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Employing ultra high pressure liquid chromatography as the second dimension in a comprehensive two-dimensional system for analysis of *Stevia rebaudiana* extracts

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ABSTRACT

Stevia rebaudiana extracts and plant materials are increasingly used as natural sweeteners. Polyphenolic and stevioside compounds contained in *S. rebaudiana* extracts were separated by comprehensive LC. A polyamine column operated in normal phase mode was used for the first dimension separation (D1), and a UHPLC C18 column operated in reversed phase mode was used for the second dimension separation (D2). The sub-2 μm column (2.1 mm × 30 mm, maintained at 70 °C) and the UHPLC pump employed for D2 elution allowed a separation/cycle time of 20 s, with a backpressure oscillating between 805 and 922 bar at 3.4 mL/min. The reduced D2 cycle time allowed 3–12 D2 samplings for each peak eluted by D1. Polyphenolic and stevioside compounds were identified by combining the information coming from the position of the compounds in the 2D plot and UV spectra with that of reference materials.

1. Introduction

Stevia rebaudiana is a perennial shrub of the *Asteraceae* (*Compositae*) family native to certain regions of South America (Paraguay and Brazil). Often referred to as "the sweet herb of Paraguay", the plant material has been used by the native population to sweeten teas for a long time. Currently, *Stevia* plant or extracts are used as sweeteners in South America, North America, Asia and in some European countries.

Clinical studies have suggested health benefits from consumption of *Stevia* components as sweeteners by patients affected by diabetes mellitus, obesity, hypertension and caries [1,2]. In addition, stevioside (the most abundant sweet component) was reported to reduce the colony-forming ability of food-borne pathogenic bacteria [3,4].

Recent toxicological studies conducted on stevioside noted that this compound did not show mutagenic, teratogenic or carcinogenic effects, and did not cause allergic reactions when used as a sweetener [5]. The *in vitro* antioxidant activities of *S. rebaudiana* leaves and callus have been investigated by Tadhani et al. This work suggested that the use of the leaf and callus extracts instead of pure stevioside as a sweetener for home consumption or food processing might increase the content of antioxidants in the final product [6].

Stevia glycosides are the compounds responsible for the sweet taste of *Stevia* plant material (Fig. 1). Stevioside was reported to be the most abundant glycoside (4–13% w/w) in plant leaves, followed by rebaudioside A (2–4% w/w), rebaudioside C (1–2% w/w) and dulcoside A (0.4–0.7% w/w) [7]. Steviolbioside, rebaudioside B, rebaudioside D, rebaudioside E and rebaudioside F were also identified in leaf extracts as minor components [8,9]. Steviol is the common aglycone backbone of these compounds. In addition to sweet glycosides, *Stevia* extracts were also reported to contain flavonoids, sterebins A to H, triterpenes, volatile oil components, pigments, gums and inorganic constituents [10–12]. *Stevia* glycosides have been separated by liquid chromatography and by electrophoresis, coupled with UV, MS and ELS detection. [7–9,13–17].

The most common approach for the separation of *Stevia* compounds is normal phase chromatography. HPLC columns functionalized with primary and secondary amines have been used for the separation of *Stevia* glycosides [7,8,14] due to their hydrogen bonding capabilities and polarity. Amino-based stationary phases were shown to provide separation for the two pairs of stevioside glycosides with the same molecular weight (stevioside and rebaudioside B, rebaudioside A and rebaudioside E) contained in most *Stevia* extracts [8], allowing simultaneous quantitation of all *Stevia* sweet components in a single HPLC–MS analysis.

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Comparing separations reported in the literature, reversed phase (RP)-HPLC showed an inferior selectivity for the separation of glycosides contained in *Stevia* compared to normal phase (NP)-HPLC [8,18,19]. RP-HPLC can be used for independent verification of quantitative data obtained by NP-HPLC, and for the simultaneous quantitation of compounds belonging to other classes.

Rajbhandari and Roberts identified six flavonoid glycosides in the leaves of *S. rebaudiana* [11]. Li et al. recently identified a new acetylated quercetin glycoside from the leaves of *S. rebaudiana* Bertoni [12]. Caffeoyl derivatives and flavonols have also been detected by capillary electrophoresis [14].

