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Article

Development of HPLC Analytical Techniques for Diterpene Glycosides from *Stevia rebaudiana* (Bertoni) Bertoni: Strategies to Scale-Up

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Stevia rebaudiana is a plant well-known as a source of dozens of high potency non-caloric natural sweeteners, many of them still uncharacterized. Herein, we describe the development of several routine HPLC (high performance liquid chromatography) analytical techniques to better separate steviol glycosides. Different stationary phases/columns were used: reverse phase C-18, amino, HILIC (hydrophilic interaction chromatography), Sepaxdiol and Synergi. The advantages and disadvantages of each method for resolving the very polar and less polar elution regions are demonstrated. Strategies for scale up of the purification process of steviol glycosides to make it more economic are also summarized, emphasizing the advantages of using high efficiency normal phase chromatography in relation with reverse phase. Additionally, we also developed an easy and useful way for predicting yields and composition of the pools resulting from the preparative chromatography. The development of these analytical and preparative liquid chromatography techniques allowed the isolation of several steviol glycosides in gram quantities at relatively low cost.

Keywords: *Stevia rebaudiana*, diterpene glycoside, HPLC technique, scale-up, enhanced normal phase preparative chromatography

Introduction

Stevia rebaudiana (Bertoni) Bertoni (Asteraceae) has been used as a sweetener for centuries by Guarani indigenous due to the occurrence of diterpene glycosides in its leaves. The plant grows up to 1 m high and is native to Brazil and Paraguay but its cultivars are now grown commercially all over the world.[1]

Diterpene glycosides with an *ent*-kaurene skeleton are recognized as the metabolites from *S. rebaudiana* with high potency sweetness. These compounds are several hundred times more sweet than sucrose.[1] The main aglycone of these glycosides is steviol and the first steviol glycosides, stevioside and rebaudioside A, were described from *S. rebaudiana* in 1930.[2] However, crude plant extracts were not employed as sweetening agents until 1971 in Japan and stevioside was accepted officially in Korea as an alternative sweetener in 1976.[3,4] Products from *S. rebaudiana* were accepted in the United States of America (USA) in 2008 and in the European Market in 2011.[5,6]

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Rebaudioside A, one of the major compounds biosynthesized in the plant is the main natural sweetener commercialized from this source. Rebaudioside A is used currently as an additive in several foods and beverages. Despite its advantages in comparison with other natural and synthetic sweeteners, this major compound has an unpleasant lingering aftertaste which has limited its broad utilization. *Stevia rebaudiana* produces dozens of steviol glycosides, mostly as yet uncharacterized. Their chemical structures differ mainly in the type, number and connection of the sugar moieties. Slight variations in the chemical structures of these glycosides surely should influence their organoleptic properties. Due to the close chemical and physical similarities of the glycosides, several analytical techniques were developed to better analyze the extract mixture of glycosides. In addition, to access these minor compounds for characterization of their structures and organoleptic properties, there is an imperative necessity to develop and improve the scale-up purification process. Scale-up for preparation of the target involves not only enhancement of the production but also containment of the costs of the process. Herein, we present the different

analytical techniques developed to monitor purification of diterpene glycosides from *S. rebaudiana* extracts as well some strategies to scale-up the purification process of the diterpene glycosides in a cost effective manner.

Experimental

Chemicals

The standards used were purified from commercial crude extracts of *Stevia rebaudiana* at Ironstone Separations Inc. in relative purity higher than 95%: dulcoside A, eugenol diglucoside, rebaudiosides A, B, C, D, E, F, M, N, O, rubusoside, steviolbioside, and stevioside. A solution of 1 mg mL^{-1} of each high purity standard was prepared in CH_3CN:H_2O (3:7) to form our standards mixture. These individual standards and other glycosides are available from Ironstone Separations Inc. as standards or in gram quantities.

