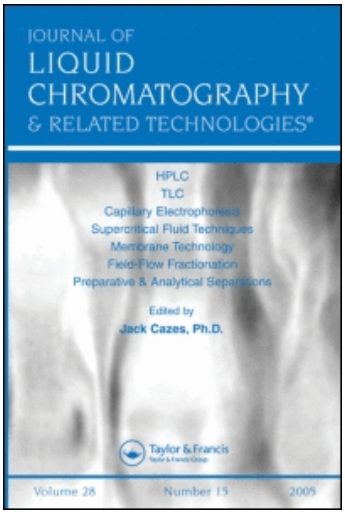

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Chemical Separation of Bioactive Licorice Compounds Using Capillary Electrophoresis

Jia Cai[a]; Paul Li[a]
[a] Department of Chemistry, Simon Fraser University, Burnaby, BC, Canada

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Chemical Separation of Bioactive Licorice Compounds Using Capillary Electrophoresis

Jia Cai and Paul Li
Department of Chemistry, Simon Fraser University, Burnaby, BC, Canada

Abstract: Four bioactive components derived from licorice root, i.e., glycyrrhizin (GL), 18α-glycyrrhetinic acid (18α-GA), 18β-glycyrrhetinic acid (18β-GA), and isoliquiritigenin (IQ) were separated by capillary electrophoresis (CE). For the first time, separation of diastereoisomers of 18α-GA and 18β-GA by CE using a chiral additive has been achieved. Simultaneous separation of the above four pharmacological active components in one run by CE was reported. Different modes of CE, such as capillary zone electrophoresis (CZE), micellar electrokinetic capillary chromatography (MECC), cyclodextrin-MECC (CD-MECC), were employed to optimize the chemical separation. Preliminary experiments started with CZE using a 50 mM sodium tetraborate buffer within a capillary tube of inner diameter 50 μm and length of 60.2 cm. A CE separation voltage of 17 kV was used. Detection was achieved at a distance of 50 cm from the capillary inlet using a diode array UV absorbance detector over the wavelength range from 190 to 300 nm. The optimum separation was achieved by 10 mM sodium tetraborate-15 mM β-CD-25 mM SC. The effects of various experimental parameters, including pH, surfactant concentration, temperature, and organic modifier, on effective separation were investigated. The conditions of pH 8.5 and temperature at 25°C have resulted in more effective separations.

Keywords: GL, 18α-GA, 18β-GA, IQ, Separation, CE

INTRODUCTION

Licorice, or liquorice, whose Latin name is *Glycyrrhiza glabra L.*, is a perennial herb which possesses a sweet taste.[1] It abounds over an extensive

Address correspondence to Paul Li, Department of Chemistry, Simon Fraser University, 8888 University Drive, Burnaby, BC., Canada V5A 1S6. E-mail: paulli@sfu.ca

area of the warm portions of Europe and Asia. Many chemical compounds have been identified in licorice. Glycyrrhizin (GL, Fig. 1a) is the main ingredient. GL has been shown to possess important pharmacological activities, such as anti-viral, anti-inflammatory, and anti-tumor activities.[2] GL comprises the disaccharide, β-D-glucurono-pyranosyl(1,2)-β-D-glucuronopyranose, linked to a triterpenoid aglycone, glycyrrhetinic acid. Glycyrrhetinic acid has two diastereoisomers, namely the α-form (18α-GA), and β-form (18β-GA) (Fig. 1b and c).[3] 18α-GA and 18β-GA possess different biological activities and physicochemical properties. Both exhibit strong anti-inflammatory effects which is similar to that of glucocorticoid.[4] However, 18α-GA has a significantly greater effect than that of 18β-GA in several experimental models.[5] Another constituent of licorice is isoliquiritigenin (IQ) whose structure is shown in Fig. 1d. IQ belongs to flavonoids.[2] IQ is considered to be effective in preventing diabetic complications and also is a very potent antioxidant toward low density lipoprotein (LDL) oxidation.

