Successful simultaneous diastereomeric separation and sensitive determination of two pairs of triterpenoidal saponins have been achieved by capillary electrophoresis (CE) using β-cyclodextrin (β-CD) as a stereoselective agent to cooperate with borate complexation. A usual technique for isolation and group separation of saponins was developed as an appropriate purification step prior to the determination of individual saponins by CE. Soyasaponin I (S1), azukisaponin V (S2), bersimidoside I (S3) and bersimidoside II (S4) could be well separated within 14 min in a fused-silica capillary (60 cm long to the detector with an additional 10 cm to the cathode; 75 μm i.d.). The background electrolyte was borate buffer (80 mM, pH 10), containing 24 mM β-CD. The separation voltage was 14 kV with a detection wavelength of 195 nm. The sample was electrokinetically injected using a voltage of 16 kV for 12 s. Methanol (70%) was used as the diluent for field-amplified sample stacking after hydrodynamic injection of short water plug (5 cm, 4 s). The method was partially validated for linearity, repeatability, reproducibility, limits of detection and limits of quantification. The correlation coefficients of the calibration curves were all >0.998, and the recoveries were from 98.23 to 96.21%.

Introduction

Saponins, a large category of secondary plant metabolites, are found in great number of plant species and in some marine organisms. Saponins have a diverse range of properties, which are found in great number of plant species and in some marine organisms. Saponins have a diverse range of properties, which include sweetness, bitterness, foaming and emulsifying properties, pharmacological, medicinal, hemolytic, as well as antimicrobial, insecticidal and molluscicidal activities (1, 2).

*Trifolium alexandrinum* is an annual plant cultivated in Egypt and its seeds are used as an antidiabetic. Isolation and structural determination of oleane triterpenoid saponins as their methyl esters from the seeds of this plant have been reported (3). The structures of saponins showed that soyasaponin I methyl ester (S1) and azukisaponin V methyl ester (S2) or bersimidoside I methyl ester (S3) and bersimidoside II methyl ester (S4) are two pairs of diastereomers, in which one compound showed the presence of galactose while the other one involved glucose (Figure 1). S1 and S2 were identified as 3-O-α-L-rhamnopyranosyl(1→2)-β-D-galactopyranosyl(1→2)-β-D-glucuronopyranosyl soyasapogenol B methyl ester (S1) and 3-O-α-L-rhamnopyranosyl(1→2)-β-D-glucuronopyranosyl soyasapogenol B methyl ester (S2), respectively. S3 and S4 were identified as 3-O-α-L-rhamnopyranosyl(1→2)-β-D-galactopyranosyl(1→2)-β-D-glucuronopyranosyl soyasapogenol B methyl ester (S1) and 3-O-α-L-rhamnopyranosyl(1→2)-β-D-glucuronopyranosyl soyasapogenol B methyl ester (S2), respectively. S3 and S4 were identified as 3-O-α-L-rhamnopyranosyl(1→2)-β-D-galactopyranosyl(1→2)-β-D-glucuronopyranosyl soyasapogenol B methyl ester (22).

As an alternative to HPLC, capillary electrophoresis (CE) is shown to be a powerful separation technique which provides high-resolution results and is becoming a standard tool for the analysis of many plant extracts (15–19). CE has many advantages over other techniques, including short analysis time, high-efficiency, technical simplicity and applicability to most analytes with small sample and reagent requirements. Kodama et al. (20, 21) developed CE methods for the analysis of some monosaccharides (mannose, galactose, fucose, glucose, xylose and arabinose) based on the chiral ligand-exchange principle, using borate as a central ion of the chiral selector after derivatization with various reagents ((S)-3-amino-1,2-propanediol, 8-aminonaphthalene-1,3,6-trisulfonate and 1-phenyl-3-methyl-5-pyrazolone). On-column complexation of saponins in *T. alexandrinum* with borate, forming anionic complexes, has been applied for the simultaneous separation of these compounds by CE with direct UV detection (22). Borate buffer was proposed for the separation of the two pairs of diastereomeric saponins S1 and S2 or S3 and S4 by complexing the diols of the sugar moieties linked to the triterpine nucleus of these saponins. Unlike Kodama group, the separations of the previously studied diastereomeric saponins were achiral. However, to date, no extensive validated analytical study on *T. alexandrinum* was found. The existing reported CE method (22) was not satisfactory for the quantification of
saponins in *T. alexandrinum*, because the concentration sensitivity detected with the electrophoresis system was poor.