HPLC (high performance liquid chromatography) analytical techniques

Analyses were performed with a Hewlett Packard Agilent 1100 Series equipped with a G1311A quaternary pump, a G1322 degasser, a G1316A oven, G1313A autosampler and a G1315A diode array detector. Acetonitrile and water for HPLC (high performance liquid chromatography) were purchased from EMD Millipore (Cincinnati, Ohio). The elution was performed with 0.01 mol L^{-1} phosphoric acid (A) and acetonitrile (B) with a flow rate set at 1 mL min^{-1}. All the analyses were performed with Phenomenex columns (Torrance, California), except Sepax-diol column, manufactured by Sepax Technologies Inc. (Newark, Delaware). After each analysis the column was washed and equilibrated appropriately.

RP-C18 method 1 (JECFA slightly modified)

The standard mixture (2 µL injection) was analyzed in a RP-C18 Luna (2) column (4.6 × 250 mm, 5 µm) as follows: 0-22 min, 32% B; 95% B over 1 min, hold for 3 min; gradient to 32% B over 1 min, hold for 6 min to equilibrate the column. The wavelength was set at 210 nm and the oven temperature at 40 °C.

RP-C18 method 2

The standard mixture (2 µL injection) was analyzed in a RP-C18 Luna (2) column (4.6 × 250 mm, 5 µm) column by using gradient elution: 0-20 min, 20-45% B; 100% B over 1 min, hold for 3 min; gradient to 20% B over 1 min, hold for 5 min to equilibrate the column. The wavelength was set at 210 nm and the oven temperature at 40 °C.

Synergi method

The standard mixture (2 µL injection) was analyzed in a Synergi Polar RP (4.6 × 150 mm, 4 µm) column as follows: 0-20 min, 15-30% B; 20-21 min, 30-95% B; hold 3 min 95% B; gradient to 15% B over 1 min, hold for 3 min to equilibrate the column. The wavelength was set at 205 nm and the oven temperature at 60 °C.

NH_2 method

The standard mixture (4 µL injection) was analyzed in an Amino Luna column (4.6 × 250 mm, 5 µm) as follows: 0-2 min, 90% B; 2-2.5 min, 90-80% B; 2.5-7 min, 80-77% B; hold for 3 min; 10-13 min, 77-75% B; 13-20 min, 75-50% B; 20-20.5 min 50-5% B; hold for 5 min; gradient to 90% B over 0.5 min, hold for 3 min to equilibrate the column. The wavelength was set at 205 nm and the oven temperature at 40 °C.

HILIC (hydrophilic interaction chromatography) method

The standard mixture (2 µL injection) was analyzed in a HILIC (hydrophilic interaction chromatography) column (4.6 × 250 mm, 5 µm) as follows: 0-5 min, 90-85% B, hold for 2 min; 7-12 min, 85-75% B; 12-15 min, 75-45% B; 15-15.5 min 5% B; hold for 2 min; gradient to 90% B over 0.5 min, hold for 3 min to equilibrate the column. The wavelength was set at 205 nm and the oven temperature at 30 °C.

Sepax-diol method

The standard mixture (2 µL injection) was analyzed in a Sepax-diol column (4.6 × 250 mm, 5 µm) as follows: 0-5 min, 87-85% B; 5-7 min, 85-75% B hold for 7 min; 14-20 min 75-65% B; 20-21 min, 5% B; hold for 3 min; gradient to 87% B over 1 min, hold for 4 min to equilibrate the column. The wavelength was set at 205 nm and the oven temperature at 30 °C.

Preparative separations

Crude extracts and/or fractions were purified by using reverse phase or silica gel columns of different sizes: semi-preparative column (1 × 25 cm; 5 µm, spherical); preparative columns (2.5 × 45 cm; 10 µm, spherical); (7.5 × 50 cm; 10 µm, spherical) and (15 × 135 cm; 37-63 µm, irregular).[7] In the purification processes using a semi-preparative and preparative columns (2.5 × 45 cm; 10 µm), solvent was pumped with two Shimadzu pumps (LC-10AS) connected in series to produce a maximum flow rate of 20 mL min^{-1}. The larger preparative columns (7.5 × 50 cm; 10 µm, spherical) were eluted at a flow rate of 200 mL min^{-1} using a Prep 250 pump (Scientific Systems,

Inc., State College, Pennsylvania). The largest preparative column (15 × 135 cm; 37-63 μm, irregular) was eluted at flow rate of 500 mL min^{-1} using a Baldor pump (Baldor Electric Co., Fort Smith, Arizona). The separations were monitored with a UV-trace set at 210 nm.