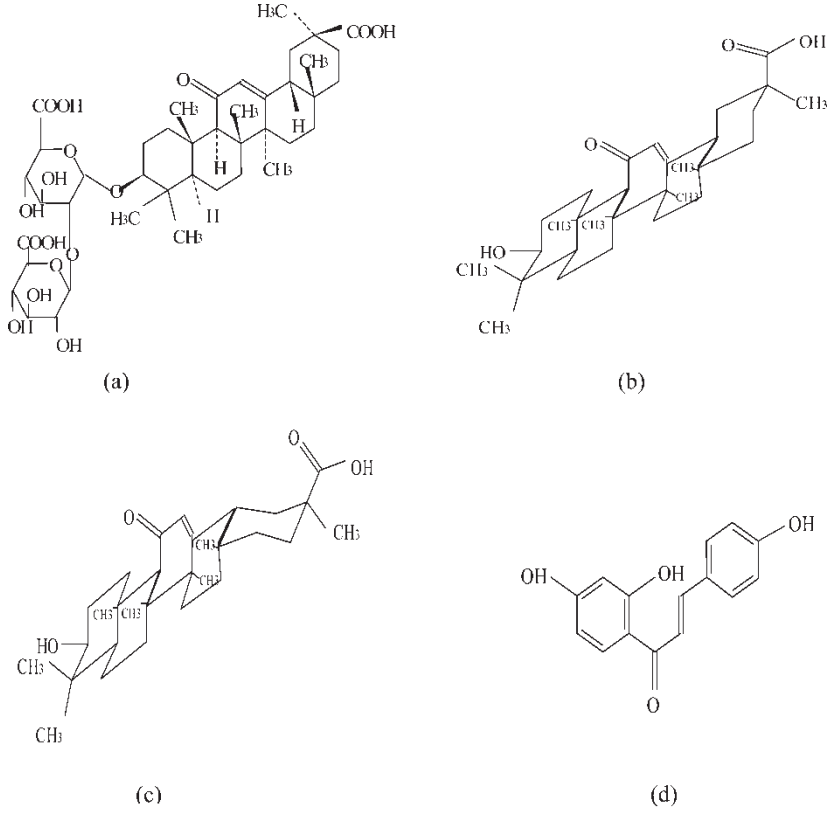

Figure 1. Structures of GL (a), 18α-GA (b), 18β-GA (c) and IQ (d).

Analytical separation and quantitation of bioactive compounds in licorice is essential to developing a scientific basis for understanding the many medical effects that have been observed clinically. Several analytical techniques have been employed for the analysis of major bioactive components in licorice root. These methods include high performance liquid chromatography (HPLC),[6–13] thin-layer chromatography (TLC),[14,15] gas chromatography (GC),[16] gas chromatography-mass spectrometry (GC-MS).[17] For 18α-GA and 18β-GA, since the biological activities of the two stereoisomers are quite different, an efficient separation method for their analysis is desirable. So far, only GC, HPTLC, and HPLC methods have been used for the analysis of the two stereoisomers. GC methods are laborious and time consuming because of the need of silyl or methyl derivatization to increase the volatility of the compounds. HPTLC is not as accurate because of the quantitation method by densitometry. Although good separation of the diastereoisomers could be achieved by HPLC, expensive chiral columns and large amounts of organic solvents should be used.

Recently, capillary electrophoresis (CE) has been shown to be very effective for the analysis of many compounds, especially for the materials with complex matrices.[18,19] CE has several advantages over other chemical separation methods, such as high separation efficiency, simplicity of operation, and low consumption of samples and solvents. Currently, several CE modes have been used to analyze licorice.[20–27] Capillary zone electrophoresis (CZE) was employed for the separation and determination of GL in licorice root and its preparations. Iwagami et al. analyzed glycyrrhizin in *Radix glycyrrhizae* and commercial oriental pharmaceutical preparations by using high performance capillary electrophoresis.[22] Zang et al. developed a capillary zone electrophoresis method to separate and determine GL in Chinese medicinal preparations.[26] Li et al. had reported a micellar electrokinetic capillary chromatography (MECC) method for the separation and determination of five licorice components, including GL, GA, and IQ.[20] Determination of GL and 18β-GA in biological fluids by MECC has been reported.[25] However, to date, separation of GL, 18α-GA, 18β-GA, and IQ in a single run simultaneously by CE has not been reported, especially for the chiral separation of 18α-GA and 18β-GA.

