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Short Communication

Borate complexation of flavonoid-O-glycosides in capillary electrophoresis

II. Separation of flavonoid-3-O-glycosides differing in their sugar moiety

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ABSTRACT

Capillary electrophoresis was found to give significantly higher efficiency, selectivity and speed than high-performance liquid chromatography for the separation of a mixture of flavonoid-3-O-glycosides differing in their sugar moiety. Boric acid running buffer (0.2 *M*, pH 10.5) was used for this electrophoretic separation. The migration order in free solution capillary electrophoresis (CE) and the selectivity of these flavonoid-3-O-glycosides can be mainly explained by *in situ* borate complexation of both the sugar moiety and the *cis*-1,2-hydroxyl groups on the flavonoid skeleton and, to a lesser extent, by the ionization of hydroxyl groups on the flavonoid skeleton due to alkaline pH conditions. The correlation of the electrophoretic mobilities with the configuration and conformation of the compounds is discussed.

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-phenylbenzopyrone and differ in the pattern of hydroxylation, degree of unsaturation and type and position of sugar links [1].

Recent improvements in capillary zone electrophoresis (CZE) are attractive for studies of natural products because of the high-resolution separations achievable with on-column UV detection [1,2]. Existing methods for the analysis of flavonoid-O-glycosides generally involve high-performance liquid chromatography (HPLC) [3–5]. However, this tech-

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nique requires gradient elution which tends to be inconvenient, time consuming and laborious. Pietta *et al.* [1] have applied micellar electrokinetic capillary chromatography (MECC) to the determination of several flavonoid drugs (quercetin-, kaempferol- and isorhamnetin-3-O-glycosides). Seitz *et al.* [6] used capillary isotachophoresis for the rapid determinations of flavonoids and phenolcarboxylic acids. Optimum separations were achieved at pH 9.5 with a leading electrolyte containing 0.015 *M* hydrochloric acid, 30% methanol and 0.2% hydroxypropylmethylcellulose. In a recent study [7], the separation of several flavonoid-7-O-glycosides, differing in their flavonoid aglycone, was achieved by CE. However, a mixture of both flavonoid-7-O-glycosides and their flavonoid aglycones was better resolved in MECC using sodium dodecyl sulphate as anionic surfactant.

In this work, a mixture of quercetin-3-O-glycosides, differing in their sugar moiety, was resolved by CE using a borate complexing running buffer. The migration order in CE and the borate complexation mechanism of these solutes were elucidated.

EXPERIMENTAL

Instrumental

Free solution capillary electrophoretic (FSCE) separations were mainly performed on a Europhor (Toulouse, France) Prime Vision instrument using a silica capillary column. A small section (1 cm length) of the polyimide coating of the capillary column was removed prior to filling to give an optical window for UV detection. Analyses were carried out at ambient temperature (Europhor). All electropherograms were recorded on a Shimadzu (Kyoto, Japan) C-R 5A integrator. A 65 cm × 50 μm I.D. silica capillary column filled with phosphate–borate buffer or boric acid–sodium hydroxide buffer served as the separation column operated at high voltages. On-column detection was effected at 270 nm at 2.0 AUFS with a rise time of 1s.

Some of the separations were performed on a Spectra-Physics (San Jose, CA, USA) Spectraphoresis 1000 instrument using a 70 cm × 375 μm O.D. × 50 μm I.D. silica capillary column. Separations were performed at 40°C at a voltage of 24.4 kV (electrical field strength 385 V/cm) and a run average current of 80 μA. For this instrument, analytes for each run were injected in the hydrodynamic mode for 1.0–3.0 s. Data were processed on an IBM PS/2 Model 70 386 computer. Software operating under IBM OS/2 was supplied by Spectra-Physics. The instrument contains a programmable, high-speed scanning, multiple-wavelength UV detector. Working in the fast scanning mode, we were able to record on-column spectra of the flavanoid-3-O-glycosides. The scanning mode was from 200 to 360 nm with a 5-nm wavelength increment.

