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Evaluation of steviol and its glycosides in *Stevia rebaudiana* leaves and commercial sweetener by ultra-high-performance liquid chromatography-mass spectrometry

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ABSTRACT

Stevia rebaudiana leaves contain non-cariogenic and non-caloric sweeteners (steviol-glycosides) whose consumption could exert beneficial effects on human health. Steviol-glycosides are considered safe; nonetheless, studies on animals highlighted adverse effects attributed to the aglycone steviol. The aim of the present study was to develop and validate two different ultra-high-performance liquid chromatography methods with electrospray ionization mass spectrometry (UHPLC-MS) to evaluate steviol-glycosides or steviol in Stevia leaves and commercial sweetener (Truvia®). Steviol-glycosides identity was preliminarily established by UV spectra comparison, molecular ion and product ions evaluation, while routine analyses were carried out in single ion reaction (SIR) monitoring their negative chloride adducts. Samples were sequentially extracted by methanol, cleaned-up by SPE cartridge and the analytes separated by UHPLC HSS C_{18} column (150 mm × 2.1 mm I.D., 1.8 μm). The use of CH_2Cl_2 added to the mobile phase as source of Cl^- enhance sensitivity. The LLOD for stevioside, rebaudioside A, steviolbioside and steviol was 15, 50, 10 and 1 ng ml^{-1}, respectively. Assay validation demonstrated good performances in terms of accuracy (89–103%), precision (<4.3%), repeatability (<5.7%) and linearity (40–180 mg/g). Stevioside (5.8 ± 1.3%), rebaudioside A (1.8 ± 1.2%) and rebaudioside C (1.3 ± 1.4%) were the most abundant steviol-glycosides found in samples of Stevia (n = 10) from southern Italy. Rebaudioside A was the main steviol-glycosides found in Truvia® (0.84 ± 0.03%). The amounts of steviol-glycosides obtained by the UHPLC-MS method matched those given by the traditional LC-NH_2-UV method. Steviol was found in all the leaves extract (2.7–13.2 mg kg^{-1}) but was not detected in Truvia® (<1 μg kg^{-1}). The proposed UHPLC-MS methods can be applied for the routine quality control of Stevia leaves and their commercial preparations.

1. Introduction

The natural sweeteners called steviol-glycosides are diterpenes extracted from a native shrub of Brazil and Paraguay, *Stevia rebaudiana* Bertoni (Stevia), now cultivated in different Asian and European countries. Its leaves contain many different components, such as labdanes, flavonoids, sterols, triterpenoids, chlorophylls, organic acids, mono-disaccharides, and inorganic salts [1]. As to steviol-glycosides, Stevia leaves mainly include stevioside (SV), whose content (4–20%) depends on the cultivar and on growth conditions [2]. Other similar elements are rebaudioside A (Ra, ∼3%); rebaudioside C (Rc, ∼1.5%); dulcoside A (Du, ∼0.5%). Traces of steviolbioside (Sb), of rubusoside (Ru), and of rebaudioside D, E, F can also be found (Fig. 1). Conversely, purified extracts obtained from Stevia leaves to be marketed mainly contain SV (>80%) or Ra (>90%). Stevioside is a non-caloric sweetener, and is considered about 300 times sweeter than solutions containing 0.4% sucrose. In different countries SV is used to sweeten foodstuffs and beverages, while in the US powdered Stevia leaves and their extracts are used only as a dietary supplement and a skin care product but not as a sweetener [3]. Actually, since December 2008, when FDA stated that purified Ra from Stevia can be considered GRAS (Generally Recognized as Safe), it has been in use to edulcorate beverages and some foods (FDA GRAS Notice GRN 000253 and GRN 000252).

Steviol-glycosides had not been approved by the European Commission due to safety concern; later, in 2008 JECFA suggested a 0–4 mg kg^{-1} BW temporary admissible daily intake (ADI) of steviol, equivalent of 0–10 mg kg^{-1} BW stevioside [4]. Scientific literature on SV and its related compounds suggests different potentially beneficial effects on human health deriving from their consumption. Recently, in spite of limits and omissions, Chatsudthipong and Muanprasat [5] reviewed scientific reports on the issue. Due to its

