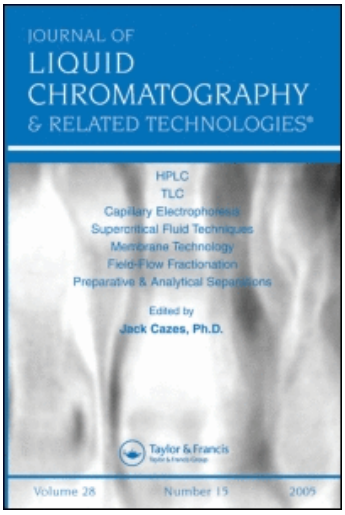

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Trihydroxyflavones from *Scutellaria baicalensis*: Separation by a Facile MEKC Technique and Comparison to an Analytical HPLC Method

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ABSTRACT

Phenolic were extracted from the roots of *Scutellaria baicalensis* Georgi (*Labiatae*) using methanol. The phenolics of the crude extract were examined by high-performance liquid chromatography (HPLC) using

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an analytical C_{18} column coupled with ultraviolet-diode array detection (UV-DAD). Chromatograms were compared with those acquired by micellar electrokinetic chromatography (MEKC) with UV-DAD. A good separation of the phenolics from the crude extract was achieved by the electrophoretic technique, and in a shorter time than by HPLC. Two dominant flavones, believed to be 5,6,7-trihydroxyflavone and 5,6,7-trihydroxyflavone-7-*O*-β-D-glucopyranosiduronate, which are commonly referred to as baicalein and baicalin, respectively, were then isolated from the crude extract using a semi-preparative HPLC method on a RP-18 column. The identities of the separated trihydroxyflavones were confirmed by NMR spectroscopies and mass spectrometry as being baicalein (1) and baicalin (2). The employment of MEKC coupled with UV-DAD as a technique to separate and to identify phenolic compounds, or their classes in natural products research, is expected to expand over the next decade.

Key Words: HPLC; MEKC; *Scutellaria baicalensis*; Flavones.

INTRODUCTION

The dried root of *Scutellaria baicalensis* Georgi (*Labiatae*) is a very old, well-known drug in traditional Chinese herbal medicine for the treatment of bronchitis, hepatitis, diarrhea, and tumors.[1,2] These roots contain a number of biologically-active flavone derivatives: baicalein (5,6,7-trihydroxyflavone, 1) and baicalin (5,6,7-trihydroxyflavone-7-*O*-β-D-glucopyranosiduronate, 2) are two such examples, which have been isolated from *S. baicalensis* and used clinically as therapeutic medicine in China.[3] In experiments on rat fed diets supplemented with polyphenolic extracts from *S. baicalensis*, a marked decrease in the contents of HDL-cholesterol and triacylglycerols was observed.[4] Gabrielska et al.[5] found that the trihydroxyflavones of *S. baicalensis*, namely baicalein (1) and baicalin (2), ensured a very satisfactory concentration-dependent protection of the liposome membrane against UV-induced oxidation. These authors reported that the antioxidative activity of an extract from *S. baicalensis* was similar to that of butylated hydroxytoluene, as confirmed in a test using an index of conjugated diene. Zhang and Shen[6] reported that baicalin was a more effective antioxidant than green tea polyphenols and vitamin E in reducing the hepatic content of 2-thiobarbituric acid reactive substances (TBARS), and better than vitamin E in lowering the serum level of alanine aminotransferase activity in bromobenzene intoxicated mice. In a food system, Pegg et al.[7] compared the TBARS values of cooked pork treated with an extract from *S. baicalensis* to meat systems treated with either a synthetic (e.g., *tert*-butylhydroquinone)

or a natural antioxidant (e.g., a rosemary extract). Results confirmed the antioxidant efficacy of constituents from *S. baicalensis* in a thermally processed food product. Part of the antioxidant activity of the flavones may be due to chelation. Wybieralska et al.[8] reported that the protective effect of baicalin against oxidation was attributable to the chelation of divalent metal cations.

