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Determination of flavonoids by micellar electrokinetic capillary chromatography

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ABSTRACT

HPCE based on cetyltrimethylammonium bromide (CTAB) or cholate micellar electrokinetic capillary chromatography (MECC) was found suitable for the separation of individual flavonoid glycosides following a rapid and simple technique of isolation, purification and group separation. Kaempferol and quercetin glycosides with varying degrees of glycosylation, and with or without additional esterification on the carbohydrate part, were included in the study. The influence of temperature and voltage as well as electrolyte, CTAB and organic modifier concentrations in the buffer on the migration order, migration times, and peak areas of the flavonoids was investigated. The method developed gives possibilities for the separation and specific determination of flavonoids isolated from vegetative parts of cruciferous plants.

INTRODUCTION

Flavonoids are plant constituents comprising one of the most numerous and widespread groups of natural products. Of the total of more than 4000 known flavonoids, about one quarter belong to the well defined group of flavone and flavonol glycosides [1]. These compounds accumulate especially in vegetative parts of plants, and often to appreciable concentrations [2]. Kaempferol, quercetin and isorhamnetin are the most common flavonol aglycones. They show great variation in the type of glycosylation and as reported during recent years with an increasing number of acyl-substituted compounds [1].

The flavonoids have attracted much attention, owing not only to their various colours and great number, but also to their biological activities, as they possess many different physiological properties [2–4]. These variations in properties are closely related to their structure, with special effects caused by the type of glycosylation [2]. Therefore, efficient methods for analysis, isolation and identification have a high priority [5,6], and major developments in recent years have been in the more general use of high-performance liquid chromatographic (HPLC) techniques [1]. However, high-performance capillary electrophoresis (HPCE) is a method that has additional possibilities and great potential as an efficient method of flavonoid analysis with the technique based on micellar electrokinetic capillary chromatography (MECC) [7,8].

This paper describes and compares the separations of flavonoids obtained with two different HPCE–MECC methods. In the first method, cetyltrimethylammonium bromide (CTAB) was used as a detergent, and in the second method,

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cholate was used as detergent in combination with taurine. The methods were compared and evaluated by studying the influences of different separation conditions on various separation parameters. The migration order of the various flavonoids is presented and discussed, and examples of analyses of flavonoids in samples of vegetative parts of cruciferous plants are presented.

EXPERIMENTAL

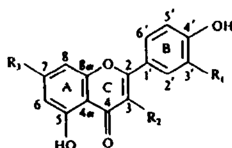
Apparatus

An ABI Model 270 A capillary electrophoresis instrument (Applied Biosystems, Foster City, CA, USA) was used with a 720 mm × 50 μm I.D. fused-silica capillary (J & W Scientific, Folsom, CA, USA). Detection was effected by measurement of UV absorption at 500 mm from the injection end of the capillary. Data processing was performed on a Shimadzu (Kyoto, Japan) Chromatopac C-R3A instrument.

Materials and reagents

The flavonoids (Fig. 1) were from the collection in this laboratory. The compounds were obtained from various cruciferous plants [2,9]. The methods applied were group separation [10,11], purification by column chromatography (CC) [2] and identification by paper chromatography (PC), thin-layer chromatography (TLC), high-voltage electrophoresis (HVE) and nuclear magnetic resonance (NMR) spectrometry [2,10].

Disodium tetraborate, disodium hydrogenphosphate, sodium cholate and taurine were from Sigma (St. Louis, MO, USA) and CTAB from BDH (Poole, UK). All chemicals were of analytical-reagent grade.



