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Simultaneous determination of flavonoid analogs in Scutellariae Barbatae Herba by β-cyclodextrin and acetonitrile modified capillary zone electrophoresis

Yuan-Yuan Li [a], Qing-Feng Zhang [a,b], Hongyan Sun [a], Nga-Ki Cheung [a], Hon-Yeung Cheung [a,*]

[a] Research Group for Bioactive Products, Department of Biology and Chemistry, City University of Hong Kong, 83 Tat Chee Avenue, Hong Kong, SAR, China
[b] Jiangxi Key Laboratory of Natural Products and Functional Food, College of Food Science and Engineering, Jiangxi Agricultural University, Nanchang 330045, Jiangxi Province, China

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ABSTRACT

A capillary zone electrophoresis (CZE) method modified by β-cyclodextrin (β-CD) and acetonitrile (ACN) was developed for simultaneous determination of seven structurally similar flavonoids in Scutellariae Barbatae Herba (SBH) and its preparations. Molecular selectivity of the analytes by β-CD was in the following order: apigenin, luteolin, quercetin, scutellarin, baicalein, rutin and wogonin, based mainly on the "molecular fit" interaction between some ligands in the C ring of the flavonoid and the cavity of β-CD. Flavonoids with hydroxyl substituent(s) at the C-ring, especially the 4′ monohydroxyl, were highly selected by β-CD although hydrophobicity of the guest molecule is the primary factor affecting the complexation. The function of acetonitrile in this study was to improve the separation of the analytes in the real SBH. The developed method was validated and applied to real samples. The principle of separation based on this CZE condition is also explained.

1. Introduction

As a micro-analytical method, capillary electrophoresis (CE) has won more and more acclaim in the analysis of natural products due to its high efficiency, good separation performance, minimum consumption of sample and environmental friendliness [1,2]. Capillary electrophoresis has previously been used in the separation of alkaloids [3], flavonoids [1], terpenoids [4], and other substances [5]. Particularly for the flavonoids, which consist of a basic 2-phenylbenzopyranone with varying degrees and types of substitutions, CE offers outstanding selectivity for differentiating isomers and analogs of the compound in a variety of matrices such as plants [6], foods [2,7], wine [8], medicinal preparations [9] and herbs [10].

Among different types of CE, capillary zone electrophoresis (CZE) is the simplest mode, in which the separation is basically dependent on the charge-to-size ratio of the analytes. However, the biggest limitation of CZE is that it lacks the ability to discriminate compounds with close charge-to-size ratios. To enhance the separation capability of CZE, cyclodextrins (CDs), proteins, surfactants such as sodium dodecylsulphate (SDS), cholate, organic solvents and polysaccharides have been added to the background electrolyte (BGE) as modifiers [11]. Cyclodextrins are oligosaccharides having a truncated cone shape with an open cavity, which can form inclusion complexes with various molecules. In particular, the cavity of β-CD is appropriate to host a wide range of chemical compounds [12]. Owing to this unique property, β-CD has been widely applied as a molecular selector in separation science [13]. In CE, CDs (including β-CD and other derivatives) have been used in the analysis of structural isomers and analogs [11,12]. There are several publications that have been reported on the application of β-CD as a modifier in flavonoid analysis. Advantages mentioned include outstanding chiral selectivity, high efficiency, good reproducibility and easy operation [10,14,15]. In most cases, the research was focused only on the optimization of separation conditions, such as the nature and the concentration of BGE and modifiers [6,9,10,15,16,17], pH value [9,18,19], separation voltage [10,20,21], electrophoretic temperature [19,20], detection mode [21,22], and others. However, the basic behavior of molecular selectivity of β-CD on flavonoids during the host–guest interaction in CE was seldomly discussed, and the optimization of separation performance in CE *via* the use of β-CD alone or a combination with other modifiers is often carried out by trial or error approaches due to the lack of a provided guidance.

Scutellariae Barbatae Herba (SBH), also called "Banzhilian", is a commonly used herb for treating cancer, inflammation and

* Corresponding author. Tel.: +852 27887746; fax: +852 27887406.
E-mail address: bhhonyun@cityu.edu.hk (H.-Y. Cheung).

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urinary diseases in traditional Chinese medicinal [23,24]. It is also used as the crude material in the manufacture of more than a hundred functional foods and drugs. Flavonoids, which are considered as the major constituents responsible for the herbal functions observed, are always chosen as the markers for quality control in the use of the herb and its preparations [25–27]. In this study, it was envisaged to establish a CE method with good separation performance, high efficiency, simple operation and low solvent consumption for simultaneous determination of the bioactive flavonoids (i.e., baicalein, scutellarin, luteolin, wogonin, apigenin, rutin, and quercetin) in SBH (structures see in Fig. 1). Besides investigating the separation parameters, more attention was paid to explain the working principle of the adopted modifiers (i.e., β-CD and acetonitrile) during the course of separation improvement. To the best of our knowledge, we are not aware of β-CD and ACN modified CZE have applied to the analysis of flavonoids in SBH [27]. In addition, this is also the first time to discuss the separation behavior of modifies for the flavonoids. The established method, as well as the proposed principle, is useful for the future application of CE on flavonoids in food and drug analysis.

2. Experimental

2.1. Reagents and materials

Acetonitrile (ACN) and methanol (HPLC grade) were purchased from Labscan Asia (Bangkok, Thailand). Borax and β-CD in ultra pure grade were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultra high purity (UHP) water was prepared using a Millipore Milli Q-Plus system (Millipore, Bedford, MA, USA). All other chemicals were of analytical grade.

Rutin, quercetin, and benzoic acid (internal standard) with purity more than 98% were purchased from Sigma (St. Louis, MO, USA). Baicalein, wogonin, scutellarin, luteolin, and apigenin (> 98% purity) were purchased from Tauto Biotech (Shanghai, China). Stock solution for each target analytes and IS were prepared at about 1.0 mg mL^{-1} with methanol and stored at −20 °C until used. A certain volume of each stock solution was mixed and then diluted with the electrophoretic medium to the desired concentration.