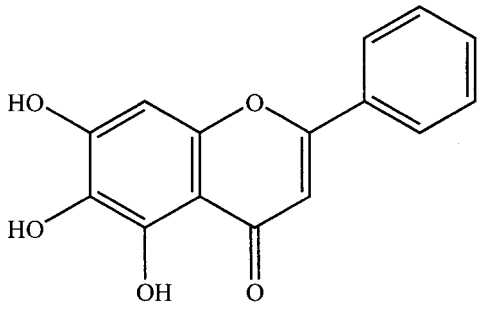
High-performance liquid chromatography (HPLC) using gradient and ion-suppression techniques have been used to separate baicalin, baicalein, wogonin-glucuronide, and wogonin from extracts of *S. baicalensis*. However, these techniques require long times for the simultaneous determination of the aglycones and their glucuronides on account of different polarities.[9] It was felt that capillary electrophoresis (CE) might solve some of the analysis problems. Since the early 1980s, the application of CE for the separation and determination of a variety of natural products has become increasingly widespread because of its minimal sample and solvent requirements, rapid analysis time, and high separation efficiency, and resolution.[3] Capillary zone electrophoresis (i.e., CZE; the most widely used mode of CE for the separation of both anionic and cationic solutes in a single analysis) and micellar electrokinetic chromatography (i.e., MEKC; a unique mode of CE that can separate neutral as well as charged solutes on account of the addition of ionic surfactants to form micelles in the running buffer) have proven to be good analytical techniques in the area of natural products research for the rapid separation of phenolic compounds such as phenolic acids,[10] isoflavones and coumestrol,[11] catechins,[12] and now possibly flavones.

The present study reports a facile, reliable, and efficient MEKC method for the separation of the antioxidative trihydroxyflavones, baicalein and baicalin, from a methanolic extract of *S. baicalensis* in comparison to analytical HPLC separation and structure elucidation of the isolated compounds (Fig. 1).

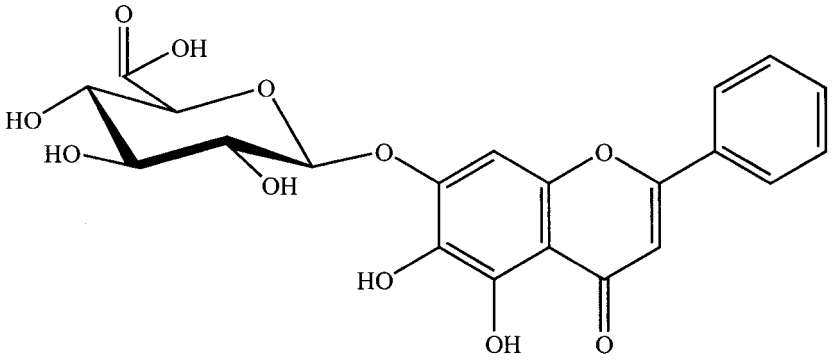
EXPERIMENTAL

Preparation of the *Scutellaria baicalensis* Extract

Plants of *S. baicalensis* were grown from authenticated seeds obtained from the Botanical Garden of Medicinal Plants herbarium at the Medical University in Wrocław, Poland, by cultivation in the University's experimental field. The seeds were sowed in sandy soil in the spring of 2000, and roots from the plants were harvested, the following September. Voucher specimens were deposited at the herbarium. Collected roots were then washed in distilled water, dried in a room with controlled humidity at room temperature



5,6,7-trihydroxyflavone
(Baicalein - Compound 1)



5,6,7-trihydroxyflavone-7-*O*-β-D-glucopyranosiduronate
(Baicalin - Compound 2)

Figure 1. Chemical structure of dominant flavones from *Scutellaria baicalensis* Georgi (*Labiatae*).

until a moisture content of 5% was achieved. The dried roots were then crushed using a laboratory mill and stored at −20°C until analyzed.

A 10 g of dry powdered roots of *S. baicalensis* was extracted three times with 100 mL of methanol for 15 min at 60°C.[13] The solution was then filtered through a Schott funnel with Whatman No. 1 filter paper and the residue was re-extracted twice more. Combined supernatants were evaporated to dryness under vacuum at 40°C using a Büchi Rotavapor/Water bath (Models

EL 131 and 461, respectively, Brinkmann Instruments [Canada] Ltd., Toronto, ON). Prepared extracts were stored at 4°C in air until further investigated.