No.	Name	R_1	R_2	R_3
1	Kaempferol	H	OH	OH
2	Kaempferol-3-glucoside	H	Glucoside	OH
3	Kaempferol-3,7-diglucoside	H	Glucoside	Glucoside
4	Rustoside	H	Xylopyranosyl-(1,2)-galactopyranosyl	OH
5	Kaempferol-3-sophoroside-7-glucoside	H	Sophoroside	Glucoside
6	Kaempferol-3-sinapoylsophoroside-7-glucoside	H	Sinapoyl-sophoroside	Glucoside
7	Kaempferol-3-(6''-carboxyglucoside)	H	6''-carboxy-glucoside	OH
8	Quercetin	OH	OH	OH
9	Quercetin-3-glucoside	OH	Glucoside	OH
10	Rutin	OH	Rutinoside	OH
11	Quercetin-3-(6''-carboxyglucoside)	OH	6''-carboxy-glucoside	OH
12	Isorhamnetin-3-(6''-carboxyglucoside)	OMe	6''-carboxy-glucoside	OH

Fig. 1. Names, structures and numbering of flavonoids used.

Procedure

Buffer preparations for the CTAB–MECC separations were performed according to Michaelsen *et al.* [12]. Samples were dissolved in water, except **1** and **8**, which were dissolved in water–dimethyl sulphoxide (1:1). Samples were introduced from the cathodic end of the capillary by 1-s vacuum injection in the CTAB system, and from the anodic end of the capillary by 1-s vacuum injection in the cholate–taurine system. Separations were performed under various separation conditions as specified in the text. On-column UV detection was at 350 nm (CTAB system) and at 250 nm (cholate–taurine system). Washings of the capillary were performed with 1.0 *M* NaOH for 5 min, water for 2 min and buffer for 5 min before each analysis. Calculations of normalized peak areas (NA), resolution (R_s) and the number of theoretical plates (N) were performed according to Michaelsen *et al.* [12].

Mixtures of flavonoids were obtained from plants homogenized and extracted in boiling methanol–water (70:30). The flavonoid extract was then purified by group separation through a weekly acidic cation exchanger, a strongly acidic cation exchanger and a weakly basic anion exchanger according to a previously described procedure [11].

RESULTS AND DISCUSSION

The names, structures and numbers of the flavonoids used in this study are shown in Fig. 1. The differences in structures between the investigated flavonoids are due to variation in the aglycones at C-3′ with R_1 as either hydrogen (kaempferol), hydroxy (quercetin) or methoxy

(isorhamnetin) groups or various glycosides attached to the 3- or 7-position of these aglycones.

The separation mechanisms which are most likely to give successful separations of these compounds in HPCE are based on differences in hydrophobic interactions for all of the compounds and differences in electrophoretic mobilities for the charged carboxy-substituted glucosides at pH values above 4.5. MECC separations of flavonoids were therefore tested with the previously described CTAB method [12–15], and the data obtained were compared with results obtained with MECC based on a cholate- and taurine-modified buffer [16].

The CTAB–MECC method results in hydrophobic interactions with flavonoid aglycones as well as ion-pairing interaction chromatography with the flavonoids containing carboxy groups and the positively charged CTAB monomers and micelles and the CTAB-coated capillary wall. This results in differential partitioning of flavonoids between the micellar and the aqueous buffer phases [13]. The neutral flavonoids migrate with the electroosmotic flow towards the anode, and they are retarded by CTAB migrating towards the cathode. The negatively charged flavonoids will migrate towards the anode owing to their electrophoretic mobility, and with electroosmotic flow increasing their velocity, whereas CTAB will decrease their velocities. In addition, complex formation of the carbohydrate parts of the flavonoids with borate in the buffer may occur [17]. This will increase the velocity of the flavonoids towards the anode.