In this work, different modes of CE, such as CZE, MECC, CD-MECC, were employed to optimize the separation of four bioactive components from licorice root extracts. Since 18α-GA and 18β-GA are diastereoisomers, their separation is more difficult. Common buffer additives, which included sodium dodecyl sulfate (SDS) or bile salts like sodium cholate (SC), were added to the buffer. A chiral selective reagent such as β-cyclodextrin (β-CD) was employed to enhance the separation of the two diastereoisomeric components. Parameters, such as a buffer pH, capillary temperature, amount of organic modifier, were varied for the optimization of the separation of the licorice components.

EXPERIMENTAL

Reagents

Glycyrrhizin (GL, CAS# 53956-04-0, 75%), isoliquiritigenin (IQ, CAS# 961-29-5, 99%), and 18α-glycyrrhetinic acid (18α-GA, CAS# 1449-05-4, 98%) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). 18β-glycyrrhetinic acid (18β-GA, CAS# 471-53-4, 97%) was obtained from Aldrich Chemical Company (Milwaukee, WI, USA). Sodium dodecyl sulphate (SDS), sodium cholate (SC), β-cyclodextrin (β-CD), and sodium tetraborate were obtained from BDH Co. (Toronto, ON). Water was purified to have a resistance of 18MΩ cm^{-1} (Barnstead water purification system, VWR Canlab, Mississauga, ON). Methanol (HPLC-grade) was supplied by BDH. All chemicals are of analytical grade unless otherwise specified.

Instrument

All experiments were carried out on a commercial CE system (MDQ, Beckman-Coulter, Fullerton, CA). CE separations were performed with the cathode at the detector end of the capillary. Uncoated fused silica capillary columns (id, 50 μm), with a total length of 60.2 cm, were used. The temperature of the capillary was kept at 25°C, unless stated otherwise, using a liquid coolant flowing through the capillary cartridge. The temperature for sample storage was set at 4°C so that the samples were prevented from evaporation before separation. The running voltage was 17 kV. The photodiode array (PDA) detector was used to collect electropherograms from 190–400 nm; data rate is 4.0 data points per second. Electrophoregrams were reported at 254 nm. Injections were performed in the hydrodynamic mode, and the injection time was set at 1 s or 5 s and injection pressure is 0.5 psi. To condition the inner capillary surface, 1 mol/L HCl, water, 0.1 mol/L NaOH, water, running buffer were used in sequence. The rinse times were 5 min, 2 min, 10 min, 2 min, 2 min, respectively.

Buffer and Standard Solutions Preparation

The running buffer was prepared by dissolving an appropriate amount of borax (sodium tetraborate) in water to form 10 to 50 mM solutions, adjusted to the desired pH by 6 M HCl. Standard solutions were made by dissolving appropriate amounts of GL, IQ, and 18β-GA in methanol to form 1 mg/mL stock solutions. Since 18α-GA was less soluble, its concentration for stock solution is 0.5 mg/mL. The standard mixture was made by adding 200 μL of each standard, then diluting to 2 mL with 10 mM sodium tetraborate running buffer.

