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ORIGINAL PAPER

Analytical evaluation of steviol glycosides by capillary electrophoresis supported with molecular docking studies

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Abstract This paper reports on a newly developed electrokinetic chromatographic method for the simultaneous separation and determination of steviol glycosides in real stevia samples by capillary electrophoresis and supported by molecular docking studies. Our results obtained using 30-mM heptakis-(2,3,6-tri-*o*-methyl betacyclodextrin) as a separating agent, suggest that at optimum experimental conditions the detection limits of 2.017×10^{-5} and 7.386×10^{-5} M and relative standard deviations ($n = 5$) of 1.10 and 1.17 were obtained for rebaudioside-A and stevioside, respectively. In addition, the molecular docking studies explained to a certain extent why the separation was successful. The calculated binding free energy results for the rebaudioside-A and stevioside complexes formed with the separating agent showed that although both ligands penetrated deeply into the hydrophobic cavity of the separating agent, the presence of additional hydrogen bonding in the case of stevioside is probably responsible for its stronger binding affinity than that of rebaudioside-A.

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Keywords Steviol glycosides (rebaudioside-A, stevioside) · Heptakis 2,3,6-tri-*o*-methyl betacyclodextrin (TM-β-CD) · Capillary electrophoresis (CE) · Molecular docking (MD)

Introduction

Stevia rebaudiana Bertoni, an herbaceous perennial shrub, belonging to the *Asteraceae* family also known as "Sweet-Leaf" has attracted economic and scientific interest due to the non-nutritive sweetness and the therapeutic properties of its leaf [1]. Japan and Korea, are the largest consumers of stevia extract consuming about 200 and 115 tons respectively, on an annual basis. In Japan, stevia replaces the artificial sweeteners like aspartame which were around since the 1970s. The stevia sweeteners are approximately 300 times sweeter than sugar [2, 3]. Lately, the use of stevia has been approved by the Food and Drug Association in South Africa with the recent promulgation (Foodstuffs, Cosmetics and Disinfectants Act, 1972, 10th September 2012) of the new sweetener regulations [4].

Stevia leaves contain diterpene glycosides, the most abundant of which are stevioside (Stv) and rebaudioside-A (Reb A) [5] as shown in Table 1. Traditionally, the dry weight percentages of glycosides present in the leaves were reported as Stv ranging from 5 to 10 %, Reb A from 2 to 4 % and with a lower percentage reported for rebaudioside-C (Reb C). On the other hand, the relative sweetness of the Stv ranges from 60 to 70 % and between 110 to 270 times sweeter than sugar, while Reb A ranges from 30 to 40 % and between 180 to 400 times sweeter than sugar, resulting in these two compounds being the sweetest compounds amongst the remaining glycosides [1]. Apart from these sweetening properties, other health benefits of steviol

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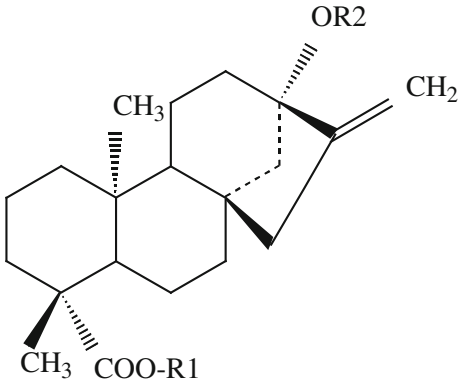
Table 1 Structure of major steviol glycosides



Compound	Abbreviation	R1	R2
Stevioside	Stv	Glu	Glu–1,2–Glu
Rebaudioside-A	Reb A	Glu	Glu—1,2—Glu | 1,3—Glu
Rebaudioside-C	Reb C	Glu	Glu—1,2—Rham | 1,3—Glu
Dulcoside-A	Dul	Glu	Glu–1,2–Rham

Glu Glucose, *Rham* rhamnose

glycosides includes antihypertensive, antihyperglycemic and anti-human rotavirus activities [6]. On the other hand, the reported drawbacks for the impure stevia glycosides include hypotension, diuresis, natriuresis and kaliuresis [7–9].

The composition of the stevia components in the leaves is highly dependent on the nature of the soil, climate and the methods used for extraction and purification [10]. A survey of the reported literature for the separation and determination of steviol glycosides in different food samples revealed that most of the analytical work was done with high-performance liquid chromatography (HPLC) [11–23], liquid chromatography coupled with tandem mass spectrometry (LC–MS) [26–28, 30] and liquid chromatography coupled with electrospray ionization mass spectrometry (LC–ESI–MS) [25, 29] but with only a few capillary electrophoretic (CE) methods [2, 7, 24]. Moreover, the above-reported chromatographic techniques require expensive experimental setup [11, 23, 25, 30] for the simultaneous separation and determination of steviol glycosides. A CE method using the micellar electrokinetic chromatography (MEKC) mode previously reported by Mauri et al. [7] used sodium dodecyl sulfate (SDS) as a charged micelle in methanol for separation of steviol glycosides. Liu and Li [2] developed subcritical fluid extraction (SubFE) method for the extraction of steviol glycosides from *Stevia rebaudiana*, suggested that CE is a valuable alternative to HPLC, but with longer migration times and poor resolution between the two peaks.