Overall, both NP-HPLC and RP-HPLC have been used when targeting the simultaneous separation/quantitation of compounds belonging to different classes contained in *Stevia* extracts [7,8,18,19]. When both orthogonal approaches were applied to the separation of the same class of compounds, a different elution pattern was observed. Neither RP-HPLC nor NP-HPLC applied alone has been capable of separating all the components of interest contained in *Stevia*.

The separations provided by the two orthogonal NP- and RP-HPLC can be combined in a single analysis by applying comprehensive two-dimensional liquid chromatography (LC × LC), which can provide enhanced separation capabilities compared to monodimensional HPLC [20–22]. In contrast to multidimensional heart-cutting separation techniques (LC–LC), in which only a selected fraction of the D1 eluent is transferred to D2 [23], in LC × LC, the entire D1 eluate is divided into fractions that are injected online onto the D2 column. Fig. 2 shows the scheme of the apparatus utilized in this work. The two two-positions valve rotate cyclically and simultaneously, and in opposite directions alternating the two loops. One loop collects the D1 eluate and the other injects the eluate collected during the previous cycle to D2.

Several combinations of D1 and D2 separation mechanisms can be applied in LC × LC [24,25]. LC × LC systems have been developed to separate molecules in biological systems [26,27], natural products [8,28–34] and other complex mixtures [35–39]. A common factor of all the LC × LC separations reported in the literature is the optimization of the D2 separation toward its shortest elution time. A shorter D2 separation time, that corresponds to the D1 collection time, allows a higher number of D2 samplings for each peak separated by D1. Most D2 separations were achieved using either short columns packed with small particles [8,27,28,30–35,37–39], or monolithic columns [8,28,29,36] which are capable of higher flow rates and lower backpressures compared to equivalent dimension standard HPLC columns. Superficially porous particles have recently been utilized for LC × LC based on their adaptability to fast chromatography, and polyphenolic antioxidants in wines and in plant extracts were separated under HPLC conditions [30–32]. In two recent studies, the application of ultra high performance liquid chromatography (UHPLC) and sub-2 μm particles for D2 separations reduced the elution time, increased the detection sensitivity and allowed more cuttings for each peak separated by D1. [33,37]. However, none of these studies took full advantage of the UHPLC capabilities. In one case, the D2 separation was achieved at the maximum backpressure of 520 bar [33], in the other of 580 bar [37]. The performances of superficially porous particles and sub-2 μm particles have been compared by Zhang et al. [40] and Fountain et al. [41].

In this work, an LC × LC method was developed for the simultaneous separation of *Stevia* components belonging to different classes. A micro-polyamine HPLC column in NP mode was chosen for the D1 separation and a sub-2 μm particle C18 column operated under UHPLC conditions in RP mode was selected for the D2 separation. The identification of the *Stevia* components was achieved by comparison with the elution and the UV spectra of reference materials.

2. Experimental

2.1. Chemicals

Standards of steviol, steviolbioside, stevioside, rebaudioside A, rebaudioside B, rebaudioside C, dulcoside A, apigenin-7-*O*-glucoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-rhamnoside, luteolin-7-*O*-glucoside and quercetin-3-*O*-rutinoside were purchased from Chromadex (Irvine, CA, USA). The structure of all these compounds is reported in Fig. 1. Phosphoric acid was purchased from Baker (Phillipsburg, NY, USA), formic acid from Sigma–Aldrich (St. Louis, MO, USA) and acetonitrile (HPLC and HPLC/MS grades) from Fischer Scientific (Fair Lawn, NJ, USA). Deionized water, 18 MΩ resistance, was produced by a Water Ultrapure unit.

The *S. rebaudiana* leaf powder, from Brazil, was purchased from Raintree Nutrition, Inc. (Carson City, NV, USA).