The capillaries were conditioned daily by washing first with 1 *M* sodium hydroxide solution (10 min) at 60°C (at ambient temperature for the Europhor instrument), then with 0.1 *M* sodium hydroxide solution (10 min) at 40°C, with water at 60°C (10 min) and with finally the electrophoretic buffer (15 min) at 40°C. Between consecutive analyses, the capillaries were flushed with water (2 min), 0.1 *M* sodium hydroxide solution (2 min), water (3 min) and finally with the electrophoretic buffer (10 min) in order to improve the migration times and peak-shape reproducibility.

Reagents

All chemicals were of analytical-reagent grade. Boric acid, sodium dihydrogenphosphate and 0.1 *M* sodium hydroxide solution (all from Fluka, Buchs, Switzerland) were used as received. Water used for dilutions and in buffer solutions was of HPLC grade (Carlo Erba, Milan, Italy).

For free solution CE under alkaline conditions, the running buffer composition was either 0.006 *M* $Na_2B_4O_7$–0.010 *M* NaH_2PO_4 (pH adjusted with 1 *M* NaOH) or 0.2 *M* H_3BO_3 (pH adjusted to 6, 7.8 or 10.5 with 1 *M* NaOH).

Authentic samples of quercitrin (quercetin-3-O-rhamnoside), peltatoside (quercetin-3-O-arabinoglucoside), isoquercitrin (quercetin-3-O-glucoside), hyperoside (quercetin-3-O-galactoside) and avicularin (quercetin-3-O-arabinoside) (Fig. 1) were obtained from Extrasynthese (Genay, France). A standard solution of *ca.* 100 ppm of each flavonoid-3-O-glycoside was prepared in dimethyl sulphoxide–methanol (80:20, v/v).

Each analyte and each solution (water, sodium hydroxide and electrophoretic buffer) were filtered prior to injection through a polypropylene filter (0.2 μm pore size, 25 mm diameter) from Whatman (Maidstone, UK).