RESULTS AND DISCUSSIONS

Selection of Running Buffer

First, an alkaline sodium borate buffer system was used for the separation of the four pharmacological components in licorice root. The results are shown in electropherograms for the standard mixture (Fig. 2). We found that 18α-GA and 18β-GA co-eluted as a single peak. It is worthwhile to note that the first peak has both negative and positive portions, which is attributed to the presence of methanol in the samples. Although methanol does not absorb UV at 254 nm very well, a disturbance in the baseline is usually observed because of the change in refractive index as the solution interface of the sample plug reaches the detector. As shown in Fig. 2, 18α-GA and 18β-GA migrate earlier than GL, while GL migrates earlier than IQ. The possible reason is attributed to the charges of these compounds in the alkaline pH. 18α-GA and 18β-GA each produces one carboxylate group, GL three carboxylate groups, and IQ three phenoate groups. Thus, the number of negative charges under alkaline conditions in descending order is GL, IQ $>$ 18α-GA, 18β-GA. Moreover, the molecular weight of GL is the greatest among the four components. Therefore, their electrophoretic mobilities (negative), which depends on the charge-to-mass ratio, are in the order of 18α-GA, 18β-GA $<$ GL $<$ IQ. As a result, their net mobilities, which are positive after combining with the positive electroosmotic mobility, are in the order of IQ $<$ GL $<$ 18α-GA, 18β-GA, and their migration times are in the order of 18α-GA, 18β-GA $<$GL $<$ IQ.

Since 18α-GA and 18β-GA were not separated, further experiments were conducted by adding 25 mM sodium dodecyl sulfate (SDS), which was above

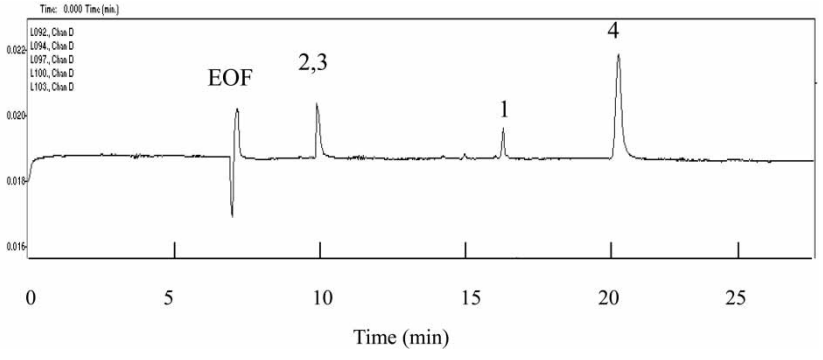

Figure 2. Electropherogram obtained for the separation of GL(1), 18α-GA(2), 18β-GA(3) and IQ(4) by CZE. Analytical conditions: 50 mM sodium tetraborate; Voltage: 17 kV; Capillary: 50 μm $\times$ 60.2 cm; distance to detector: 50 cm; Wavelength: 254 nm; temperature: 25°C.

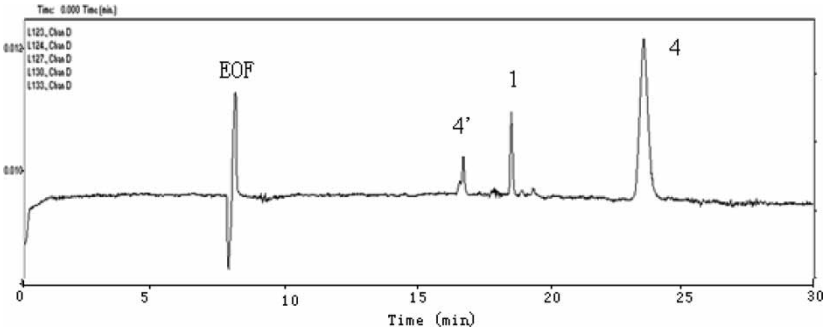

Figure 3. Electropherogram obtained for the MECC separation of GL(1), 18α-GA(2), 18β-GA(3) and IQ(4). Analytical conditions: 50 mM sodium tetraborate + 25 mM SDS. For other conditions, see Fig. 2.

its critical micelle concentration (CMC) of 8 mM, to the running buffer, leading to the use of the micellar electrokinetic capillary chromatography (MECC) mode. As shown in Fig. 3, the migration order for GL and IQ was the same as in Fig. 2. Unfortunately, within a run time of 30 min, no peaks showed up for 18α-GA and 18β-GA. Apparently, their migration times were much longer than 30 min.

In order to achieve the separation of 18α-GA and 18β-GA, a chiral selector cyclodextrin (CD) was added to the running buffer in the MECC mode. CD is usually used as an effective chiral additive in CE because of its ability to form inclusion complexes with a variety of molecules.[28] Because of the low cost of β-CD (versus γ-CD) and the great cavity size (versus α-CD), β-CD was selected in this work. Figure 4 shows the electropherograms with a final concentration of 15 mM β-CD added to the buffer.