In modern years, a growing interest in the use of stevia in the natural food market has prompted the development of a fast, reliable, cost effective and reproducible analytical method to determine the sweetening components in *Stevia rebaudiana*. Accordingly, in this paper, we present a modern eco-friendly method for the separation of steviol glycosides using electrokinetic chromatography–capillary electrophoresis (EKC–CE) in the presence of TM-β-CD as a separating agent. However, a detailed mechanism for the separation of steviol glycosides by TM-β-CD remains unclear. Fortunately, molecular modeling methods have recently been proposed as powerful tools to obtain information about the interactions involving CD complexes [31]. For this purpose, and to the best of our knowledge, the molecular docking (MD) studies were utilized in this study for the first time, to better understand the mechanism of the separation between steviol glycosides and TM-β-CD.

Experimental

Apparatus

All analytical experiments were performed using Agilent Technologies 7100 CE system equipped with a diode array detector (DAD), an auto-sampler and a temperature controller (15–60 ± 0.1 °C). Instrument control and data analysis were carried out by Agilent Chemstation software installed on a personal computer. A fused silica capillary with 50 µm inner diameter and 363 µm outer diameter with total and effective lengths of 64.5 and 56 cm, respectively, was employed (Agilent Technologies, SA). For pH measurements, a pH meter (CRISON micro pH 2000) calibrated with a precision of 0.1 pH units was used. All samples were sonicated before analysis using an Ultra sonic (Labcon 5019 U model) supplied by Lasec (Durban, SA).

Materials

Individual standards of Reb A and Stv with 98 % analytical quality were obtained from (Ganzhou Julong High Tech Industrial Co., Ltd, China). Deionized water was generated from an aqua MAX^{TM}—basic 360 series water purification system from TRILAB SUPPORT (Durban, SA). Sodium dihydrogen orthophosphate, heptakis 2,3,6-tri-*o*-methyl betacyclodextrin (TM-β-CD), Sodium hydroxide, Hydrochloric acid and methanol were purchased from Capital Lab Supplies CC (KwaZulu-Natal, SA). All solutions and samples were prepared in deionized water filled in vials with disposable syringes filtering through a 0.45-µm pore

size and 25-mm diameter syringe filters containing cellulose acetate as filter medium supplied from Anatech Instruments (Pty) Ltd. (Durban, SA) before analysis.

Reagents

3-mM stevia standard solutions, each containing 98 % of Reb A and Stv were prepared quantitatively by weighing an equivalent amount in a 5-mL volumetric flask and diluting with deionized water. 30-mM TM-β-CD was prepared by dissolving 0.4288 g in 50-mM phosphate buffer in a 10-mL volumetric flask at 60 °C. 50-mM phosphate buffer was prepared by dissolving 0.780 g of sodium dihydrogen orthophosphate with adequate amount of deionized water in a 100-mL volumetric flask, and then adjusted to pH 8.0 with 1.0 M NaOH.

Capillary electrophoresis procedure

The new capillaries were first conditioned by flushing for 15 min with 1 M NaOH and then rinsed for 5 min with deionized water and 15 min with phosphate buffer at 30 °C. At the beginning of each working day, the capillary was cleaned and conditioned as follows: (i) 2 min rinse with deionized water (ii) 2 min rinse with 0.1 M NaOH (iii) 2 min rinse with deionized water and (iv) 2 min with phosphate buffer at 1,000 mbar. The samples were injected at a pressure of 50 mbar for 4 s; between runs the capillary was flushed with 1 M NaOH for 2 min and with water for 2 min; and finally with the separation buffer for 3 min to generate a stable electro-osmotic flow (EOF) in the capillary. The detection wavelength was set ranging from 200 to 240 nm.

The resolution (R_s) between Reb A and Stv was calculated using the expression:

$$R_s = \frac{2(t_2 - t_1)}{w_1 + w_2}$$

where t_1 is the migration time of first elution compound (Reb A), t_2 is the migration time of second elution compound (Stv), w_1 is the peak width of first elution compound at the base (Reb A), w_2 is the peak width of second elution compound at the base (Stv).

Procedure for real sample analysis

Real stevia samples used in this study were purchased from a local supermarket in different forms containing steviol glycosides, tablet samples (Green Canderel, Merisant Company 2, Czech Republic), powder samples (Stevia, Dis-Chem Pty Ltd, SA) and liquid samples (Tantalize, Delite Foods, SA). All standard solutions were kept at 4 °C in a refrigerator for stability. All the samples were used without any further purification. Specifically, in the preparation of a tablet sample, one tablet was ground into fine powder and dissolved in the equivalent of 5-mL deionized water. Powdered samples were prepared by dissolving the equivalent amount in a 5-mL volumetric flask with deionized water. Liquid samples were prepared directly by dissolving the equivalent amount in deionized water. Before the analysis, all the samples were diluted up to optimized dilution level (100-fold) to avoid possible interferences from the other substances.

