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Short Communication

Borate complexation of flavonoid-O-glycosides in capillary electrophoresis

I. Separation of flavonoid-7-O-glycosides differing in their flavonoid aglycone

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ABSTRACT

Capillary electrophoresis was found to give significantly greater efficiency, selectivity and speed compared with high-performance liquid chromatography for the separation of mixtures of flavonoid-O-glycosides. The migration behaviour of selected flavonoid-O-glycosides and their flavonoid aglycones in free solution capillary electrophoresis and micellar electrokinetic capillary chromatography (MECC) was investigated. A mixture of flavonoid-7-O-glycosides and their flavonoid aglycones was resolved by MECC. A 70 cm × 50 μm I.D. fused-silica capillary column and a 50 m*M* sodium dodecyl sulphate–20 m*M* Tris–HCl running buffer (pH 7.1) were used for these electrophoretic separations. Under these neutral conditions, the retention mechanism is based on hydrophobic interactions between flavonoids and the hydrophobic core of the micelles. Free zone capillary electrophoresis of borate complexes was more appropriate for the separation of a mixture of flavonoid-O-glycosides. In that case, the migration behaviour of these selected flavonoid-O-glycosides was explained by ionization of some hydroxyl groups and mainly by borate complexation of the sugar moiety. The identity of each flavonoid-O-glycoside was determined by using a fast-scanning multiple-wavelength UV detector and by recording its on-column UV spectrum in the range 190–360 nm.

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].

In micellar electrokinetic capillary chromatography (MECC), a surfactant is added to a buffer at a concentration above its critical micelle concentration. Micelles provide both ionic and hydrophobic sites of interaction, so this technique is suitable for the separation of ionic and uncharged solutes. Since the first description of MECC by Terabe and coworkers [2,3], many applications have been reported, such as to drug substances [4], acidic solutes [5],

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ionic and non-ionic catechols [6–8], cationic solutes [9] and glucosinolates and desulphoglucosinolates [10].

The existing method used for the analysis of flavonoid-O-glycosides in very complex matrices is generally high-performance liquid chromatography [11–13]. Nevertheless, Seitz *et al.* [14] used capillary isotachophoresis for the rapid analysis of flavonoids and phenolcarboxylic acids in phytopharmaceutical industry. In addition, MECC has recently been investigated for the determination of flavonoid drugs, such as quercetin-, kaempferol- and isorhamnetin-3-O-glycosides [1]. In this paper, the separation of a mixture of flavonoids and flavonoid-7-O-glycosides was studied using capillary electrophoresis (CE) and MECC techniques; the migration behaviour and solubilization mechanism of these neutral and ionizable solutes were explained.

EXPERIMENTAL

Instrumental

All open-tube electrokinetic capillary chromatographic separations were performed on a Spectra-Physics (San Jose, CA, USA) Spectraphoresis 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 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. Using the fast scanning mode, we were able to record on-column spectra of the compounds. The scanning mode ranged from 200 to 360 nm in 5-nm increments. Electrokinetic separations were performed at 60°C at 25 kV (electrical field strength 350 V/cm), a run average current of 45 μA and a capillary resistance of 0.18 GΩ. Analytes were injected in the hydrodynamic mode using a 0.75 p.s.i (1 p.s.i. = 6894.76 Pa) vacuum for 1–5 s.

All free solution capillary electrophoretic separations were performed on a Europhor (Toulouse, France) Prime Vision instrument using a 65 cm × 50 μm I.D. silica capillary tube. A small section (1-cm length) of the polyimide coating of the capillary column was removed prior to filling to obtain an optical window for UV detection. Analyses were carried out at ambient temperature with the Europhor instrument. All electropherograms were recorded on a Shimadzu (Kyoto, Japan) C-R 5A integrator. The on-column detector was operated at 270 nm at an absorbance range of 2.0 a.u.f.s. and a rise time of 0.1 s.