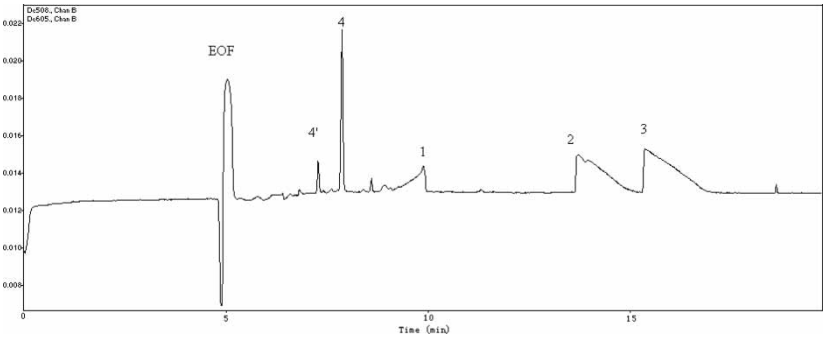

Figure 4. Electropherograms obtained for the MECC separation of GL(1), 18α-GA(2), 18β-GA(3) and IQ(4). Analytical conditions: 50 mM sodium tetraborate + 25 mM SDS + 15 mM β-CD. For other conditions, see Fig. 2.

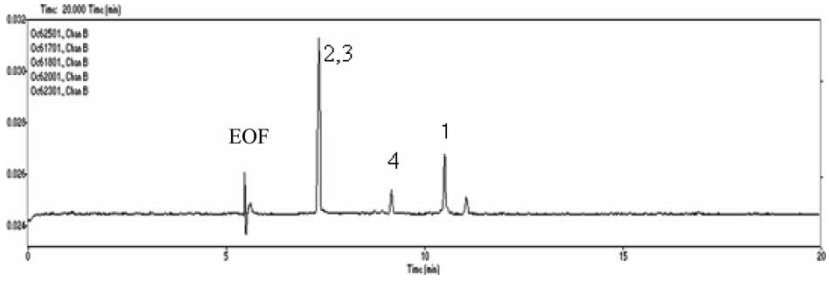

Figure 5. Electropherogram obtained for the separation of GL(1), 18α-GA(2), 18β-GA(3) and IQ(4) by MECC (SC). Analytical conditions: 10 mM sodium tetraborate + 25 mM SC; For other conditions, see Fig. 2.

Although under the CD-MECC mode, four components of licorice were separated, the efficiency of the method was not satisfactory because of the tailing peaks of 18α-GA and 18β-GA and fronting peak of GL.

Thereafter, we studied another micelle-sodium cholate SC or bile salt[29] in order to obtain a desirable separation of the four components. Figure 5 shows that 25 mM SC in 10 mM borate buffer at pH 8.5 was used. 18α-GA and 18β-GA migrated earlier than GL and IQ, which were opposite to their behavior in the SDS based buffer system. This suggests that 18α-GA and 18β-GA did not interact with SC very much. In order to improve the separation of the two diastereoisomers, β-CD was added. A sufficient separation for the four components was obtained in about 10 min (see Fig. 6a). The migration order (in increasing migration time) is 18α-GA, 18β-GA, IQ, and GL. In contrast to the SDS-mediated system, a faster separation was now obtained under the same capillary length and voltage conditions, the separation time decreased from 30 mins to 10 mins. The efficiency and resolution were improved, especially for GL.

Optimization of Analytical Conditions

Since the buffer system involving SC produced good separation resolution and peak symmetry, this buffer system was then optimized in terms of pH, temperature, and MeOH content.