Analytical HPLC

Analytical HPLC was carried out using the Waters HPLC system, which consisted of the following components: the Waters 600 controller, a 600E multisolvent delivery system, a 996 photodiode array detector, a 715 Ultra Wisp sample processor, and Millenium software. Conditions of separation entailed a prepacked LUNA analytical C_{18} column (5 μm, 4.6 × 250 mm; Phenomenex, Torrance, CA), a mobile phase of methanol : water : acetic acid (10 : 9 : 1; v/v/v), a flow rate of 1 mL/min, an injection volume of 20 μL, and the detector being set at 280 nm.

MEKC

The MEKC of the crude methanolic extract from *S. baicalensis* and of compounds 1 and 2 (i.e., baicalein and baicalin, respectively, as confirmed below by structure elucidation techniques) isolated by semi-preparative HPLC was performed using a Hewlett-Packard (subsequently Agilent Technologies) 3D CE instrument equipped with UV-DAD and an HP Chem-Station software package. The running buffer employed for MEKC consisted of 150 mM boric acid, 15 mM heptakis(2,6-di-*O*-methyl)-β-cyclodextrin and 75 mM sodium dodecyl sulphate (SDS) (adjusted to a pH of 8.5 with NaOH).[14] An uncoated fused-silica capillary column (50 μm i.d. × 72 cm) was filled with the aqueous buffer solution and allowed to equilibrate with the inner walls of the capillary. A small volume (ca. 50 nL) of the analysis solution was injected electrokinetically into the anodic end of the capillary. Separation was achieved by applying a 30 kV potential across the capillary via platinum electrodes immersed in the buffer reservoirs at each end of the system. The capillary temperature was held at 35°C. Photodiode detection was performed at 220 nm with DAD scanning between 190 and 400 nm for identification purposes. SDS and heptakis(2,6-di-*O*-methyl)-β-cyclodextrin were obtained from Sigma–Aldrich Canada Ltd. (Oakville, ON).

Semi-Preparative HPLC

Semi-preparative HPLC was carried out using the Waters HPLC system detailed above. Conditions of separation entailed a prepacked semi-preparative

RP-18 column (5 μm, 10 × 250 mm; Agilent Technologies, Wilmington, DE), a mobile phase of methanol : water : acetic acid (12 : 7 : 1; v/v/v),[2] a flow rate of 4 mL/min, an injection volume of 500 μL and the detector being set at 280 nm.

Structure Elucidation of Isolated Compounds 1 and 2

Mass spectra were recorded using a VG analytical mass spectrometer (Manchester, UK). Depending upon the nature of the compound, electron impact (EI) and chemical ionization (CI) modes were employed. The conditions for EI were the following: ion source temperature, 200°C; ionization energy, 70 eV; mass range, 50–850 amu; and pressure, 1×10^{-7} mbar. For CI, ammonia was used as the ionization reagent and the mass spectral conditions were as follows: ion source temperature, 175°C; ionization energy, 50 eV; mass range, 33–850 amu; and pressure, 5×10^{-5} mbar. Positive identifications were based on authentic standard compounds.

Nuclear magnetic resonance (NMR) spectra were obtained at 300 and 500 MHz on Bruker AMX-300 and Avance 500 (Bruker Analytik GmbH, Rheinstetten, Germany) spectrometers, respectively. ^{1}H and $^{13}C\{^{1}H\}$ NMR data were collected at room temperature in DMSO-d_6 or in DMSO-d_6/D_2O mixtures. Deuterated dimethyl sulfoxide (DMSO-d_6) was obtained from Sigma-Aldrich Canada Ltd., while deuterated water (D_2O) was acquired from Cambridge Isotope Laboratories (Andover, MA).

RESULTS AND DISCUSSION

Analytical HPLC of the crude methanolic extract of *S. baicalensis* resulted in the detection of 6 main peaks at the 280 nm wavelength over a 30 min analysis period (Fig. 2). Two of the well-resolved peaks from the chromatogram at retention times of 10.14 and 25.99 min were believed to be baicalein and baicalin. Semi-preparative HPLC of the crude methanolic extract resulted in the detection of ca. 10 peaks at the 280 nm wavelength over a 40 min analysis period. The dominant peaks with retention times of 19.20 and 23.10 min were suspected as being baicalein and baicalin. During subsequent runs of the crude extract, eluent was collected at the 19.20 and 23.10 min retention times, solvent removed in vacuo, and products then labeled as compounds 1 and 2, respectively.