2.2. Standard solutions and sample preparation

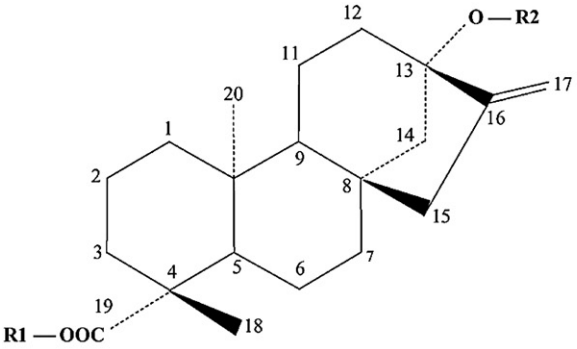
Stevia glycoside stock solutions at the concentration of 1 mg/mL were prepared by dissolving the reference materials in 80:20 acetonitrile/water (v/v). A mixed solution of *Stevia* glycosides at a concentration of 143 μg/mL each was prepared by combining known amounts of the stock solutions and diluting with 80:20 acetonitrile/water to final concentration. One gram of *Stevia* powder was placed in a 20 mL screw cap test tube and was extracted by vortex mixing for 10 min at ambient temperature with 10 mL of 80:20 acetonitrile/water. The tube was subsequently centrifuged at 5000 rpm for 5 min and a portion of the supernatant was filtered through a 0.45 μm PTFE membrane (Whatman, Florham Park, NJ, USA).

2.3. LC × LC apparatus

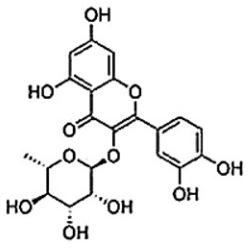
LC × LC separations were carried out using the combination of an Agilent capillary liquid chromatograph (Agilent Technologies, Wilmington, DE) for D1 and an Agilent UHPLC for D2 as shown in Fig. 2. D1 separations were achieved using a 1200 capillary binary pump (G4226A) equipped with a 1200 degasser (G1379B) and an Agilent 1290 Infinity autosampler (G1376A). D2 separations were achieved using an Agilent 1290 Infinity UHPLC binary pump (G4220A) equipped with a 1290 Infinity column heating compartment (G1316C) and a 1290 Infinity PDA detector (G4212A) operated at 80 Hz. High pressure and low pressure fluidic connections were made with Dionex Viper capillary connectors (Sunnyvale, CA, USA). LC × LC modulation was achieved by a combination of two VICI high speed/high pressure two-position six-ports valves with a micro-electric actuator (model C72VX-6696EH, 1034 bar, VICI, Houston, TX). The volume of the two collecting loops was 20 μL. Data acquisition and system control, including operating the two switching valves by external contact closures, were provided by Agilent Chemstation for LC 3D Systems (Rev.B.04.02). LC × LC data visualization and re-elaboration into two- and three-dimension chromatograms were achieved using the Chromsquare ver. 1.1 software (Chromaleont, Messina, Italy).

2.4. LC × LC chromatographic conditions

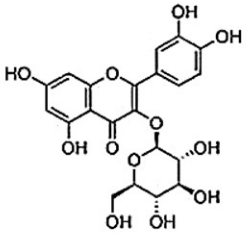
D1 separation was achieved using a YMC-Pack PA-G column (YMC, Allentown, PA, USA, 1.0 mm × 250 mm, 5 μm, PG12S05-2510WT) maintained at ambient temperature, and a gradient of water (A) and acetonitrile (ACN, B), both adjusted to pH 3.0 with H_3PO_4 (0.004% v/v), as follows: 0–50 min, from 5 to 25% A; 50–85 min, from 25 to 70% A; 85–100 min 70% A isocratic; 100–101 min, from 70 to 5% A. The flow rate was 20 μL/min, yielding 26 bar of backpressure at the injection time, and the injection



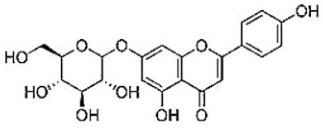
Compound	R_1	R_2
Steviol	H	H
Steviolbioside	H	β-Glc-β-Glc(2-1)
Stevioside	β-Glc	β-Glc-β-Glc(2-1)
Rebaudioside A	β-Glc	β-Glc-β-Glc(2-1) β-Glc(3-1)
Rebaudioside B	H	β-Glc-β-Glc(2-1) β-Glc(3-1)
Rebaudioside C	β-Glc	β-Glc-α-Rha(2-1) β-Glc(3-1)
Dulcoside A	β-Glc	β-Glc-α-Rha(2-1)



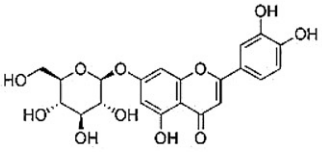
Quercetin-3-O-rhamnoside



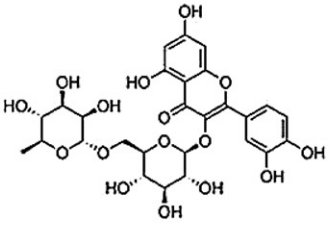
Quercetin-3-O-glucoside



Apigenin-7-O-glucoside



Luteolin-7-O-glucoside



Quercetin-3-O-rutinoside

Fig. 1. Structures of *Stevia rebaudiana* glycosides and polyphenols investigated in the work.

volume was 5 μL. The pH of the mobile phase was continuously monitored over the entire 2D analysis.