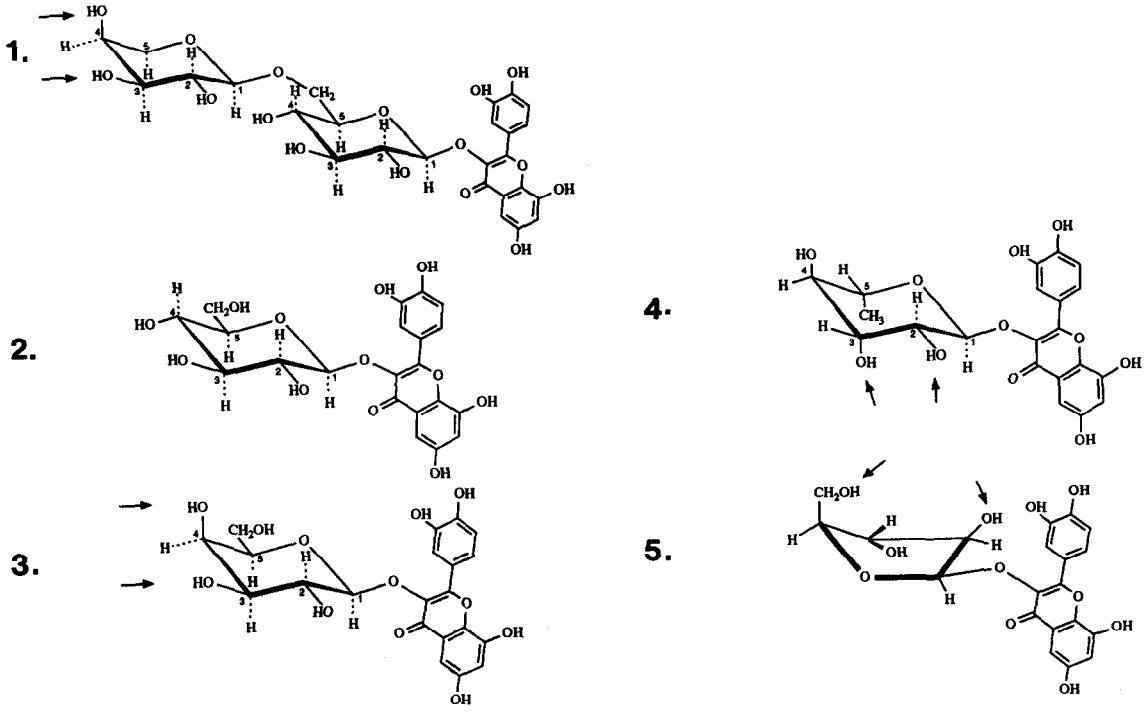

Fig. 1. Structures of quercetin-3-O-glycosides. 1 = Peltatoside [quercetin-3-O-arabinoglucoside; 3-[(6-O-α-L-arabinopyranosyl-β-D-glucopyranosyl)oxy]-2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4*H*-1-benzopyran-4-one}; 2 = isoquercetrin {quercetin-3-O-glucoside; 3-[(β-D-glucofuranosyl)oxy]-2-(3,4-dihydroxylphenyl)-5,7-dihydroxy-4*H*-1-benzopyran-4-one}; 3 = hyperin {quercetin-3-O-galactoside; 3-[(β-D-galactopyranosyl)oxy]-2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4*H*-1-benzopyran-4-one}; 4 = quercitrin {quercetin-3-O-rhamnoside; 3-[(6-deoxy-α-L-mannopyranosyl)oxy-2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4*H*-1-benzopyran-4-one}; 5 = avicularin {quercetin-3-O-arabinoside; 3-[(α-L-arabinofuranosyl)oxy]-2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4*H*-1-benzopyran-4-one}; Arrows indicate hydroxyl groups occurring as boration sites on the sugar molecule, but the same boration sites in the 3′,4′-positions on the flavonoid skeleton are not shown.

RESULTS AND DISCUSSION

Flavonoids commonly occur in plants as the 3-O-glycosides in which one flavonoid hydroxyl group is bound to a sugar by an acid-labile hemiacetal bond [8]. Some quercetin-like-glycosides have a carbohydrate component represented either by a monosaccharide residue such as 3-O-D-galactosyl (hyperin) or by a disaccharide residue such as 3-O-rutinosyl (rutin). Further, several O-methylated derivatives of quercetin and their glycosides are also found in nature, and on hydrolysis yield molecules of saccharides and a molecule of flavonoid, such as tamarixetin (the 4-methyl ether of quercetin).

For the compounds shown in Fig. 1, the same flavonoid aglycone (quercetin) is found in association with either a monosaccharide (β-D-glucose, β-D-galactose, α-L-rhamnose, α-L-arabinose) or a disaccharide (α-L-arabino-β-D-glucoside).

Influence of the nature of the running electrolyte

A standard mixture of the five quercetin-3-O-glycosides was resolved by free solution CE using running buffers having the constant pH (10.5) but differing in their chemical nature, either phosphate–borate buffer (Fig. 2a) or boric acid–NaOH buffer (Fig. 2b). In the alkaline and none-complexing phosphate–borate buffer, the electrophoretic mobility of each flavonoid-3-O-glycoside increases with ionization of the hydroxyl groups located on the flavonoid skeleton. However, the ionization of the flavonoid skeleton is not great enough to allow dif-

