{mc}, $t_{m(quercetin)}$], the repeatability was less than 0.6% R.S.D for migration times and better than 0.74% R.S.D for apparent capacity factors. The day-to-day reproducibility was low (4.3% R.S.D.) for the migration time but better for capacity factor (0.89% R.S.D). Thus, the identification of a complex mixture in MEKC should be performed by selecting capacity factor rather than migration time.

Two factors (pH and SDS concentration) have been initially selected in order to determine the optimum resolution region. As expected, the apparent capacity factor of each hydroxylated flavonoid decreases with an increasing buffer pH (Fig. 3). The migration order between 4′-OH and 7-OH,4′-OCH_3 flavone is affected by the change in the pH near 8.0. The migration behaviour of 7-OH,4′-OCH_3 mainly depends on hydrophobicity at lower pH and on molecular ionisation at higher pH. For nonionic compounds, SDS concentration would have been certainly more important than pH while for these charged analytes, pH is the main determining factor on the capacity factor compared to SDS concentration in the 7–9 pH range buffer. Indeed, these analytes are partially ionized at these pH values and large changes in apparent charge and consequently partition coefficients between aqueous and micellar phases occur with pH modifications. So, changes in the pH from 7.0 to 9.0 should be selected during an optimization process for the MEKC analysis of these flavonoids.

At pH 8, an increase in SDS concentration leads to an increase in apparent capacity factor (Fig. 4a). A higher micellar concentration enhances the partitioning of each flavonoid towards the SDS pseudo-stationary phase. For neutral solutes, the theory predicts that a linear relationship exists between the capacity factor and the SDS concentration and that this curve has an *x*-intercept close to the CMC value. For charged analytes, the migration phenomenon does not only involve the partitioning mechanism between the aqueous phase and the micellar phase, but also its electrophoretic migration. In such a case, the calculation of partition coefficient requires the determination of the corrected capacity factor (k'_{corr}) defined according to the following relationship [21]:

$$k'_{corr} = \frac{t_m - t'_m}{t'_m\left(1 - \frac{t_m}{t_{mc}}\right)} \quad (2)$$

where t_m and t'_m are the migration times of the solute in MEKC and CZE conditions, respectively, and t_{mc}

Table 2
Repeatability and reproducibility standard deviations of quercetin migration data

	Mean value	Repeatability standard deviation (%)	Reproducibility standard deviation (%)
t_0	3.17 min	0.3	2.5
t_m (quercetin)	6.26 min	0.6	4.3
t_{mc}	9.08 min	0.4	2.9
k'	2.87	0.74	0.89

Fused-silica capillary: 70 cm×50 μm I.D; applied voltage: +15 kV; temperature: 25°C; UV detection at 250 nm; electrolyte: 20 m*M* $Na_2B_4O_7$, 50 m*M* SDS, pH 8 (adjusted with 1 *M* CH_3COOH); hydrodynamic injection time: 1 s; solute concentration: 15 μg/ml. EOF marker: methanol; micelle marker: anthracene.