Due to the small internal diameter of the capillary and the small injection amount, detection sensitivity was the major drawback of CE, especially when coupled with UV detector. Two general approaches have been adopted to improve sensitivity. One method used more sensitive detectors, such as fluorescence, electrochemical or mass spectrometry detectors, and the other employed on-line concentration strategies, such as stacking and sweeping. Stacking is a phenomenon through which sample ions accumulate at the boundary, which separates the low conductivity sample plug and the high-conductivity background electrolytes. The simplest technique for sample stacking is field-amplified sample stacking (FASS) \(^{(23-27)}\).

Experimental

**Instrumentation**

Separation was performed in fused-silica capillary covered with a polyamide-coating layer (Polymicro Technologies, Phoenix, AZ, USA), 60 cm (long to the detector) with an extra 10 cm to the cathode with 75 μm i.d. and 300 μm o.d. The detector was a Jasco UV/VIS 875-CE, equipped with a CE-UV cell cartridge (Japan Spectroscopic, Tokyo, Japan) operated at 195 nm. A detector window was fashioned in the capillary column 60 cm from the injection end by burning off a section (~0.5-cm long piece) of the polyamide-coating layer on the capillary column. Both ends of the tube were separately dipped in the anodic and cathodic solutions, having the same composition as the carrier solution, and the surface of these electrode solutions were adjusted to the same level. A model HCZE-30 PNO 25-LDS high-voltage power supply was used to apply voltage up to 30 kV (Matsusada Precision Devices, Japan). The high-voltage end of the capillary was enclosed in a plexiglass box for safety reasons. Hydrodynamic injection of water was performed by gravity, placing the inlet of the capillary into the water vial and raising the vial 5 cm higher than the capillary outlet for 4 s, allowing the short plug of water to siphon into the capillary. The sample was electrokinetically injected using a voltage of 16 kV for 12 s. Electropherograms were processed and recorded on a chromatopack integrator C-R6A (Shimadzu, Kyoto, Japan). The cathode and anode electrolytes and the capillary run buffer was 80 mM borate buffer, pH 10 containing 24 mM β-CD in the final solution. This was freshly prepared before each set of analysis, degassed by sonication and filtered through a 0.45-μm (Millipore, Bedford, MA, USA) filter before use. All electrophoretic separations were carried out at 14 kV and capillary temperature was ambient (23°C).

**Capillary conditioning**

Each new capillary was conditioned by rinsing with 1 M sodium hydroxide, ultrapure water and running electrolyte for 20, 20 and 30 min, respectively. At the start of each working day, the capillary was washed with ultrapure water for 5 min, 0.1 M HCl for 5 min, 0.1 M NaOH for 10 min, ultrapure water for 5 min and then equilibrated with running electrolyte for 3 min. Between analysis the capillaries were rinsed with 0.1 M NaOH for 3 min followed by ultrapure water for 2 min and then equilibrated with running electrolyte for 3 min. At the end of the working day, the capillary was washed with ultrapure water for 5 min, and the capillary ends were dipped in a vial containing ultrapure water.
**Materials and reagents**

All chemicals were obtained at the highest purity available from the manufacturer and were used without additional purification. Sodium hydroxide, sodium tetraborate, hydrochloric acid, β-CD, methanol and acetonitrile were purchased from Yoneyama Yakuhin Co (Osaka, Japan). All reagent solutions and buffers were prepared with distilled deionized water purified with a Milli-Q ultrapure water system (Millipore). Standard solutions of S1–S4 were prepared in methanol at a concentration of 1 mg/mL and suitably diluted with the same solvent. Diaion HP-20 was obtained from Mitsubishi Chemical Corporation (Tokyo, Japan).

**Standard solution and calibration**

Stock standard solutions of the four saponins (1 mg/mL) were prepared by dissolving an accurately weighed amount of each saponin in 70% methanol. The stock standard solutions were stored frozen at −20°C until required. The standard solutions for calibration were prepared daily by serial dilutions of appropriate volumes of stock standard solutions to produce saponin solutions in the concentration range of 25–300 μg/mL and stored at 5°C before being injected into the electrophoresis system.