Results and Discussion

HPLC analytical techniques

Stevia rebaudiana extracts are a complex mixture of dozens of diterpene glycosides, most of them still uncharacterized. They are derivatives of steviol with glucose, rhamnose, xylose, 6-deoxy-glucose and fructose as the most common carbohydrate units attached to the aglycone, mainly at positions C-13 and C-19. Slight structural differences due to differing sugars and several possibilities of connectivity of the sugars, cause many of these compounds certainly to co-elute in a single HPLC method. Therefore, to better verify the identity of a known or new target analyte, and even a potential fraction, different analytical techniques were developed (Figure 1). A mixture of fourteen standards (Table 1) was analyzed using six different HPLC methods. Although eugenol diglucoside is not a diterpene glycoside, it was included in the standard mixture since it is found very commonly in our experience in Stevia crude plant extracts.

The JECFA (Joint FAO/WHO Expert Committee on Food Additives) method is a method previously described and accepted for diterpene glycoside analyses from *S. rebaudiana*.[8] This method was our starting point for analyzing diterpene glycosides and was put in place in our facility with slight modification (Figure 1a). However, because of the limitations of the JEFCA and the slightly modified JEFCA method, other methods using a RP-C18 column (Figure 1b), Synergi (Figure 1c), NH_2 (Figure 1d), HILIC (Figure 1e) and Sepax-Diol column (Figure 1f) were developed and optimized.

The JECFA method (Figure 1a) showed a good resolution in the less polar region (rebaudioside A-rebaudioside B) however, the more polar glycosides co-elute. To better resolve the polar region, an alternative RP-C18 method with a gradient elution was developed (Figure 1b). This method is most useful for minor diterpene glycosides eluting between rebaudiosides M and A; there are some polar compounds still co-eluting, e.g., rebaudiosides E and O and rebaudiosides D and N. Selection of a more polar column led to development of the Synergi method. Although the five minor polar glycosides elute close together, this method was useful for analyzing the polar region for compounds with earlier retention times to rebaudioside E, and compounds with retention times between rebaudiosides N and A (Figure 1c). Development of the amino method showed two important regions, between rebaudioside F and A, and rebaudioside A and E, where uncharacterized glycosides could be identified with confidence. The HILIC method (Figure 1e) was the method where the best resolution of all the standards was obtained. However, broad peaks and lack of reproducibility of retention times for the glycosides were observed. These disadvantages were improved by using a similar analytical method with a Sepax-Diol column (Figure 1f); the same order of elution was observed. Although rebaudioside M and D co-eluted, new very polar compounds, chemically close to rebaudioside N and O should be separated as well as compounds with retention between steviolbioside and dulcoside A. All the methods developed for verifying the identity of each individual diterpene glycoside showed advantages and disadvantages. In our experience, the combination of methods which best resolved in the range of retention times of interest, gave us a better understanding of the identity and/or purity of the fractions and individual analytes obtained during the purification process.

Strategies to scale-up

Stationary phases

Two main approaches were developed for scaling-up the purification process of diterpene glycosides from *S. rebaudiana*: utilization of reverse phase (C-18) and normal phase (Silica gel) stationary phases. The strategies presented herein have also been applied for purifying other plant metabolites with interest to the pharmaceutical industry.

Reverse phase chromatography is perceived to have economic advantages over normal phase chromatography due to the usual practice of replacement of the normal phase adsorbent after one or at most a few uses whereas the reverse phase adsorbent can be used for hundreds of separations. The gravitation to reverse phase technology for preparative separations has occurred even in spite of the recognized advantages of normal phase chromatography. Normal phase chromatography has significantly greater capacity *per* column run and ease of compound recovery from the organic solvent of the mobile phase, both very important advantages for production scale separations.