The cholate–MECC separation is based on hydrophobic interaction with all flavonoids and ion repulsion of the flavonoids with carboxy groups and the negatively charged cholate monomers and micelles. This also results in differential partitioning of flavonoids between the micellar and the aqueous buffer phases. Compared with the likely spherical form of CTAB micelles with the charged groups situated on the surface of the micelle and a hydrophobic interior [18], cholate micelles have been suggested to form rod-like or cylindrical micelles with the hydrophobic part situated on the surface and the hydrophilic portions turned inward [19]. The effect of the zwitterion taurine in the system is less clear. Taurine may affect the degree of binding of the counter ions to the cholate micelles, and thereby affect the critical micelle concentration (CMC) [18]. The high ionic strength of the buffer due to taurine may also result in increased aggregation number of the micelles and decreased CMC values [18,20]. The association of taurine with either the capillary surface or the negatively charged flavonoids will probably not have a large influence on the separation, as both the capillary surface and the flavonoids will still be negatively charged. According to the theory, neutral flavonoids will migrate with the electroosmotic flow towards the cathode, and the cholate monomers and micelles migrating towards the anode will have a retarding effect on the flavonoids. The negatively charged flavonoids will migrate towards the anode owing to their electrophoretic mobility, and the cholate monomers and micelles migrating towards the anode will change their velocity depending on the effect of hydrophobic interaction compared with ion repulsion. Finally, the electroosmotic flow will decrease their velocity. Owing to the size of the electroosmotic flow compared with the influence of the other mechanisms involved, flavonoids will migrate towards the cathode end of the capillary.

Migration order

The separations of flavonoids in the CTAB and the cholate–taurine systems are shown in Fig. 2. The migration order of the compounds in the CTAB system was **5**, **6**, **4**, **3**, **10**, **2**, **9**, **7**, **8** and **1**. The migration order was apparently not affected by changes in 1-propanol, electrolyte or CTAB concentration as well as changes in temperature or voltage, whereas the separations and migration times were affected by changes in these parameters (see below). The migration order of the compounds in the cholate–taurine system was **5**, **6**, **10/11**, **12**, **4**, **8**, **1**, **2** and **9**. However, in this system the migration order of **8**, **1**, **2** and **9** was affected by changes in temperature, voltage and concentration of 1-propanol (see below). The best separations were generally obtained with the cholate–taurine system. The separation efficiency, expressed as the number of

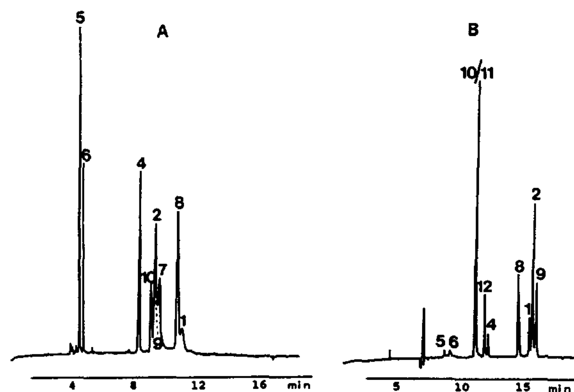


Fig. 2. (A) Electropherogram of flavonoids in the CTAB system. Separation conditions: temperature, 50°C; voltage, −26 kV; buffer composition, 12 m*M* borate–20 m*M* phosphate–40 m*M* CTAB adjusted to pH 7.0, 4% 1-propanol added; UV detection at 350 nm. (B) Electropherogram of flavonoids in the cholate–taurine system. Separation conditions: temperature, 40°C; voltage, 25 kV; buffer composition, 35 m*M* cholate–100 m*M* phosphate–500 m*M* taurine, 6% 1-propanol added; UV detection at 250 nm. Numbers as in Fig. 1.

theoretical plates per metre of capillary (N/m), and resolution (R_s) are given in Table I for the separation obtained with the cholate–taurine system under the conditions as described in Fig. 2B. The N/m values obtained were high, with values between 150 000 and 225 000, and the R_s values ranged from 1.21 for **1–2** to 12.91 for **4–8**.

Compounds **5** and **6** are the most hydrophilic compounds, and they migrate with the highest velocities in both systems. The 2″-sinapoylsophoroside group of **6** makes this compound more hydrophobic than **5** with the unsubstituted sophoroside group, and **5** therefore migrates faster than **6** in both the CTAB and the cholate–taurine systems (Fig. 2). The presence of a 3-glucoside in both kaempferol and quercetin (*i.e.*, **2** and **9**, respectively) compared with the unsubstituted kaempferol and quercetin (*i.e.*, **1** and **8**, respectively) resulted in decreases in migration times of 2 min in the CTAB system. In the cholate–taurine system the positions of kaempferol and quercetin compared with their glucosides depended on the applied voltage, temperature and 1-propanol concentration (see below).