Effect of pH

The pH value of the electrolyte solution is an important parameter in CE separation.[30] Figure 6 shows the effect of buffer pH on the electrophoretic mobility of 18α-GA, 18β-GA, IQ, and GL obtained in the pH range 8.5 to 10.0. Although increasing pH did result in an increase in separation efficiency and resolution, this was achieved at the expense of a long analysis time. While

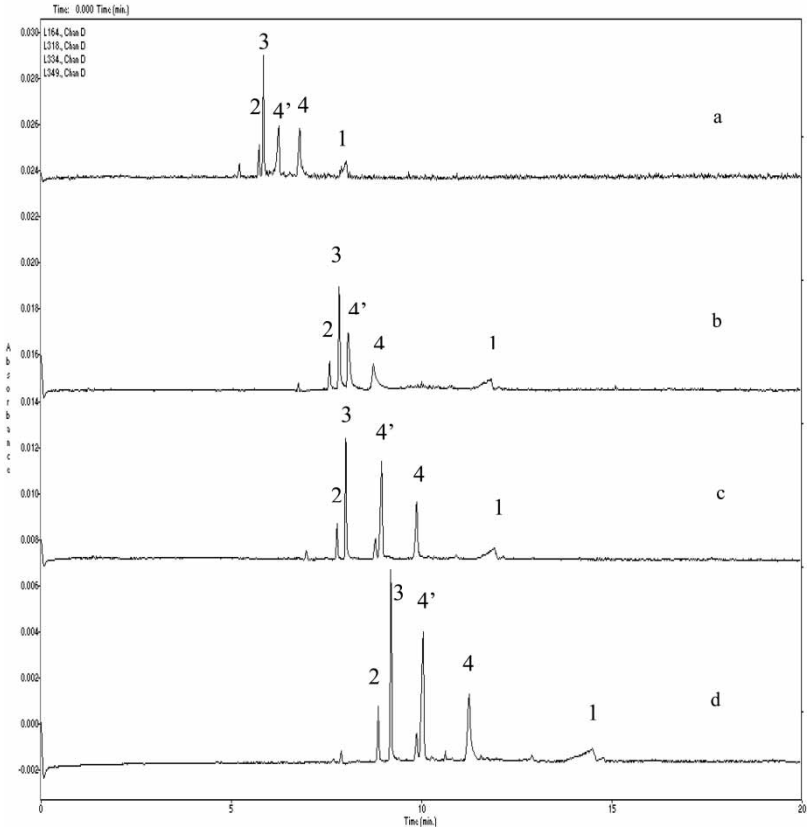

Figure 6. Electropherograms obtained to study the effect of different pH values of run buffer on the separation of GL(1), 18α-GA(2), 18β-GA(3) and IQ(4, 4'). Analytical conditions: 10 mM sodium tetraborate + 25 mM SC + 15 mM β-CD. For other conditions, see Fig. 2. (a) pH 8.5; (b) pH 9.0; (c) pH 9.5; (d) pH 10.0.

separation resolution is enhanced with the pH increment, a sufficient separation has been obtained at pH 8.5 with adequate efficiency and fast analysis speed. It is interesting to note that a small peak appeared in front of peak 4 may be attributed to the degradation of IQ. Despite this, separation of the four components was achieved successfully. Therefore, for the separation 18α-GA, 18β-GA, GL, and IQ, buffer with pH 8.5 was considered to be the optimal pH.

Effect of Temperature

It has been reported that the capillary temperature considerably affects resolution, efficiency, and analysis time.[31] Therefore, the temperature effect was

investigated at 5 temperatures between 20°C and 40°C, using the buffer system of 10 mM sodium tetraborate- 25 mM SC-15 mM β-CD buffer (pH 8.5). As shown in Fig. 7, the migration time of all components decreased with increasing capillary temperature. For instance, at 20°C analysis was completed in 10 min; whereas analysis was completed within 7 min at 40°C. This behavior is attributed to the viscosity decrease at an elevated temperature, resulting in higher electrophoretic and electroosmotic mobilities, presumably with the latter being to a greater extent. Additionally, from the resolution calculations, we found that, at 25°C, the resolution for 18α-GA and 18β-GA is the greatest. Hence, the use of capillary temperature at 25°C was considered as optimal for rapid separation and adequate efficiency.