Because of the perception that normal phase adsorbents are not reusable or of a very limited useful life, less expensive poor quality normal phase adsorbents are typically employed in preparative column packings. The poorer quality normal phase adsorbent is usually of irregular shaped particles and possesses a wide particle size distribution which together

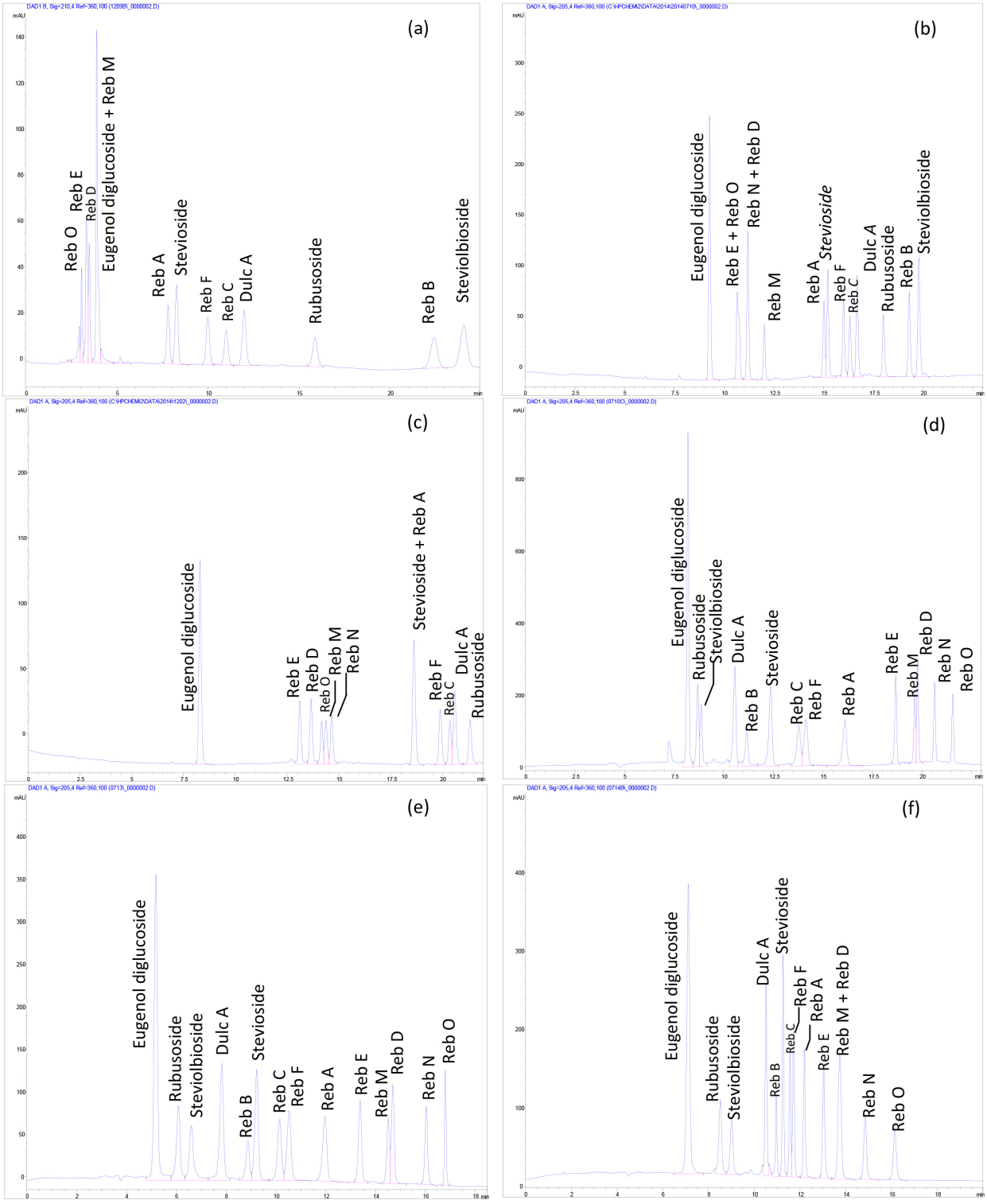
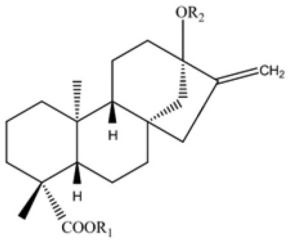


