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Continuation of Reversed-Phase HPLC Analysis Studies of Steviol Glycosides Isolated From *Stevia rebaudiana* Bertoni

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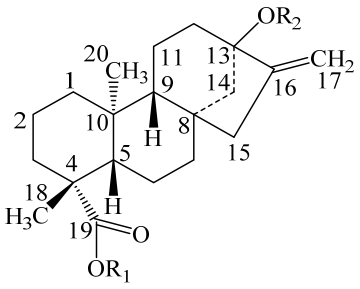
Abstract

Additional High Performance Liquid Chromatography (HPLC) studies were performed on the nine sweet steviol glycosides reported in Joint Expert Committee on Food Additives (JECFA) namely rebaudioside A, steviolbioside, stevioside, rubusoside, rebaudioside B, rebaudioside C, rebaudioside D, rebaudioside F, and dulcoside A isolated from the leaves of *Stevia rebaudiana*. Using Reversed-Phase (RP) HPLC method, individual retention times and area for nine naturally occurring *ent*-kaurane diterpene glycosides of *S. rebaudiana* have been determined at five different temperatures: 40, 45, 50, 55, and 60 ºC at three different pH 2.4, 2.6, and 2.8. HPLC results suggested that temperatures 50 and 55 ºC at pH 2.4 would be ideal condition for better separation of steviol glycosides.

Keywords: *Stevia rebaudiana*, asteraceae, diterpene glycosides, reversed-phase HPLC, temperature, pH

1. Introduction

Recently we have reported a qualitative Reversed-Phase (RP) High Performance Liquid Chromatography (HPLC) method for the sweet steviol glycosides isolated from *Stevia rebaudiana* Bertoni namely rebaudioside A, steviolbioside, stevioside, rubusoside, rebaudioside B, rebaudioside C, rebaudioside C, rebaudioside D, and dulcoside A at temperatures 20, 40, 60, and 79 ºC (Chaturvedula & Zamora, 2014) using a condition closely associated with JECFA (pH 2.63) (JECFA, 2010). Experimental results indicated that the temperatures between 40 and 60 ºC were ideal for the separation of nine steviol glycosides. In continuation to our research studies on the isolation of new natural steviol glycosides from *S. rebaudiana* and using them as possible natural sweeteners or sweetness enhancers (Chaturvedula & Zamora, 2014; Chaturvedula, 2014; Chaturvedula et al., 2013; Chaturvedula & Prakash, 2011), we are also engaged in developing analytical methods for steviol glycosides (Chaturvedula, Mubarak, & Prakash, 2012). In this paper, we are describing a qualitative HPLC method to find out precise temperature suitable for the separation of steviol glycosides between 40 and 60 ºC, as well as finding out an ideal pH of the buffer utilized in the mobile phase for the nine steviol glycosides rebaudioside D (**1**), rebaudioside A (**2**), stevioside (**3**), rebaudioside F (**4**), rebaudioside C (**5**), dulcoside A (**6**), rubusoside (**7**), rebaudioside B (**8**), and steviolbioside (**9**) reported in Joint Expert Committee on Food Additives (JECFA) (Figure 1).



Compound	R_1	R_2
1	Glcβ1-2Glcβ1-	Glcβ1-2(Glcβ1-3)Glcβ1-
2	Glcβ1	Glcβ1-2(Glcβ1-3)Glcβ1-
3	Glcβ1	Glcβ1-2Glcβ1
4	Glcβ1	Xylβ1-2(Glcβ1-3)Glcβ1-
5	Glcβ1	Rhaα1-2(Glcβ1-3)Glcβ1-
6	Glcβ1	Rhaα1-2Glcβ1-
7	Glcβ1	Glcβ1-
8	H	Glcβ1-2(Glcβ1-3)Glcβ1
9	H	Glcβ1-2Glcβ1

Glcβ: D-Glucopyranosyl

Rhaα: L-Rhamnopyranosyl

Xylβ: D-Xylopyranosyl

Figure 1. Structures of steviol glycosides **1-9**

In this paper we are describing reversed-phase HPLC methods for the separation of nine steviol glycosides **1-9** at five temperatures 40, 45, 50, 55, and 60˚C at three different pH conditions: 2.4, 2.6 and 2.8.