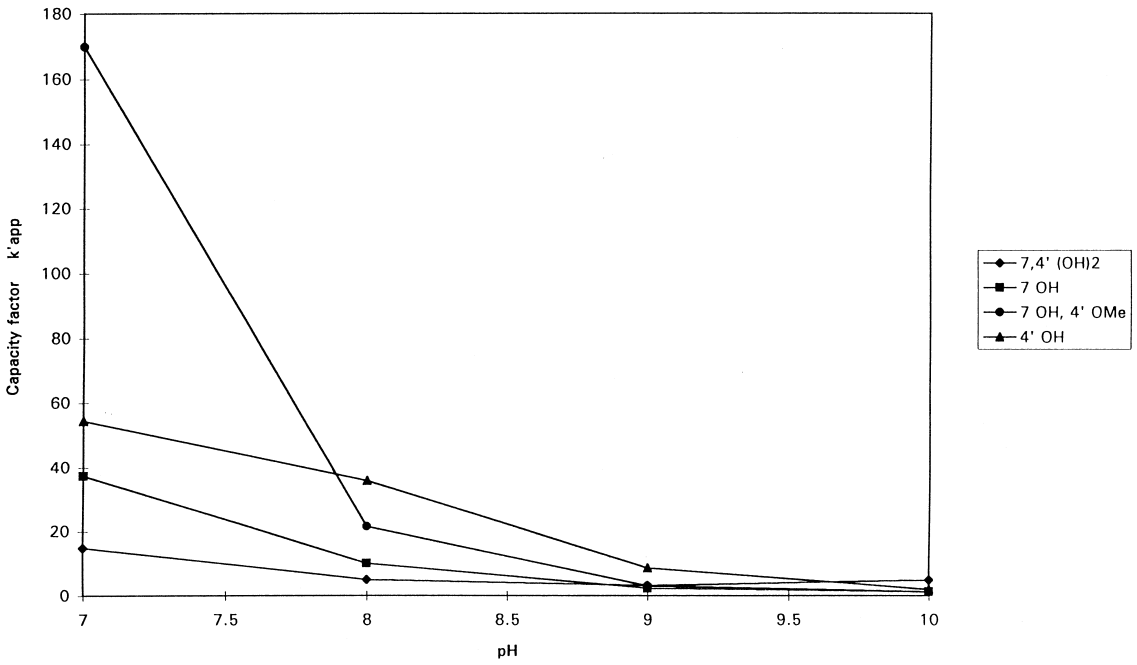


Fig. 3. Dependence of apparent capacity factors of four hydroxylated flavonoids versus buffer pH in MEKC. Fused-silica capillary: 44 cm×50 μm I.D; applied voltage: +20 kV; temperature: 30°C; UV detection at 250 nm; electrolyte: 20 m*M* $Na_2B_4O_7$, 20 m*M* SDS (pH value adjusted with 1 *M* CH_3COOH), hydrodynamic injection time: 1 s; solute concentration: 15 μg/ml.

the migration time of the micelle marker. This formula will be correct only for identical electroosmotic mobility values in MEKC and CZE modes. In our experiments, the electroosmotic mobility changed only by 2.3% between MEKC and CZE conditions. Fig. 4b reports the influence of increasing micellar SDS concentration upon the corrected capacity factor of three hydroxylated flavones at pH 8. Finally, the corrected capacity factor (k'_{corr}) of an ionized molecule is found to be linear in MEKC versus the micellar concentration, according to the expression [3]:

$$k'_{corr} = K\nu[(SDS) - CMC] \quad (3)$$

where K is the partition coefficient of the solute between aqueous and micellar phase and ν the partial specific volume of the micelle (0.25 l/mol for SDS [19,20]).

The linear correlation coefficients are all greater than 0.9978 and are shown in Table 3. The partition coefficient of each flavonoid has been deduced from the slope of its linear curve given in Fig. 4b. The 4′-OH flavone will be more easily partitioned towards the SDS micellar phase than the 7-OH flavone due to its quite neutral character at pH 8 (−0.08 as apparent charge). The molecules of 7,4′-$(OH)_2$ flavone will be less incorporated in SDS micelles at pH 8 than the 4′-OH flavone molecules as expected from their respective apparent charge (−0.72 and −0.08, respectively). The additional methoxy group on ring B (solute 5) contributes to a better inclusion in SDS micelles at pH 8 (compare *K*-values of 7-OH,4′-OCH_3 and 7-OH flavonoids). The experimental CMC values are relatively dispersed (5.5–9.6 m*M*) but coherent with the literature (8.1 m*M*, [22]).

Finally, Fig. 5 reports the separation of the standard mixture of seven methoxylated and hydroxylated flavonoids by MEKC; the introduction of