D2 separation was achieved using a custom packed Agilent Zorbax RRHD SB-C18 UHPLC column (2.1 mm × 30 mm, 1.8 μm, 1200 bar), maintained at 70 °C, and a gradient of the same eluents used for D1 as follows: 0.00–0.01 min, 5% B isocratic; 0.01–0.27 min, from 5 to 70% B; 0.27–0.28 min, from 70 to 5% B; 0.28–0.33 min, 5% B isocratic. The total time of separation including reconditioning was 0.33 min, corresponding to 20 s. The flow rate was 3.4 mL/min, and the pressure before starting the gradient cycle was 883 bar. The PDA signal was acquired from 200 to 400 nm with 80 Hz sampling rate, no reference, 0.025 s time constant, and using a 1 μL detector cell. Single wavelength chromatograms were extracted at 210 nm for detection of stevioside glycosides, and at 280 nm for other compounds. The LC × LC modulation time was 20 s. All the analyses were run in triplicate.

3. Results and discussion

D1 and D2 separations were at first optimized independently, then combined and tuned together. Unknown compounds separated in the *S. rebaudiana* extract were identified by comparison with reference materials.

3.1. First dimension separation

NP chromatography was chosen for D1 separation based on the lack of availability of UPLC/UHPLC amino columns that could be used for D2 separation, on the lower adaptability of these columns to fast chromatography (needed for D2 separation) and on the higher selectivity of amine columns for the *Stevia* glycosides compared to RP columns. Polyamine columns (weak anion exchangers) in combination with aqueous-organic mobile phase show some

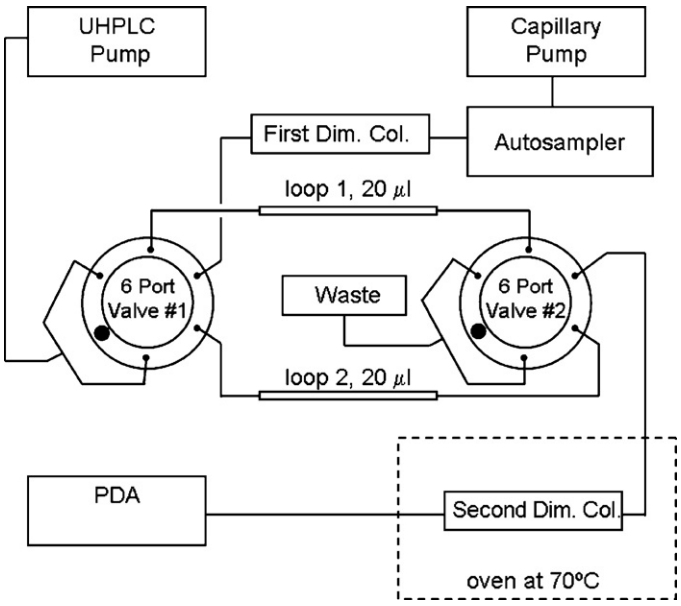


Fig. 2. Scheme of the apparatus employed for the 2D-LC separations. The two two-positions valve rotate cyclically and simultaneously, and in opposite directions alternating the two loops. One loop collects the D1 eluate and the other injects the eluate collected during the previous cycle to D2.