Four batches of SBH (WH01 to 04) were collected from different cultivation sites in China (i.e., Guangdong, Sichuan, Hunan and Henan). All the collected herbs were identified as

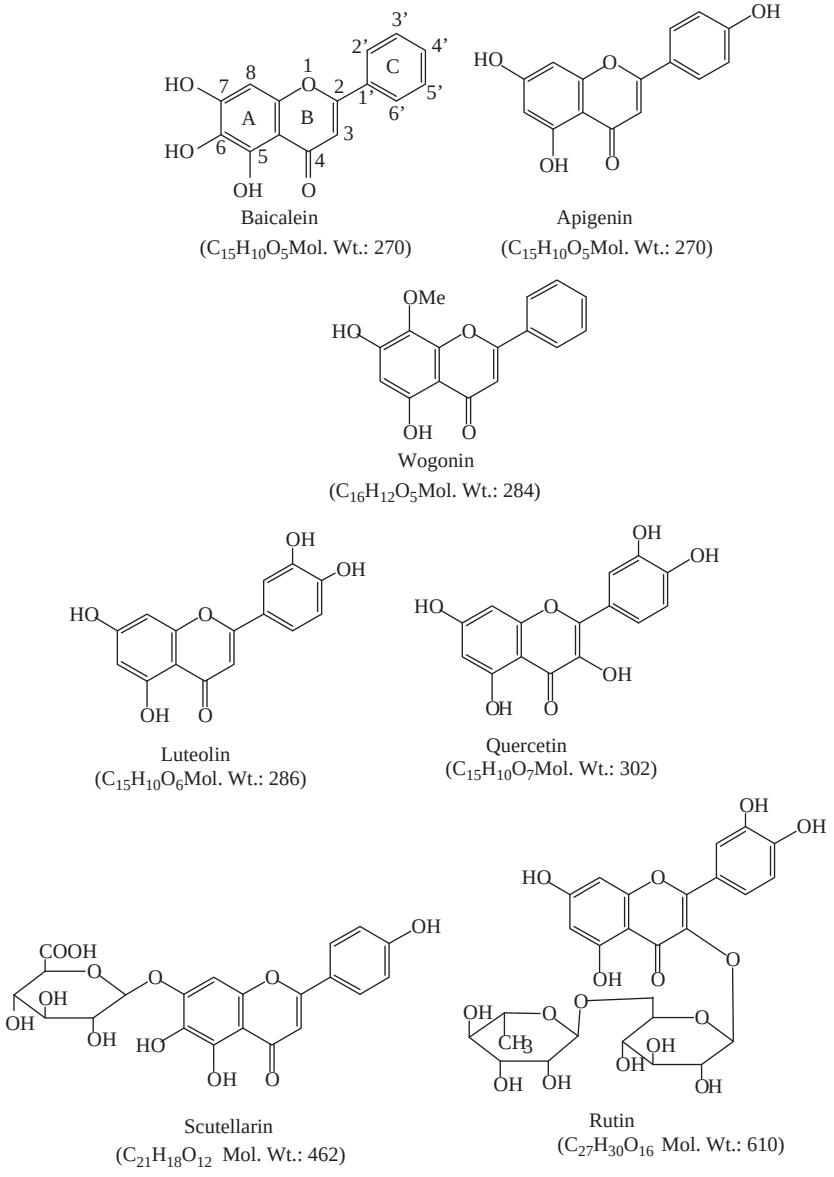


Fig. 1. Structure and molecular weight of the investigated flavonoids.

Scutellaria barata D. Don. by Dr. Zhang Zhi-Feng (Associate Professor of the Ethnic Pharmaceutical Institute, Southeast University of Nationalities). Granular extract products of another four batches of SBH were purchased from the Festival Walk's Supermarket (Kowloon Tong, Hong Kong) and Chinese pharmacy stores in Hong Kong and in Taiwan.

2.2. Apparatus

Capillary electrophoresis was carried out on a P/ACE MDQ electrophoresis system with photodiode array detection (DAD) (Beckman Instruments, Fullerton, CA, USA). The fused-silica capillary tube with size 50 μm (I.D.) × 60.2 cm (50 cm active length) was also purchased from Beckman. The pH was measured with a HI 8424 Microcomputer pH meter (Hanna Instruments, Porto, Portugal). Plant samples were sonicated in a Transsonic TS 540 Tank (Lab-Line Instruments, Troisdorf, Germany).

2.3. Sample preparation

Five hundred milligram of accurately weighted herbal powder (for the SBH granules weighed 200 mg) was immersed in 25.0 mL 50% methanol for 1 h, and then sonicated for 30 min. After filtration (with filter paper), 1 mL of the filtrate was diluted to 2 mL using the electrophoretic medium containing 20 μg mL^{-1} benzoic acid (IS). Then, the solution was filtered (with 0.22 μm pore size membrane filter) before injection.

2.4. Electrophoretic procedure

The capillary tube was rinsed in the following order, 0.1 M NaOH (for five min), the running UHP water (five min) and the medium (five min) before its daily use. The running electrophoretic medium was composed of 50 mM borax, 1 mM β-CD and 2% acetonitrile (v/v). The pH value of the medium was 9.28 without adjustment. The separation voltage was 25 kV, and the capillary temperature was 25 °C. Samples were loaded by pressure injection for 10 s at 0.5 psi. The detection wavelength was set at 214 nm. For the purified standard solutions, the capillary was rinsed with UHP water (1 min) and fresh medium (2 min) between every run. For the herb samples, the capillary was rinsed between every run with water (2 min, in reverse rinsing mode), acetonitrile (1 min, in reverse rinsing mode), UHP water (1 min), 0.1 M NaOH (2 min), water (2 min) and then fresh medium (2 min). The capillary tube was cleaned daily with UHP water (2 min), acetonitrile (10 min), UHP water (5 min), 0.1 M NaOH (30 min) and UHP water (30 min) after use. The prepared buffer was stored in a sealed container, and the running buffer was replaced after every three runs (about 2 h).

2.5. Parameters optimization for CE

In this study, the effect of concentration (10–80 mM) of borax background electrolyte was investigated. In a similar way, the modifiers, β-CD and ACN, were investigated in the range from 0 to 5 mM (0, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3, 4 and 5 mM) and 0 to 12% (v/v, 0, 2%, 4%, 5%, 7%, 10%, 12%), respectively. The electrophoretic behavior of individual analytes under specific condition was evaluated by electrophoretic mobility (EM) or absolute electrophoretic mobility (AEM) [28], which were calculated from the following equation:

$$\mu_{ep} = \mu - \mu_{eo} = \frac{L_d L_t}{V}\left(\frac{1}{tm} - \frac{1}{teo}\right) \quad (1)$$

where μ_{ep} is the EM of analyte tested, μ is the apparent mobility, μ_{eo} is the electroosmotic mobility, t_m is the migration time measured directly from the chromatogram, t_{eo} is the migration time of the electro-osmotic flow (EOF) which was recorded at the organic solvent peak, L_t is the total length of capillary, L_d is the length of capillary between injection and detection, and V is the applied voltage.