MEKC, a hybrid of electrophoresis and chromatography, is the only electrophoretic technique that can be used for the separation of neutral solutes/analytes as well as charged ones. In this work, SDS was the surfactant

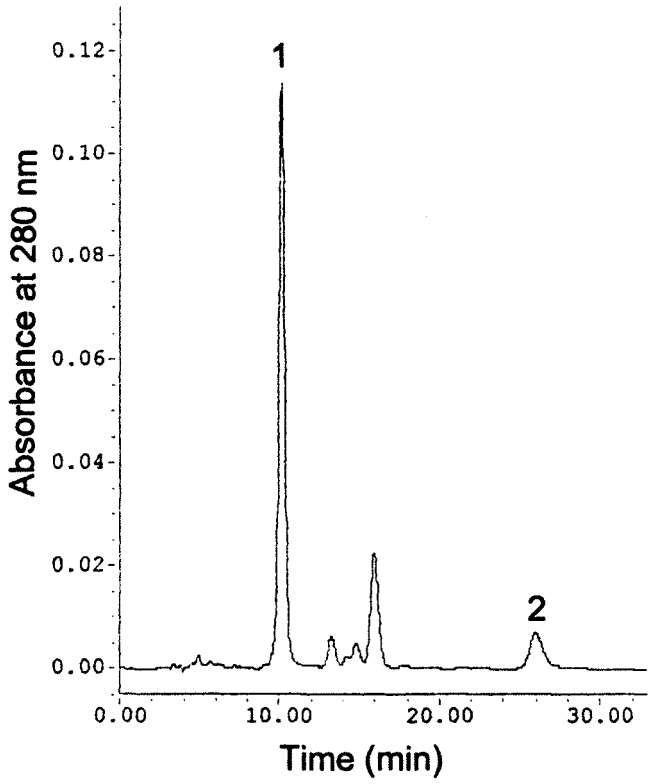


Figure 2. Analytical HPLC chromatogram of a crude methanolic extract of *Scutellaria baicalensis* Georgi (*Labiatae*).

employed as the micelle-forming species; its concentration in the running buffer (75 mM) was well above the critical micelle concentration of 8 mM.[15] It is uncertain, however, as to the ionization state of the hydroxyl moieties of compounds 1 and 2 in the buffer (pH 8.5); i.e., whether they are mostly charged or protonated (i.e., neutral compounds). Because SDS is an anionic surfactant and carries a large negative charge, the micelles migrate "upstream" towards the anode, which is in the opposite direction to the electroosmotic flow (EOF). The velocity of the EOF is faster than the migration of the micelles, and the net movement is, thus, in the direction of the cathode. For neutral species, it is the partitioning in and out of the hydrophobic interior of the micelles that affects the analytes' retention in the capillary. In other words, the hydrophobic nature of compounds 1 and 2 influences their separation. Compounds 1 and 2 eluted from the capillary at a time that was determined

by their affinity for the micelles, which are migrating upstream, relative to their affinity for the aqueous moving phase, which is flowing at the rate of the EOF. This separation process, governed by polarity, is completely analogous to HPLC; however, with SDS micelles in solution, the stationary phase is actually moving.[15] Additionally, the presence of heptakis(2,6-di-*O*-methyl)-β-cyclodextrin in the running buffer complicates the system, as further potential interactions can occur between charged cyclodextrin molecules and analytes. The cyclodextrins form a truncated cone, with an internal cavity of hydrophobic character varying in diameter depending on the actual cyclodextrin employed. Selectivity is brought about by stereospecific inclusion of the analyte into the cavity of cyclodextrins, whereby the complex formed is primary stabilized by hydrophobic interactions.[16]

The electropherogram of compound 1 (i.e., whose chemical structure was elucidated to be baicalein, see below), isolated by semi-preparative HPLC, was characterized by a well-resolved peak (i.e., no tailing) with a migration time of 8.92 min [Fig. 3(A)]. As the CE unit has a photodiode-array detection