characteristic features of a HILIC system, probably including a contribution by an ion-exchange mechanism. Primary and secondary amine columns provided a similar pattern of separation. The secondary amine column YMC-Pack PA-G provided more reproducible retention times and higher stability when eluting with a high water mobile phase, compared to the primary amine column YMC-Pack NH_2. According to our preliminary results, the most reproducible and suitable separation for mono-dimensional chromatography was achieved with a Dionex Acclaim Mixed-Mode WAX-1 column. In particular, the partial RP character of this stationary phase provided retention and separation of Steviol that was unretained by amine functionalized columns (data not shown). However, the secondary amine column YMC-Pack PA-G, due to its retention properties, was chosen over a mixed mode column for hyphenation with the second dimension RP column. The D1 elution profile and retention times were observed to be highly dependent on the exact pH of the mobile phase, probably as a consequence of the anion exchange interactions affecting the ionization of weakly acidic compounds. Both solvents were prepared fresh daily and the pH was carefully adjusted to 3.0. During each 20 s modulation cycle, at the D1 flow rate of 20 μL/min, 6.7 μL of D1 eluent were transferred to D2. We used 20 μL collecting loops in this separation. It is possible that the use of smaller loops might have resulted in a slight improvement in the focusing of the second dimension injection. However, van der Horst et al. have demonstrated that for ensuring true comprehensiveness, the size of the loop must be significantly larger than the volume of the fraction being transferred because of the parabolic flow profile in the loop [42].

3.2. Second dimension separation

D2 separation was achieved by RP chromatography using an Agilent Zorbax RRHD Stable Bond C18 column. The chromatographic system was capable of operating at a backpressure of 1200 bar up to 2 mL/min flow rate, decreasing to 800 bar at 5 mL/min. Elution parameters were optimized as a compromise between the chromatographic separation, modulation time, maximum column operating temperature with negligible degradation, and highest operating UHPLC flow rate before reaching the maximum system backpressure. The Agilent Zorbax RRHD Stable Bond C18 column was preferred based on the separation of *Stevia* extract components, its stability with high water acidic solvents (at up to pH 2), its ability to operate at backpressures up to 1200 bar, and at temperatures up to 90 °C. Earlier method development relied on the separation provided by a commercially available 2.1 mm × 50 mm RRHD SB-C18 column. A modulation time of 30 s was achieved by setting the column temperature to 80 °C, the flow rate of 2.5 mL/min, and using an elution gradient of water (pH 3) in ACN from 5 to 90% over 18 s. During the gradient, the backpressure fluctuated from 802 to 928 bar. D2 separation could be further sacrificed to reduce the modulation time, and the elution temperature was decreased to reduce noticeable column degradation after less than 50 h of operation. In order to obtain null or negligible degradation, we decreased the D2 column compartment temperature to 70 °C. As a negative consequence, a lower elution temperature caused a higher system backpressure. The reduction of the modulation time was achieved by replacing the 2.1 mm × 50 mm SB-C18 column with a custom packed 2.1 mm × 30 mm SB-C18 column, by increasing flow rate to 3.4 mL/min, and decreasing the maximum organic gradient concentration to 70% ACN. Although the system could be operated at the maximum of 3.5 mL/min, 3.4 mL/min was preferred to avoid random system overpressure failures. During the elution gradient the highest system backpressure was observed in correspondence with the 20% ACN content in water, and then the backpressure quickly decreased about 200 bar in few seconds. The eluting solvent was heated at 70 °C through a heat exchanger before entering the D2 column, and chilled to the detector cell temperature afterward.

3.3. Overall optimization of HPLC × UHPLC system

The most challenging aspect in combining an NP and an RP elution system into an LC × LC apparatus is that the strong eluting solvent of one becomes the weak eluting solvent in the other, and vice versa. The result is the difficulty in achieving D2 peak focusing when transferring to D2 an aliquot of the strong eluting D1 effluent. In the current study, peak focusing and band broadening issues were avoided by applying a D1 flow rate of 20 μL/min and a 20 s modulation time, resulting in 6.7 μL of D1 eluent injected onto the D2 stream at 3.4 mL/min each cycle.

The two high speed six ports switching valves configuration was chosen over a single high torque 8–10 ports valve to decrease the rotation time to as low as 129 ms as stated by the manufacturer. Rotation time is a critical parameter when performing very rapid separations in the second dimension of a comprehensive LC system.