A thermodynamic double-reciprocal equation (Eq. (2)) was used to evaluate the formation constant (K) between the individual analyte and β-CD [29,30]

$$\frac{1}{\mu_{ep} - \mu_f} = \frac{1}{(\mu_c - \mu_f)K[\mathrm{CD}]} + \frac{1}{\mu_c - \mu_f} \quad (2)$$

where μ_{ep} is the EM of analyte tested, μ_f is the EM of analyte in the free state (does not form complex with CD), μ_c is the EM of analyte in complexed state and [CD] is the concentration of β-CD. A straight line was derived by plotting $1/(\mu_{ep} - \mu_f)$ versus 1/[CD] for each analyte. The correlation (R) of the straight line can reflect whether the interaction between individual analyte and β-CD is a fit for a 1:1 ratio or not. The value of K and μ_c was calculated from the intercept and the slope of the straight line. All the experiments were carried out in triplicate.

2.6. The procedure of HPLC

The hydrophobicity comparison of seven flavonoids was carried out on an Agilent 1260 system (Agilent, USA) equipped with an on-line degasser, and a binary bump, a diode array detector (DAD). The column adopted was Waters XBridge ™ C_{18} (4.6 mm × 250 mm i.d., 5 μm) fitted with a C_{18} guard column (Waters, Ireland). The mobile phase consisted of (A), aqueous solution at pH 2.5 adjusted with formic acid and (B), ACN. The optimum isocratic elution condition was A:B=7:3. The detection wavelength was at 280 nm. In reverse phase chromatography, an analyte with a higher retention factors (k') represents a greater hydrophobicity [8].

The k of the seven flavonoids was calculated with the following equation:

$$k' = \frac{tR}{tm} - 1 \quad (3)$$

where t_m is the retention time of the mobile phase or un-retained solute, and t_R is the retention time of the analyte.

2.7. Method validation

The calibration curve was derived by plotting the peak area ratio between individual analyte and IS versus the concentration ratio between them. The limit of detection (LOD) and quantification (LOQ) for each analyte were determined at a signal-to-noise ratio (S/N) of about 3 and 10, respectively.

The method precision was evaluated by analyzing the mixed standards (with 5 and 25 μg mL^{-1} for each analyte) in six replicates. The precision, including the peak area ratio precision and migration time precision, was defined as relative standard deviation (RSD, %) calculated from the six independent assays.

The sample recovery was performed by adding known amounts of individual standards into an accurately weighed mixed sample mentioned above. The mixed sample was extracted and analyzed using the method mentioned above. For each concentration, three replicate experiments with the whole analysis process were performed. Recovery was calculated with the following equation: recovery (%)=100 × amount found/(original amount+amount spiked).

3. Results and discussion

3.1. Influence of the background electrolyte (BGE) concentration on the analytes

A borax solution was applied as the BGE because of the easy formation of negatively charged flavonoid–borate complexes [31–34]. The influence of borax concentration on the separation was investigated with results showed in Fig. 2A. With the increase of borax concentration, a longer migration time was observed and the peak resolution was improved gradually. However, thorough separation cannot be achieved by using BGE alone, even with high concentrations. Additionally, high borax concentration (80 mM) may cause various negative effects, i.e., peak broadened, low analytical efficiency, and big baseline noise, which were unbeneficial to get satisfactory performance. Fig. 3A shows the effect of