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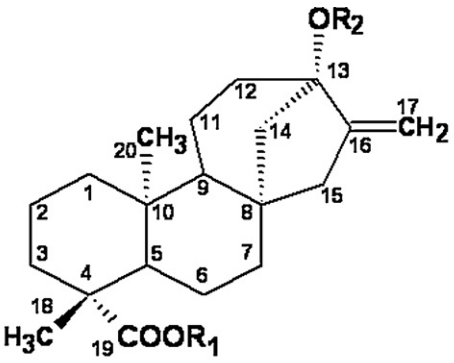

Compound	R_1	R_2	$[M-H]^-$	$[M+Cl^{35}]^-$
Steviol (ST)	H	H	317	
Stevioside (SV)	β-Glc	β-Glc-β-Glc(2→1)	803	839
Rebaudioside B (Rb)	H	β-Glc-β-Glc(2→1)-Glc(3→1)	803	839
Rebaudioside A (Ra)	β-Glc	β-Glc-β-Glc(2→1)-β-Glc(3→1)	965	1001
Rebaudioside E (Re)	β-Glc-β-Glc(2→1)	β-Glc-β-Glc(2→1)	965	1001
Steviolbioside (Sb)	H	β-Glc-β-Glc(2→1)	641	677
Rubusoside (Ru)	β-Glc	β-Glc	641	677
Rebaudioside C (Rc)	β-Glc	β-Glc-β-Glc(2→1)-β-Rha(3→1)	949	985
Dulcoside A (Du)	β-Glc	β-Glc-α-Rha(2→1)	787	823
Steviol glucoside	H	β-Glc	479	515
Steviol glucoside	β-Glc	H	479	515

Fig. 1. Structures of steviol and its glycosides found in *Stevia rebaudiana* leaves from southern Italy.

wide and even increasing consumption, the toxicology of SV has been extensively studied, and related data indicating its being not toxic, not mutagenic, and not carcinogenic were lately reassessed [6–8]. It was also clearly demonstrated that high concentrations of the sweetener Ra administered in the diet of rats over 90 days were not associated with any signs of toxicity [9].

On the other hand, some authors have observed histopathological changes in the liver of rats treated with SV [10]; low oral toxicity in mice, rats, and hamsters [11]; lesions in DNA from peripheral blood, as well as from liver, brain, and spleen cells [12]. Such adverse effects have been ascribed to aglycone steviol (ST) formed by the intestinal microflora activity [13] and not to SV. Therefore, it can be said that the determination of SV, Ra and ST was carefully pursued through different methods as indicated in scientific literature, including enzymatic hydrolysis and chemical detection [14], GC [15], overpressure TLC [16], densitometry [17], HPLC [18–21], capillary electrophoresis [22]. A qualitative LC-TOF method was also proposed to evaluate steviol-glycosides [23], together with a validated HPTLC procedure with densitometric detection [24] and a NIRS procedure for the quantification of steviol-glycosides [25]. Recently, a semi-quantitative determination of ST-glycosides was performed by desorption electrospray ionization mass spectrometry [26]. As to steviol quantification, Minne et al. [27] validated an RP-LC method with fluorometric detection after derivatization by a coumarin by-product. This paper shows the results yielded on the determination of Stevia sweeteners and ST in leaves from southern Italia and in a commercial sweetener named Truvia®. With the aim of qualitative and quantitative evaluation of Stevia sweeteners and ST, we validated an LC-SIR-MS method. Steviol-glycosides identity was established by molecular weight comparison, MS/MS fragmentation study and, when the standard was available, by co-chromatography. Moreover, dichloromethane (CH_2Cl_2) was added to the mobile phase as source of chlorine to enhance sensitivity of steviol-glycosides analysis in negative ESI. The observed outcomes were also compared with the results obtained by using the assay method reported in the JECFA monographs 5 [4]. For the routine determination of ST and its glycosides, a purification step based on solid phase extraction was developed in order to reduce possible interference from hydrophilic compounds, and to extend the column life.

2. Experimental

2.1. Chemicals

Stevioside, rebaudioside A, steviolbioside and steviol were purchased from Chromadex (Laguna Hills, CA, US); their purity was higher than 98%. Ammonium acetate, ammonium formate and dichloromethane were from Merck (Darmstadt, D). Methanol and acetonitrile LC-MS grade were obtained from Sigma–Aldrich (Milan, I). Water was obtained from a MilliQ apparatus (Millipore, Milford, MA, US). Dried Stevia leaves samples ($n = 10$) harvested in different fields were kindly provided by San Demetrio organic farm (Specchia, LE, I). Truvia® single-serve sachets were purchased at the site http://www.truviastore.com.