The performance of the system was evaluated by calculating its theoretical peak capacity. The peak capacity n_c of a one-dimensional separation system, utilizing a gradient elution, can be calculated from the equation [43]:

$$n_c = 1 + \frac{t_g}{(1/n)\sum_1^n w}$$

in which t_g is the gradient run time, n is the number of peaks selected for the calculation, and w is the average width of the n peaks. Total peak capacity $n_{c,tot}$ of the LC × LC system is calculated by multiplying the mono-dimensional peak capacities of D1 and D2. The D1 peak capacity of our system was 68, that of D2 was 51, and of the overall LC × LC system was 3468. However, the theoretical peak capacity does not characterize the real performance of a 2D separation system, especially since it does not account for the loss of resolution during fraction modulation. For this reason, we considered several other useful and realistic approaches. We calculated the practical peak capacity according to Liu et al. and obtained a value of 1850 [44].

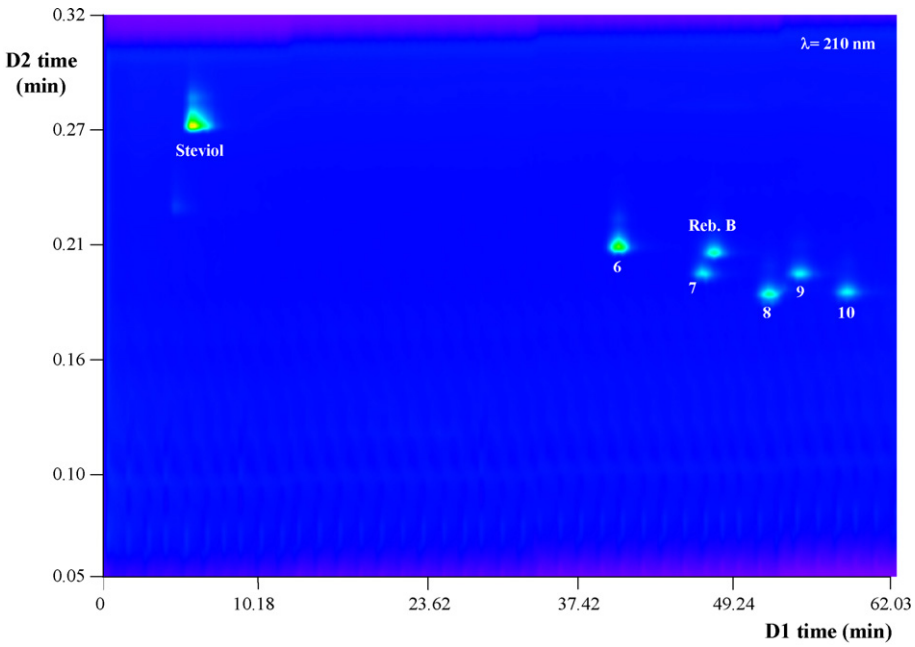


Fig. 3. LC × LC separation of *Stevia* glycoside reference materials monitored at 210 nm, obtained using a YMC-Pack PA-G column (1.0 mm × 250 mm, 5 μm) in the first dimension and a custom packed Agilent Zorbax RRHD SB-C18 UHPLC column (2.1 mm × 30 mm, 1.8 μm, 1200 bar), in the second dimension. Peak assignment as in Table 1.

A minimum of three D2 samplings was achieved for each D1 peak by operating the 1 mm I.D. D1 column at the flow rate of 20 μL/min, far lower than the optimal ~60 μL/min flow rate for this column diameter, and by applying a long shallow elution gradient.

3.4. HPLC × UHPLC analysis of Stevia extracts

The LC × LC separation was first optimized for the separation of the *Stevia* glycosides utilizing the available reference materials, then the experimental conditions were refined to separate the compounds identified in the following section. Fig. 3 shows the LC × LC separation of available *Stevia* glycoside reference materials, monitored at 210 nm. Fig. 4 shows the separation of a *Stevia* extract monitored at 210 nm. Peak labeling is reported in Table 1. The D2 separation of the D1 dead volume is affected by a local wrap around phenomenon, and it could be eliminated by changing the end of the D2 gradient from 65% to 90% ACN. This change can be applied when quantitation of steviol is desired, but it would affect the separation of all the other compounds. *Stevia* glycosides do not contain any conjugated double bond or aromatic rings or chromophore absorbing at wavelengths higher than 210 nm, thus their UV absorption is limited to 190–210 nm. The other compounds identified in this work, in contrast, showed a strong UV absorption at higher UV wavelengths caused by the presence of a conjugated/aromatic system in their structures. Although these compounds can be detected at 190–210 nm, the more selective 280 nm wavelength was preferred. Fig. 5 shows the separation of a *Stevia* extract monitored at 280 nm. The PDA signal was acquired from 190 to 400 nm to