Molecular docking methodology

Due to the absence of the X-ray structure for heptakis 2,3,6-tri-*o*-methyl betacyclodextrin (TM-β-CD) the atomic coordinates of β-cyclodextrin_hemolysin complex crystal (pdb id: 3M3R) were retrieved from the protein data bank [32] and were used as a reference to construct the 3D structure of TM-β-CD. The molecular mechanics force field (MMFF) was considered for the development of partial atomic charges. The unreasonable bond distances and angles of both ligands (Stv and Reb A) were adjusted by optimization using the Forcite module in Materials Studio (MS) [33]. The initial structure of TM-β-CD was energetically minimized using the Builder module in Discovery Studio (DS) 3.1 [34]. Docking studies were performed using the CDOCKER module of DS. CDOCKER is a grid-based molecular docking method where the receptor is held rigid while the ligands are allowed to flex during the refinement process. The ionic states of both ligands were determined at pH 8.0 followed by a conformational search using the Prepare Ligands and Conformations modules in DS [34], resulting in 40 and 22 conformations generated for Stv and Reb A, respectively. The lowest energy conformation for each ligand was further used for docking simulations. Prior to docking, a binding sphere of dimensions 30.4, 32.5 and 21.0 Å covering the whole TM-β-CD was created using the Define and Edit binding site modules in DS. Different ligand conformations were generated using molecular dynamics method and were refined further by grid-based (GRID 1) simulated annealing and a final grid-based minimization. The pose showing the highest docking score (CDOCKER energy) was considered for the binding energy (BE) calculations. The higher negative value of BE indicates a stronger binding and therefore a more favorable binding of the steviol glycoside to TM-β-CD.

Results and discussion

Electrokinetic studies of the host–guest complexation

The interaction of the analytes (Reb A and Stv) with the separating agent (TM-β-CD) depends on the complex

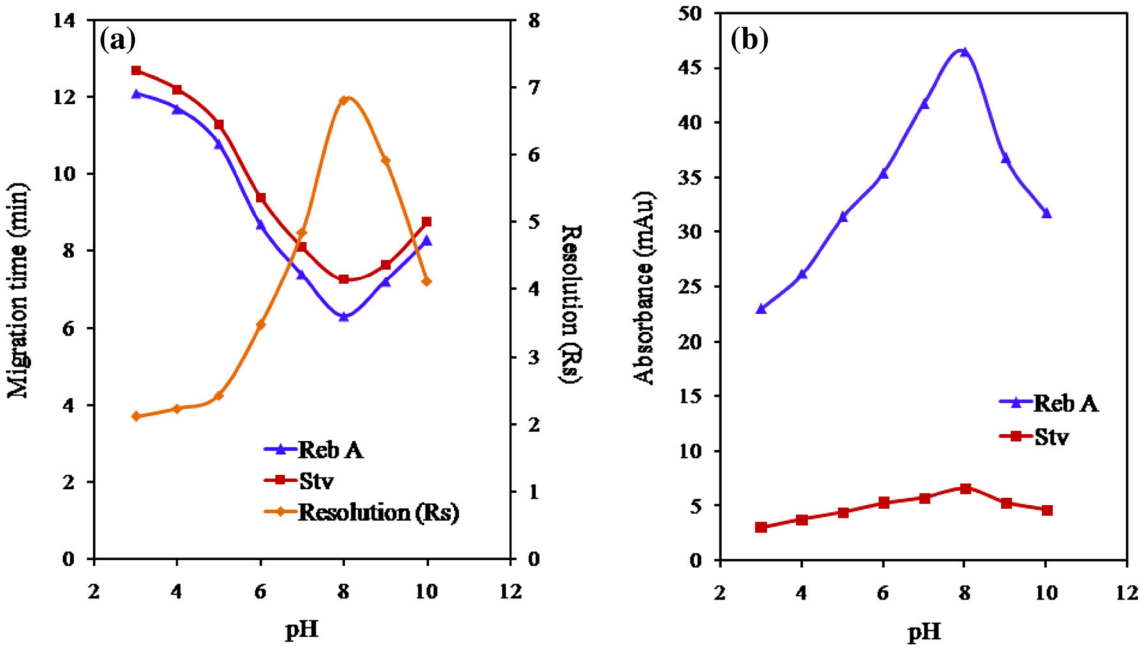


Fig. 1 Effect of buffer pH on **a** migration time and resolution **b** absorbance (conditions: 50-mM phosphate buffer, 30 mM TM-β-CD, 18 kV applied voltage, 30 °C temperature, 200 nm wavelength)

formation inside the capillary along with the EOF of the buffer. If the separating agent was directly bound to the capillary surface as a modifier, then the net velocity of the complex would be zero, but if the separating agent was directly added to the buffer, then the net velocity of the complex would not be zero. Accordingly, three different injection methods were employed for a better understanding of the interaction between the analytes and TM-β-CD. In first method, the analytes were injected followed by the separating agent. While in the second method, the separating agent was injected before the analytes. Finally, in the third method, the analytes were sandwiched by the separating agents.