2.2. Extraction and purification by SPE

2.2.1. Steviol-glycosides

Two hundred grams of dried Stevia leaves (105 °C, 2 h) were first finely grinded into a fine powder, and then passed through a 500 μm (35 mesh) filter. In order to optimize the extraction conditions (leaves amount:solvent volume ratio), the powder was divided into different quantities, from 50 to 800 mg, and sonicated with 20 ml of methanol for 10 min. The mixture was centrifuged at 1500 × *g* for 5 min, and the supernatant transferred into a 25 ml

flask, while the solid residue was extracted twice as described above. Before being injected in the chromatographic system, the volume obtained for every extract was set up by methanol, diluted with methanol, and centrifuged at 4000 × *g* for 1 min.

The alcoholic extract (1 ml) was diluted with water (2 ml); the resulting solution was loaded on a 3 ml HLB Oasys 100 mg SPE cartridge (Waters, Milford, MA, US) pre-activated with methanol (3 ml) and then washed with water (5 ml). Further, the SPE cartridge was then sequentially washed with 3 ml each of water and 40% methanol in water; the steviol-glycosides were eluted from the cartridge using 3 ml of 70% methanol in water and the volume then adjusted to 10 ml by methanol. The solution obtained was serially diluted and centrifuged at 4000 × *g* for 1 min before injecting 2 μl from it in the UHPLC-MS. About 1 g of dried Truvia® powder was sonicated with 15 ml of water and after 10 min the volume was adjusted to 20 ml by water. The solution was diluted, filtered through a 0.22 μm filter and 2 μl injected in the UHPLC-MS system.

2.2.2. Steviol

Preliminarily, dried Stevia leaves divided into quantities with different weights, from 0.2 to 2 g, were sonicated with 15 ml of methanol for 15 min. The obtained mixture was centrifuged at 1500 × *g* for 5 min, and then the solid residue re-extracted using the same procedure twice. Extract solutions were evaporated in vacuum and the dry residue dissolved in 5 ml of methanol; 1 ml from this solution was then diluted 1:1 with water and loaded on a pre-activated 100 mg HLB cartridge (Waters). The SPE cartridge was washed with 3 ml of 70% methanol in water, while ST was eluted from the cartridge using 3 ml of methanol. Steviol containing fraction was evaporated in vacuum and the dry residue suspended in 1 ml of methanol, centrifuged at 4000 × *g* for 1 min before injecting 5 μl in the UHPLC system.

About 1 g of dried Truvia® powder was sonicated with 7 ml of methanol and after 10 min the volume was adjusted to 10 ml by methanol. One milliliter of this solution was diluted 1:1 with water and loaded on a SPE cartridge as described above. Steviol containing fraction was evaporated in vacuum and the dry residue suspended in 0.1 ml of methanol.

2.3. Method validation

The UHPLC-MS methods developed were validated for the following parameters.

2.3.1. Sensitivity

External standards were used to quantify SV, Ra, Sb and ST in Stevia leaves. Calibration curves were constructed for each standard at eight concentration levels; four independent determinations were performed at each concentration and regression analysis was employed to determine the linearity of the calibration graphs. The amounts of Du and Rc in Stevia leaves were evaluated by SV and Ra calibration, respectively, and the resulting data corrected by molecular weight ratios. LLOQ was defined by the lowest injected inter-day concentration whose RSD% resulted to be <20% [28]. LOD was defined by the lowest concentration the assay can differentiate from background levels (S/N ratio > 3).

2.3.2. Accuracy

The accuracy (matrix effect) of the procedure was determined by a recovery test according to the published method [29]. Briefly, three raw samples of Stevia leaves (Sample 4, ~0.1 g) were spiked with different amounts of SV and Ra (1–10 mg), Sb (25–100 μg) and ST (2–10 μg). The spiked samples were extracted under optimized conditions, purified by SPE and the recovery rates for each step were calculated for SV, Ra, Sb and ST. Each sample was extracted in triplicate and analyzed in duplicate.

2.3.3. Specificity

The peak identity related to SV, Ra, Sb and ST was confirmed by co-chromatography with authentic standard, while Rc, Du and ST-glucosides were identified by molecular weight evaluation. Quantitative analysis regarding steviol-glycosides was performed following the ions corresponding to $[M+Cl^{35}]^-$ and $[M+Cl^{37}]^-$.