**Column chromatography for isolation of saponins**

The dried seeds of *T. alexandrinum* were defatted with hexane and then extracted with methanol. The extract was partitioned with ethylacetate and water. The aqueous fraction was subjected to Diaion HP-20 column chromatography and eluted with water, 50% methanol and methanol, respectively. Preparative polyamine-HPLC (YMC Co., Ltd. Tokyo, Japan) and aqueous acetonitrile 87% as a solvent system was used to isolate S1 and S2 from methanolic extract fraction as their methyl ester, while aqueous acetonitrile 80% was used to isolate S3 and S4 from the 50% methanolic extract fraction as their methyl ester.

**Results**

Carbohydrates are a huge family, encompass neutral and ionized sugars. Neutral sugars have received the most attention in analyses. Mannose, galactose, fucose, glucose, xylose and arabinose are examples of neutral sugars. Alkaline borate buffers (pH 8–12) can transform these molecules to more negatively charged complexes (28–31). In the present work, borate buffer was proposed to play a key role in the separation of the two pairs of diastereomeric saponins S1 and S2 or S3 and S4 (22). The variations of the electrophoretic mobility of S1 and S2 or S3 and S4 as a function of these variables were further investigated in the newly FASS-CE-UV method. The migration times of the tested saponins were prolonged and the resolution of S1 and S2 or S3 and S4 was obviously increased initially, and when the pH was >10.0, the noise of the baseline increased to an unacceptable level. As shown in Figure 2A and B, the influence of the pH and borate concentration

**Influence of the pH and borate concentration**

The effects of pH and borate concentration were the key parameters as they affected the separation between S1 and S2 or S3 and S4 (22). The variations of the electrophoretic mobility of S1 and S2 or S3 and S4 as a function of these variables were further investigated in the newly FASS-CE-UV method. The migration times of the tested saponins were prolonged and the resolution of S1 and S2 or S3 and S4 was obviously increased initially, and when the pH was >10.0, the noise of the baseline increased to an unacceptable level. As shown in Figure 2A and B, the influence of the pH and borate concentration

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Effect of borate buffer pH on the electrophoretic mobility of S1 and S2 (A) and S3 and S4 (B). CE conditions: borate buffer (80 mM), applied voltage (14 kV), ambient temperature (23°C), fused-silica capillary, 60 cm (effective length) × 75 μm i.d., sampling 12 s, 16 kV (pre-injection of water plug for 4 s) and detection wavelength (195 nm).
electrophoretic mobility decreased and the mobility difference between \( S_1 \) and \( S_2 \) or \( S_3 \) and \( S_4 \) is improved with increasing buffer pH up to 10.0. A convenient compromise between the advantageous effect of high pH on the separation on one hand and the acceptable migration time and the noise of the baseline on the other hand was found to be at pH 10. The electrophoretic mobility (\( \mu_{\text{ep}} \)) of the saponins was calculated according to the formula: (32)

\[
\left( \frac{L_t}{V} \right) \left( \frac{1}{t_m} - \frac{1}{t_{eo}} \right)
\]

where \( \mu_{\text{ep}} \) is the electrophoretic mobility of the analyte tested, \( t_m \) the migration time measured directly from the electropherogram, \( t_{eo} \) the migration time of the electro osmotic flow marker, \( L_t \) the total length of the capillary, \( L_d \) the length of the capillary between injection and detection and \( V \) is the applied voltage.

The variation of the electrophoretic mobility of \( S_1 \) and \( S_2 \) or \( S_3 \) and \( S_4 \) as a function of borate concentration was studied over the range of 10–100 mM at pH 10. As shown in Figure 3A and B, the electrophoretic mobility of saponins decreased quite drastically with increasing borate concentration from 10 to 50 mM, and then decreased gradually from 60 to 100 mM. The optimum borate concentration for the separation of \( S_1 \) and \( S_2 \) or \( S_3 \) and \( S_4 \) was found to be 80 mM at pH 10, which combined a sufficient resolution with a moderate analysis time. Figure 4 shows representative capillary electropherograms of each pair of the diasteromers \( S_1 \) and \( S_2 \) or \( S_3 \) and \( S_4 \).