Figure 1. HPLC methods developed for diterpene glycosides from *Stevia rebaudiana* at Ironstone: (a) JECFA modified; (b) RP-C18 column; (c) Synergi; (d) NH_2; (e) HILIC; (f) Sepax-Diol.

gives poor chromatographic performance for the packed bed. High quality normal phase adsorbents are available with spherical particles and narrow particle size distributions. As quality normal phase adsorbent costs about $5,000 *per* kilogram and as the adsorbent is perceived not to be reusable, development of normal phase preparative chromatographic processes has largely been avoided or not considered. With the technologies outlined below, normal phase

Table 1. Chemical structures of the steviol glycoside standards used in the HPLC methods



Name	R_1	R_2
Rubusoside	Glcβ$_1$–	Glcβ–
Steviolbioside	H	Glcβ(1-2)–Glcβ$_1$–
Dulcoside A	Glcβ$_1$–	Rhaα(1-2)–Glcβ$_1$–
Rebaudioside B	H	Glcβ(1-2)[Glcβ(1-3)]–Glcβ$_1$–
Stevioside	Glcβ$_1$–	Glcβ(1-2)-Glcβ$_1$–
Rebaudioside C	Glcβ$_1$–	Rhaα(1-2)[Glcβ(1-3)]–Glcβ$_1$–
Rebaudioside F	Glcβ$_1$–	Xylβ(1-2)[Glcβ(1-3)]–Glcβ$_1$–
Rebaudioside A	Glcβ$_1$–	Glcβ(1-2)[Glcβ(1-3)]–Glcβ$_1$–
Rebaudioside E	Glcβ(1-2)–Glcβ$_1$–	Glcβ(1-2)–Glcβ$_1$–
Rebaudioside M	Glcβ(1-2)[Glcβ(1-3)]–Glcβ$_1$–	Glcβ(1-2)[Glcβ(1-3)]–Glcβ$_1$–
Rebaudioside D	Glcβ(1-2)–Glcβ$_1$–	Glcβ(1-2)[Glcβ(1-3)]–Glcβ$_1$–
Rebaudioside N	Rhaα(1-2)[Glcβ(1-3)]–Glcβ$_1$–	Glcβ(1-2)[Glcβ(1-3)]–Glcβ$_1$–
Rebaudioside O	Glcβ(1-3)Rhaα(1-2)[Glcβ(1-3)]–Glcβ–	Glcβ(1-2)[Glcβ(1-3)]–Glcβ$_1$–

chromatographic processes can be developed providing cost savings to users through better performance, higher capacity, easier product recovery, less costly solvent recovery, and less costly solvent disposal. This technology thus provides an economic and convenient approach to the isolation and purification of larger quantities of potentially interesting natural products for confirmation of their bioactivities. For example, our high performance preparative silica gel column (7.5 × 50 cm; 10 μm, spherical) was packed in 2009 in our facility and we have performed hundreds of separations until now without re-packing. Each chromatography run is followed by column regeneration and re-equilibration to the next mobile phase composition which restores the performance qualities of the column. More details regarding the improvement of the performance of the columns packed with silica gel stationary phase and their use in multiple column runs have been well summarized by McChesney.[7,9]

Mobile phases

The most used mobile phase for purifying steviol glycosides in reverse phase chromatography are mixtures of acetonitrile/acidified water in different ratios depending of the analytes. In a routine separation of a complex mixture of steviol glycosides by using a reverse phase high efficiency preparative column (7.5 × 50 cm; 10 μm), around 15 L of solvents are pumped at 200 mL min^{-1} *per* run. The solvent mixtures after processing the fractions are recovered (ca. 70% in acetonitrile) and re-used in the next chromatography after adjusting the ratios based on the density of the mixtures. Thus, we are able to save solvents and make the purification process of these compounds at large scale more economic. However, compound recovery from the organic solvent of the mobile phase used in normal phase chromatography is easier than from the water containing mobile phase used in reverse phase chromatography. At production scale, the energy required to recycle the normal phase organic solvents is significantly less than that of reversed phase aqueous solvents. The waste disposal costs are reduced for the normal phase organic solvents because of their usually higher BTU content.