2.3.4. Precision

Intra- and inter-day precision of the assay was verified by analyzing spiked samples 3 times for 5 consecutive days. Peak purity and identity were confirmed by UHPLC-MS(MS). Precision was confirmed by evaluating standard deviations of the amounts and of retention time.

2.3.5. Ruggedness and robustness

The ruggedness of the proposed UHPLC-MS methods was estimated by two analysts evaluating the amounts of steviol and ST-glycosides in a sample of dried Stevia leaves. Each analyst performed twelve tests, and standard and extract solutions were injected in triplicate. Robustness was estimated by varying several chromatographic conditions such as flow-rate ±0.1 ml min^{-1}, column temperature ±3 °C, organic strength and pH ±10%, cone voltage ±2 eV and capillary voltage ±0.2 kV. Data were analyzed by Wilcoxon test considering significant a level of $p > 0.05$.

2.3.6. Stability studies

The powder of Stevia leaves and solutions of the standards in methanol were stored at 4 and −20 °C, respectively and their stability evaluated up to 30 days. Moreover, standard solutions and Stevia leaves extracts were placed in the autosampler at 20 °C and their stability evaluated overnight.

2.4. Comparative method for the quantitative analysis of steviol-glycosides

The chromatographic system we used consists of a mod. 2695 Alliance (Waters, Milford, MA, US) equipped with a diode array detector mod. 2996 (Waters). Our analyses were carried out by a 5 μm YMC Polyamine II column (250 mm × 4.6 mm, Waters) maintained at 30 °C; the gradient profile was as follows: 20% B for 5 min, from 20 to 25% B in 10 s and then 25% B for 15 min. The eluents were (A) CH_3CN and (B) water, and the flow-rate was 2 ml min^{-1}. For peak identification, chromatographic were acquired in the 190–350 range and integrated at 205 nm.

2.5. Quantitative analysis of steviol-glycosides by UHPLC-MS

The chromatographic system was a mod. Acquity (Waters) coupled to a mod. Quattromicro triple quadrupole mass spectrometer (Micromass, Beverly, MA, US). The analyses were carried out in gradient mode by a 1.8 μm C_{18} HSS column (150 mm × 2.1 mm, Waters) maintained at 80 °C, and the flow-rate was 0.5 ml min^{-1}. The eluents were: (A) 2 mmol l^{-1} ammonium acetate pH 6.5, (B) 0.1% CH_2Cl_2 in CH_3CN, and the gradient was as follows: 40% B for 1.5 min, from 40 to 65% B in 10 s, 65% B for 30 s, from 60 to 85% B in 10 s and then 85% B for 1.5 min.

Routine analyses were carried out in single ion reaction (SIR) mode monitoring the ions with $(m/z)^-$ corresponding to $[M+Cl^{35}]^-$ and $[M+Cl^{37}]^-$. In detail we have: $(m/z)^-$ 551 and 553 for steviol-monoglucosides, $(m/z)^-$ 677 and 679 for steviolbioside, $(m/z)^-$ 823 and 825 for dulcoside A, $(m/z)^-$ 839 and 841 for stevioside, $(m/z)^-$ 985 and 987 for rebaudioside C, $(m/z)^-$ 1001 and 1003 for rebaudioside A. The capillary voltage was set to 3.0 kV while the cone

voltage was specific for each compound. The source temperature was 130 °C and the desolvating temperature was 380 °C.

2.6. Quantitative analysis of steviol by UHPLC-MS

The chromatographic system consisted of a UHPLC – mod. Acquity (Waters) – equipped with a triple quadrupole mass spectrometer mod. Quattromicro (Micromass). A 1.8 μm HSS C_{18} column (100 mm × 2.1 mm, Waters) was used for the separation at a flow-rate of 0.6 ml min^{-1}. The column was maintained at 60 °C and the isocratic separation was performed using a solution containing 5 mmol l^{-1} ammonium acetate pH 6:CH_3CN (45:55, v/v) as eluent. Mass spectrometer operated in negative SIR mode monitoring the ions with $(m/z)^-$ 317, with a dwell time of 0.1 s. The capillary voltage was 2.7 kV, and the cone voltage was 36 V. The source and desolvating temperature was 120 and 350 °C, respectively.