Changing the 3-glucoside in **2** to the 3-diglycoside xylopyranosylgalactopyranosyl in **4** decreased the migration time in the CTAB system by 1 min, whereas in the cholate–taurine system the migration time decreased by 4.6 min (Fig. 2). Changing the 3-glucoside in **9** to the 3-diglycoside rutinoside in **10** also resulted in a minor change in the migration times in the CTAB system and a large change in the cholate–taurine system (Fig. 2). The change in hydrophobicity of these compounds apparently has a much larger effect in the cholate–taurine system than in the CTAB system.

Replacing hydrogen at C-3′ in **1** or **2** with the hydroxy group at C-3′ in **8** or **9** had little

TABLE I

NUMBER OF THEORETICAL PLATES PER METRE IN THOUSANDS (N/m) AND RESOLUTION (R_s) FOR FLAVONOIDS ANALYSED IN THE CHOLATE–TAURINE SYSTEM

Numbers as in Fig. 1 and separation conditions as in Fig. 2B.

Parameter	No.								
	5	**6**	**10/11**	**12**	**4**	**8**	**1**	**2**	**9**
N	139	77	151	185	181	150	170	227	210
	Pair								
	5–6	**6–10/11**	**10/11–12**	**12–4**	**4–8**	**8–1**	**1–2**	**2–9**	
R_s	2.82	11.75	4.87	1.84	12.91	4.41	1.21	1.69	

influence on migration times in both systems except for **1** and **8** in the cholate–taurine system. Replacing the hydroxy group at C-3′ in **11** with the methoxy group at C-3′ in **12** resulted in an increased migration time in the cholate–taurine system, probably owing to the increased hydrophobicity of **12** compared with **11**.

Finally, the presence of a carboxy group at the glucoside in **7** compared with **2** gave a small increase in migration time in the CTAB system. This is caused by the ion-pairing effect with CTAB, as reported for other compounds [12,15]. The presence of a carboxy group at the glucoside in **11** compared with **9** gave a very large decrease of 5 min in the migration time in the cholate–taurine system. Ion repulsion between the negatively charged cholate and the carboxy group of **7** and **11** can explain these results, as the influence of the hydrophobic interaction then decreases and the compounds migrate faster.

Separation conditions

Increasing voltages gave large non-linear decreases in migration times in both systems (Fig. 3). Further, the migration order of some of the flavonoids changed with increasing voltage in the cholate–taurine system. At a voltage of 15 kV **8** was in a position just after **9**, and at 20 kV **8** and **9** migrated together after **2** and in front of **1**, and finally, at 25 kV **8** had moved to a position in front of **2**, **9** and **1**. These results were obtained with separation conditions as described in Fig. 2B except for a buffer containing 4% 1-propanol. The general decrease in migration time with higher voltages is caused by an increase in electroosmotic flow, and the velocities of **7**, **11** and **12** are further affected owing to their negative charge. Increasing temperature in the capillary due to insufficient heat removal at high voltages may influence the interaction of analytes with micelles [15], and this can also cause the change in migration order seen for some of the compounds. The concave curves and the change in migration order may therefore be caused by a combination of the described effects. The *NA* values of **6** increased slightly, those of **1**, **4**, **5**, **9** and **12** were constant and those of **8** and **10/11** decreased with increasing voltage. Changes in *NA* values are probably caused by changes in the relative response factors of the flavonoids due to the changed interaction with cholate–taurine.

The migration times decreased markedly with increasing temperature in both systems. The decreases were linear for the CTAB system except for values obtained at 35°C, whereas in the cholate–taurine system the decreases were non-linear with a decreasing rate of reduction at higher temperatures (Fig. 4). The non-linear decrease in the cholate–taurine system indicates increased interaction of flavonoids with cholate–taurine at higher temperatures.