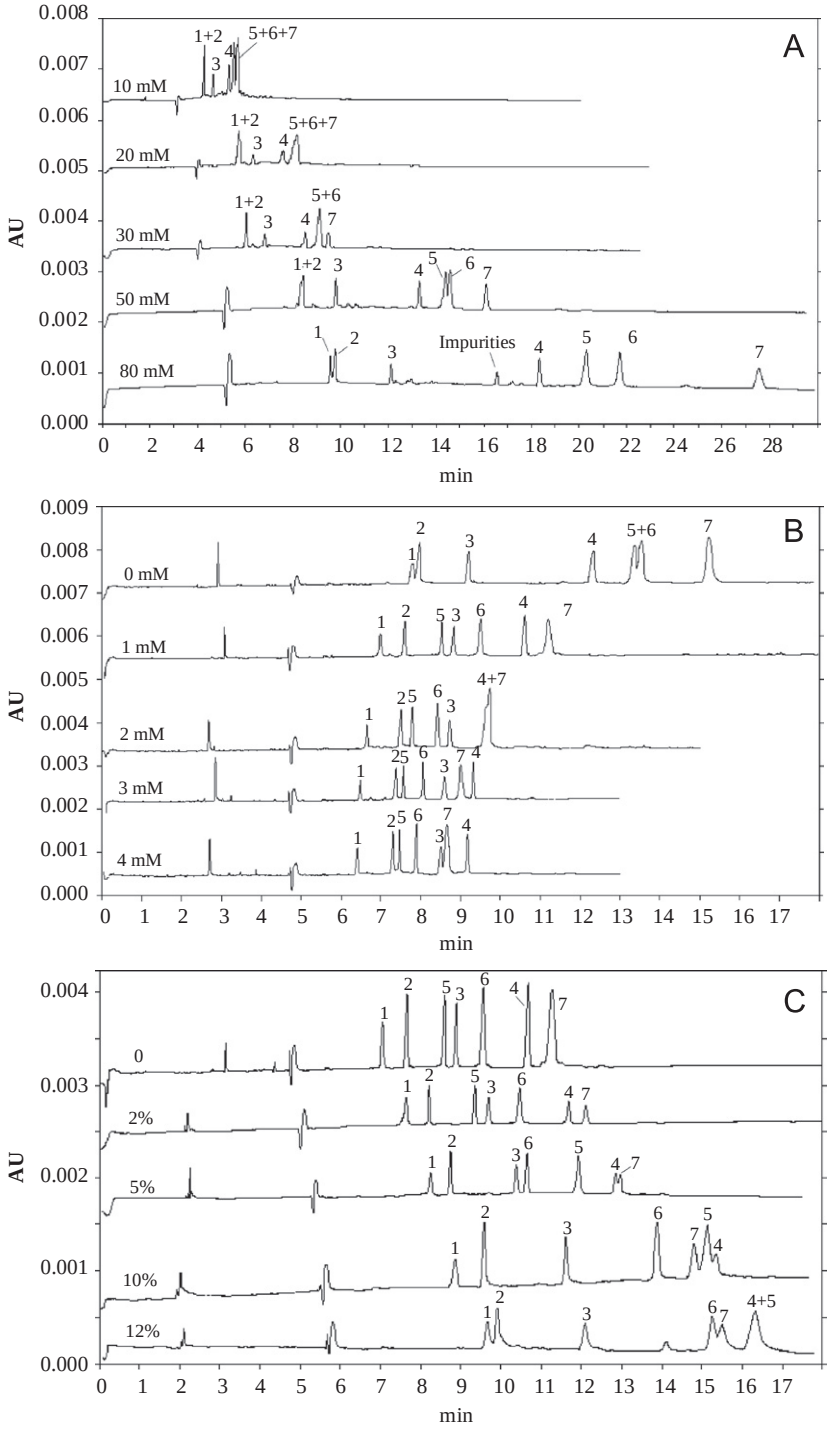


Fig. 2. Effects of borax (A), β-CD (B) and acetonitrile (C) addition on the migration time of the flavonoids. The electrophoretic procedure was the same as described in Section 2.4. The composition of electrophoretic medias were: (A) borax solutions (10–80 mM) without modifier; (B) borax 50 mM with different concentrations of β-CD (0–5 mM); (C) borax 50 mM, β-CD 1 mM and different concentrations of acetonitrile (0–12%, v/v). Peaks: 1=baicalein; 2=wogonin; 3=rutin; 4=scutellarin; 5=apigenin; 6=luteolin; 7=quercetin.

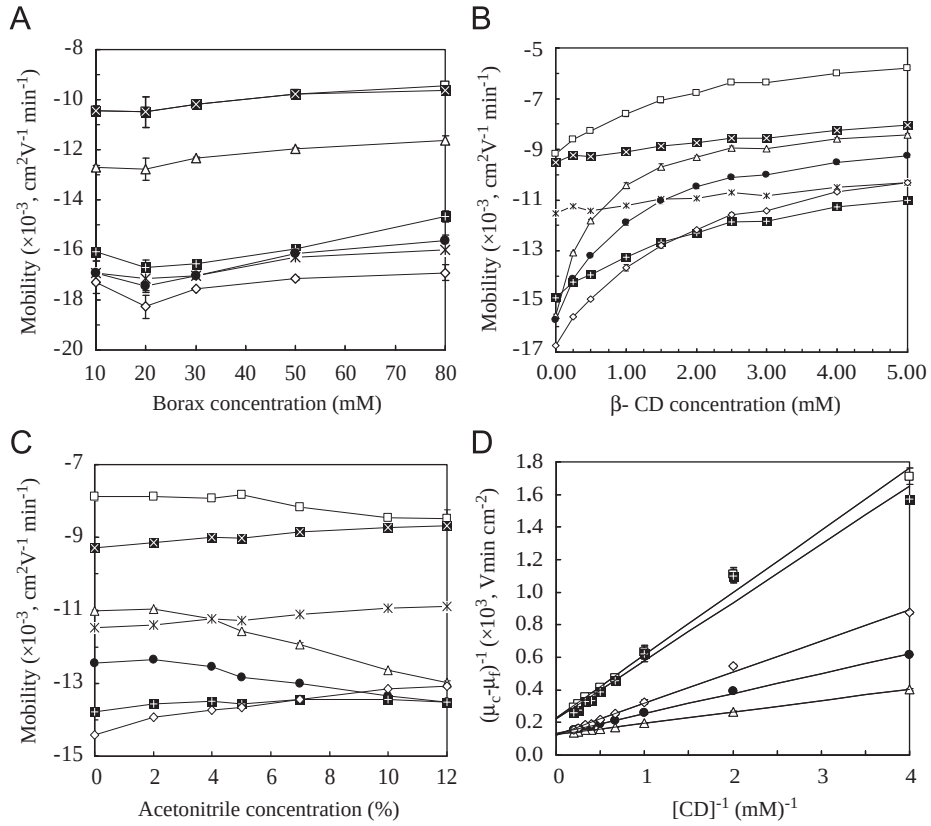


Fig. 3. Effects of borax, β-CD and acetonitrile addition on the electrophoretic mobility of flavonoids.(A) electrophoretic mobility plotted against the concentration of borax; (B) electrophoretic mobility plotted against the concentration of β-CD; (C) electrophoretic mobility plotted against the concentration of acetonitrile; (D) double-reciprocal plot of the electrophoretic mobility for calculation of the formation constant (*K*) between the analytes and β-CD. Symbols: (baicalein), (wogonin), (scutellarin), (rutin), (luetolin), (apigenin), (quercetin).

borax concentration on the electrophoretic mobility (EM) (Eq. (1)). It was found that borax concentration displayed no selective influence on the analytes. This indicates that the increase of migration time in higher BGE was caused by the reduction of Zeta potential in the electrolyte (presented as the increase of t_{EOF}) instead of the alternation of ionization in the analytes. With an aim to achieve better separation performance, borax solution with middle concentration (50 mM) was adopted as the BGE in the study. Moreover, some electrophoretic modifiers (i.e., β-CD and organic solvent) would be introduced into the electrolyte to improve the separation performance.

3.2. Influence of β-CD on analytes in CE

3.2.1. Influence of β-CD on the electrophoretic behavior

The influence of β-CD on the separation of the flavonoids with structural similarities was investigated. Fig. 2B shows the effect of increasing β-CD concentration, a shorter migration time and rearranged order of migration was observed. The migration time of baicalein, apigenin, scutellarin, luteolin and quercetin was found to decrease more significantly, while the migration time of wogonin and rutin seemed to remain constant. Fig. 3B further indicates that the enhancing magnitude of EM varied from analyte to analyte. All these results suggest that the molecular selectivity of β-CD on analytes was a key factor responsible for the different magnitudes of EM, thus resulting in separation improvement. In this study, all the analytes got baseline separation when β-CD was at 1 mM, while the performance became worse when the concentration was continually increased.