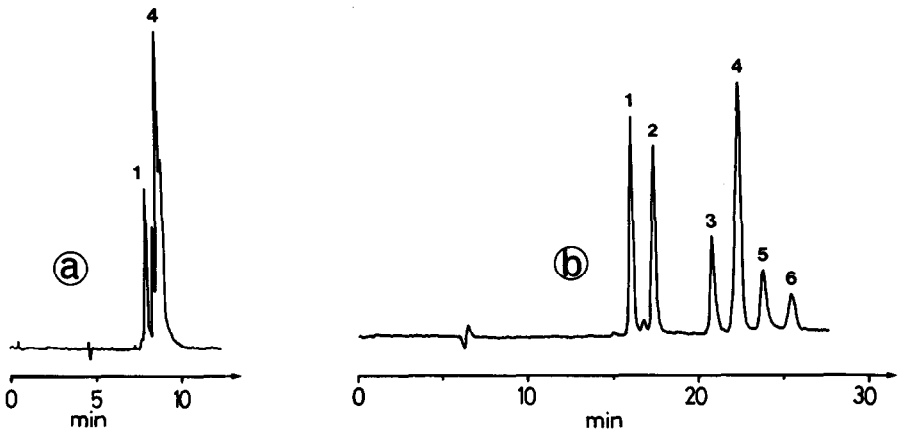

Fig. 2. Influence of the complexing nature of the running buffer in free solution CE on the separation of flavonoid-3-O-glycosides. Voltage, +24.4 kV; capillary, 65 cm × 50 μm I.D.; detection wavelength, 270 nm. Peaks 1–5, as in Fig. 1; 6 = impurity. Running buffer: (a) 0.006 *M* $Na_2B_4O_7$–0.010 *M* NaH_2PO_4 (pH 10.8); (b) 0.2 *M* H_3BO_3 (pH 10.5).

ferentiation among the electrophoretic mobilities of the five compounds (Fig. 2a). The ionization constants of common monosaccharides or disaccharides are in the pK_a range 11.9–12.5, according to Lee and Bunker [9]. More alkaline conditions are required to induce hydroxyl group ionization and different electrophoretic mobilities of sugars.

Another analytical approach involves a complex

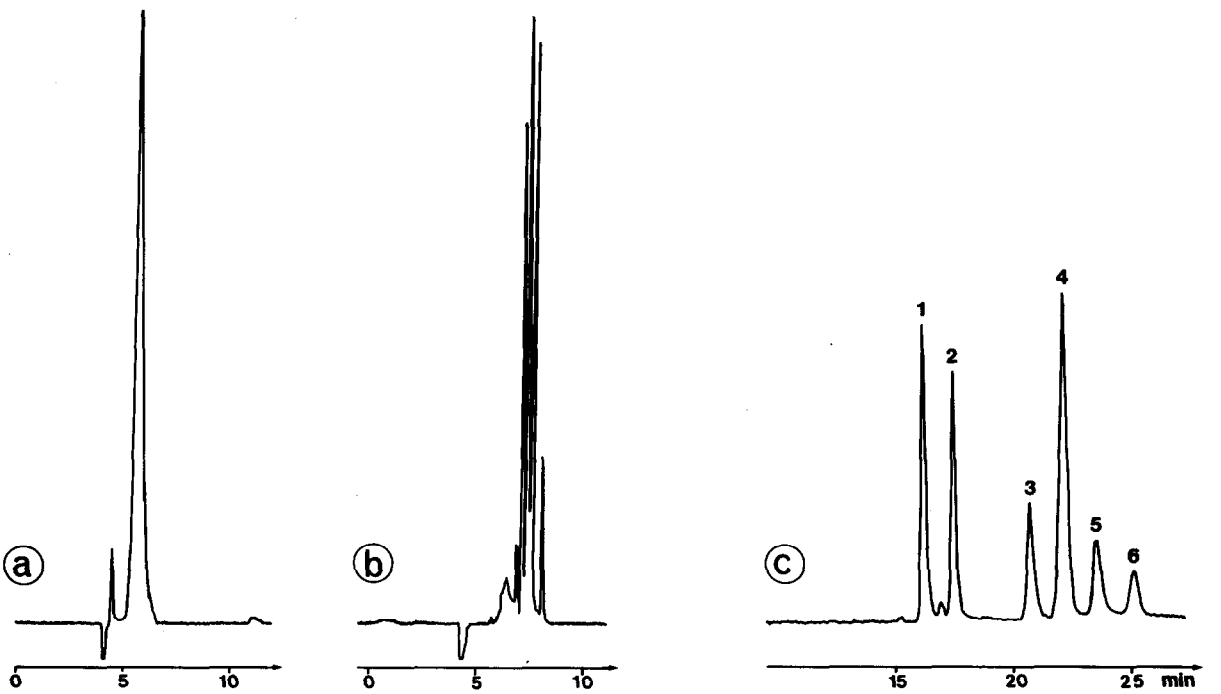

Fig. 3. Influence of the running buffer pH in free solution CE on the separation of flavonoid-3-O-glycosides. Conditions as in Fig. 2, except running buffer, 0.2 *M* H_3BO_3. Peaks 1–5, as in Fig. 1; 6 = impurity. (a) pH 6.0; (b) pH 7.8; (c) pH 10.5.