The flavonoids **1** and **8** moved from positions after **2** and **9** to positions in front of **2** and **9** when the temperature increased (Fig. 5). These

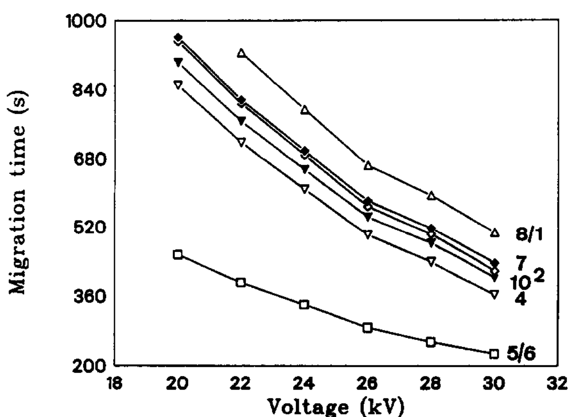


Fig. 3. Influence of applied voltage on migration times of flavonoids in the CTAB system. Numbers as in Fig. 1. Other separation conditions as in Fig. 2A.

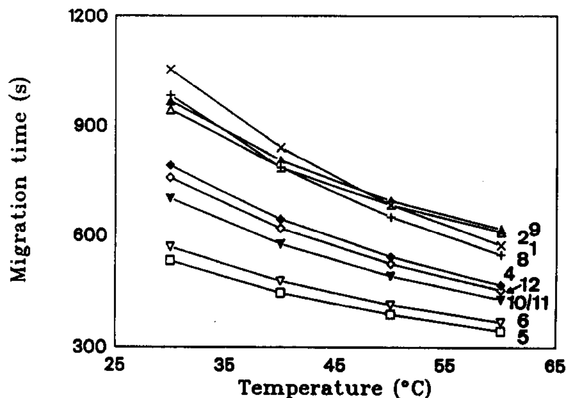


Fig. 4. Influence of temperature on migration times of flavonoids in the cholate–taurine system. Numbers as in Fig. 1. Other separation conditions as in Fig. 2B except 4% of 1-propanol in the buffer.

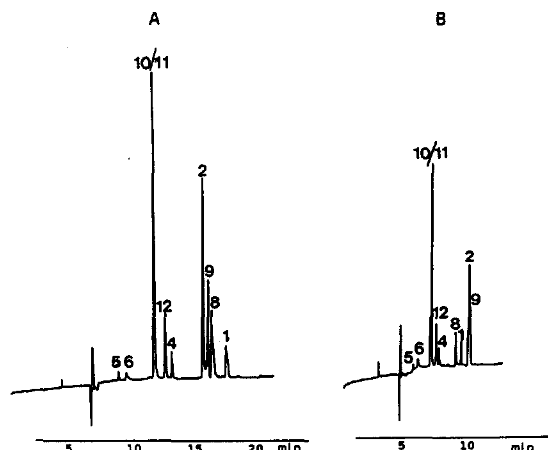


Fig. 5. Influence of temperature on migration order of flavonoids in the cholate–taurine system. (A) 30°C; (B) 60°C. Numbers as in Fig. 1. Other separation conditions as in Fig. 2B except 4% of 1-propanol in the buffer.