3.2.2. Complexation and structural selectivity of β-CD

To further explore the molecular selectivity of β-CD, a double-reciprocal equation (Eq. (2)) was applied to analyze the complexation in CE [16,29,30]. According to this equation, if analyte binds to β-CD at 1:1 ratio, a straight line could be derived by the plotting of $1/(\mu_{ep}-\mu_f)$ versus 1/[CD] [16,29,30]. Experimental data in Fig. 3D and Table 1 show that five of the seven analytes (i.e., quercetin, luteolin, baicalein, scutellarin and apigenin) displayed good straight-line correlations ($R > 0.98$). This phenomenon suggests that their complexation fits the dynamic mathematical model, and the calculated formation constant (*K*) reflects the degree of inclusion between the analyte and β-CD. Table 1 also shows that the *K* value for apigenin, luteolin, quercetin, scutellarin and baicalein was 1880 ± 65, 1076 ± 5, 649 ± 12, 618 ± 22 and 599 ± 37, respectively. In general, the higher the *K* value, the more stable the complex formed, thus resulting in a greater alteration of EM. However, the *K* value for wogonin and rutin could not be calculated because the alteration of EM was almost negligible even in the presence of β-CD.

In the complexation process between host and guest, the *K* value is mainly affected by two parameters according to the literature: (1) the hydrophobicity of the analyte [12,35]; (2) the "molecular-fit" principle which is decided by the molecular feature [12,35]. To investigate the relationship between the *K* value and the hydrophobicity of analytes, retention factor (k') of an analyte was experimentally determined by reverse-phase high performance chromatography as described in Section 2.6 and was calculated based on Eq. (3). As shown in Table 1, the two most hydrophilic compounds, i.e. scutellarin and rutin, displaying a relatively low *K* also have the lowest k' value. These figures

Table 1
Formation constants (K) of analyte and β-CD, hydrophobicity of analyte (k'), electrophoretic mobility of analyte in free and complexed states ($n=3$).

Analyte[a]	K (L mol^{-1})[a]	k'[b]	r ($n=9$)[c]	μ_f[d] ($\times 10^{-3}$)	μ_c[d] ($\times 10^{-3}$)	M_f/M_c[e]	μ_c/μ_f
Apigenin	1880 ± 65 (827)[g]	4.85	0.999	-15.556 ± 0.062	-7.617 ± 0.054	0.19	0.490 ± 0.002
Luteolin	1076 ± 5 (697)[g]	4.55	0.999	-15.739 ± 0.027	-8.172 ± 0.043	0.20	0.519 ± 0.003
Quercetin	649 ± 12 (1284)[g]	3.81	0.997	-16.746 ± 0.031	-8.7258 ± 0.111	0.21	0.521 ± 0.006
Scutellarin	618 ± 22 (420)[g]	0.55	0.987	-14.871 ± 0.019	-10.348 ± 0.109	0.29	0.696 ± 0.008
Baicalein	599 ± 37 (50)[g]	6.33	0.996	-9.189 ± 0.039	-4.831 ± 0.185	0.19	0.526 ± 0.020
Wogonin	–[f] (67) [g]	7.28	–[f]	-9.520 ± 0.037	–[f]	–[f]	–[f]
Rutin	–[f] (250) [g]	0.29	–[f]	-11.196 ± 0.023	–[f]	–[f]	–[f]

[a] K, formation constant calculated from Eq. (2). Order of the analyte from top to bottom in the table is arranged according to the K value.
[b] k', retention factor which was used to evaluate the hydrophobicity of the analyte.
[c] r, correlation coefficient ($n=9$) calculated on basis of the plots shown in Fig. 2B and D.
[d] The unit for μ is cm^2 V^{-1} min^{-1}.
[e] M_f is the molecular size of analyte in the free state (i.e., the molecular weight of analyte shown in Fig. 1). M_c is the molecular size of analyte in the complexed state, i.e., M_f + the molecular weight of β-CD.
[f] Unable to form complex with β-CD.
[g] Number in the brackets in Table 1 refers to the K value reported in the previous host-guest studies. The data are derived from the following literatures: [36] (quercetin and rutin), [37] (baicalein), [38] (wogonin), [39] (apigenin), [40] (luteolin) and [41] (scutellarin).

confirm that the hydrophobic aglycone rather than the hydrophilic glycoside was more preferable by β-CD. Our conclusion is not without supports as Alvarez-Parrilia et al. also reported that the K of quercetin which is the aglycone of rutin was about 6 fold greater than that of rutin [36]. However, the exceptional high k', low K value for baicalein and wogonin, suggests that hydrophobicity is probably not the only determination factor. In a previous guest–host study, it was reported that these two compounds did not show good affinity to β-CD as well [37,38].