2.7. Calibration curves

The calibration curves were obtained from SV, Ra, Sb and ST mother solutions prepared by dissolving 10 mg of each dried standard (105 °C, 2 h) in 10 ml methanol. The working solutions were prepared in methanol in the range of 0.01–10 μg ml^{-1} for SV, Sb, Ra and 1–100 ng ml^{-1} for ST. Mothers and working solutions were stored at −80 and −20 °C, respectively. Calibrations were performed following the ion corresponding to $[M+Cl^{35}]^-$.

3. Results and discussion

3.1. Sample preparation

3.1.1. Steviol-glycosides

Three subsequent extractions were preliminarily applied to extract ST-glycosides present in the samples of Stevia leaves, and the extracted amount following the first and second extraction was 95.4 ± 1.1 and 4.7 ± 0.4%, respectively. In the third extract only small amounts (≈0.3%) of SV were found, thus for the routine analysis two extractions were performed. The extraction procedure was linear in the range of 50–500 mg dried Stevia leaves, thus about 100 mg were used for the routine analysis of ST-glycosides. For intra-day precision, five aliquots of the same sample were extracted one-time and analyzed by the proposed method. Percentage RSD of method precision was in the range of 3.1–5.3%. The inter-day precision was evaluated on three different days and the %RSD was in the range of 3.7–6.1%.

3.1.2. Steviol

The extracted amount following the first and second extraction was 88.6 ± 2.1 and 11.4 ± 0.5%, respectively. Steviol was not detected in the third extract, thus for the routine analysis two extractions were performed. The extraction procedure was linear in the range of 0.6–1.5 g dried Stevia leaves, thus about 1 g was used for the routine analysis of ST. For intra-day precision, three aliquots of the same sample were extracted two-times and analyzed. Percentage RSD of method precision was in the range of 6.3–9.4%. The inter-day precision was evaluated in three different days and the %RSD was in the range of 7.2–10.3%.

3.1.3. SPE of steviol and its glycosides

Different Silica-C_{18} and polymeric sorbents were tested to determine which combination of sorbent and eluent could provide the best results. For their handiness, versatility and stability, polymer sorbents were preferred to traditional C_{18}-based SPE material for the purification of Stevia sweeteners and ST from Stevia leaves extract. All the steviol-glycosides were eluted from the cartridge by a solution containing 70% of methanol in water, and the recoveries for all the analytes were in the range of 93–103%. Regarding ST, the methanolic fraction contained 93.1 ± 1.8% of the loaded steviol, while for the spiked solutions the recovery was in the range of 95–103%.

Table 1
Recovery test of the UPLC-MS assay.

Compounds	Added (mg)	Detected (mg)	Recovery (%)	RSD (%)	Error (%)
SV	0	4.1		6.1	
	2.5	6.6	100	5.5	0.6
	5	8.9	98	4.1	2.0
	10	14.2	101	3.2	0.6
Ra	0	1.3		7.7	
	1	2.2	101	6.5	0.7
	2.5	3.6	98	5.3	2.4
	5	6.5	102	3.3	1.6
	Added (μg)	Detected (μg)	Recovery (%)	RSD %	Error %
Sb	0	300			
	25	315	99	6.3	1.2
	50	340	100	5.2	0.3
	100	387	99	3.9	0.4
ST	0	4		4.5	
	2	5.4	95	2.8	4.7
	5	8.5	101	1.3	0.7
	10	13.1	99	1.0	0.2

The added (*x*-axis) and detected amounts (*y*-axis) were plotted to calculate the slope (m) and the intercept (q) values; these data were then employed to find the theoretical data corresponding to the added amount. The equations were the following:

$$\text{SV}: m = 1.008,\ q = 4.04;\ \text{Ra}: m = 1.004,\ q = 1.18;\ \text{Sb}: m = 0.889,\ q = 296.6;\ \text{ST}: m = 0.926,\ q = 3.814$$

$$\text{Recovery\%} = \left(\frac{\text{detected amount}}{\text{theoretical amount}}\right) 100$$

$$\text{Error\%} = \left[\frac{\text{detected amount} - \text{theoretical amount}}{\text{theoretical amount}}\right] 100$$