**Applied voltage**

The electrophoretic mobility is directly proportional to the field strength, so the use of the highest voltages possible will result in the shortest times for the separation. Experimentally, an increased voltage resulted in a decreased migration time for all saponins. The resolution was relatively constant, with a declining tendency, when the voltage was increased from 10 to 20 kV. The limiting factor here is the Joule heating. The optimum voltage was determined by performing runs at increasing voltages until deterioration in resolution was observed. Above 15 kV an even shorter migration time could be achieved, but the baseline became inclined and more noise was observed. The optimum voltage for the separation of \( S_1 \) and \( S_2 \) or \( S_3 \) and \( S_4 \) was found to be 14 kV, which combines sufficient resolution with a less base line noise.

**Optimization of the pre-injection plug**

It was reported that introducing a short plug of water before electrokinetic injection could provide a high electric field...
strength from the beginning of the injection \( (33) \). The basic functions of short water plug were as follows: (i) making the boundary between the sample solution and the background electrolyte much clearer for a higher analytical sensitivity, (ii) reducing the electricity discrimination, (iii) concentrating the analytes and (iv) achieving good reproducibility. Experimentally, the pre-injection plug was injected by hydrodynamic mode, by placing the inlet of the capillary into the water vial and raising the vial 5 cm higher than the capillary outlet, for 1–10 s allowing the short plug of water to siphon into the capillary followed by electrokinetic injection of sample dissolved in 70% methanol with 16 kV for 12 s. According to the results, 4 s for the pre-injection plug provided the highest peak height. At higher injection times, the length of water plug became too long and worsened the efficiency of separation.

**FASS of electrokinetic injection of samples**

To improve the detection sensitivities, FASS was introduced in this system. In FASS mode, the sample solution was of lower conductivity than the running buffer. Theoretically, the amount of stacking is proportional to the conductivity difference between the running buffer and the sample solution. This difference is caused by the concentration drop between the two solutions; the larger the drop in concentrations, the narrower the peak and the greater the amount of stacking. A borate buffer of 80 mM concentration at pH 10 as background electrolyte was found to give maximum peak height enhancement for the four saponins. The injection voltage and the injection time were the factors that could be adjusted to increase the stacking amount. These two factors were studied to search for their optimum values. The influence of the electrokinetic sample injection voltage was investigated over the range of 5–20 kV. Saponins were injected with different voltages in FASS mode (pre-injection of water plug for 4 s). Increasing the injection voltage enhanced the peak heights and they attained their maximum at 16 kV. When the injection voltage was \( >16 \text{ kV} \), the peak heights did not increase significantly and the baseline became noisy. This could be caused by excessive Joule heating and bubble formation under the high electric field strength conditions. So the injection voltage was set at 16 kV. The injection time was also tested over the range of 5–20 s. The peak heights increased linearly from 5 to 12 s. However, when injection time was \( >12 \text{ s} \), the resolution was reduced because of the peak-broadening. These results might be explained as the high electric field of the sample solution zone could produce the partially Joule heat, which would...
generate the bubble and broaden the peaks. Therefore, 12 s of electrokinetic injection at 16 kV of the sample was selected to achieve an efficient sample stacking as well as acceptable repeatability.

**Cyclodextrin-modified CE**

It was observed that, the selectivity of the method under alkaline borate electrolyte (80 mM, pH 10) and applied voltage (14 kV) was not sufficient to separate S1 and S3 or S2 and S4 unless a stereoselective agent was introduced in the running electrophoretic buffer. Cyclodextrins (CDs) have been frequently used as electrolyte modifiers to affect the separation of compounds with closely related structures (34–36). For this purpose, β-CD was used as a stereoselective agent in the electrolyte background and its concentration was found to be the key role in the separation of all four saponins. The effect of β-CD concentration on the electrophoretic mobility of the tested saponins was investigated in the range of 4–28 mM (Figure 5). It was observed that, adding a higher amount of β-CD increased the electrophoretic mobility of the saponins, and this was evident in the case of S1 and S2. The electrophoretic mobility of S1 and S2 was faster than that of S3 and S4, when the β-CD concentration was >16 mM. The optimum β-CD concentration for the simultaneous separation of S1, S2, S3 and S4 was found to be 24 mM, which combines sufficient resolution with a less base line noise (Figure 6).