Results from this study revealed that no separation was achieved in the first method, due to the higher mobilities of the analytes; hence there was an insufficient interaction between the analytes and the separating agent. However, in the second method, the EOF of the analytes was blocked by the slow moving TM-β-CD leading to some interaction and separation being observed. The weaker hydrophobic interactions between Reb A and TM-β-CD resulted in a less stable complex, whereas Stv formed a more stable complex with TM-β-CD, due to the stronger hydrophobic interactions with two more hydrogen bonds as confirmed by the molecular docking calculations. On the other hand, the less stable Reb A-(TM-β-CD) complex having a higher EOF and a higher velocity was eluted first. While in the third method, the hydrophobic interactions between the analytes and the hydrophobic cavity of TM-β-CD were very high due to the presence of TM-β-CD on either side of the analyte (sandwich). Therefore, the resulting complexes were more stable, with longer migration times than the corresponding complexes formed in the second method but

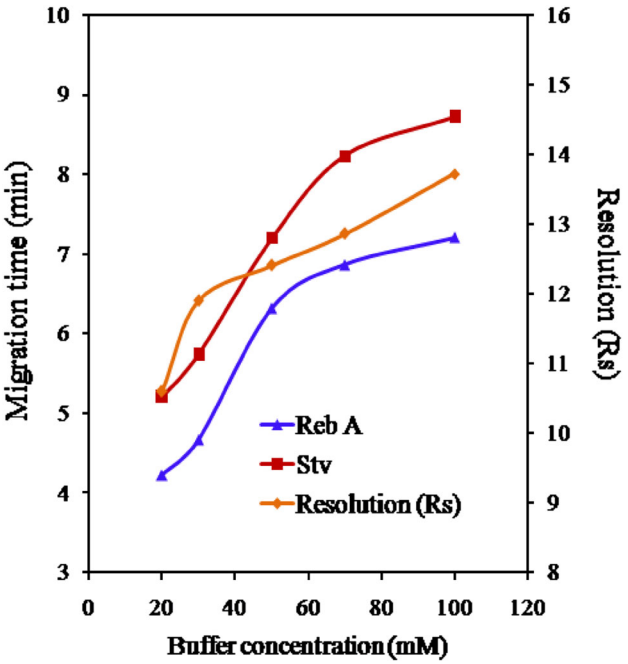


Fig. 2 Effect of buffer concentration on migration time and resolution (conditions: 30 mM TM-β-CD with pH 8.0, 18 kV applied voltage, 30 °C temperature, 200 nm wavelength)

with a similar elution order observed. For this purpose, the second method was selected, but with the corresponding parameters re-optimized in accordance with the host–guest molecular docking studies undertaken to evaluate the separation mechanism.

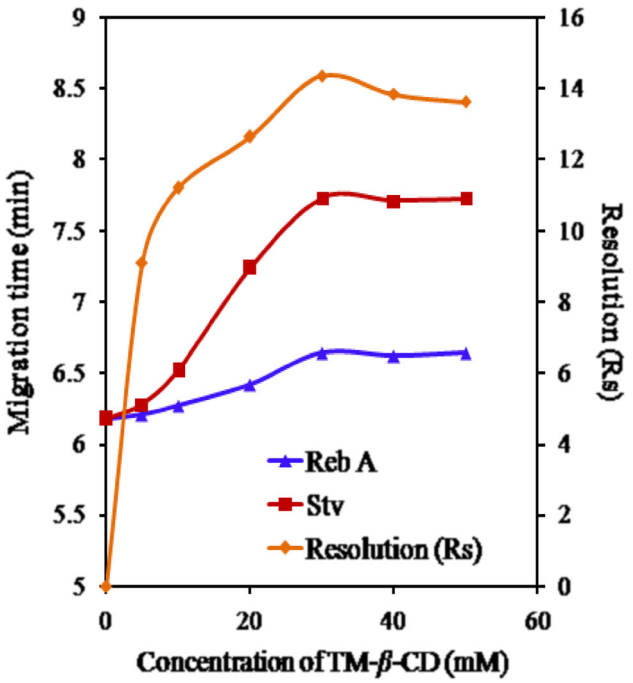


Fig. 3 Effect of concentration of TM-β-CD on migration time and resolution of compounds (conditions: 50-mM phosphate buffer with pH 8.0,18 kV applied voltage, 30 °C temperature, 200 nm wavelength)