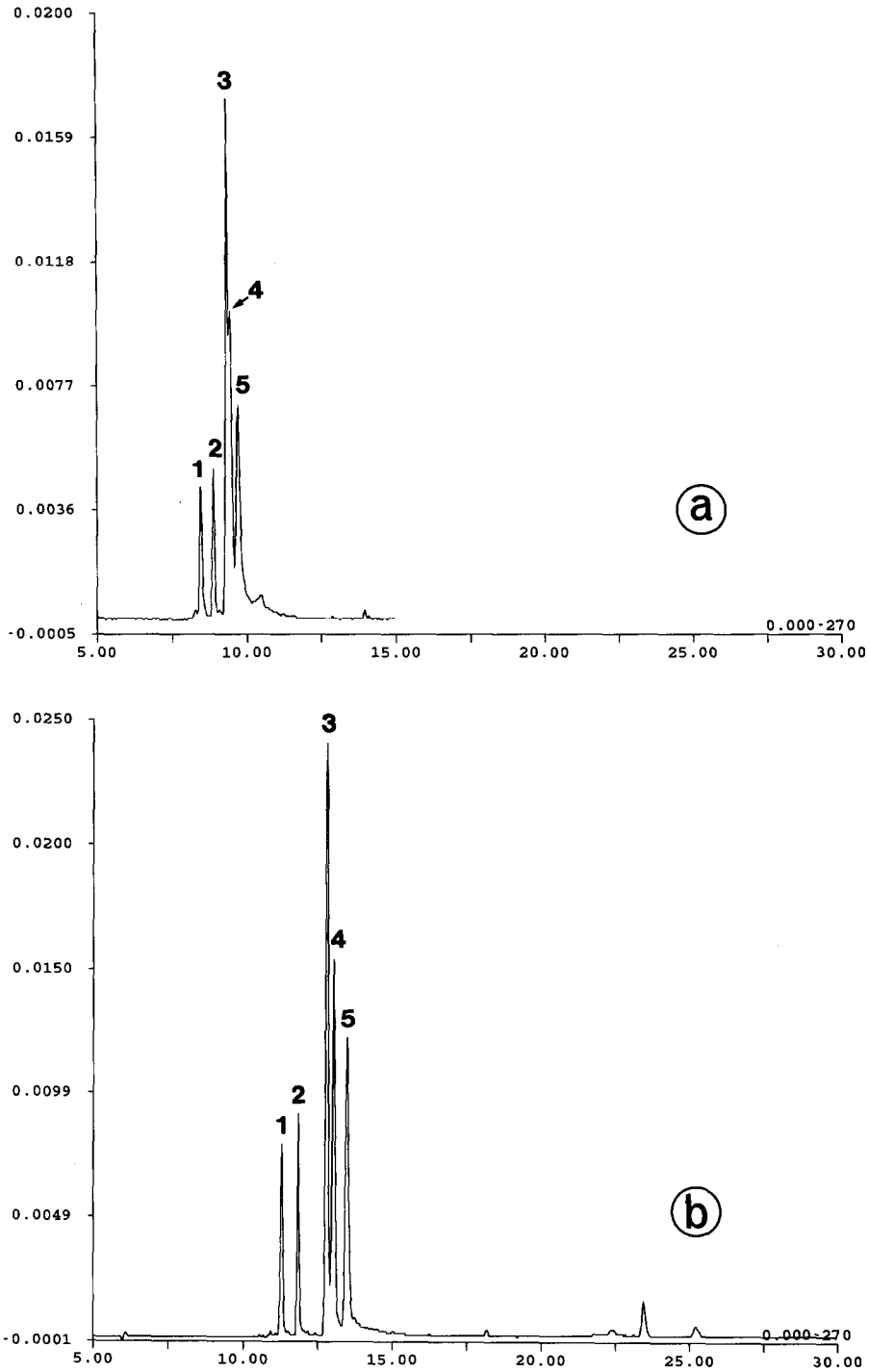

Fig. 4.