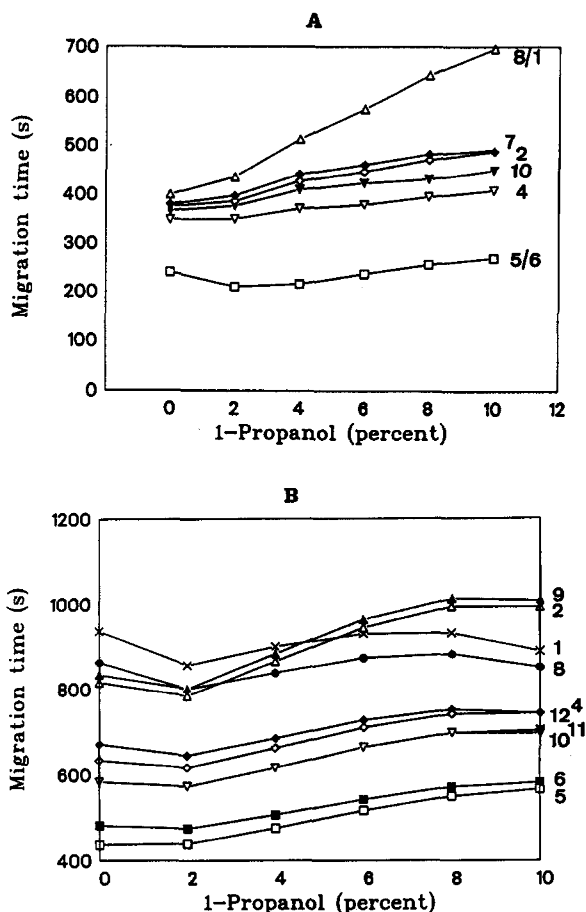


Fig. 6. Influence of 1-propanol concentration on migration times of flavonoids in (A) the CTAB system and (B) the cholate–taurine system. Numbers as in Fig. 1. Other separation conditions as in Fig. 2.

changes in migration order are probably also caused by changes in the interaction between analytes and cholate at high temperatures. The best separations with the CTAB system were obtained at 30 or 50°C and with the cholate–taurine system at 30 or 60°C.

The influence of the presence of 1-propanol in the buffer on the migration times of flavonoids depended strongly on the analyte (Fig. 6). Increasing the concentration resulted in decreasing migration times for most analytes when going from 0 to 2% 1-propanol in both systems, and from 2 to 8% of 1-propanol all migration times increased except for **1** in the cholate–taurine system. Finally, changing from 8 to 10% of 1-propanol resulted in increasing migration times in the CTAB system, whereas for **2**, **4**, **5**, **6**, **9**, **10**, **11** and **12** the migration times were constant in the cholate–taurine system but decreased for **1** and **8**.

The migration times generally increased less in the cholate–taurine system than in the CTAB system with increasing 1-propanol concentration. This may be explained by the likely ability of the cholate micelles to tolerate high concentrations of organic solvents [19]. The helical structure of the cholate micelles with a hydrophobic surface, compared with the normal spherical form of micelles [*e.g.*, sodium dodecyl sulphate (SDS)] with a hydrophobic interior, is believed to make these cholate micelles less vulnerable to disassociation by the reduction of the polarity of the buffer solution, which occurs when the alcohol is added [19].

The migration order also changed for some of the compounds. Flavonoids **1** and **8** shifted from positions after **2** and **9** to positions in front of **2** and **9** when the concentration of 1-propanol increased (Fig. 7). Further, flavonoids **4** and **12** separated well at low concentrations of 1-propanol, whereas with 10% 1-propanol in the buffer they migrated as one peak (Fig. 7).

Increasing the amount of organic modifier in the buffer causes decreases in the electroosmotic flow [21], and the decreases become larger with

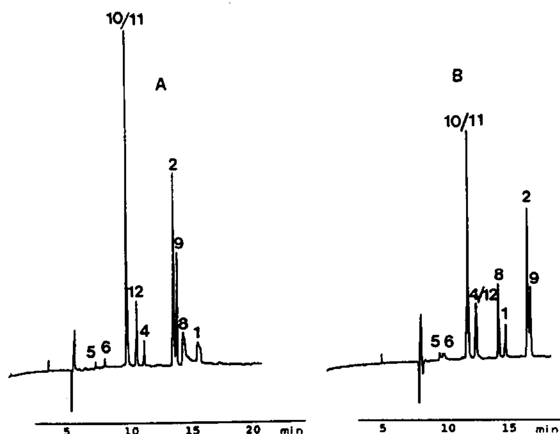


Fig. 7. Influence of 1-propanol concentration on the migration order of flavonoids in the cholate–taurine system. (A) 0 and (B) 10% 1-propanol. Numbers as in Fig. 1. Other separation conditions as in Fig. 2B.