**Partial method validation**

A series of sample analysis was performed to partially validate the performance of the method, linearity, precision, accuracy, limit of detection (LOD) and quantification (LOQ).

**Linearity and sensitivity**

The calibration curves for saponins were constructed with seven concentrations (simultaneously prepared) ranging from 25 to 300 μg/mL. Calibration curves were constructed by plotting the measured peak heights versus concentrations. Each concentration was repeated three times; this approach provided information on the variation in peak height values between samples of the same concentration. The linearity of the calibration graph was validated by the high values of the correlation coefficients (>0.998). The equations for the best-fit straight line were determined by the following linear regression analysis: \( Y = a + bC \), where \( Y \) is the peak height and \( C \) denotes the concentration in μg/mL of saponins. Characteristic parameters of the linear calibration curves are shown in Table I. The LOD and LOQ were determined according to ICH guidelines for validation of analytical procedures (37). The LODs were found to be 7.70, 7.14, 7.69 and 7.57 μg/mL for S1, S2, S3 and S4, respectively (Table I). LOQs were found to be 23.33, 21.64, 23.30 and 22.94 μg/mL for S1, S2, S3 and S4, respectively (Table I).

**Precision**

Precision of the method was determined for both the intra- and interday validation of the assay and expressed as the relative standard deviation (RSD %) and the relative error (RE %) of the mean measured concentration. Intra- and interday precision and accuracy were established at three concentration levels (50, 100 and 200 μg/mL) and five replicate analyses of each sample were analyzed on a single assay day (intra-), and on five consecutive days (interday) (Table II). Repeatability (intraday RSD) was excellent being in the range of 1.71–2.16% and the mean RE ranged from 1.77 to 3.13%. Reproducibility (interday RSD) was in the range of 2.12–2.60% and the mean RE ranged from 2.36–3.79%. Repeatability and reproducibility of saponins with high and low concentrations were <3.8%, indicating a reliable measurement using the proposed method (Table II). The determination of the analytical recovery assessed the accuracy of the method. Recoveries for all samples were from 98.23–96.87% for the intraday and from 97.64 to 96.21% for the interday studies. RE was evaluated by back-calculation and expressed as the percent deviation between concentration found and concentration added according to the following:

\[
RE = \left(\frac{\text{concentration added} - \text{concentration found}}{\text{concentration added}}\right) \times 100
\]

**Discussion**

Complexation of diols with borate was strongly dependent on the pH and the borate concentration, thus both parameters were adjusted for the optimization of the separation. The results clearly showed that, increasing the pH of the running electrolyte resulted in a more negative net charge, which is reflected in an increased selectivity and separation efficiency. Besides, the variation of the electrophoretic mobility as a function of borate concentration reflected the binding strength of the saponins to the borate buffer. Borate of higher concentration converted each saponin into a negatively charged complex form and provided

---

**Table I**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
<th>S4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibration range (μg/mL)</td>
<td>25–300</td>
<td>25–300</td>
<td>25–300</td>
<td>25–300</td>
</tr>
<tr>
<td>Detection limit (μg/mL)</td>
<td>7.70</td>
<td>7.14</td>
<td>7.69</td>
<td>7.57</td>
</tr>
<tr>
<td>Quantitation limit (μg/mL)</td>
<td>23.33</td>
<td>21.64</td>
<td>23.30</td>
<td>22.94</td>
</tr>
<tr>
<td>Regression equation (Y): slope (b)</td>
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<td>76.69 x 10^-2</td>
<td>76.83 x 10^-2</td>
<td>76.72 x 10^-2</td>
</tr>
<tr>
<td>Standard error of the slope</td>
<td>1.02 x 10^-2</td>
<td>1.09 x 10^-2</td>
<td>1.01 x 10^-2</td>
<td>1.08 x 10^-2</td>
</tr>
<tr>
<td>Intercept (a)</td>
<td>-50.54 x 10^-2</td>
<td>63.41 x 10^-2</td>
<td>-98.20 x 10^-2</td>
<td>3.70 x 10^-2</td>
</tr>
<tr>
<td>Standard error of the intercept</td>
<td>193.62 x 10^-2</td>
<td>196.03 x 10^-2</td>
<td>182.45 x 10^-2</td>
<td>194.88 x 10^-2</td>
</tr>
<tr>
<td>Correlation coefficient (r²)</td>
<td>0.9991</td>
<td>0.9990</td>
<td>0.9990</td>
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</tr>
</tbody>
</table>