Optimization of separation conditions

Effect of pH on resolution and absorbance

The pH of the running buffer plays a pivotal role in the separation mechanism because it affects the EOF, zeta (ζ) potential and the overall charge of the analytes [35]. Accordingly, in this study, the influence of pH ranging from 3.0 to 10.0 using a 50-mM phosphate buffer on the migration times and resolution of Reb A and Stv were investigated. Poor resolution and longer migration times with noisy baseline were observed at pH values ranging from 3.0 to 3.9, whereas good resolutions on the peaks were observed at pH 4.0. A further increase in pH from 4.0 to 8.0 resulted in shorter migration times, greater absorbances and good resolution between Reb A and Stv. At higher pH values, the deprotonation of the hydroxyl groups was favored [36, 37], as it promotes the complex formation between the negatively charged oxygen atoms in steviol glycosides with the hydrogen atoms in TM-β-CD, resulting in a better resolution and absorbances as shown in Fig. 1a, b. However, at pH >8.0, no significant differences in resolution capacities and migration times were observed. Consequently, 50-mM phosphate buffer at pH 8.0 was selected as the running buffer in this study.

Effect of buffer concentration

The viscosity coefficient of the solution, diffusion coefficient of the analytes and the zeta (ζ) potential of the inner

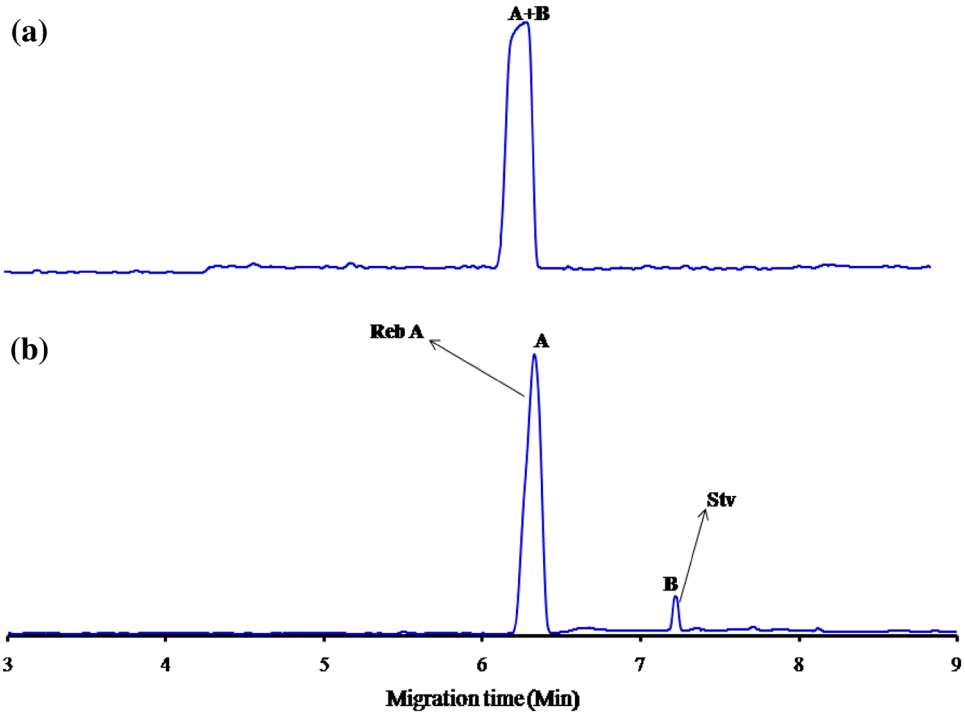


Fig. 4 Electropherograms showing the effect of TM-β-CD on the resolution of steviol glycosides **a** without TM-β-CD and **b** with TM-β-CD of *A* Reb A and *B* Stv (conditions: 50-mM phosphate buffer, 30 mM TM-β-CD with pH 8.0, 18 kV applied voltage, 30 °C temperature, 200 nm wavelength)

Table 2 Analytical figures of merit for the separation of Reb A and Stv

Sample	$y = a + bx$	Correlation coefficient	LOD (M)	LOQ (M)
Rebaudioside-A	$y = 12{,}669x + 27.05$	0.9935	2.017×10^{-5}	6.114×10^{-5}
Stevioside	$y = 4{,}079.6x - 16.46$	0.9850	7.386×10^{-5}	2.2881×10^{-4}

Regression equation ($y = a + bx$), where y is the peak areas of Reb A and Stv, x is the concentration (mM) of Reb A and Stv, r is the correlation coefficient, LOD's & LOQ's is calculated according to signal-to-noise ratio $S/N = 3$ and $S/N = 10$ respectively

Table 3 Analytical data for the determination of steviol glycosides from the spiked samples

Sample	Concentration added (mM)					
	1 mM		2 mM		3 mM	
	Found	Recovery ± RSD[a]	Found	Recovery ± RSD[a]	Found	Recovery ± RSD[a]
Rebaudioside-A	0.00392	97.51 ± 1.10	0.00746	92.78 ± 1.32	0.01195	99.08 ± 1.28
Stevioside	0.00384	95.52 ± 1.25	0.00789	98.13 ± 1.68	0.0102	84.57 ± 1.17