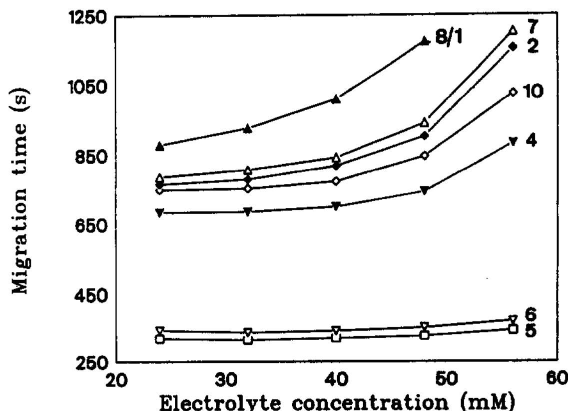


Fig. 8. Influence of electrolyte concentration on migration times of flavonoids in the CTAB system. Numbers as in Fig. 1. Other separation conditions as in Fig. 2A.

increasing length of the alcohol chain [22]. Further, the presence of an organic modifier also results in more of the analyte being present in the aqueous phase than in the micellar phase. Both effects are probably involved here as the migration times first decrease and then increase with higher amounts of organic modifier. The best separations were obtained with 4 or 6% 1-propanol in the buffer.

The *NA* values generally decreased for all compounds and the decrease was largest for **1**, **2**, **9** and **10/11**. The changes in *NA* values are probably caused by changes in the relative response factors of the flavonoids and changes in the viscosity of the buffer [15].

1-Propanol was chosen as a modifier because its boiling point is close to that of water. Other modifiers with lower boiling points were also tested, but the migration times from consecutive analyses of identical samples changed considerably owing to continuous changes in the buffer composition, as the modifier evaporated at a higher rate than water from the buffer.

The effects of increasing electrolyte concentration [borate–phosphate (3:5)] were investigated with the CTAB system. The migration times of **5** and **6** were unaffected, whereas the migration times showed non-linear increases for the other compounds (Fig. 8). The changes were greatest when going from 48 to 56 m*M*. A combination of decreasing electroosmotic flow with increasing electrolyte concentration [23,24] and changed interaction of flavonoids with CTAB [8] can explain the observed results. Also, a possible effect of the concentration and the equilibrium of the borate–glycoside complex formation [15,17] is a likely explanation of the observed results. An electrolyte concentration of 32 m*M* gave the best separation of the investigated flavonoids.

Finally, increasing CTAB concentration gave increased migration times for all of the flavonoids (Fig. 9). This result is expected owing to

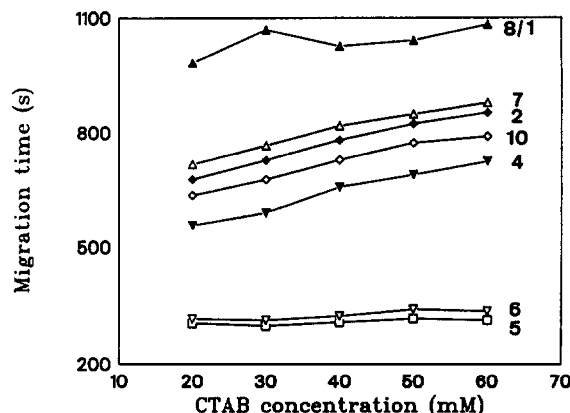


Fig. 9. Influence of CTAB concentration on migration times of flavonoids in the CTAB system. Numbers as in Fig. 1. Other separation conditions as in Fig. 2A.

an increase in the ratio of the micellar phase to that of the aqueous phase and possible changes in the electroosmotic flow [12]. The separation of the compounds studied was slightly improved for **5** and **6** and reduced for **2** and **7** and for **7** and **8/1** with increasing CTAB concentration. The best separation was obtained with 30 or 40 m*M* CTAB in the buffer.