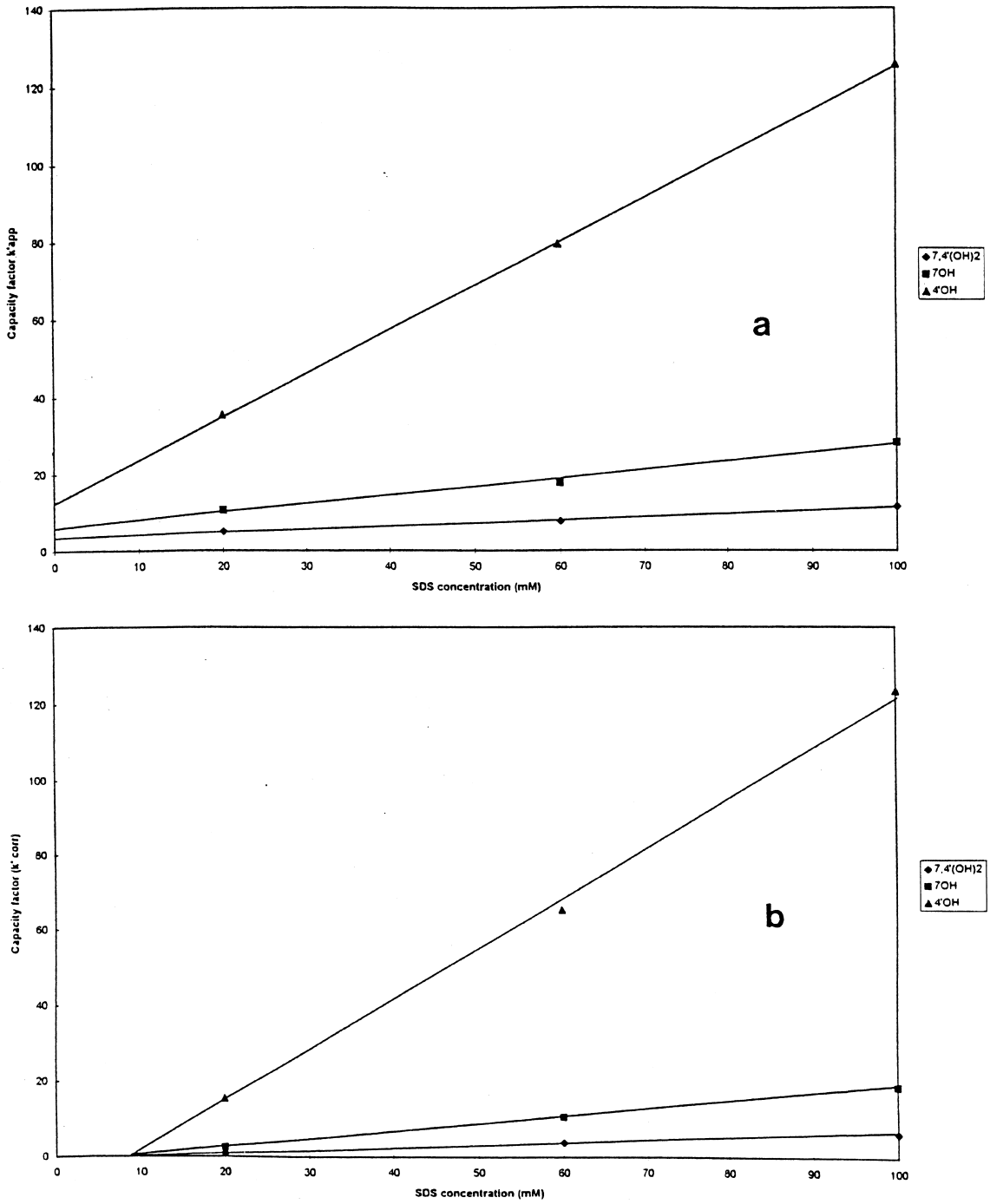


Fig. 4. Influence of SDS concentration on capacity factors in MEKC. Fused-silica capillary: 44 cm×50 μm I.D; applied voltage: +20 kV; temperature: 30°C; UV detection at 250 nm; electrolyte: 20 m*M* $Na_2B_4O_7$, SDS, pH 8 (adjusted with 1 *M* CH_3COOH), hydrodynamic injection time: 1 s; solute concentration: 15 μg/ml. (a) k'_{app} values (as defined in Eq. (1)), (b) k'_{corr} values (as defined in Eq. (2)).

Table 3
Partition coefficients between aqueous and SDS micellar phases of three hydroxylated flavonoids

Flavonoid	7,4′-$(OH)_2$	7-OH,4′-OCH_3	7-OH	4′-OH
K	300	1600	800	5400
pK_a	7.78; 9.27	7.78	7.78	9.13
Correlation coefficient	0.9978	0.9993	0.9999	0.9982

Fused-silica capillary: 44 cm×50 μm I.D; applied voltage: +20 kV; temperature: 30°C; UV detection at 250 nm; electrolyte: 20 m*M* $Na_2B_4O_7$, SDS, pH 8 (adjusted with 1 *M* CH_3COOH); hydrodynamic injection time: 1 s; solute concentration: 25 μg/ml. *K*-values are deduced from Fig. 4b and according to Eq. (3).