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 0.1 *M* sodium hydroxide (10 min) at 40°C, water at 60°C (10 min) and the electrophoretic buffer (15 min) at 40°C. Between two analyses, the capillary tubes 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 reproducibility of the migration time.

Reagents

All chemicals were of analytical-reagent grade. Tris(hydroxymethyl)aminomethane (Sigma, St. Louis, MO, USA), hydrochloric acid (Sigma), boric acid (Fluka, Buchs, Switzerland), sodium dihydrogenphosphate (Fluka) and the surfactant sodium dodecyl sulphate (SDS) (Fluka) were used without further purification. Water used for dilutions or as buffer solution was of HPLC grade (Carlo Erba, Milan, Italy).

For free solution capillary electrophoresis under alkaline conditions, the electrophoretic medium composition was either 0.03 *M* NaH_2PO_4 (adjusted to pH 10.5 by addition of 0.1 *M* NaOH) or 0.2 *M* H_3BO_3 buffer (adjusted to pH 10.5 by addition of 0.1 *M* NaOH).

For MECC separations under neutral conditions, the electrophoretic medium consisted of SDS micelles in Tris–HCl buffer. Stock solutions to prepare pH buffer were 0.1 *M* Tris and 0.1 *M* HCl. The buffer (pH 7.1) was prepared by mixing these two stock solutions in the proportions 50:45.7 (v/v) and diluting to 100 ml and adjusting the pH to 7.1 with hydrochloric acid. The aqueous buffer composition was 0.050 *M* Tris–0.046 *M* HCl with a 0.050 *M* surfactant concentration. Methanol was used to determine the retention time of a neutral unretained solute and anthracene the migration time of the micelles.

Authentic samples of diosmin, hesperidin, isorhoifolin, hesperitin, linarin and diosmetin were obtained from Extrasynthese (Genay, France). A standard solution of each flavonoid or flavonoid-7-

O-glycoside was prepared in dimethyl sulphoxide–methanol (80:20, v/v) at a concentration of *ca.* 100 ppm for each of these solutes. Finally, this solution was filtered through a Whatman (Maidstone, UK) polypropylene filter (0.2 μm pore size, 25 mm diameter) prior injection.

RESULTS AND DISCUSSION

Flavonoids commonly occur as flavonoid-O-glycosides in which one flavonoid hydroxyl groups is bound to a sugar by an acid-labile hemiacetal bond [15]. For our solutes, an identical disaccharide, rutinose (6-O-α-rhamnosyl-D-glucose), is found in association with flavonoids (Fig. 1).

Free solution capillary electrophoresis under no complexing conditions

The separation mechanism in free solution capillary CE is based on differences in the electrophoretic mobilities of species. Flavonoid compounds are weak acids with ionization constants in the pH range 10–12; their apparent charge depends on their pK_a values and on the pH of the running buffer. At neutral pH, these flavonoid-O- glycosides could not be resolved by CE. Because of their phenolic nature, an alkaline buffer system was chosen in order to ensure an adequate degree of dissociation of the flavonoids. A 30 m*M* phosphate running buffer (pH 10.5) was used for this electrophoretic separation (Fig. 2). Under these conditions, anionic species moved toward the cathode with the velocity of electrophoretic migration minus that of electrosmotic flow.

Linarin with one free hydroxyl group has a smaller mobility than the the other three flavone-O-glycosides with two free hydroxyl groups; this almost undissociated analyte migrates only with electrosmotic flow. Linarin and diosmin give sharp and symmetrical peaks well resolved from each other. Diosmin and hesperidin differ only in a carbon–carbon double bond; their effective mobilities are too close for them to be resolved.