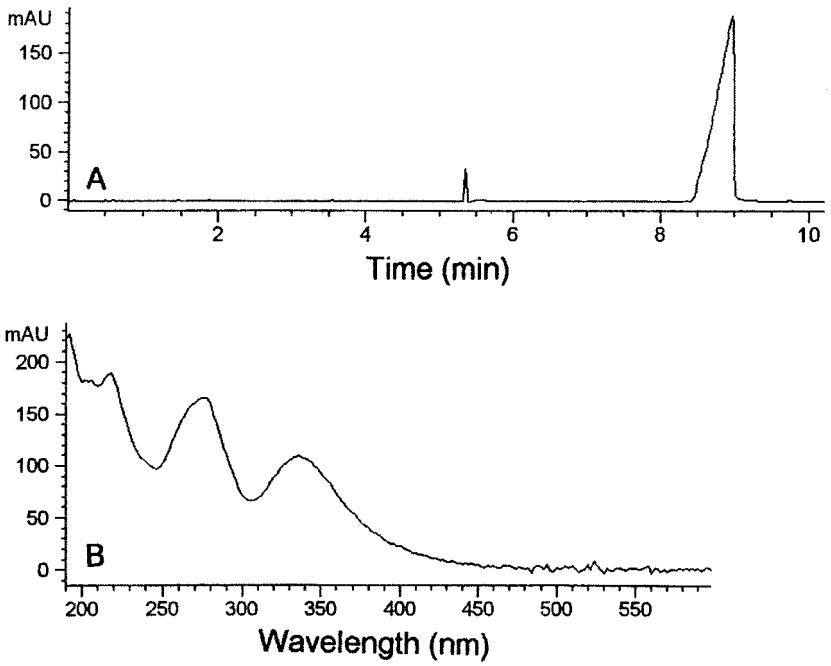


Figure 3. Electropherogram (A) and UV-DAD spectrum (B) of baicalein (1), isolated by semi-preparative HPLC.

feature, additional information about the chemical nature of separated compounds is provided. In this case, the UV-DAD spectrum of baicalein (1) showed two maxima at 280 and 338 nm [Fig. 3(B)]. The electropherogram of compound 2 (i.e., whose chemical structure was elucidated to be baicalin, see below), isolated by semi-preparative HPLC, showed a well-resolved peak (i.e., no tailing) with a migration time of 9.49 min [Fig. 4(A)], which was slightly later than that of baicalein (1). The UV-DAD spectrum of the isolated compound exhibited a maximum at 284 nm with a shoulder at 314 nm [Fig. 4(B)].

The crude methanolic extract from the roots of *S. baicalensis* was subjected to MEKC using the operating conditions established for compounds 1 and 2. The electropherogram thus obtained for the crude extract is depicted in Fig. 5(A). There were three well-resolved peaks of interest with resolutions >2: the dominant one (peak 3) had a migration time of 9.53 min and its UV-DAD spectrum [Fig. 5(B3)] matched that of compound 2 (i.e., baicalin). Gabrielska et al.[5] prepared extracts from hairy root cultures of *S. baicalensis* with methanol and obtained products with a 5,6,7-trihydroxyflavone-7-*O*-β-D-glucopyranosiduronate content of ca. 75%. Thus, it was no surprise that baicalin (2) was the main flavone identified in the electropherogram from