The use of SDS–MECC for the separation of flavonoids has been reported [7]. However, compared with the separations reported here, the flavonoids migrated very close to each other and in the time interval from only 10 to 13 min. In our experiments flavonoids migrated with migration times between 4 and 11 min in the CTAB system and between 8 and 16 min in the cholate–taurine system. In both experiments not all flavonoids were separated with one set of separation conditions. Owing to the increased separation window the methods with CTAB or cholate–taurine may give better separations than the SDS method. Finally, isotachophoretic HPCE analyses of flavonoids have also been reported [4]. However, the separations obtained and the separation capacity of the isotachophoretic method make this method less promising than the CTAB and cholate–taurine methods.

Examples of analyses of flavonoids isolated and purified by group separation of plant extracts are shown in Fig. 10. The group separation procedure applied for the purification of flavonoids yields a sample of neutral compounds [11]. As seen in the electropherograms, the isolated compounds can be detected at either 350 or 250 nm, and it is therefore likely that most of the isolated compounds are flavonoid glycosides or other phenolics. The leaves of rapeseed (*Brassica napus*) and the vegetative parts of broccoli (*Brassica oleracea*) contained many possible flavonoids (Fig. 10A, C and D), whereas the vegetative parts of yellow mustard (*Sinapis alba*) contained only two possible flavonoids in large amounts (Fig. 10B). Good separations were obtained in both systems, although the separations were not identical. However, it is not possible to choose one system or the other from the separations obtained.

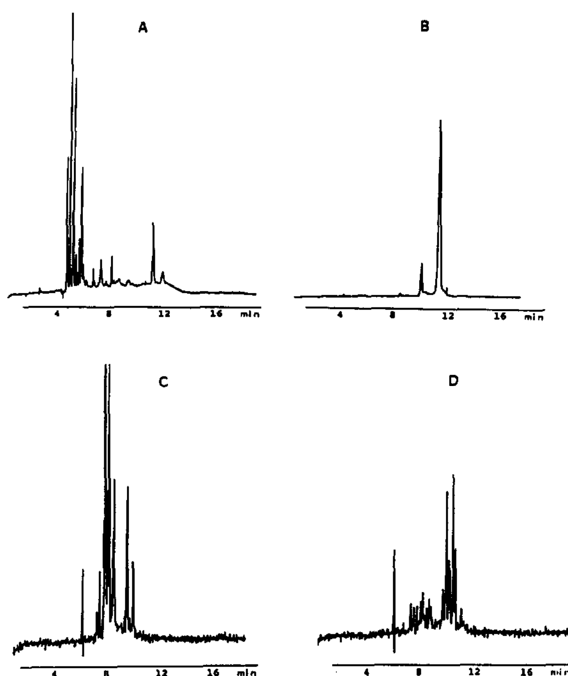


Fig. 10. Electropherograms of samples of neutral flavonoids from plants. (A) Leaves of rapeseed (*Brassica napus*) analysed in the CTAB system; (B) vegetative parts of yellow mustard (*Sinapis alba*) analysed in the CTAB system; (C) leaves of rapeseed (*Brassica napus*) analysed in the cholate–taurine system; (D) vegetative parts of broccoli (*Brassica oleracea*) analysed in the cholate–taurine system.

CONCLUSIONS

MECC–HPCE proved its value for analyses of flavonoids. Both the CTAB and the cholate–taurine systems can be applied to the separation of flavonoids, but the cholate–taurine system generally gave better separtions of the investigated flavonoids. The investigated separation systems resulted in different migration orders of some of the flavonoids. The migration order of flavonoids in both systems could be explained by the differences in the hydrophobicity and charge of the compounds. Further, the change in hydrophobicity of some of the flavonoids had a much larger effect on migration times in the cholate–taurine system than in the CTAB system. The separation parameters were much affected by the separation conditions in both systems, but the effects depended strongly on the system. Finally,

analyses of samples of flavonoids prepared from various plants showed the practical analytical use of both system for the analysis of flavonoids.

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