Hence, the resolution of this separation cannot

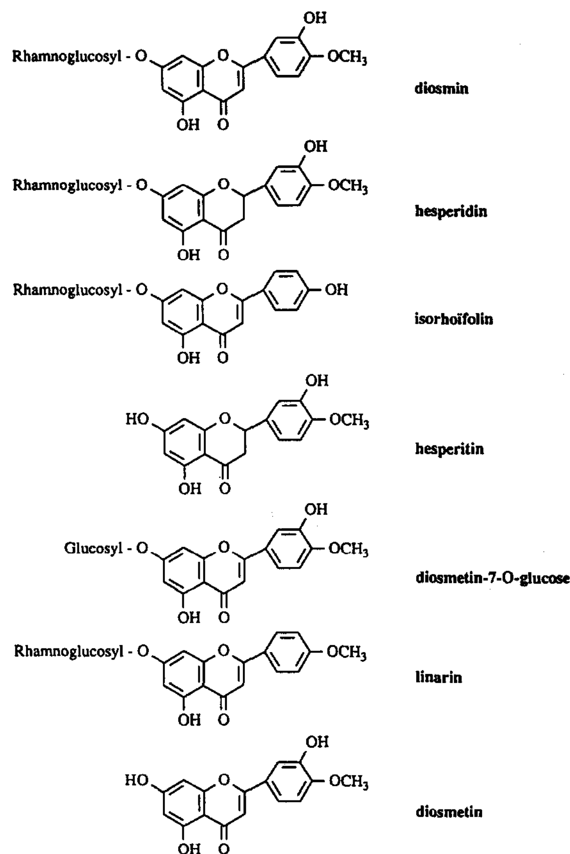


Fig. 1. Structures of flavonoids and flavonoid-7-O-glycosides.

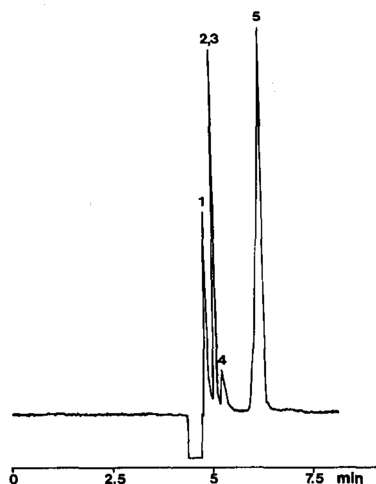


Fig. 2. Separation of a mixture of two flavonoids and four flavonoid-7-O-glycosides by free zone CE. Applied voltage, + 25 kV; capillary, 65 cm × 50 μm I.D., running electrolyte, 30 m*M* NaH_2PO_4 (pH 10.5); current, 43 μA; detection wavelength, 270 nm. Solutes: 1 = linarin; 2 = diosmin; 3 = hesperidin; 4 = impurity; 5 = isorhoifolin.