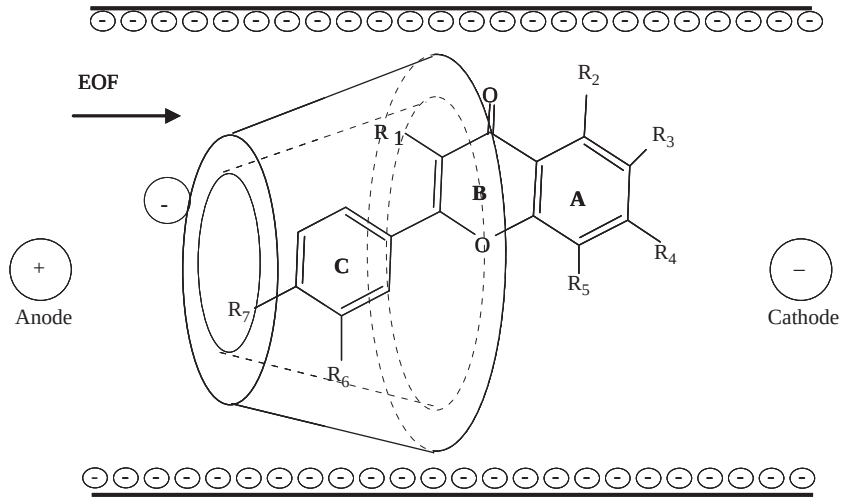
Fig. 1 reveals that baicalein and wogonin contain nil hydroxyl substitution in the C-ring, while other aglycones, such as apigenin, luteolin and quercetin, contain hydroxyl(s) moiety in the ring. Consequently, these latter molecules have a lower hydrophobicity but have much higher K values than that of the former two molecules. In addition, compound, which has a monohydroxyl substitution, such as apigenin, also presented a greater K than those with dihydroxyl substitutions (quercetin and luteolin). This phenomenon indicates that complexation is affected by both the number and the position of hydroxyl substitution in the C-ring. According to some previous studies on host–guest complexation, it has been shown that the C-ring of flavonoids was the moiety inserted into the cavity of β-CD [37,38]. Thus, ligand interaction between the C-ring of flavonoid and the host molecule plays some roles in the complexation. The greater affinity of the C-ring with hydroxyl substitution to β-CD than that without hydroxyl substitution could be explained by the volume and polarizability, i.e. molar refraction (R_m) of the hydroxyl moiety against the proton. As the R_m value of the hydroxyl moiety is much larger than that of the proton, the van der Waals force of the former is also stronger than that of the later [42]. The aforesaid explanation has been observed in another similar study using naphthalenesulfonate derivatives as the analysts in cyclodextrin-mediated CE [43,44]. The greater K of apigenin (monohydroxyl) than that of quercetin and luteolin (dihydroxyl) could be explained as a consequence of the steric hindrances of hydroxyl substation on the C-ring [44]. Based on the observations described above together with the explanations based on ligand interaction in host-guest complexation, a possible inclusion mode between flavonoid aglycone and β-CD for CE is deduced as illustrated in Fig. 4. The structural selectivity of β-CD on flavonoids can be explained as a "molecular-fit" interaction between the C-ring and the cavity of β-CD. Flavonoid with mono-hydroxyl substitution in the C-ring, especially the 4′-hydroxyl, is a favorable target of β-CD molecule and can be strongly and selectively included by β-CD in CE.

3.2.3. *Influence of β-CD on the molecular mass and charge for the analytes*

The calculated μ_c (Eq. (2)) is defined as the EM of the analyte-CD complex in a state in which complete complexation has taken place [45]. Thus, the value of μ_c/μ_f reflects the ratio of EM between the complexes and the free-state molecules. From a general recognition of the CD modified CZE, the increased mass of the forming analyte-CD-complex is a key factor responsible for the change of the effective EM [12]. Therefore, the value of μ_c/μ_f is speculated to be equal to that of M_f/M_c (the ratio of molecular weights between free and complex states). However, as shown in Table 1, the values of μ_c/μ_f were found to be about 2–2.5 fold higher than that of M_f/M_c. This result indicates that besides increasing the molecular weight for analytes, the ionization of the analyte-CD complex might also be involved in the complexation in the CE method [46].

3.3. *Influence of acetonitrile (ACN)*

The addition of organic solvent in the running buffer was investigated with respect to improvement of separation in CE [36,47]. The effect of ACN on the migration behavior is shown in Fig. 2C. Migration time increased with the increase in the presence of ACN. The peak order was found to alter remarkably when the ACN concentration was higher than 5%. However, the separation performance was not improved but became worse with continually increasing ACN. The influence of ACN on the EMs (Fig. 3C) further reveals that the low concentration of ACN ($<5\%$, v/v) had no selective influence on the EM. However, when concentration of ACN was higher, the selectivity became obvious, e.g., the EM of luteolin, apigenin and baicalein decreased significantly, while that of others were almost not affected. Hence, the increase of migration time caused by ACN was most likely due to its inhibitory effect on the viscosity and dielectric constant of running buffer [47]. The ACN-induced alternation in EM for some analytes can be explained as a result of the competition between ACN and analyte molecule for occupying the hydrophobic cavity of the β-CD molecule [35,47]. The data of selectivity suggest that the binding strength of luteolin, apigenin and baicalein to β-CD was quite weak, which was easily weakened by ACN. In this study, the introduction of ACN in the running buffer seems insignificant to further improve the separation of the standard solution, since the baseline separation had been achieved when β-CD was adopted as the sole modifier. However, it is worthy to note that using a small amount of ACN (2%, v/v) was very effective



Aglycones	R_1	R_2	R_3	R_4	R_5	R_6	R_7
Apigenin	H	OH	H	OH	H	H	OH
Luteolin	H	OH	H	H	H	OH	OH
Quercetin	OH	OH	H	OH	H	OH	OH
Baicalein	H	OH	OH	OH	H	H	H
Wogonin	H	OH	H	OH	OMe	H	H

Selectivity of β-CD on flavonoid aglycones with different –OH substitution in C ring:

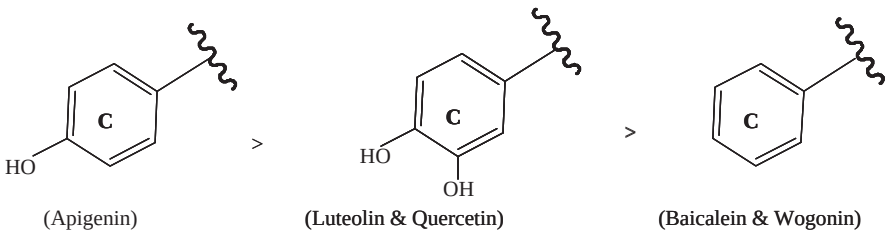


Fig. 4. Possible inclusion mode of flavonoid aglycone–β-CD complex in the background electrolyte (50 mM borax solution) and the structural selectivity of β-CD on C ring.

to improve the peak resolution in real herbal samples (shown in Fig. 5, panels c and d). Therefore, 2% ACN (v/v) was added as another electrophoretic modifier working together with β-CD.

3.4. Method validation

As commonly known, the injected volumes in CE typically vary from 5–50 nL, which is too small to insure the precision. Herein, an internal standard method was adopted to counteract the variance of injection volume. According to the result of our preliminary experiment, benzoic acid, which can be well separated from the investigated analytes but which is not present in the SBH samples, was selected as the internal standard. The calibration curves for the investigated flavonoids were established under the optimum condition with six concentrations. As shown in Table 2, the correlation coefficients (r) of the area ratio equations were all >0.999. The good linearity implies the reliability of the determined result in the defined concentration range. The LOQ and LOD values of the individual analytes were on the basis of the signal-to-noise ratios (S/N) of 3 and 10, respectively. As to the investigated flavonoids, the LODs and LOQs of the flavonoids ranged from 0.08 to 0.31 $\mu g\,mL^{-1}$ and 0.24 to 0.93 $\mu g\,mL^{-1}$, respectively. The method precision was investigated through the relative standard deviation (RSD, %) of the migration time and peak ratio of the analytes with both low and high concentrations. The results shown in Table 2 indicate the good precision and reproducibility of the developed method. As shown in Table 3, the recoveries of the investigated compounds ranged from 94.73 to 105.08% with RSD between 0.86 and 4.04% indicating good quantitative performance of the developed method.