[a] Relative standard deviation for five individual determinations

surface of the capillary are mainly dependent on the concentration of running buffer which in turn affects the peak resolution, migration time and the absorbance of the analytes. Accordingly, in this experiment, different concentrations of the buffer ranging from 20 to 100 mM were examined on the resolution of Reb A and Stv. Figure 2 shows the influence of the buffer concentration on the resolution and migration time of Reb A and Stv. At low concentrations (<50 mM of phosphate buffer), low instrumental current was observed at an applied voltage of approximately 18 kV. However, at higher concentrations of phosphate buffer (>50 mM), joule heating becomes more pronounced resulting in a negative effect on the LOD values. The maximum resolution values were obtained with a 50-mM phosphate buffer at pH 8.0 and therefore chosen as the running buffer for further investigation in this work.

Effect of applied voltage

The applied voltage mainly affects the resolution, migration time and the peak shapes of the analytes. Shorter migration times with a good resolution between analytes were possible with higher voltages applied, as the EOF increases with increasing voltage. However, higher voltages are not preferred due to the increased joule heating generated from the capillary walls. In this investigation, the effect of the applied voltages ranging from 12 to 20 kV was examined. Application of a higher voltage 20 kV resulted in a noisy baseline with a maximum peak resolution and poor detection limits. However, the use of a lower voltage (12 kV) resulted in good baseline resolved peaks, but with longer migration times. Consequently the optimum voltage for this analysis was chosen at approximately 18 kV.

Table 4 Determination of Reb A and Stv in food samples

Sample	Available form	Active ingredients	Stevia glycoside concentration (g) Found	RSD[d]
Green Canderel[a]	Tablet	Rebaudioside-A stevioside	0.0119	1.15
			0.0112	1.53
Stevia[b]	Powder	Rebaudioside-A stevioside	0.0118	1.46
			0.0102	1.57
Tantalize[c]	Liquid	Rebaudioside-A stevioside	0.0125	1.55
			0.0114	1.87

[a] Merisant Company 2, Sarl, Czech Republic

[b] Dis-Chem Pty Ltd, South Africa

[c] Delite Foods, South Africa

[d] Relative standard deviation for five individual determinations

Effect of cassette temperature

In this study, the effect of cassette temperature ranging from 20 to 40 °C on baseline resolution was examined. As the capillary temperature increases the viscosity of the buffer decreases and the EOF of the buffer increases, resulting in shorter migration times but with good resolutions achieved. As a consequence of this, the optimum temperature of 30 °C was selected to sustain a baseline resolution with a shorter migration time and good peak shapes for both Reb A and Stv.

Effect of sample injection time

The effect of sample volume on the separation of Reb A and Stv was studied by changing the injection times ranging from 2 to 10 s. It was observed that the resolution between the two peaks decreased while the migration times

of the two analytes increased with an increase in injection times of the samples. Increasing the injection time beyond 4 s resulted in a peak broadening, hence lowering of the resolution between the two peaks. Therefore, 4 s was selected as an optimum injection time to enhance the separation of Reb A and Stv.

Effect of concentration of TM-β-CD

Figure 3 shows the influence of the concentration of TM-β-CD ranging from 5 to 50 mM. The separations of steviol glycosides with and without TM-β-CD are depicted in Fig. 4a, b respectively. Clearly, the migration times and the resolution between Reb A and Stv reached a maximum value at a concentration of 30 mM, and for this reason 30 mM of TM-β-CD was chosen as the optimum concentration.

Repeatability and reproducibility

Repeatability and reproducibility in terms of relative standard deviation of this method were studied on three types of stevia real samples described above. The reproducibility mainly depends on the dissociation of the silanol groups present on the inner walls of the capillary. To achieve a good reproducibility between the consecutive runs, the capillary has to be equilibrated. For this purpose, the capillary was flushed sequentially with deionized water for 2 min, 1 M NaOH for 2 min to refresh the silanol groups, and finally with the separation buffer for 2 min. Repeatability was evaluated by performing five replicates with the pH 8 buffer solution resulting in the percentage relative standard deviations (%RSD $n = 5$) of ±1.13 and ±1.43 % for migration time and 0.94 and 1.38 % for peak area.

Calibration curve and detection limit

For calibration studies, 98 % of Reb A and Stv standards was used with different concentrations ranging from 1 to 5 mM. The detection limits were calculated using individual standards at the following optimum conditions: 50-mM phosphate buffer, 30-mM TM-β-CD with pH 8.0, 18 kV applied voltage, 30 °C cassette temperature and 4 s of sample injection. From the calibration plots, the analytical figures of merit for the separation of Reb A and Stv are represented in Table 2.