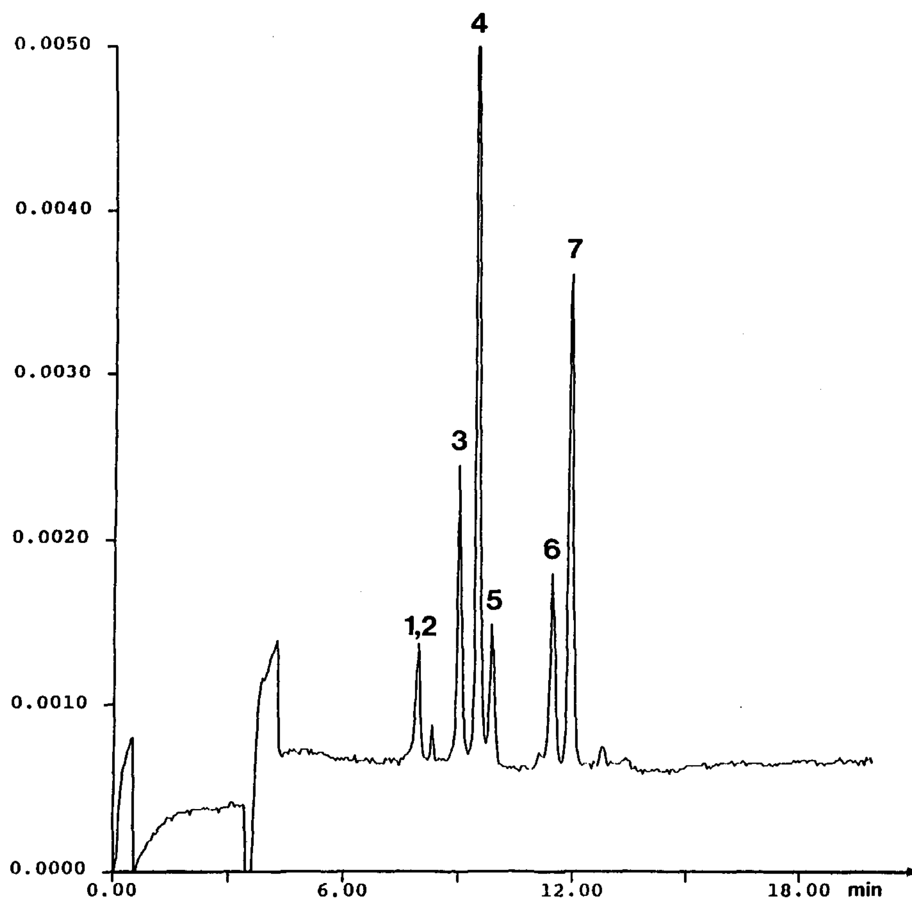


Fig. 3. Separation of a mixture of flavonoids and their flavonoid-7-O-glycosides by MECC. Applied voltage, 25 kV; capillary, 70 cm × 50 μm I.D.; buffer, Tris–HCl–50 m*M* SDS (pH 7.1); detection wavelength, 280 nm; current, 45 μA. Solutes: 1 = diosmin; 2 = hesperidin; 3 = isorhoifolin; 4 = hesperitin; 5 = diosmetin-7-O-glycoside; 6 = linarin; 7 = diosmetin.

be improved in CE simply by controlling the pH of the alkaline buffer.

Micellar electrokinetic capillary electrophoresis

A 50 m*M* SDS solution with a neutral running buffer (pH 7.1) was used for the electrophoretic experiments. Under such neutral conditions, only hydrophobic interactions between these flavonoid compounds, which have weak acids properties, and SDS micelles explain the migration order in MECC. For instance, the less hydrophobic flavonol-3-O-glycoside (rutin) migrates faster than its flavonoid aglycone (quercetin).

A mixture consisting of one flavone (hesperitin), one flavonone (diosmetin) and five flavonoid-7-O-glycosides (diosmin, hesperidin, isorhoifolin, linarin, diosmetin-7-O-glycoside) migrated to the cathode within 12 min (Fig. 3). The selectivity obtained by MECC is great enough to resolve these solutes, except for diosmin and hesperidin. This method offers the opportunity of the separation of both flavonoids and flavonoid-7-O-glycosides in a single run, without a gradient approach such as in reversed-phase HPLC.

The more hydrophilic flavonoid-7-O glycosides (diosmin and hesperidin) migrate faster than their hydrophobic flavonoid aglycones (diosmetin and hesperitin, respectively). Indeed, the effect of glycosylation is to render these flavonoids more hydrophilic and water soluble (see the migration order of

diosmin, diosmetin-7-O-glycoside and diosmetin). Two flavonoid 7-O-glycosides with a slight structure difference (methoxy or hydroxyl group in the 4′ position) have different migration times: a methoxy group (see the 4′-OCH_3 group in linarin) clearly enhances the hydrophobicity compared with a hydroxyl group (see the 4′-OH group in isorhoifolin). Nevertheless, when a hydroxyl group is close to a methoxy group, the total hydrophobicity of the molecule decreases (compare the migration times of diosmin with those of linarin and isorhoifolin) [16].