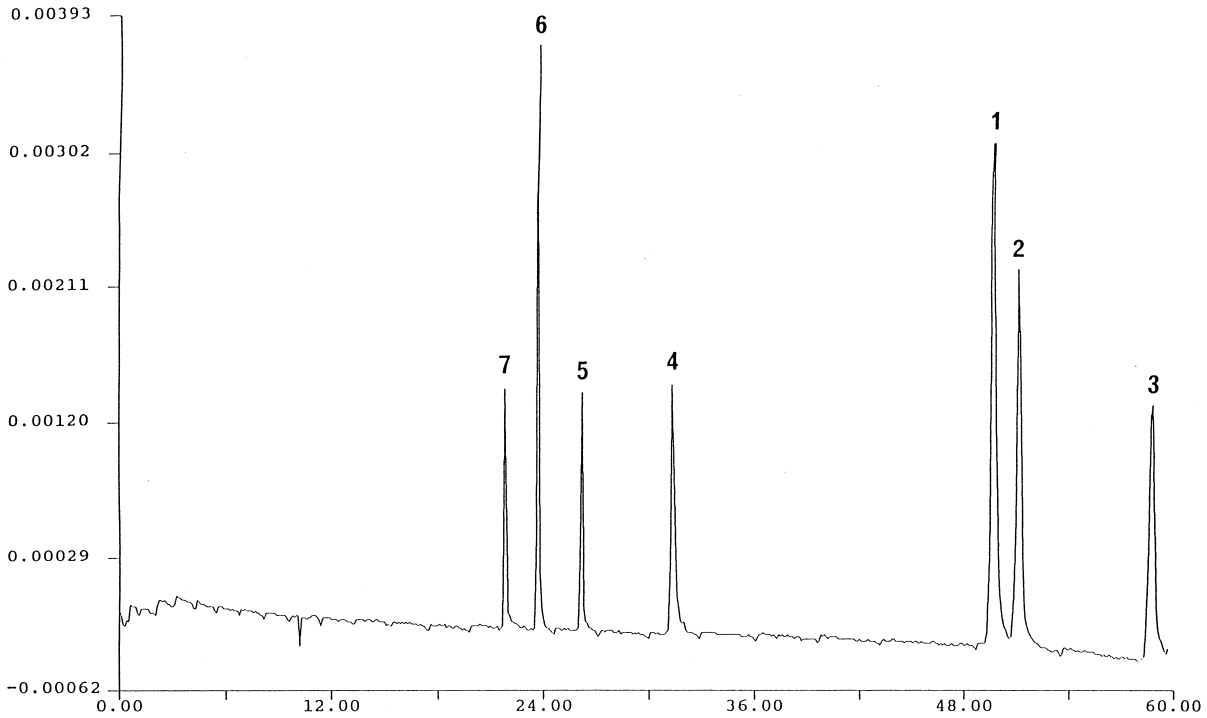


Fig. 5. Separation of a standard mixture of seven methoxylated and hydroxylated flavonoids by MEKC. Fused-silica capillary: 70 cm×50 μm I.D; applied voltage: +20 kV; temperature: 30°C; UV detection at 250 nm; electrolyte: 20 m*M* $Na_2B_4O_7$, 25 m*M* SDS, 20% methanol, pH 8 (adjusted with 1 *M* CH_3COOH), hydrodynamic injection time: 1 s; solute concentration: 15 μg/ml. Solute numbers referred to are given in Fig. 1.

25 m*M* SDS and 20% methanol into the sodium tetraborate buffer improves the resolution of these neutral and charged analytes compared to that obtained by CZE (Fig. 1); nevertheless, the addition of methanol caused an increase in separation time.

Further studies are being undertaken in our laboratory to investigate the influence of buffer pH upon partition coefficients of these charged hydroxylated flavonoids in order to determine the log *P* values of these flavonoids.

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