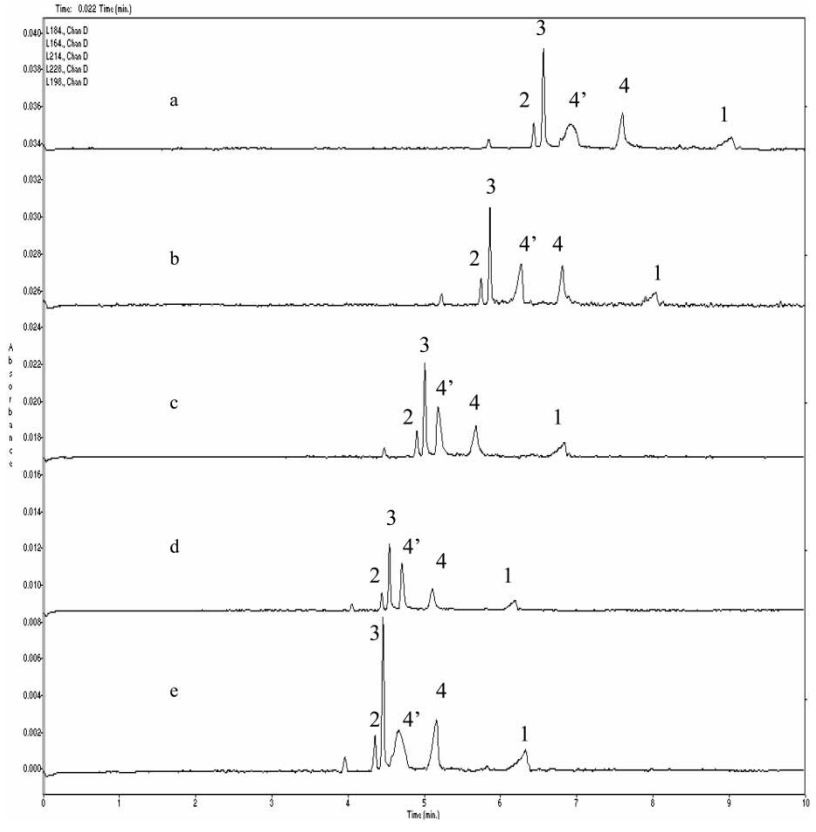

Figure 7. Electropherograms obtained to study the effect of different temperatures of capillary on the separation of GL(1), 18α-GA(2), 18β-GA(3) and IQ(4,4'). Analytical conditions: 10 mM sodium tetraborate + 25 mM SC + 15 mM β-CD; pH 8.5; temperature of capillary at (a) 20°C; (b) 25°C; (c) 30°C; (d) 35°C; (e) 40°C. For other conditions, see Fig. 2.

Effect of Organic Modifier

The effect of using organic modifiers in the CE buffers has been vastly studied. The effect of the addition of methanol to the buffer on the separation of four components was shown in Fig. 8. As illustrated, with an increase in the proportion of methanol (0–20%) contained in the buffer solution, the migration time of all components increased significantly, which was attributed to a decrease in EOF. Moreover, 18α-GA and 18β-GA coeluted and was not resolved once MeOH was present in the running buffer. However, the peak shape for GL was improved with a symmetric peak replacing a fronting