However, a difficult problem concerns the resolution of two compounds that differ in the occurrence of a carbon–carbon double bond. For example, diosmin and hesperidin, which differ only in a carbon–carbon double bond, are not resolved with this MECC system, whereas their flavonoid aglycones, diosmetin and hesperitin, respectively, have very different migration times. This specific behaviour, strongly dependent on surfactant type, is the unsaturation contribution, which distinguishes flavones from flavanones. In fact, there were differences in molecular structure, the flavones being planar and the flavanones partially planar.

The retention mechanism in MECC with a neutral running buffer is mainly based on hydrophobic interactions between flavonoids and the hydrophobic core of micelles. In contrast, working under alkaline conditions in MECC should induce hydrophobic interactions with the micelles and also electrophoretic mobility of ionized flavonoid aglycone. In that event, some interactions should occur between anionic solutes and SDS micelles.

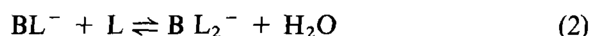
Complexation of flavonoid-7-O-glycosides with boric acid

By adding boric acid to the background electrolyte, the retention time of each flavonoid-O-glycoside is increased as they react with boric acid to form borate complexes with negative charge. It is well known that certain neutral polyhydroxy compounds or sugars react with borate ions to form borate complexes which are negatively charged ions [14,17,18].

The influence of different borate buffers as background electrolytes on the electrophoretic mobilities of underivatized mono- and oligosaccharides has recently been studied [19]. The very low UV absorbance of carbohydrates makes photometric detection very difficult without a derivatization step. However, by adding borate to the running buffer, absorbance in the low UV range (195 nm) becomes slightly increased (for hexose, pentose and the main common disaccharides, the increase factor is *ca.* 5–15). Hence, the enhanced UV absorption of sugars in the presence of borate allows a more sensitive UV detection than that in pure water and consequently their electrophoretic separation in a capillary electrophoretic system using a borate complexing buffer.

The complex formation can be described by the following equations [19]:

$$B^- + L \rightleftharpoons B\,L^- + H_2O \quad (1)$$



$$BL^- + L \rightleftharpoons B\,L_2{}^- + H_2O \quad (2)$$

where L is the polyol ligand and B^- represents tetrahydroxyborate, $B(OH)_4{}^-$. Further, alkaline borate anion solutions (pH 8–12) contain not only $B(OH)_4{}^-$ or $(B_3O_3(OH)_5)^{2-}$ but also $(B_4O_5(OH)_4)^{2-}$. Hoffstetter-Kuhn *et al.* [19] mentioned some very interesting structural characteristics of polyol–borate and sugar–borate complexes: polyols can form 1:1 and 1:2 complexes with borate; hydroxyl groups on adjacent carbon atoms but also those on alternate carbon atoms occurred in a complexation mechanism; the complex is stabilized by a

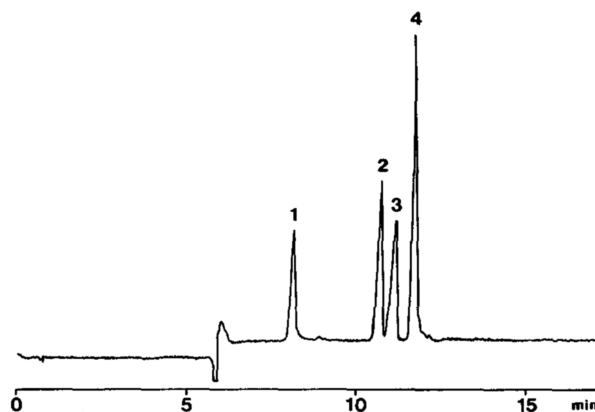


Fig. 4. Separation of a mixture of four flavonoid-7-O-glycosides by free zone CE with borate complexing buffer. Applied voltage, + 25 kV; capillary, 65 cm × 50 μm I.D.; current, 62 μA; running buffer, 200 m*M* boric acid (pH 10.5); detection wavelength: 270 nm. Solutes: 1 = linarin; 2 = diosmin; 3 = isorhoifolin; 4 = hesperidin.