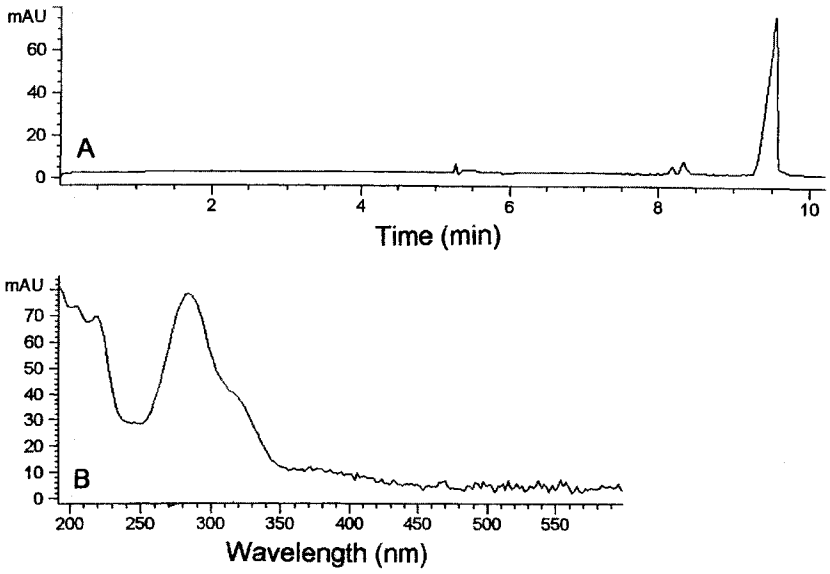


Figure 4. Electropherogram (A) and UV-DAD spectrum (B) of baicalin (2), isolated by semi-preparative HPLC.

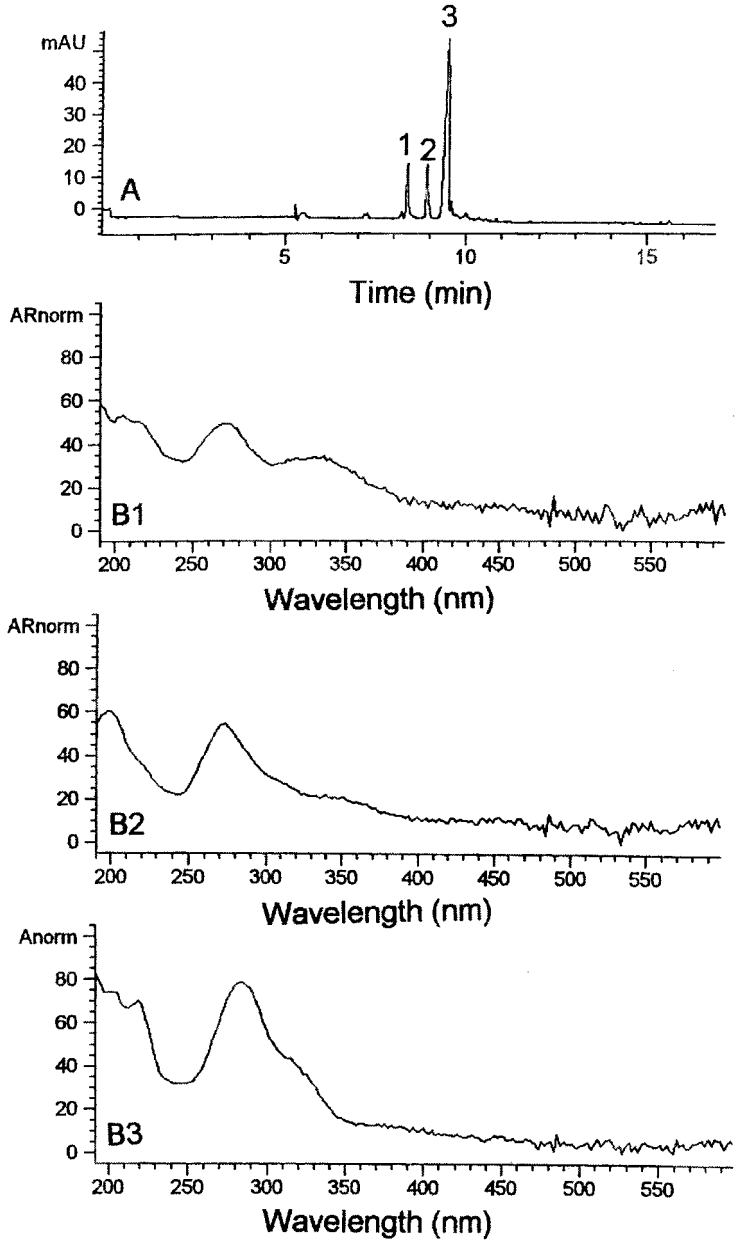


Figure 5. Electropherogram of a crude methanolic extract of *Scutellaria baicalensis* Georgi (*Labiatae*) (A) and UV-DAD spectra (B1, B2, B3) of the separated flavones.

