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Short communication

Beaming steviol glycoside analysis into the next dimension

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ABSTRACT

Nine state-of-the-art reversed phase (RP) columns for ultra-high performance liquid chromatography were tested for the separation of steviol glycosides. The main criteria were resolution of the critical peak pair rebaudioside A and stevioside and the retention time of rebaudioside D. Three columns yielded a resolution of 2 or more of the critical peak pair and two of them showed sufficient retention of rebaudioside D, namely 1.62 and 1.84 min corresponding to retention factors of 0.98 and 1.24. The separation of nine steviol glycosides was possible in 11 min with UV and MS compatible, buffer-free eluents at moderate temperature. The presented method is proposed to be adopted as a new official method.

1. Introduction

High-performance liquid chromatography (HPLC) is the technique of choice for the analysis of steviol glycosides although the separation of the two main steviol glycosides stevioside and rebaudioside A is not easy to achieve on reversed phase (RP) columns.

The most recent official method, published by JECFA, proposed a C18 column with the dimensions 4.6 mm × 250 mm and 5 µm particle size for the analysis of nine steviol glycosides (Joint FAO WHO Expert Committee on Food Additives, 2010). Some scientific articles present improved variations of this method with the same column dimensions and particle size (Chaturvedula & Zamora, 2014; Tada et al., 2013). Even recent publications use columns of such dimensions, which leads to poor resolution (Soufi et al., 2016) or co-elution (Molina-Calle, Sánchez de Medina, Delgado de la Torre, Priego-Capote, & Luque de Castro, 2016) of rebaudioside A and stevioside. Smaller columns (e.g., 2.1 mm × 150 mm) with smaller particles (< 2 µm) are generally more efficient due to higher peak capacity. This recent development in HPLC is called ultra high-performance liquid chromatography (UHPLC) and had its breakthrough at the Pittcon exhibitions 2003 (presentation of the first commercial sub-2 µm particle column by Agilent) and 2004 (presentation of a HPLC apparatus with a pressure capacity of 1000 bar called Acquity UPLC by Waters) (Majors, 2015). In the meantime, a wide variety of columns with sub-2 µm particles is available.

Nevertheless, only a few articles were published dealing with the analysis of steviol glycosides on UHPLC columns with varying success. Gardana, Scaglianti, and Simonetti (2010) used a HSS C18 column (Waters; 2.1 mm × 150 mm, 1.8 µm) running a very fast separation gradient of less than 3 min at 80 °C column temperature, although the manufacturer of the column guarantees column stability only up to 45 °C. Several steviol glycosides were not separated and therefore not quantifiable by UV but by MS on different *m/z* traces. Rebaudioside D was not mentioned. The authors stated that the HSS C18 column showed the best performance among other HPLC and UHPLC columns tested in this article.

Cabooter, Ruis, Jooken, Meesschaert, and Desmet (2010) tested BEH C18 columns (Waters) in varying length by coupling up to four 100 mm columns (2.1 mm diameter, 1.7 µm). Among these trials, the 200 mm option gave the best result, i.e., a sufficient resolution and a reasonable separation time, using gradient elution. Steviol glycosides eluted between 26 (rebaudioside A) and 31 min (steviolbioside). Rebaudioside D is not mentioned.

Espinoza et al. (2014) employed a HSS C18 column (Waters; 2.1 mm × 150 mm, 1.8 µm) and a 12 min-gradient, in order to detect

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enzymatically modified steviol glycosides. It was not the scope of the study to analyze the native steviol glycosides and in fact, their separation was incomplete.

Wang et al. (2015) used a HSS-T3 column (Waters; 2.1 mm × 100 mm, 1.8 µm) with a gradient of 8.5 min. BEH C18 and BEH Shield columns (same dimensions; all manufactured by Waters) were also tested. Only five steviol glycosides were considered: rebaudiosides A and D, stevioside, dulcoside A and steviolbioside.

In cooperation with the group of Prof. Morlock, I used a CSH C18 column (Waters, 2.1 mm × 150 mm, 1.7 µm) applying gradient elution (Morlock, Meyer, Zimmermann, & Roussel, 2014). Within 20 min the same nine steviol glycosides as those mentioned in the JECFA method were separated. This kind of column is included in the present article and was re-tested with isocratic elution.

The aim of this investigation was to develop a fast UHPLC method on a RP column able to separate the nine steviol glycosides mentioned in the JECFA document. The eluents should be UV and MS compatible, so that the same method can be used for either detector unlike the JECFA method that uses a phosphate buffer. Other conditions should be as simple as possible, i.e., avoiding complex eluent mixtures and high column temperatures.

2. Material and methods

2.1. Chemicals and samples

Gradient grade (for sample dissolution) and MS grade acetonitrile (ACN) (for the eluent) and MS grade water were purchased from Th. Geyer (Renningen, Germany).