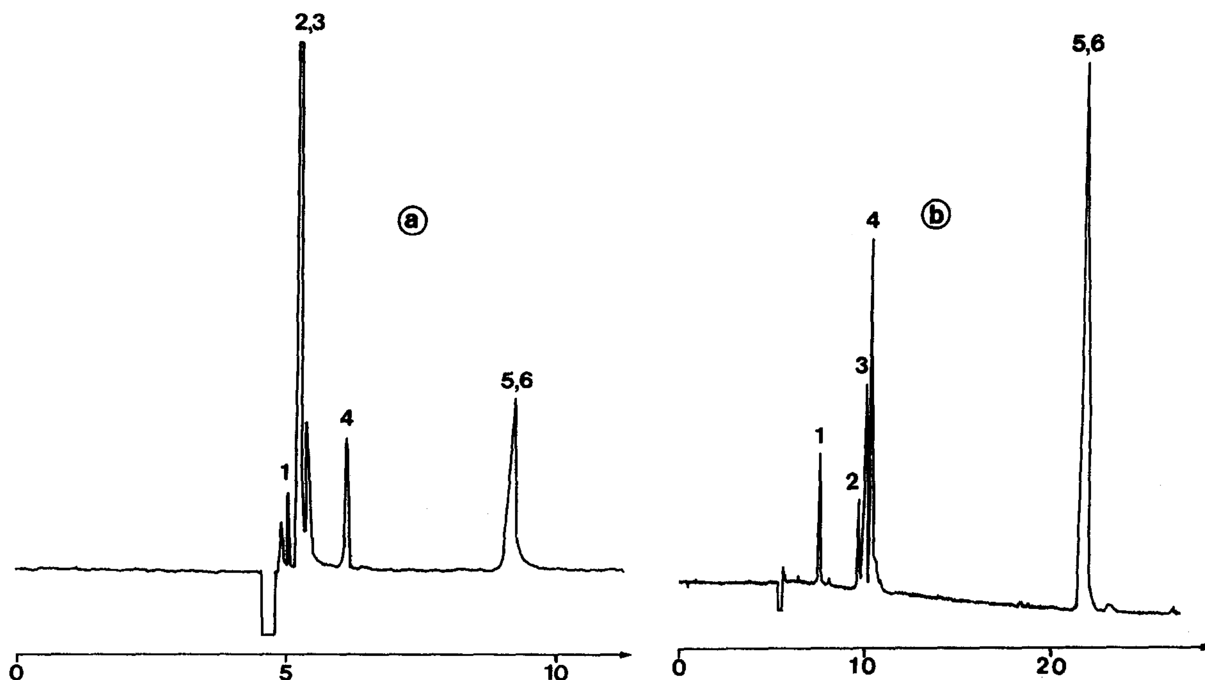


Fig. 5. Separation of a mixture of flavonoids and their flavonoid-7-O-glycosides by free zone CE. Applied voltage, + 25 kV; capillary, 65 cm × 50 μm I.D.; detection wavelength: 270 nm; current, 62 μA. Solutes: 1 = linarin; 2 = diosmin; 3 = isorhoifolin; 4 = hesperidin; 5 = diosmetin; 6 = hesperetin. (a) With no complexing buffer (30 m*M* NaH_2PO_4, pH 10.5). (b) With complexing buffer (200 m*M* boric acid, pH 10.5).

large number of hydroxyl groups; and *cis*-1,2-diols are preferred in complexation over *trans*-1,2-diols.

Hence the possibilities for borate complexation of a sugar are numerous, including α- and β-pyranose forms, α- and β-furanose forms, hydrated open-chair form and carbonyl open-chain form. By adding borate to running buffer, saccharides are complexed in different ways according to their different forms.