*Note: Y = a + bC, where C is the concentration and Y is the peak height.*
ultaneous separation of all four saponins in one run could not be explained, as charge to mass ratio for carbohydrates, the ligand in the cis–diol configuration is more favorable than the saponin because of the structure of the galactopyranosyl unit. The molecular masses of S1, S2, S3, and S4 were found to be 1480.3, 1328.1, 1468.6 and 1424.5 μg/mL for S1, S2, S3, and S4, respectively. Also, LODs were found to be 454.00, 416.57, 445.26 and 441.60 μg/mL for S1, S2, S3, and S4, respectively. The enhancement factor, 20-fold, was calculated by comparing the results of LOD and LOQ with that carried out using FASS in the proposed method. With the use of electrokinetically injected voltage of 16 kV for 12 s (pre-injection of water plug for 4 s), the LODs for saponins were in the range of 7.14–7.70 μg/mL, while LOQs were in the range of 21.64–23.33 μg/mL, and the analytical run time was <14 min.

**Conclusions**

A simple and reliable CE analytical method has been developed and validated for the routine quantification of two pairs of oleanene triterpenoidal diastereomeric saponins (S1–S4) in *T. alexandrimum*. A dual mechanism involving both inclusion of the saponins into the cavity of the β-CD and the formation of borate complexes was employed to achieve a very effective tool for separation. To the best of our knowledge, this is the first FASS-CE-UV method, for the quantification of oleanene triterpenoidal diastereomeric saponins, that was characterized by excellent resolution, good repeatability and favorable detection limits. This stacking CE method provided a sensitivity enhancement of ~20-fold in comparison with our previous results.

**Table II**

Intra- and Interday Validation for the Proposed CE Method

<table>
<thead>
<tr>
<th>Saponin</th>
<th>Concentration (μg/mL)</th>
<th>Recovery Intraday (%)</th>
<th>RSD Intraday (%)</th>
<th>RE Intraday (%)</th>
<th>Recovery Interday (%)</th>
<th>RSD Interday (%)</th>
<th>RE Interday (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>50</td>
<td>98.23 ± 1.82</td>
<td>1.77</td>
<td>2.55</td>
<td>97.45 ± 2.35</td>
<td>2.36</td>
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<td></td>
<td>100</td>
<td>96.16 ± 1.76</td>
<td>1.84</td>
<td>2.36</td>
<td>97.64 ± 2.12</td>
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<td></td>
<td>200</td>
<td>97.84 ± 2.16</td>
<td>2.16</td>
<td>2.74</td>
<td>97.26 ± 2.60</td>
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</tr>
<tr>
<td>S2</td>
<td>50</td>
<td>97.28 ± 1.71</td>
<td>2.72</td>
<td>3.25</td>
<td>96.75 ± 2.13</td>
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<td>97.63 ± 1.74</td>
<td>2.37</td>
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<td>97.15 ± 2.22</td>
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<td>2.14</td>
<td>2.76</td>
<td>97.24 ± 2.42</td>
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<tr>
<td>S3</td>
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<td>2.55</td>
<td>3.12</td>
<td>96.82 ± 2.26</td>
<td>3.12</td>
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</tr>
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<td></td>
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<td>98.01 ± 1.82</td>
<td>1.99</td>
<td>2.46</td>
<td>97.54 ± 2.16</td>
<td>2.46</td>
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<tr>
<td></td>
<td>200</td>
<td>97.75 ± 1.94</td>
<td>2.25</td>
<td>2.99</td>
<td>97.01 ± 2.38</td>
<td>2.99</td>
<td></td>
</tr>
<tr>
<td>S4</td>
<td>50</td>
<td>97.24 ± 1.81</td>
<td>2.76</td>
<td>3.12</td>
<td>96.88 ± 2.23</td>
<td>3.12</td>
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<tr>
<td></td>
<td>100</td>
<td>98.01 ± 1.95</td>
<td>1.99</td>
<td>2.68</td>
<td>97.32 ± 2.37</td>
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<tr>
<td></td>
<td>200</td>
<td>96.87 ± 2.01</td>
<td>3.13</td>
<td>3.79</td>
<td>96.21 ± 2.57</td>
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</tbody>
</table>