2. Materials and Methods

2.1 Reagents and Chemicals

Steviol glycoside standard kit containing rebaudioside A, steviolbioside, stevioside, rubusoside, rebaudioside B, rebaudioside C, rebaudioside C, rebaudioside D, and dulcoside A was obtained from Chromadex (P/N KIT-00019568- 010, Irvine CA). HPLC grade acetonitrile and water were obtained from Fischer Scientific (Fair Lawn, NJ), and Pharm Co., (Brookfield, CT). Phosphoric acid (49%-51%) HPLC grade was acquired from Sigma-Aldrich (Bellfonte, PA) whereas Sodium phosphate (monobasic) laboratory grade was obtained from Scientific Strategies (Oklahoma, OK).

2.2 Mobile Phase Preparation

All solvents utilized for reversed-phase HPLC mobile phase preparation were degassed at least fifteen minutes before starting experimentation. The method employed for separation of steviol glycosides **1-9** was an isocratic binary solvent mobile phase system with a 32:68 mixture of acetonitrile and 10 mmol/L sodium phosphate buffers. Buffer solutions were prepared for each pH 2.4, 2.6, and 2.8 using the method described earlier (Chaturvedula & Zamora, 2014).

2.3 Standard Preparation

Each standard steviol glycoside **1-9** was prepared separately at a concentration of 10 mg/ml. The dilutions for all steviol glycosides (**1-7**) were made using the phosphate buffer, but the two compounds steviolbioside (**8**) and rebaudioside B (**9**) were prepared dissolving in methanol. The mixture of standard steviol glycosides **1-9** was

prepared in such way that final dilutions were made to set the concentration at 1.0 mg/mL of each compound in the mixture.

2.4 Instrumentation and Conditions

An Agilent (Wilmington, DE) 1100 HPLC System, including a quaternary pump, a temperature controlled column compartment with additional 6 port switching valve, an auto sampler and VWD absorbance detector, was used for separation of steviol glycosides **1-9**. The detector was set-up at UV 210 nm. The data acquisition was done using a Chemastation A 10.02 software. The column used for HPLC analysis was a reversed-phase C18 (2) 100 A Phenomenex (Torrance CA) (Length: 250 mm, inner diameter 4.6 mm, particle size: 5 µm); pH was measured using meter Metler Toledo seven compact pH/ion S220 (Switzerland). Branson Ultrasonic Cleaner Model 2510 (Maplewood, NJ) was used for degassing HPLC solvents.

2.5 Analysis Procedure

For the RP-HPLC method, the column was flushed with 50 mL of 90% ACN to waste before use and the samples were bracketed with standards by injecting them at the beginning and at the end of a run for accuracy of their retention times. 1 µL of steviol glycoside mixture (**1-9**) has been injected at temperatures 40, 45, 50, 55, and 60˚C also three different pH values 2.4, 2.6 and 2.8. All the nine steviol glycosides were detected under UV at 210 nm. The two compounds rebaudioside A (**2**) and stevioside (**3**) were injected once at the beginning and once at the end of the sequence for consistency and reproducibility of HPLC chromatograms.

3. Results and Discussion

In this study, we have used RP-HPLC method under isocratic binary solvent mobile phase system (32:68; acetonitrile and phosphate buffer) at temperatures 40, 45, 50, 55, and 60 ºC. Further, the pH of the buffer used in the HPLC method was tested between 2.0 to 4.0 and results found that pH 2.4, 2.6 and 2.8 gave good separation compared to others. Hence, we were describing the HPLC analysis of compounds 1-9 at temperatures 40, 45, 50, 55, and 60 ºC under three pH conditions 2.4, 2.6 and 2.8. Although the steviol glycosides **1-9** are weak UV absorbers, they give an adequate signal at 210 nm to meet the required quantitation limit (LOQ) of 0.5 mg/L. Duplicate runs were performed at temperatures 40, 45, 50, 55, and 60˚C at each pH 2.4, 2.6 and 2.8; results found almost identical.