The separation of flavonoid-O-glycosides can be performed in CE with a borate running buffer at pH 10.5 (Fig. 4). The ionization constants of the main mono- or disaccharides are in the range 11.9–12.5, according to Lee and Bunker [17]. Hence we may assume that the sugar moiety of the flavonoid-7-O-glycoside is not ionized at the pH value of the running buffer. Well resolved peaks were obtained and identified by comparison with authentic specimens.

The mixture of flavonoids and their glycoside derivatives has also been resolved by CE at the same pH value with a non-complexing buffer (Fig. 5a) or a complexing buffer (Fig. 5b); in both instances, the chromatogram was divided into two parts on the basis of retention times: the first part consisted of the heterosides (compounds 1–4) and the second part contained the flavone diosmetin and the flavanone hesperitin. Under these both conditions, flavonoid-O-glycosides migrate faster than flavones. Indeed, diosmetin, which contains three free hydroxyl groups, has a greater electrophoretic mobility than diosmin at this alkaline pH. Nevertheless, no separation occurred between the two flavonoids diosmetin and hesperitin, even with complexing running buffers.

Spectral identification

The instrument contains a programmable, high-speed, scanning, multiple-wavelength UV detector. Using the fast scanning mode we were able to record on-column spectra of the compounds; the scanning mode ranged from 200 to 360 nm in 5-nm increments. It is well known that UV spectra of fla-

vonoid glycosides include important structural information concerning the aglycones. The precise position and relative intensities of these maxima give valuable information on the nature of the flavonoid aglycone and its oxygenated pattern [15]. Table I reports the UV absorption maximum wavelengths for these solutes resolved by MECC and by using a multiple-wavelength UV detector.