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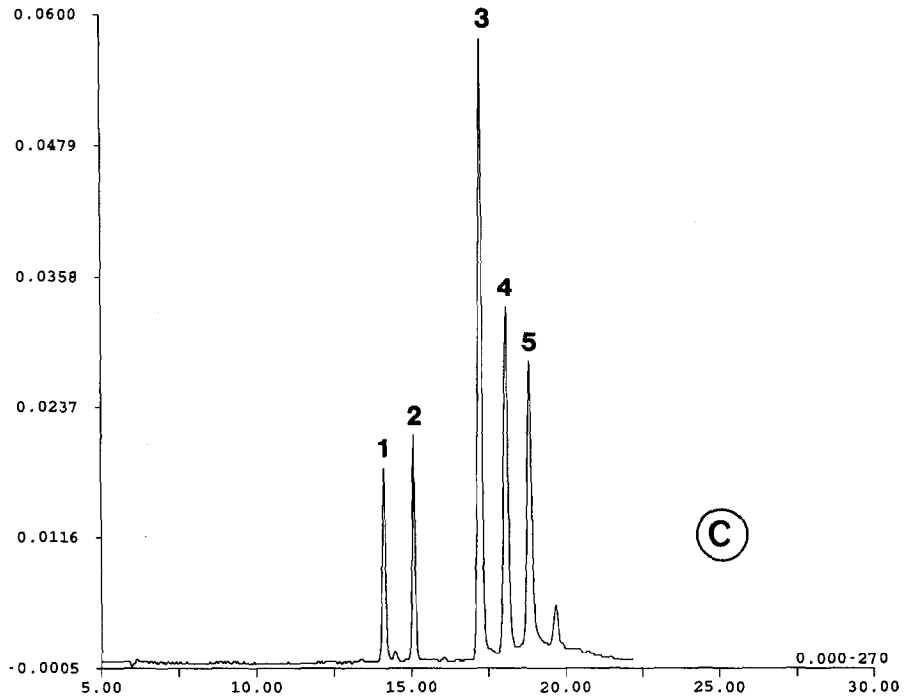

Fig. 4. Influence of boric acid concentration in free solution CE on the separation of flavonoid-3-O-glycosides. Voltage, +20 kV; capillary, 70 cm × 50 μm I.D.; running buffer, H_3BO_3 (pH 10.5); detection wavelength, 270 nm; temperature, 40°C. Peaks 1–5, as in Fig. 1. Boric acid concentration: (a) 0.05; (b) 0.10; (c) 0.20 *M*.

formation equilibrium by adding to the electrophoretic buffer a complexing agent of the saccharide or the flavonoid skeleton (Fig. 2b); the retention time of each solute is increased as they react with boric acid to form a borate anionic complex.

Influence of borate buffer pH

The different borate buffers tested in CE to resolve the flavonoid-3-O-glycosides had the same borate concentration (0.2 *M*) but different pH values (6–11). The complex formation reaction is a strongly pH-dependent equilibrium (Fig. 3). displacement of equilibria 2 and 3 towards the complex formation becomes greater at higher pH values, inducing a greater electrophoretic mobility and resulting in retardation of migration.

Influence of borate concentration

The effect of borate concentration on the CE selectivity was studied at pH 10.5 using several electrolyte systems of different borate concentrations (0.05–0.2 *M*). The migration time of each flavonoid-3-O-glycoside and the overall resolution increased with increasing concentration of boric acid (Fig. 4). Borate of higher concentration converts each flavonoid-O-glycoside into a charged complex form and provides separations due to the differences in the charge-to-mass ratio. The optimum buffer solution for the separation of these five quercetin-3-O-glycosides contained 0.2 *M* borate at an alkaline pH (10.5), which combines sufficient resolution with a moderate analysis time. Separation was complete and all peaks gave larger numbers of theoretical plates, as shown in Table I.