The retention times and percent areas for all steviol glycosides reported in JECFA including rebaudioside D (**1**), rebaudioside A (**2**), stevioside (**3**), rebaudioside F (**4**), rebaudioside C (**5**), dulcoside A (**6**), rubusoside (**7**), rebaudioside B (**8**), and steviolbioside (**9**) at temperatures 40, 45, 50, 55, and 60 ºC under each pH 2.4, 2.6 and 2.8 were identified and are given in Figures 2-5.

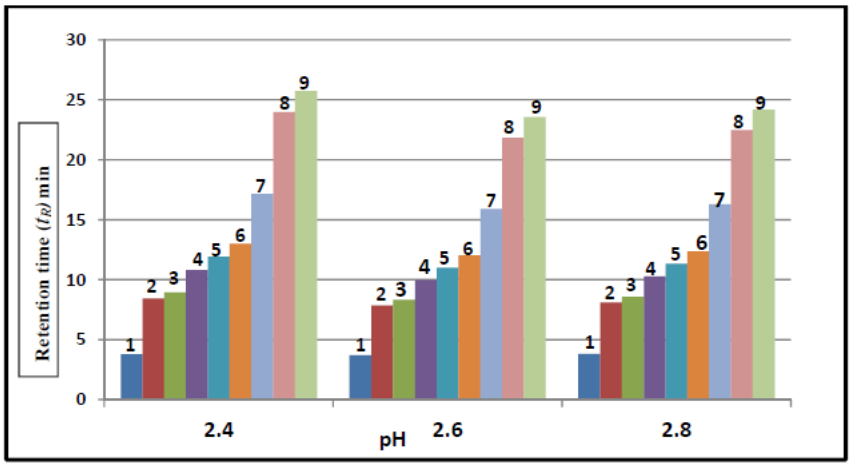


Figure 2. Retention Times (t_R) for steviol glycosides **1-9** at 50 ºC under pH 2.4, 2.6 and 2.8

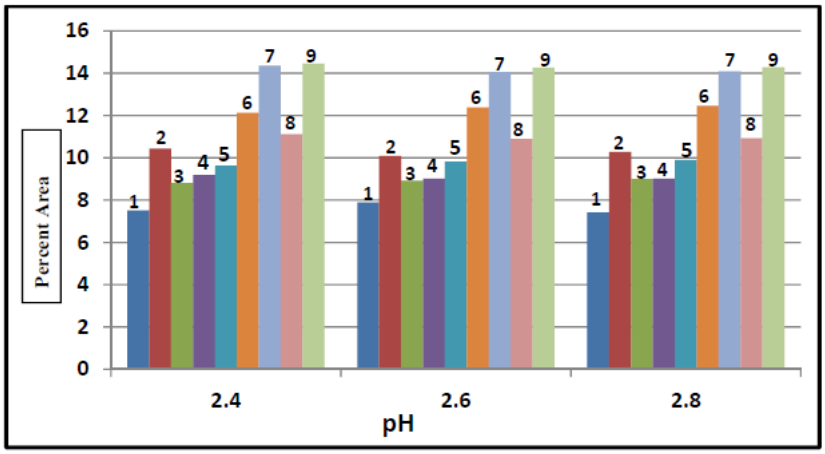


Figure 3. Percent Area of steviol glycosides **1-9** at 50 ºC under pH 2.4, 2.6 and 2.8