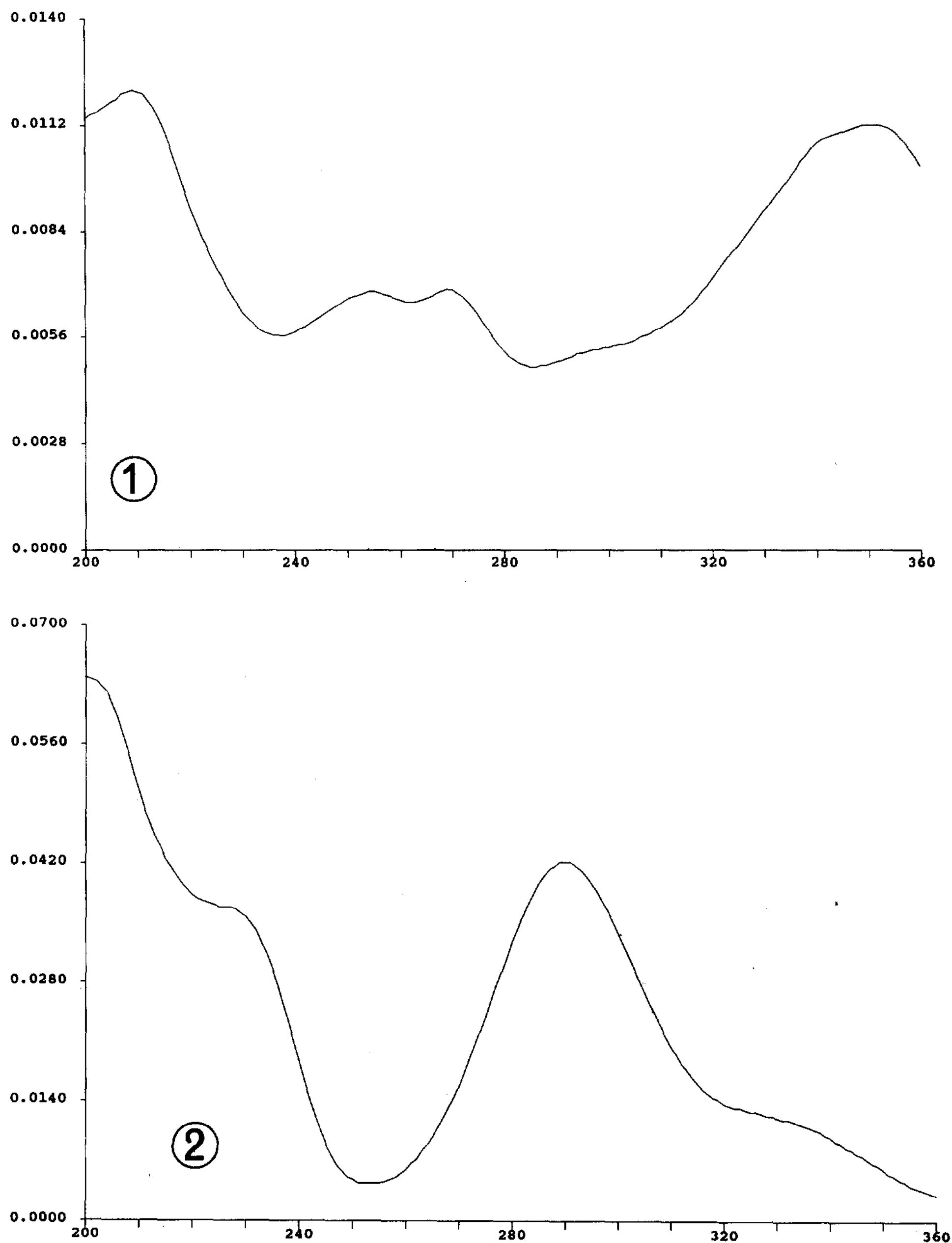


Fig. 6. Full on-line UV spectra of selected flavonoids and their glycoside derivatives in the 190–360 nm range. Resolution, 5 nm; bandpass, 1 nm. Solutes: 1 = diosmetin; 2 = hesperitin.

TABLE I

UV ABSORPTION MAXIMA FOR FLAVONOID-7-O-GLYCOSIDES AND FLAVONOID AGLYCONES

Compound	λ_{max} (nm)
Flavanones	
Hesperitin	290, 228 (shoulder), 200
Hesperidin	285, 225 (shoulder), 200
Flavones	
Diosmetin	351, 269, 255, 209
Diosmetin-7-O-glucose	350, 269, 208
Diosmin	348, 270, 207
Linarin	333, 273, 209
Isorhoifolin	336, 269, 200

Fig. 6 shows that the UV spectrum of a flavone (diosmetin) typically consists of two absorption maxima near 250–280 nm (band I) and 310–350 nm (band II); for a flavanone (hesperetin), two absorption maxima appear in the range 275–295 nm (band I) and a shoulder near 300–330 nm (band II). These two flavonoids, which differ only in the presence of a carbon–carbon double bond, have very different features in their UV spectra. Glycosylation of the 7-hydroxyl group appears generally to cause small band shifts to shorter wavelength (Table I). The nature of the sugar in the glycoside has no effect on the position of bands but induces different relative intensities of these maxima.

CONCLUSIONS

Capillary electrophoresis is a useful technique for investigating the composition of flavonoids and flavonoid-O-glycoside mixtures. Micellar electrokinetic capillary chromatography has been used for the separation of both flavonoid-O-glycosides and their flavonoid aglycones. Under neutral pH conditions, the retention mechanism involves mainly hydrophobic interactions with the micelles. This determination of flavonoid-O-glycosides is rapid and reproducible, and may serve as a valuable means for assessing quality in the pharmaceutical industry. The identity of each flavonoid O-glycoside was determined by recording its on-column UV spectrum using a high-speed, scanning, multiple-wavelength detector.

Further investigations are in progress for a better understanding of the mechanism of insertion of a flavonoid-O-glycoside into the interior of micelles in MECC, and of borate complexation of flavonoids and flavonoid-O-glycosides in CE.

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