Flavonoid-O-glycoside complexation by borate anions

The 1,2-dihydroxy compounds form borates under alkaline conditions, as detailed in Fig. 5. Boric acid (B^0) is a Lewis acid and can bind a hydroxyl ion to form the borate anion (B^-). Both boric acid and borate can react with a suitable dihydroxy com-

TABLE I

ELECTROPHORETIC PARAMETERS OF FLAVONOID-3-O-GLYCOSIDES

Applied voltages, 21.5 kV; field strength, 307 V/cm; electroosmotic flow, $5.62 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1}; electrolyte, 0.2 *M* H_3BO_3 (pH 10.5).

Quercetin-3-O-X X =	Efficiency[a]	Asymmetry factor	Electrophoretic mobility ($\times 10^4$) (cm^2 V^{-1} s^{-1})
Arabinoglucoside	96 080	0.89	3.19
Glucoside	146 750	0.78	3.35
Galactoside	98 220	0.52	3.63
Rhamnoside	75 850	0.84	3.72
Arabinoside	80 630	0.95	3.80

[a] Expressed as theoretical plate number.

pound (L), resulting in the boric acid ester (B^0L) and the borate monoester (B^-L), respectively. Subsequently, the two esters can react with another dihydroxy compound to give the borate diester (B^-L_2) [10,11].

The formation of complexes between borate and hydroxy compounds was first examined by Böeseken [12] by monitoring the changes in conductivity and pH that occur on adding compounds to boric acid or borax solutions, and by measuring the electrophoretic mobility of hydroxy compounds in borax solutions.

Spectroscopic methods offer physical means of examining chemical equilibria and measuring directly the concentrations of species in solution; Raman spectroscopy has been used to study borate complex formation [13]. Henderson *et al.* [14]

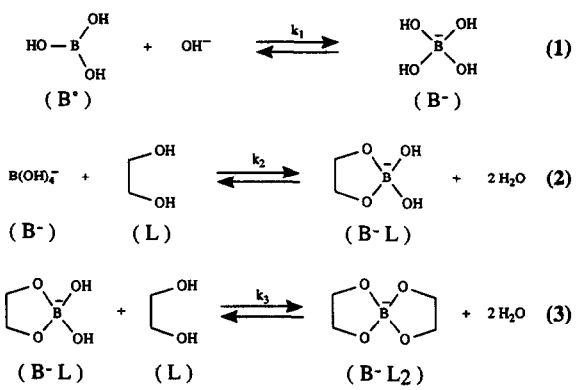

Fig. 5. Equilibria between boric acid, borate and a diol in alka line aqueous medium [10,11].

showed by ^{11}B NMR spectroscopic studies that the interconversion of boric acid and tetrahydroxyborate (borate) anions in aqueous solution is pH dependent. The interaction of 1,2-diols with borate anions at pH 12 induces the formation of 1:1 and 1:2 anionic complexes, as deduced from the existence of discrete ^{11}B resonances for these anions in the equilibrating systems. Increased substitution in 1,2-diols enhances their complexing ability.

The very stable borate complexes of ketoses and aldoses have been the subject of numerous studies. First, various aldoses have been derivatized to their 3-methyl-1-phenyl-2-pyrazolin-5-one derivatives or N-2-pyridylglycamines and then measured by free solution CE as their borate complexes [15,16]. In another instances, carbohydrates can be converted *in situ* into anionic borate complexes.

Chapelle and Verchère [17] showed that *cis*-1,2-diol groups complex borate more strongly than any other diol systems in polyhydroxy compounds. This study demonstrated also that in all 1:2 borate–sugar complexes, the borate anion was bound to two vicinal hydroxyl groups in a furanose ring, based on the fact that *cis*-1,2-cyclopentanediol formed more stable complexes than its *cis*-1,2-cyclohexanediol homologue. This effect is so large that the free sugars are forced into the furanose form by complexation, although they mainly adopt the pyranose structure when uncomplexed. For free sugars, the ability of a sugar to mutarotate creates a favourable situation for equilibration to a *cis*-1,2-glycol, which then forms a borate complex. Those sugars which can mutarotate and equilibrate to furanose forms will have the greatest potential to forming stable borate

complexes. Sterically, the most favourable configuration for complex formation is a *cis*-oriented pair of hydroxyl groups at C-2 and C-4. For instance, glucose does not have vicinal *cis*-OH groups in the pyranose form, so complex formation is assumed to take place mainly in the open-chain form [18].