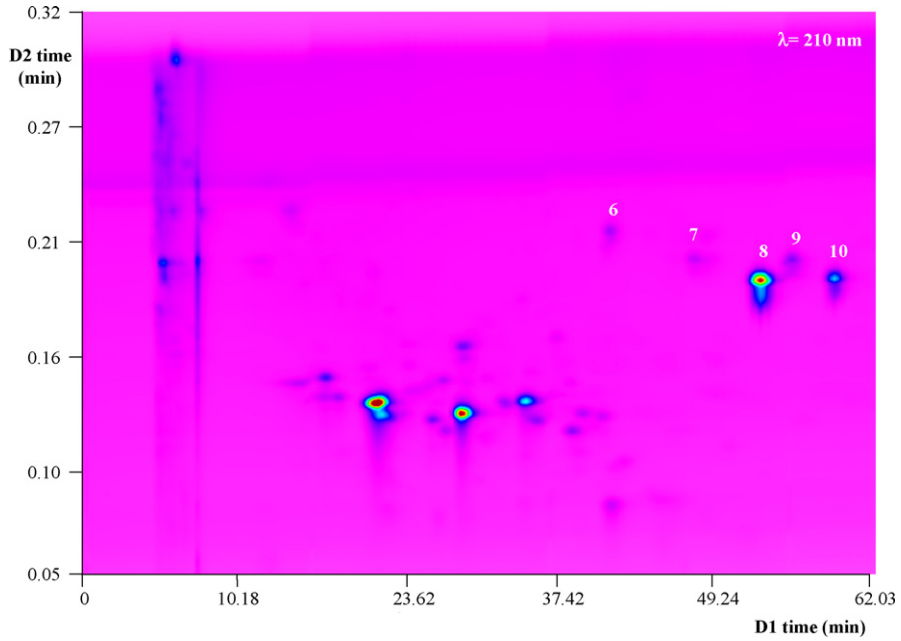


Fig. 4. LC × LC separation of a *Stevia* extract monitored at 210 nm, obtained using the same combination of columns employed for Fig. 3. Peak assignment as in Table 1.

Table 1
LC × LC-PDA profile of *Stevia rebaudiana* extract.

Peak	Molecular formula	UV–vis (nm)	Compounds	D1 t_R (min)*	D2 t_R (min)*
1	$C_{21}H_{20}O_{11}$	256,354	Quercetin-3-*O*-rhamnoside	19.45	0.13
2	$C_{21}H_{20}O_{12}$	256,354	Quercetin-3-*O*-glucoside	24.13	0.13
3	$C_{21}H_{20}O_{10}$	256,354	Apigenin-7-*O*-glucoside	24.28	0.15
4	$C_{21}H_{20}O_{11}$	256,344	Luteolin-7-*O*-glucoside	31.20	0.14
5	$C_{27}H_{30}O_{16}$	256,354	Quercetin-3-*O*-rutinoside	36.95	0.13
6	$C_{32}H_{50}O_{13}$	210	Steviolbioside	40.27	0.22
7	$C_{38}H_{60}O_{17}$	210	Dulcoside A	48.38	0.20
8	$C_{38}H_{60}O_{18}$	210	Stevioside	53.44	0.19
9	$C_{44}H_{70}O_{22}$	210	Rebaudioside C	56.85	0.20
10	$C_{44}H_{70}O_{23}$	210	Rebaudioside A	60.43	0.19

* Mean of three replicates.