3.5. Flavonoids analysis in different SBH samples

The optimized conditions were applied to analyze the flavonoids in the SBH collected from different locations of China, and its products (extracts) made by different manufacturers. The identification of peaks in herbal samples was performed by comparing the UV spectra and the migration time with those of standards, and also by spiking the sample solutions with the corresponding standard substances (Fig. 5A, panels c–e). The representative electrophoretograms of the SBH and the product (extract) are shown in Fig. 5A and B. The calculated contents of the analytes for all samples are summarized in Table 4. It was found that scutellarin, apigenin, baicalein, luteolin and wogonin were the major compounds in the whole herbs of SHB, while quercetin and rutin were very rare. The fact that the content of the investigated analytes varied remarkably from location to location reveals that the quality of the herb was controlled by a series of complicated parameters, i.e., habitat environment,

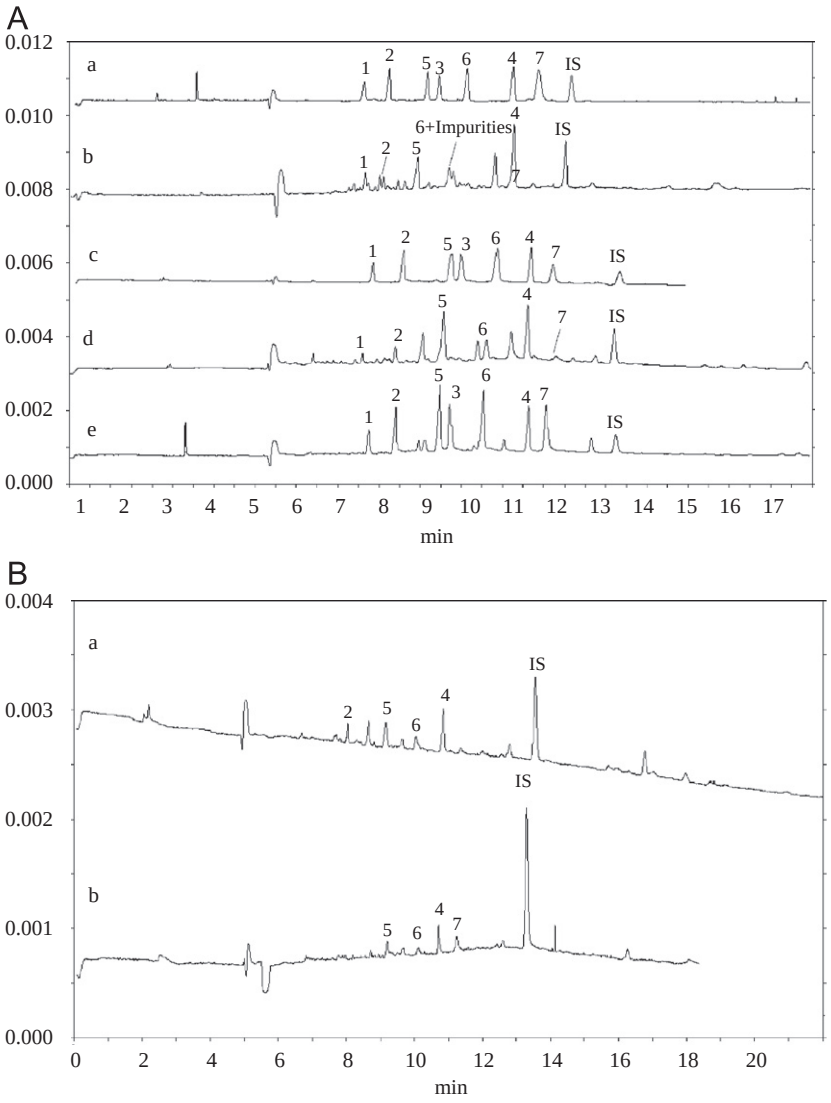


Fig. 5. Representative electropherograms of standard solutions and real samples of SBH herb and products. (A) Electropherograms of standard solutions (a and c), SBH herb samples (batch 01) (b and d) and SBH sample spiked with standards (e). Panels (a) and (b) were run with electrolyte comprised by 50 mM borax, 1 mM β-CD; (c), (d) and (e) were run with electrolyte comprised by 50 mM borax, 1 mM β-CD and 2% acetonitrile (v/v). (B) Electropherograms of the SBH products from brand 1 (a) and brand 4 (b). The electrolyte was comprised by 50 mM borax, 1 mM β-CD and 2% acetonitrile (v/v). Peaks: 1=baicalein; 2=wogonin; 3=rutin; 4=scutellarin; 5=apigenin; 6=luteolin; 7=quercetin. Other conditions were the same as described in Section 2.4.

Table 2
Linear regression, detection limit and precision for flavonoids in the developed CE method [a] (n=6).