The present quercetin-3-O-glycosides have one flavonoid hydroxyl group bound to a sugar by an acid-labile hemiacetal bond [8]. Consequently, the sugars maintained their unique natural form (furanose or pyranose) during borate complexation processes. Hence, complex formation cannot involve the open-chain form of the sugar. The magnitude of the borate complexation depends on the number of boration sites on sugar moiety and consequently on the sugar configuration.

In this borate complexation mode, flavonoid-3-O-glycosides migrated in the order quercetin-O-disaccharide (α-L-arabinoside-β-D-glucoside) and then quercetin-O-monosaccharides (β-D-glucoside, β-D-galactoside, β-L-rhamnoside and α-L-arabinoside). We tried to elucidate for this series of quercetin-3-O-glycosides the relationship between the migration order and the nature of the sugar moiety. The migration order depends on the structural preference for the formation of the borate complex and also on the relative molecular mass of the borate–solute complex (Fig. 1). Indeed, the electrophoretic mobility of an anionic borate–solute complex depends on its net charge and on its mass.

The α-L-arabinofuranoside unit has obviously no *cis*-1,2-diol system but can probably form a borate complex between the 2- and 5-hydroxyl groups [19]. *cis*-1,2-Glycols in a furanose system are known to form stronger borate complexes, whereas those in a pyranose system show little affinity to form borate complexes [20]. As indicated in Fig. 4c, quercetin-3-O-rhamnoside (4) migrates more slowly than quercetin-3-O-galactoside (3); this is probably due to the formation of a more stable complex at the 2- and 3-hydroxyl groups in quercetin-3-O-rhamnoside rather at the 3- and 4-hydroxyl groups in quercetin-3-O-galactoside [19] or a weaker charge density for compound 3. Quercetin-3-O-galactoside (3) migrates more slowly than quercetin-3-O-glucoside (2) because of the favourable structure of the galactopyrannosyl unit for formation of a borate complex (presence of the 3,4-*cis*-diol system). The quercetin-3-O-disaccharide (1) has a lower electrophoretic mobility than the quercetin-O-monosaccharides because of its lower charge density. However, this disccharide has *cis*-3,4-hydroxyl groups which can form diol complexes with borate.

In order to determine the electrophoretic mobility of a solute by free solution CE, we must also consider the influence of electroosmosis on the mobility measurements. The electrophoretic mobility is defined as $u_{ep} = L_d L_t V (1/t_0 - 1/t_m)$, where L_d is the length of the capillary from the inlet to the detector, L_t the total length of the capillary, t_m the migration time, t_0 the migration time for a neutral marker (methanol) and V the applied voltage. The electrophoretic mobility of a borate–diol complex is affected by the complex stability constants [16], and consequently this parameter depends on the disposition of hydroxyl groups, as indicated in Fig. 1.

The number of theoretical plates was fairly high (from 147 000 for quercetin-3-O-glucoside to 76 000 for quercetin-3-O-rhamnoside), but the separation appears to be kinetically retarded owing to the complexation with the borate anion. As an indication of peak asymmetry, the B/A ratio taken at 10% of the peak maximum was determined for these compounds; the values were less than unity from 0.95 for quercetin-3-O-arabinoside to 0.52 for quercetin-3-O-galactoside).

CONCLUSIONS

The formation of sugar–borate complexes is of particular value in the CE of flavonoid-O-glycosides having the same flavonoid aglycone but differing in their sugar moiety. In this borate complexation mode, flavonoid-3-O-glycosides migrated in the order quercetin-O-disaccharide (α-L-arabinoside-β-D-glucoside) and then quercetin-O-monosaccharides (β-D-glucoside, β-D-galactoside, β-L-rhamnoside and α-L-arabinoside). The magnitude of the borate complexation depends on the number of boration sites on sugar moiety and also of their charge density. The formation of a borate complex from a flavonoid-O-glycoside is facilitated by an alkaline buffer and the resolution *versus* boric acid concentration is increased. The migration order, which depends on the nature of the sugar moiety, has been explained for this series of quercetin-3-O-glycosides.

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