A steviol glycoside mix originating from proficiency testing of the International Stevia Council (Brussels, Belgium) realized by LGC Standards (Bury, Lancashire, United Kingdom) was used for method development. The sample contains the rebaudiosides A, C, D, F, stevioside, steviolbioside, dulcoside A and rubusoside. The total steviol glycoside content in dry matter was reported as 87 g/100 g with stevioside (52 g/100 g) and rebaudioside A (22 g/100 g) as the main components. 25 mg of that sample were dissolved in 25 ml ACN + water (1 + 1). Additionally, a steviol glycoside standard mix (LGC Standards) with the same eight steviol glycosides, plus rebaudioside B, with a concentration of 50 mg/l of each compound was analyzed. Peak resolution was calculated as difference of retention times divided by the sum of peak widths at half-height multiplied by 1.18.

2.2. UHPLC-UV-MS

The apparatus was the same as in a previous work (Feuereisen et al., 2014). Injection volume was 2 µl. The column was kept at 40 °C. Detection wavelength was 200 nm. The UV chromatograms were used to calculate peak separation. In addition, MS was used to ensure peak identity: MS in selected ion monitoring (SIM) mode after negative ionization was used to confirm peak identity applying the same MS conditions as in a previous study (Zimmermann, 2011). Isocratic elution with 28–36% ACN in water in steps of 2% was applied to all columns at a flow of 0.35 ml/min. When a promising resolution of stevioside and rebaudioside A was achieved, further eluent compositions around the composition with the best resolution were tested in steps of 0.5%. The tested columns are listed in Table 1.

3. Results and discussion

The basic conditions were chosen in order to enable UV detection at 200 nm, which is necessary for steviol glycosides, and MS detection. Therefore, formic acid and formate buffers were excluded and the common eluent mix of ACN and water was chosen.

Further, manual workload and costs could be minimized by:

1. buffer-free eluents: mixing of buffers and pH adjustment is time-consuming; larger rinse volumes are required; the ion source of a MS detector has to be cleaned more often;
2. moderate column temperature, i.e., 40 °C: higher column temperatures decrease column lifetime and require longer equilibration times.

These conditions limited the parameters to be optimized to column choice and ACN/water ratio. Among the sub-2 µm RP18 columns available, those columns were selected that are similar to the HPLC column of the JECFA method (Joint FAO WHO Expert Committee on Food Additives, 2010), e.g., Luna Omega, HSS-T3, or that have particular modifications, e.g., Nucleodur Polartec and Pyramid, CSH C18 or BEH Shield RP18.

The aim of a baseline separation of nine steviol glycosides was achieved with three of the tested columns even under the self-given restrictions mentioned above. The other columns might be useful under varied conditions like different eluent composition (e.g., mixed with methanol) or modifiers (e.g., acids or buffers). But it was not the aim of this study to find the optimal conditions for each column.

The main criterion was resolution between stevioside and rebaudioside A. They are the main steviol glycosides in the stevia leaves and the major components of most sweeteners. Secondly, the retention time (RT) of rebaudioside D was considered. This compound should not be eluted too early, in order to avoid possible interferences with weakly retained matrix components. On all columns, resolution between stevioside and rebaudioside A tended to be higher, when the percentage of ACN increased. This led to shorter RT of rebaudioside D. The trade-off is to choose the lowest possible ACN percentage allowing an acceptable resolution between stevioside and rebaudioside A, in order to increase RT of rebaudioside D.

Unfortunately, a gradient elution could not resolve this dilemma (data not shown). Thus, Fig. 1 showing resolution between rebaudioside A and stevioside and RT of rebaudioside D summarizes the performances of the various columns. RT can be compared directly instead of comparing retention factors, because the dead time is the same for all columns (0.82 min) due to the identical dimensions and flow. Moreover, a retention factor > 1 is recommended to indicate a sufficient retention on the column.

A resolution between rebaudioside A and stevioside greater than 2 was achieved on three columns: Luna Omega, Cortecs C18+ and HSS-T3. Among them, the Luna Omega column yielded a resolution of 2.13 at 32% ACN, which is the highest resolution in this study. The resolution was relatively robust, i.e., in the range of 31–34% ACN the resolution was always above 2. RT of rebaudioside D was 1.62 to 1.28 min corresponding to retention factors of 0.98 and 0.56, respectively. In order to increase retention of rebaudioside D, 30% acetonitrile was used as eluent leading to a rebaudioside A/stevioside resolution of 1.90 and a RT of rebaudioside D of 1.81 min corresponding to a retention factor of 1.21. Back-pressure was between 710 and 720 bar at 34 and 30% acetonitrile, respectively.

The HSS-T3 column barely reached a resolution of 2 between the critical peak pair at 31.5% ACN. Under these conditions, rebaudioside D had a RT of 1.84 min equivalent to a retention factor of 1.24 and a back-pressure of 690 bar. Interestingly, the Cortecs-T3 column is the solid-core version of the HSS-T3, but had a different behavior. In fact, the manufacturer stated upon request that not only the structure of the particles but also the chemistries of the HSS-T3 and the Cortecs-T3 columns are slightly different. Also the Cortecs C18+ column reached the resolution of 2.0 at 32% ACN, but RT of rebaudioside D was only 1.21 min.

Elution on the Luna Omega column was further optimized by applying increasing ACN percentage after the elution of stevioside, in order to accelerate elution of the following compounds, and a rinse step with 100% ACN was introduced. The resulting gradient was: 0–7 min: 30% ACN; 10 min 42% ACN; 11–13 min: 100% ACN; 14–16 min: 30% ACN.