*Average of five determinations.*

separation due to the differences in the charge-to-mass ratio. The magnitude of the borate complexation depends on the number of boration sites on the sugar moiety and consequently on the sugar configuration. In this borate complexation mode, diastereomeric saponins migrated in the order of S2 and S1 or S4 and S3 (Figure 4). This set of results stated that, the electrophoretic mobility and migration order of the tested saponins depend only on the structural preference for the formation of the borate complex. If another electrolyte was employed, such as phosphate or acetate instead of the borate electrolyte, separation of S1–S2 and S3–S4 was not possible.

We tried to elucidate the relationship between the migration order and the nature of the sugar moiety. For the cyclic form of carbohydrates, the ligand in the cis–dihol configuration is more likely to interact with the borate anion than the trans–dihol form. S1 migrated more slowly (low electrophoretic mobility) than S2 because of the favorable structure of the galactopyranosyl unit in the formation of the borate complex (presence of the 3,4-cis–dihol system). The β-D-galactopyranosyl (S1) had cis–dihol system and could form a stronger borate complex between the 3- and 4-hydroxyl groups, while those in β-D-glucopyranosyl system (S2) showed less affinity than S1 to form borate complexes as it did not have cis–dihol system. Also, S4 had a higher electrophoretic mobility than the saponin S3 because of the favorable structure of the galactopyranosyl unit in the formation of the borate complex (presence of the 3,4-cis–dihol system, S3). The electrophoretic mobilities of S1–S3 and S2–S4 are practically identical without β-CD, despite the differences in molecular masses, due to the presence of the two sugar moieties linked to the C-22, which enable them to form borate complexes at these sites. This can be explained by the fact that increasing of molecular masses of S3 and S4 is accompanied by increasing of their negative charges that were resulted from the additional borate complex sites due to the interaction of trans–dihol forms of the sugar moieties, which will result in almost equivalent charge to mass ratio for S1 and S3 or S2 and S4.

Unfortunately, our investigations showed that a complete simultaneous separation of all four saponins in one run could not be achieved under alkaline borate electrolyte (80 mM, pH 10) and applied voltage (14 kV) unless β-CD was introduced in the running electrophoretic buffer. Thus, the combination of β-CD inclusion–complexation and borate complexation could be a useful approach for the separation of the studied saponins. The β-CD concentration in the electrolyte background was found to be crucial for the resolution of saponins. It was clear that by adding a higher concentration of β-CD the electrophoretic mobility of the saponins was increased, and this was evident in the case of S1 and S2 (Figure 5). The electrophoretic mobility of S1 and S2 was faster than that of S3 and S4, when the β-CD concentration was >16 mM which caused a reverse in migration order. Saponins that can enter the β-CD cavity will move faster than the free saponins, owing to the increase in the molecular mass and, thus, decrease in the charge density. In contrast, the electrophoretic mobility of S3 and S4 was not significantly affected after the addition of β-CD. This revealed that they did not form inclusion-complexes with β-CD probably because of their unique molecular structures. Thus, it can be deduced that the effect of molecular mass became more influential when β-CD was employed for the separation of S1–S3 and S2–S4 (Figures 5 and 6). The presence of sugar moieties, linked at C-22, may explain this behavior (Figure 1). This phenomenon indicated that, the baseline separation of the four saponins could be achieved with the addition of β-CD at a concentration of 24 mM (Figure 6).
making this approach attractive for the quantitative determination of the studied diastereomers. Partially, validation of the method confirms its applicability for the determination of saponin species in real sample. In addition, the short analysis time along with greatly reduced solvent consumption associated with this method made it a viable alternative to hyphenated techniques combining HPLC and tandem mass spectrometry (HPLC–MS/MS).

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