Interference study

To test the selectivity of the developed method, the effect of foreign species was performed in this study. According

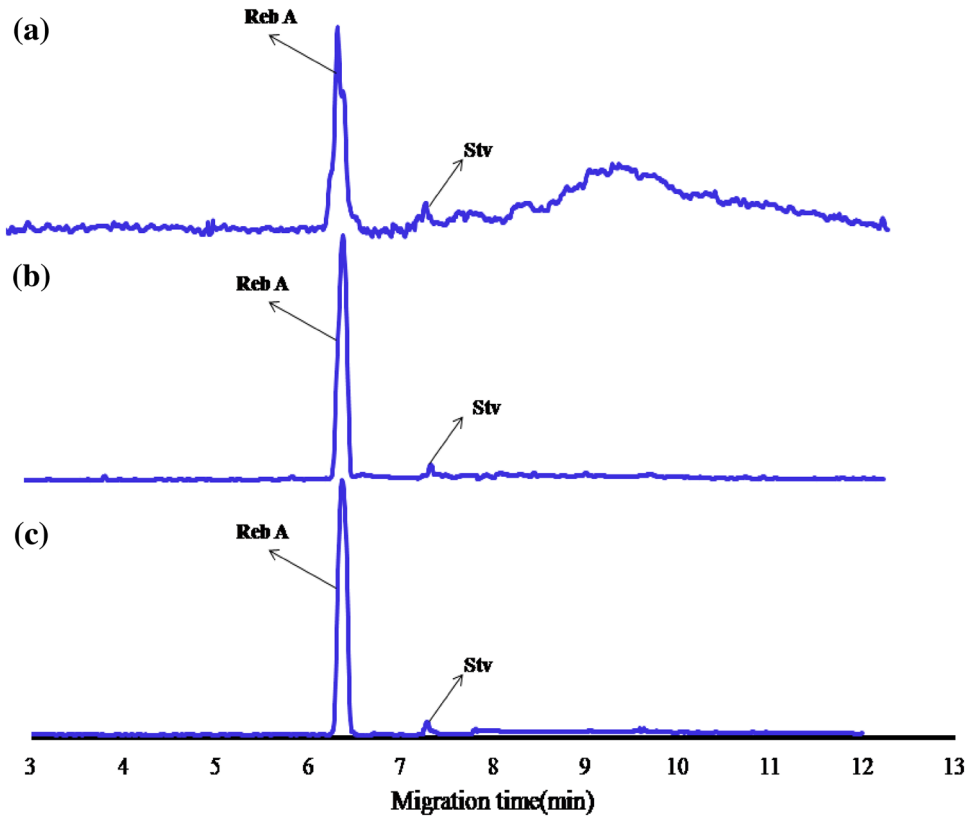


Fig. 5 Electropherograms for Reb A and Stv in real samples **a** tablet **b** powder **c** liquid samples (conditions: 50-mM phosphate buffer, 30 mM TM-β-CD with pH 8.0, 18 kV applied voltage, 30 °C temperature, 200 nm wavelength)

to the manufacturer's label, the tablet, liquid and powder samples contain lactose, fructose, citric acid, sorbic acid, natural flavors, colorants, wheat and gluten along with steviol glycosides. To discriminate the interferences from other foreign species, the dilution method was adopted to determine the optimum dilution level that would minimize the amount of fructose, lactose, citric acid, sorbic acid in the capillary while maintaining a measurable amount of steviol glycosides. The following dilutions: 2-, 10-, 50-, 100-, 500- and 1,000-fold were tested. A 100-fold dilution was optimized to measure steviol glycosides for all the studied real samples without any interference.

Performance evaluation of present method with reported methods

The performance of the developed method was assessed by comparing with those reported in the literature [2, 7, 16–18, 20]. From the data presented in Tables 3, 4 it is clear that the present method is fast, reliable and reproducible with shorter migration times and better resolution capacity in contrast to the reported methods [2, 7]. The selectivity pattern was superior to most of the cited references; however, it offers a better limit of detection than some of the previous reports [2, 7].

Analytical applications

The developed method worked well under laboratory conditions and was successfully applied to Reb A and Stv with no significant separation observed in the absence of the separating agent (TM-β-CD) (Fig. 4). To evaluate the accuracy of the developed method, real stevia samples in tablet, powder and liquid forms as described in the reagents section were purchased and analyzed. The obtained electropherograms for the real samples are shown in Fig. 5. The percentages of Reb A and Stv ranged from 92.78 to 99.08 % and 84.57 to 98.13 % while their % RSD was 1.10 and 1.17 %, respectively, are depicted in Tables 3, 4.