Mobile phase a

One of the most common mobile phases for separating non-glycosylated steviol derivatives using a high efficiency normal phase (7.5 × 50 cm, 10 μm) are mixtures of *n*-heptane: waEtOAc and/or *n*-heptane:waEtOAc:MeOH where "wa" is an abbreviation for "wet acidified" which is a solvent approaching saturation or saturated with water and which has 0.1 to 1% acetic acid added. The solubility of water in EtOAc is approximately 3.3% v/v at room temperature.[7] However, the detection of the steviol glycosides is generally set at 210 nm, below the UV absorbance cut off wavelength of ethyl acetate. An alternative mobile phase to solve this problem is the use of methyl *tert*-butyl ether (MTBE) instead of ethyl acetate. MTBE is less polar than EtOAc and

the solubility of water is approximately 1.4% v/v at room temperature, hence, the solvent composition ratios should be adjusted by scouting by TLC.[9] For example, during the separation process of isosteviol from a mixture of steviol and other isomers, the optimal retention factors were observed with *n*-heptane:waEtOAc (8:2 v/v) whereas for the optimum separation the rate of MTBE was increased in the mixture *n*-heptane: waMTBE (7:3 v/v).

Mobile phase b

Separation of the glycosides requires a more polar mobile phase. For example, separation of rebaudioside C from a concentrate prepared from commercial extract by partial removal of the major glycosides, rebaudioside A and stevioside, by crystallization of commercial extract from 10% aqueous methanol required "reb C mobile phase", 100:18:14; EtOAc: MeOH: H_2O with 0.1% HOAc. The enriched mother liquors were chromatographed by normal-phase chromatography. There were five large-scale column runs. The loads were nominally 150 g of mother liquor dried onto 450 g of Celite 545 and packed into a 7.5 × 35 cm load column. The normal-phase column was 15 internal diameter × 135 cm length packed with 10 kg of 37-63 μm flash silica gel. The columns were eluted at 500 mL min^{-1} with 16 L of mobile phase collected as a forerun followed by 70 1 L fractions. The fractions were analyzed by HPLC and pools selected by column analysis (see below). Six main pools were obtained (pool 1: rubusoside/steviolbioside, pool 2: dulcoside A/ rebaudioside B; pool 3: rebaudioside C/stevioside; pool 4: rebaudioside C/F; pool 5: rebaudioside A and pool 6: "regeneration" pool (polar glycosides - rebaudiosides D/E/M/N/O). The rebaudioside C/stevioside pool was crystallized from methanol to reduce stevioside concentration and the rebaudioside C rich mother liquors were crystallized from water to provide rebaudioside C in > 95% purity and in multi-gram quantities. Ultimately more than 300 g of high purity rebaudioside C were produced.

Prediction of yield and composition of a chromatographic pool

In the present section some strategies for pooling fractions with similar chromatographic profile and prediction of the yield of the pools are presented. One requirement is that the fraction volumes are consistent, and each fraction is analyzed by HPLC using whatever method is most appropriate. The large normal phase chromatography from a mixture of steviol glycosides in which, rebaudioside A, stevioside and rebaudioside C are the major compounds is used as the example (details above). The fractions were 1 L and aliquots from each fraction were analyzed using the RP-C18 method 2 (Figure 1b) described above. Herein, we restricted the chromatographic analysis to fractions from 10 until 65. In Supplementary Information section are shown the retention times of each diterpene glycoside in the mixture and the area of each peak *per* fraction.

The area was plotted *versus* the fraction number and retention times and/or name of the target, thus the chromatographic profile after the separation process was generated (Figure 2). Thus, we are able to predict the yield and composition of the selected pool (Table 2).