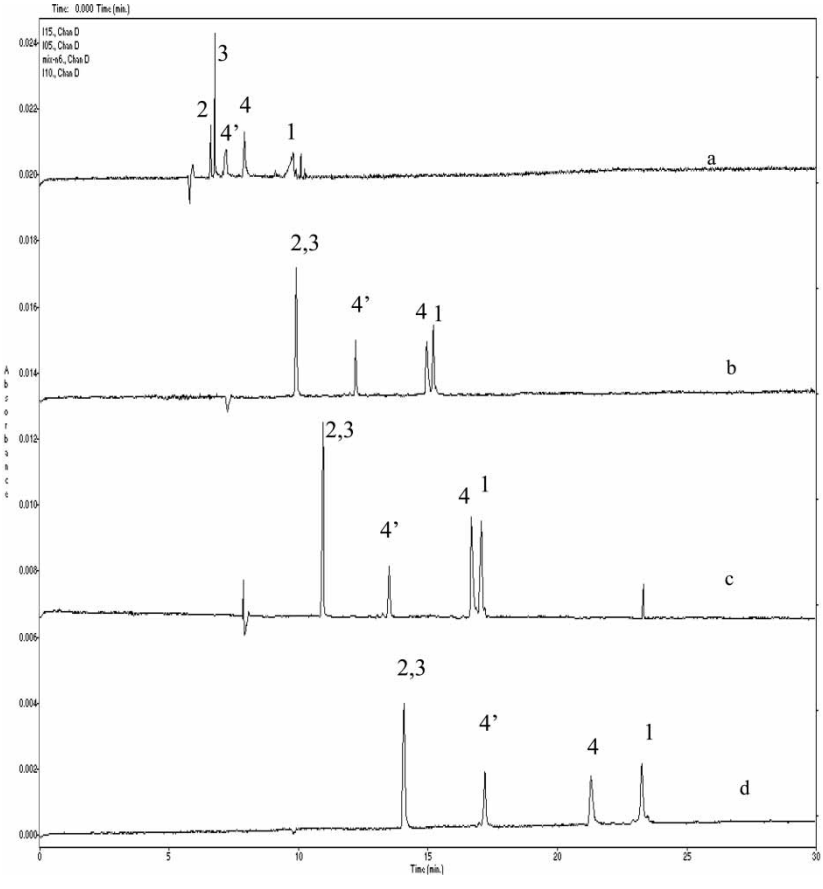

Figure 8. Electropherograms obtained to study the effect of different percentage of methanol in run buffer on the separation of GL(1), 18α-GA(2), 18β-GA(3) and IQ(4,4'). Analytical conditions: 10 mM sodium tetraborate + 25 mM SC + 15'mM β-CD; pH 8.5; temperature at 25°C; content of methanol: (a) 0%; (b) 10%; (c) 15%; (d) 20%. For other conditions, see Fig. 2.

peak when MeOH was added. Nevertheless, we found that the use of MeOH provided no advantage for our separation of the 2 diastereoisomers.

CONCLUSION

Separation of GL, 18α-GA, 18β-GA, and IQ derived from licorice by various modes of capillary electrophoresis has been investigated in this study. Although, separation of the components in licorice samples has been previously reported, most methods focus on one or two of the four components by HPLC or GC, and CE methods are seldom employed. In addition, since 18α-GA and 18β-GA are two diastereoisomers, separation for them was more difficult. To date, no literature has been found to report their separation by CE. In the present research, for the first time, the simultaneous separation of the above four components in one CE run has been reported. Furthermore, different CE modes have been investigated to optimize the separation conditions.

Preliminary experiments started with the CZE mode with 50 mM sodium tetraborate buffer. Methanol was used as an EOF marker. Only GL and IQ were separated. The MECC mode was then employed to achieve separation of the four components. Two kinds of micelle systems have been investigated. 18α-GA and 18β-GA, which presumably interacted with SDS strongly, did not show up within the usual 30 min analysis time. Increasing SDS concentration worsened the situation by prolonging the analysis time. As a chiral selector, the addition of β-CD improved the separation of 18α-GA and 18β-GA, but they migrated very slowly, and with tailing peaks. Hence, the separation was performed on another micelle system: SC. By using the SC micelle system, the analysis time was decreased greatly from 30 min to 10 min. In order to achieve chiral separation, β-CD was also added to the buffer. A complete separation was obtained by using 50 mM sodium tetraborate-15 mM β-CD-25 mM SC. Moreover, the effects of pH, temperature, and the amount of organic modifier on separation, were investigated systematically. The optimum analytical conditions were pH 8.5 at 25°C, and without the use of an organic modifier (MeOH).

In both buffer systems, the peak shape of GL was not symmetric. It has been found that the addition of methanol improved peak shape, but the separation 18α-GA and 18β-GA was lost. The optimized CE conditions will be used to analyze the 4 bioactive components in licorice root samples in future studies.

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