Computational discussion

To get a deeper understanding of the host–guest interactions of Reb A and Stv with TM-β-CD, molecular docking simulations were performed using the CDOCKER module of Discovery Studio 3.1 [34]. The docked complexes of Reb A and Stv with TM-β-CD are diagrammatically depicted in Fig. 6a, b, respectively. In contrast, a closer inspection of Fig. 5 reveals that both ligands (Reb A and Stv) penetrated the cavity of the TM-β-CD, thereby stabilizing their geometries, probably due to the hydrophobic interactions between their aliphatic functional groups and the hydrophobic cavity of the TM-β-CD ring. Apart from these hydrophobic interactions, two additional hydrogen bonds between the hydroxyl (–OH) groups and the oxygen atoms of the glucopyranose rings were observed in the case of Stv (Fig. 6b; Table 5), which accounts for their stronger interaction with the TM-β-CD than the Reb A (Fig. 6a) as evidenced by its lower CDOCKER energy (CDE score = −278.4). Moreover, it is believed that the more folded docked conformation of Stv (Fig. 6b) brought the hydroxyl groups (−OH) in the vicinity of the side chains of the TM-β-CD and facilitated the hydrogen-bonded interactions between them. The conformation of Reb A (Fig. 6a) on the other hand, was comparatively extended and simply penetrated through the cavity of the TM-β-CD. Thus, the proton acceptor/donor sites of Reb A were not close enough to form hydrogen bonds with the cyclodextrin. Finally, the computed binding energy (energy of complex-energy of ligand-energy of TM-β-CD) of Stv was found to be lower than Reb A, thus confirming the stronger interaction of the former with TM-β-CD than the later (Table 5). Moreover, the lower migration times of Stv

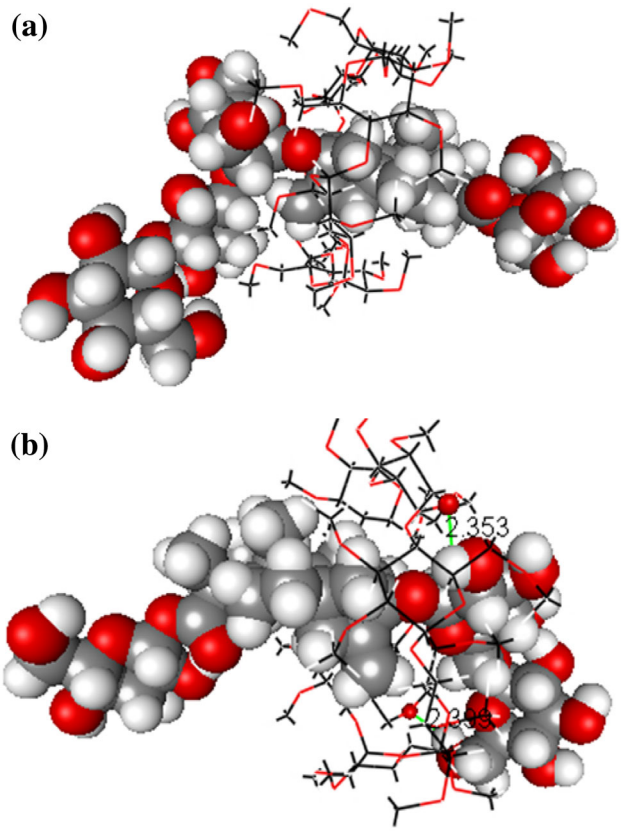


Fig. 6 Docked conformation of **a** Reb A and **b** Stv with TM-β-CD. Both ligands are shown in CPK format, while the TM-β-CD is shown in line format. Oxygen atoms of TM-β-CD participating in hydrogen bonding are shown in ball format and the hydrogen bonds are presented in *green*

Table 5 Docking results of Reb A and Stv with TM-β-CD using docking studies

Compound	CDocker energy (CDE) score	Binding energy (kcal mol^{-1})	Migration time (min) in CE	Number of H-bonds	H-bond distance (Å)
Stevioside	−278.4	−34.9	7.21	2	2.39, 2.35
Rebaudioside-A	−175.4	−25.4	6.32	0	–

observed in our CE experiments can also be explained on the basis of its stronger interaction with TM-β-CD (Table 5), resulting in its slower movement toward the anodic end of CE system compared to the Reb A–TM-β-CD complex, under the influence of EOF.

Conclusions

In this study, the simultaneous separation and determination of stevia glycosides with TM-β-CD were successfully implemented using the EKC–CE method, supported with molecular docking (MD) studies. Our results showed that the present method is a fast, efficient and facile method for the simultaneous separation and quantification of Reb A and Stv in real stevia samples. This study revealed that the good baseline resolution along with the reproducibility and repeatability renders this method to be more superior to those reported by HPLC methods, suggesting that the inclusion of a separating agent, TM-β-CD greatly improved the separation efficiency of stevia glycosides. Moreover, the MD studies complimented the experimental results by highlighting the stability and the stronger interaction of Stv with TM-β-CD than with Reb A, thus supporting the order of elution obtained under experimental CE conditions. To the best of our knowledge, this is the first attempt at using the CE-MD approach for the simultaneous separation and determination of stevia glycosides in real samples, which could have a special significance to the food flavoring industry.

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