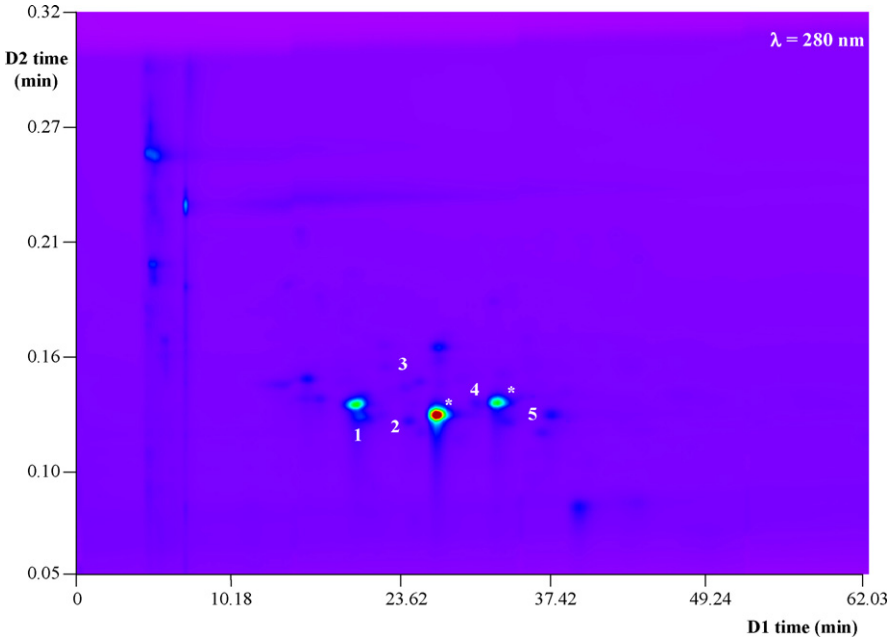


Fig. 5. LC × LC separation of a *Stevia* extract monitored at 280 nm, obtained using the same combination of columns employed for Figs. 3 and 4. Peak assignment as in Table 1. Two additional peaks, labeled with *, may be caffeoyl derivatives as suggested by their UV spectra.

collect the UV spectra of the compounds investigated. Maximum absorbance wavelengths are reported in Table 1. The RSD values for the retention times (mean of 3 replicates) were lower than 5% for both D1 and D2.

3.5. Identification of Stevia active components

Table 1 reports the identification of the *Stevia* constituents on the basis of their retention times on the two columns separately, and by comparison of their diode array spectra with those of reference materials.

Peaks 6–10 represent the *Stevia* glycosides tentatively identified in the extract, all detected at 210 nm. The chromatographic pattern shown in Fig. 4 is characterized by the presence of two main peaks, stevioside (peak 8) and rebaudioside A (peak 10). Steviolbioside (peak 6), dulcoside A (peak 7), rebaudioside C, (peak 9), well separated from the rest of the matrix, are present in lower concentration as already reported [8].

Compounds labeled 1–5 in Fig. 5, which migrate ahead of the *Stevia* glycosides, were characterized as flavonoid compounds. All of them were detected at 280 nm. Peaks 1, 2, 3 and 5 showed UV maxima at 256 and 354 nm, while peak 4 showed UV maxima at 256 and 344 nm. As with *Stevia* glycosides, the identification of the flavonoid components was achieved by comparison of their retention times in both dimensions and UV spectra with those of reference materials. As a consequence, peaks 1–5 were, tentatively, identified as quercetin-3-*O*-rhamnoside, quercetin-3-*O*-glucoside, apigenin-7-*O*-glucoside, luteolin-7-*O*-glucoside and quercetin-3-*O*-rutinoside, respectively.

Two additional peaks, labeled with * in Fig. 5, may be caffeoyl derivatives as suggested by their UV spectra, characterized by two maxima, at 250 and 350 nm and a shoulder at 325 nm [31]. For reliable identification of these compounds, however, more powerful detection methods such as ^{1}H NMR or MS instruments providing adequate MS^n experiments are needed.

4. Conclusions

The comprehensive NP × RP LC system developed in this work allowed the separation of components contained in *S. rebaudiana* extracts. Specifically, we report the separation and identification of 10 glycosides from the extracts.

To the best of our knowledge, this is the first work that reports use of UHPLC conditions in the second dimension performed on a short octadecylsilica column with sub-2 μm particles for separation of *Stevia* components. Peak focusing and band broadening suppression on the top of the D2 column were successfully achieved by decreasing to 6.7 μL the volume of high organic D1 eluent which was subsequently diluted due to the high flow rate employed in the D2 during each modulation cycle.

The platform investigated can be employed for the separation and identification of components of *S. rebaudiana* and those in other complex extracts. Additional work is required to develop a quantitation method for *Stevia* glycosides and other components based on this separation platform. Additionally, more work will be required to optimize the extraction procedure for components of particular interest.

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