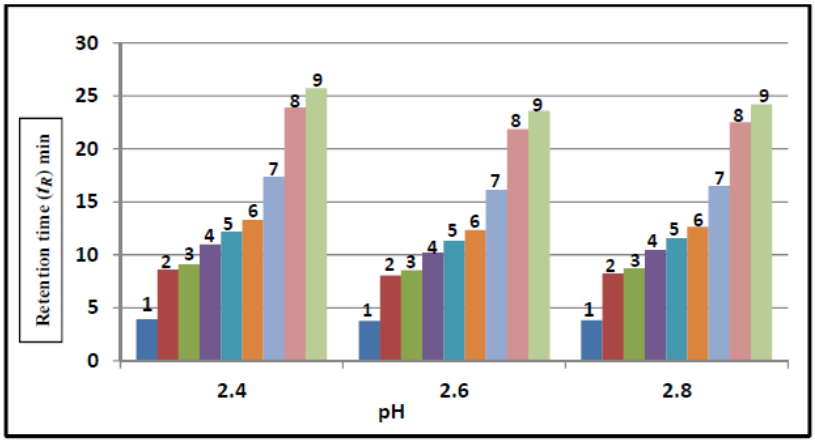


Figure 4. Retention Times (t_R) of steviol glycosides **1-9** at 55 ºC under pH 2.4, 2.6 and 2.8

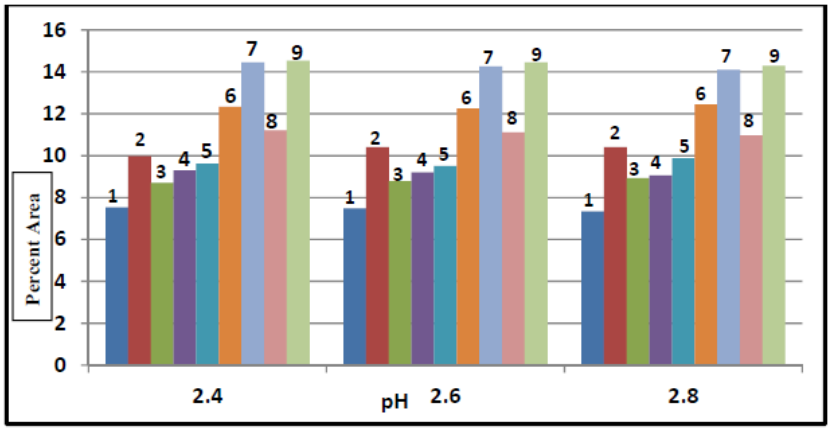


Figure 5. Percent Area of steviol glycosides **1-9** at 55 ºC under pH 2.4, 2.6 and 2.8

A close observation of the retention times from Figures 2 and 4 indicated that the four steviol glycosides rebaudioside D (**1**), rubusoside (**7**), rebaudioside B (**8**), and steviolbioside (**9**) were having retention times not close to the other five steviol glycosides **2-6**, and are clearly separated at all temperatures under pH 2.4, 2.6, and 2.8. The other five steviol glycosides rebaudioside A (**2**), stevioside (**3**), rebaudioside F (**4**), rebaudioside C (**5**), and dulcoside A (**6**) were having good separation at higher temperatures 50, 55 and 60 ºC. Further analysis of

Figures 2 and 4 indicated that the three compounds rebaudioside A (**2**), rebaudioside F (**4**), and rebaudioside C (**5**) having four β-D-glucopyranosyl units, three β-D-glucopyranosyl units and one β-D-xylopyranosyl unit, and three β-D-glucopyranosyl units and one α-L-rhamnopyranosyl unit respectively were better separated at temperatures 50, and 55 ºC under pH 2.4. Likewise, the two compounds stevioside (**3**), and dulcoside A (**6**) having three β-D-glucopyranosyl units, and two β-D-glucopyranosyl units and one α-L-rhamnopyranosyl unit respectively were better separated at pH 2.4 at temperatures 50, and 55 ºC. The above results suggested that steviol glycosides having identical number of sugar units attached to the C-19 acid and C-13 hydroxyl groups may be separated better at temperatures 50, and 55 ºC under the pH 2.4.

4. Conclusion

We are herewith reporting a qualitative HPLC method for the identification of nine steviol glycosides rebaudioside D (**1**), rebaudioside A (**2**), stevioside (**3**), rebaudioside F (**4**), rebaudioside C (**5**), dulcoside A (**6**), rubusoside (**7**), rebaudioside B (**8**), and steviolbioside (**9**) based on the RP-HPLC method using UV detection system. From the above experiments it has been concluded that temperatures 50 and 55 ºC would be ideal under pH 2.4 for separation of steviol glycosides having identical number of sugar units attached to the C-19 acid and C-13 hydroxyl groups.

Acknowledgement

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