this study. The signal denoted as peak 1 is that of compound 1 (i.e., baicalein), but it shifted slightly. Its migration time was earlier at 8.37 instead of 8.92 min, as that observed for the compound isolated by semi-preparative HPLC. Nevertheless, the UV-DAD spectrum of this peak [Fig. 5(B1)] was similar to that of baicalein (1). Another signal due to a phenolic compound was recorded with a migration time of 8.90 min [Fig. 5(A), peak 2]. The UV-DAD spectrum of this compound was somewhat like that of baicalin (2), as it had a maximum at 276 nm, but it also exhibited a shoulder at 340 nm [Fig. 5(B2)]. Although it was not identified by further spectroscopic techniques, the compound is most probably a glycoside of a trihydroxyflavone.

Chen et al.[3] reported the electropherograms of baicalein, baicalin, and quercetin standards purchased from Sigma–Aldrich by CZE using cyclic voltammetry detection. In their investigation the migration times for baicalein and baicalin were 6.28 and 7.41 min, respectively, which are ca. 2 min earlier than those observed in the present study. This suggests that hydrophobic interactions were taking place between the SDS micelles and the trihydroxyflavones of the extract. With stronger affinity for the nonpolar interior of the micellar "pseduostationary phase," there is a longer retention of baicalein and baicalin in the capillary; the micelles are trying to flow in the opposite direction to the EOF. Inclusion complex formation between charged cyclodextrin molecules and baicalein or baicalin may have also assisted with the chromatographic separation. The electropherogram of an ethanolic extract from Scutellariae Radix reported by Chen et al.[3] was not as well resolved as the flavones separated in the electropherogram of the crude methanolic extract from the roots of *S. baicalensis* in the present investigation. This indicates that MEKC, based on the conditions described in the Experimental, was more effective than CZE in the separation of trihydroxyflavones from the extracts. However, a direct comparison between these two CE techniques cannot be made: the CZE separations by Chen et al.[3] were performed using a shorter length of fused-silica capillary (25 μm i.d. × 40 cm), as well as a different separation voltage, buffer, and mode of detection than those employed in this study. These differences may be partially, if not totally, responsible for the variations in the observed migration times.

A phenolic acid from rapeseed and two of its derivatives, sinapic acid, sinapine, and glucopyranosyl sinapate, were separated by MEKC with the identical buffer as that used in the present study; similar migration times and resolutions were reported.[10,14] Moravcová and Kleinová[11] showed CZE data in which baseline separations of the isoflavones, daizein and genistein, were achieved using an uncoated fused-silica capillary with migration times of 18.50 and 19.32 min, respectively.

The two compounds isolated by semi-preparative HPLC were labeled as 1 and 2. The EI mass spectrum showed the molecular ion radical, $M^{+\bullet}$, at an

m/z of 270 (100%—relative intensity) and a $[M + H]^+$ peak at an m/z of 271 (16.3%), which is typical for baicalein. No EI mass spectrum could be acquired for compound 2; hence, the softer ionization technique of CI was employed. The CI mass spectrum exhibited a $[M + H]^+$ peak at an m/z of 447 (20.5%), thereby indicating that compound 2 has a molecular weight of 446 Da, which is characteristic of baicalin. Proton and carbon assignments from ^1H and $^{13}C\{^1H\}$ NMR spectra, as well as heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond coherence (HMBC) experiments, agreed with the mass spectra that compounds 1 and 2 were baicalein and baicalin, respectively. Further details concerning the elucidation of the chemical structures for these compounds are provided elsewhere.[17]

CONCLUSION

In conclusion, the migration times required to separate the dominant flavones from an extract of *S. baicalensis* by MEKC with UV-DAD were shorter than those by analytical HPLC. MEKC was found to offer a quicker analysis with high separation efficiency and less sample handling than conventional HPLC methods. The identities of two of the separated trihydroxyflavones were determined by mass spectral and NMR data as baicalein (1) and baicalin (2). It is anticipated that the employment of MEKC, coupled with UV-DAD as a technique to separate and assist in identifying phenolic compounds or their classes in natural products research, will rapidly expand over the next decade.

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