Following these strategies, most of the known compounds reported from *Stevia rebaudiana* and other novel targets still undergoing characterization have been purified by Ironstone. Several of them are available in gram quantities (Table 3).

Table 2. Prediction yield and composition of the pools

SG	Reb A	Stev	Reb F	Reb C	Dulc A	Unknown	Rub	Reb B	Steviolbioside	ΣTAC *per* ap / mAU
RT	14.8	15	15.8	16.1	16.5	16.8	17.8	19.1	19.6	
ΣTAC *per* p	17878	19456	2671	16548	2582	2374	4735	4160	1702	
Pool 30-33										
ΣTAC *per* sp	0	4605	0	378	128	434	0	159	0	5704
R. Pur / %	0	80.73	0	6.63	2.24	7.61	0	2.79	0	
Yield / %	0	23.67	0	2.28	4.96	18.28	0	3.82	0	
Pool 54-65										
ΣTAC *per* sp	16976	0	425	456	0	0	0	0	0	17857
R. Pur / %	95.07	0	2.38	2.56	0	0	0	0	0	
Yield / %	99.88									

SG: Steviol glycosides; Reb: rebaudioside; RT: retention times; ΣTAC *per* p: total area *per* peak (mAU) = $\sum_{i=1}^{n}$ fri, fr: area of a peak in the fractions, n: total number of fractions; ΣTAC *per* sp: total area of selected pool (mAU) = $\sum_{i=m}^{n}$ fri, m: first fraction of the pool, n: last fraction of the pool; ΣTAC *per* ap: total area of all peaks in a pool (mAU); R. Pur: relative purity.

Table 3. Diterpene glycosides made available by Ironstone Separations Inc.

Reb C / g	Reb D / g	Reb E / g	Reb F / g	Reb G / g	Reb H / g	Reb I / g	Reb J / g	Reb M / g
363.50	17.25	4.19	6.00	3.53	1.5	0.41	1.1	26.04
Reb N / g	Reb O / g	Stevioside / g	Dulc A / g	Rubusoside / g	Steviol / g	Iso Reb A[a] / g	Iso Stevioside[a] / g	
7.68	10.18	140.00	6.50	6.50	2.00	2.00	2.00	

Reb: rebaudioside; [a]Iso Stevioside and Iso Rebaudioside A were obtained through chemical modification but both steviol glycosides, with an exocyclic double bond, have been already isolated from *Stevia rebaudiana* commercial extract.[10,11]

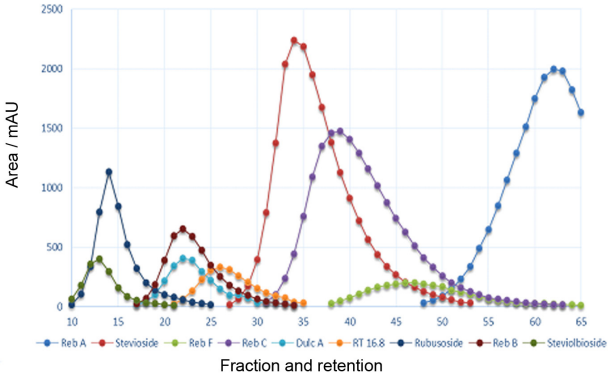


Figure 2. Chromatographic profile of the separation process.

Conclusions

The development of six HPLC analytical techniques led us to better understand the regions where less and very polar steviol glycosides are best resolved: JECFA modified between (rebaudioside A-B), the alternative RP-C18 method (rebaudioside M-A); Synergi Polar RP (earlier than rebaudioside E and between rebaudioside N-A; amino (rebaudioside F-A and rebaudioside A-E); HILIC and Sepax-Diol (rebaudioside D-O). We also showed that the scale up of the purification process of steviol glycosides using large normal phase chromatography should be more economic and efficient than reversed phase chromatography, supported by the economic isolation of several steviol glycosides in gram quantities.

Supplementary Information

Supplementary data are available free of charge at http://jbcs.sbq.org.br as PDF file.

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