Analyte[a]	Linear regression data			LOD (μg mL^{-1})	LOQ (μg mL^{-1})	Precision (RSD, %)[b]	
	Linear range (μg mL^{-1})	Regression equation[c]	R[d]			Migration time	Peak area ratio
Baicalein	1.55–124	y=0.3695x+0.0045	0.9995	0.31	0.93	0.03, 0.08	1.88, 3.51
Wogonin	1.75–140	y=0.7698x−0.0678	0.9998	0.17	0.53	0.02, 0.12	2.54, 3.57
Apigenin	1.70–136	y=0.7807x−0.052	0.9994	0.17	0.53	0.08, 0.10	1.02, 3.15
Rutin	1.58–126	y=0.5317x−0.0512	0.9993	0.24	0.72	0.05, 0.12	2.69, 1.83
Luteolin	1.89–151	y=1.1788x−0.1791	0.9997	0.13	0.39	0.15, 0.12	2.40, 2.30
Scutellarin	2.31–185	y=0.6445x−0.1291	0.9994	0.23	0.69	0.16, 0.11	1.65, 2.67
Quercetin	0.82–130	y=1.9589x−0.1335	0.9999	0.08	0.24	0.26, 0.20	2.06, 1.69

[a] Conditions: 50 mM borax containing 1 mM β-CD and 2% acetonitrile without adjustment of pH. The voltage was 25 kV and the effective length of the capillary was 50 cm. Order of the analyte in the table from top to bottom is arranged according to the peak number (from 1 to 7) in Figs. 2 and 5 (the same order in Tables 3 and 4, hereinafter).
[b] Analyte concentrations was 5 and 25 μg mL^{-1}, respectively.
[c] In the regression equations, y was the area ratio between analytes and IS, while x was the concentration ratio between analytes and IS.
[d] R, squares of correlation coefficients for the standard curves.

collection season, and geographic location. The commercial products of SHB contained scutellarin, apigenin and luteolin as the common dominant active compounds, whereas, the contents of the functional flavonoids in these so-called high concentrated SHB granules were not higher than those in the herb. Content of the flavonoids also significantly varied among the products

Table 3
Recoveries for the assay of flavonoids in SBH ($n=3$).

Analyte	Original (μg)	Spiked (μg)	Found (μg)	Recovery (%)[a]	RSD (%)[b]
Baicalein	45.41	38.58	88.89	105.83	1.34
		48.21	90.76	96.94	3.32
		57.86	105.86	102.50	3.02
Wogonin	176.61	144.21	328.87	102.51	1.49
		180.26	368.98	103.39	1.89
		216.31	396.88	101.01	2.03
Apigenin	595.43	480.17	1086.68	101.03	2.86
		600.21	1239.34	103.66	1.64
		720.34	1324.58	100.67	1.21
Rutin	0.00	163.26	165.68	101.48	2.12
		204.07	206.86	101.37	3.01
		244.88	236.98	96.77	1.68
Luteolin	189.23	160.18	358.68	102.66	3.86
		200.22	388.78	99.83	1.16
		240.26	438.86	102.18	2.12
Scutellarin	865.39	680.40	1568.86	101.49	1.28
		850.50	1676.46	97.70	1.36
		1020.60	1898.52	100.67	2.46
Quercetin	15.14	16.21	30.23	96.44	3.28
		20.26	36.42	102.89	6.68
		24.31	40.46	102.56	2.38

[a] Recovery (%)=100 × amount found/(original amount+amount spiked).
[b] RSD (%)=100 × SD/mean.

Table 4
The content of the investigated flavonoids in different SBH samples.

Batch	Origin/manufacture	Compounds[a]							Total
		Baicalein	Wogonin	Apigenin	Rutin	Luteolin	Scutellarin	Quercetin	
WH01	Guangdong	0.18 ± 0.04	0.70 ± 0.03	2.36 ± 0.04	nd[b]	0.75 ± 0.05	3.43 ± 0.39	0.06 ± 0.02	7.47 ± 0.36
WH02	Sichuan	0.22 ± 0.03	0.53 ± 0.01	1.77 ± 0.14	0.94 ± 0.03	0.44 ± 0.02	1.64 ± 0.15	nd	5.54 ± 0.09
WH03	Hunan	0.52 ± 0.15	0.87 ± 0.02	2.83 ± 0.30	nd	0.72 ± 0.02	3.93 ± 0.25	nd	8.87 ± 1.40
WH04	Henan	nd	0.71 ± 0.08	1.97 ± 0.30	nd	1.02 ± 0.06	8.22 ± 0.21	nd	11.92 ± 0.11
Extr.01	Brand 1	nd	1.09 ± 0.11	1.35 ± 0.04	nd	0.84 ± 0.06	2.54 ± 0.04	nd	5.83 ± 0.18
Extr.02	Brand 2	nd	0.83 ± 0.08	0.79 ± 0.04	nd	0.59 ± 0.08	1.26 ± 0.01	nd	3.47 ± 0.19
Extr.03	Brand 3	nd	0.98 ± 0.04	1.78 ± 0.02	nd	0.66 ± 0.06	3.26 ± 0.02	nd	6.68 ± 0.16
Extr.04	Brand 4	nd	nd	0.62 ± 0.07	nd	0.61 ± 0.04	1.14 ± 0.08	0.11 ± 0.03	0.07 ± 0.07

[a] mg g^{-1}, each value is expressed as mean ± SD ($n=3$).
[b] nd, not detected in the sample.

produced by different manufacturers. This might be related to the different qualities of the raw material and the distinctive processing technology of each factory.

4. Conclusions

A CZE method facilitated by β-CD and ACN was established for simultaneous determination of seven flavonoids with similar structures in SBH. During the course of method development, the selectivity principle of β-CD for the investigated flavonoids was systematically investigated and discussed. It was concluded that, first, the "molecular fit" principle is a prerequisite factor deciding the selectivity. The flavonoid aglycones with hydroxyl substitution in C-ring, especially with 4′ monohydroxyl, are most strongly influenced by β-CD; second, the hydrophobicity of the analyte is also a factor affecting the complexation affinity to β-CD, since the cavity of β-CD is less preferred to include the hydrophilic glycoside; and third, the alteration of electrophoretic mobility caused by β-CD is not only ascribed to the increased mass, but also enhanced ionization of the analyte–CD complex formed. The influence of ACN on the electrophoretic behavior reveals that a low concentration of ACN ($<5\%$) can prolong the migration time of analytes without selectivity, while higher ACN concentration ($>5\%$) can selectively weaken the complexation, thus weakening the separation performance. In this study, 2% ACN in the electrolyte was beneficial to improve the peak resolution for real sample.

The optimal electrophoretic buffer in this study was found to consist of 50 mM borax, 1 mM β-CD and 2% ACN (v/v). The established method, which proved to be precise, reproducible and reliable, has been successfully applied to analyze the SBH and its preparation, and the obtained results well reflected the quality distinction among herbs collected from different locations, as well as the preparations from various manufactures. The good separation performance of this method are easily operated, highly efficient and solvent-saving, which is suitable for wide-scale and flexible determination of flavonoid analogs in biological

samples. The discussed principle and experiences in this study is useful for providing guiding theories to CE method development for analyzing flavonoid analogues in future.

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