3.2. Method validation

3.2.1. Sensitivity

The calibration curve was linear in the range of 0.05–10 μg ml^{-1} for SV, 0.1–10 μg ml^{-1} for Ra, 0.025–10 μg ml^{-1} for Sb, and 5–100 ng ml^{-1} for ST. The LLOD for SV, Ra, Sb and ST was 15, 50, 10 and 1 ng ml^{-1}, respectively. The equations of calibration curve were as follows:

$$\text{SV}: Y = 3950X + 25 \quad (R^2 = 0.999)$$
$$\text{Ra}: Y = 1440X + 17 \quad (R^2 = 0.998)$$
$$\text{Sb}: Y = 7605X + 38 \quad (R^2 = 0.999)$$
$$\text{ST}: Y = 13.3X - 1 \quad (R^2 = 0.997)$$

where Y = peak area of the ion corresponding to $[M+Cl^{35}]^-$, X = μg ml^{-1} for SV, Ra and Sb and X = ng ml^{-1} for ST.

3.2.2. Accuracy

The percentage mean recovery values of the extraction for SV, Ra, Sb, and ST from spiked dried leaves samples was in the range of 93–103 and of 89–94%, respectively (Table 1).

3.2.3. Specificity

Peak identity was confirmed by RT comparison with authentic standard (SV, Ra, Sb and ST), molecular weight and $[M+Cl^{35}]^-/[M+Cl^{37}]^-$ evaluation.

3.2.4. Precision

The intra- and inter-day precision (n = 5) was evaluated by analyzing the spiked samples in triplicate; for SV-glycosides and ST

the repeatability resulted to be in the range of 1.8–3.5% and lower than 4.2%, respectively. Regarding inter-day precision, the %RSD varies from 2.2 to 3.8%, and shows lower than 5.7% for ST-glycosides and ST, respectively. The %RSD of the retention times was lower than 0.5%. For SV and Ra linearity was tested in the range of 40–180 mg g^{-1} dried leaves.

3.2.5. Ruggedness and robustness

The results relative to the effect of an external factor on the degree of reproducibility of the UHPLC-MS methods were compared statistically and there was no significant difference in the amount of steviol and its glycosides in the analyzed Stevia leaves extracts ($p = 0.530$, $Z = 0.627$). Regarding robustness, slight variations in buffer composition and pH, flow-rate and column temperature did not change the peak shape and resolution. Moreover moderate variations in cone (±2 eV) and capillary (±0.2 kV) voltage did not influence significantly the quantization of steviol and its glycosides. Thus, the proposed methods were found to be very robust and rugged.

3.2.6. Stability studies

The mean of steviol-glycosides recovered from the dried Stevia leaves stored at 4 °C for up to 30 days were consistent (>95%) and steviol was not found indicating that its glycosides were not hydrolyzed. Standard solutions and Stevia leaves extracts in methanol resulted stable in the autosampler at 20 °C overnight (RSD < 2.1%). Standard solutions stored at −20 °C for up 30 days showed a slight reduction (about 3.6%) for all the analytes.

3.3. Optimization of UHPLC-MS conditions for the analysis ST-glycosides

In a preliminary phase, different sub-2 μm and traditional HPLC columns were tested in order to optimize the condition of separation. The tested LC narrow-bore columns (150 mm × 2.1 mm, 3.5 μm) were Sunfire C_{18} (Waters), Symmetry C_{18} (Waters), Atlantis C_{18} (Waters), X-Bridge C_{18} (Waters), Luna C_{18}(2) (150 mm × 2.0 mm, 3 μm, Phenomenex, Torrance, CA, US), Luna C_{18}(2) (100 mm × 2 mm, 2.5 μm) and Hydro C_{18} (150 mm × 2 mm, 4 μm, Phenomenex). The UHPLC columns tested were BEH C_{18} (100 and 150 mm × 2.1 mm, 1.7 μm), HSS and HSS SB C_{18} (150 mm × 2.1 mm, 1.8 μm) and Hilic (100 mm × 2.1 mm, 1.7 μm) from Waters. The separation efficiency and peak shape obtained through the UHPLC columns were better than 2.5, 3.5 and 4 μm columns; for steviol-glycosides the best performance was achieved by a 150 mm × 2.1 mm HSS C_{18} column.

Regarding MS conditions, the determinations were carried out in negative ion mode as the analytes signal was about 10 times higher than in positive ion mode. The higher sensitivity in negative mode was due to CH_2Cl_2 added to the mobile phase as source of chlorine. For all the steviol-glycosides, the LC-MS analysis showed the presence of molecular ion chlorine adduct $[M+Cl^{35}]^-$ and $[M+Cl^{37}]^-$, while in MS/MS fragmentation these compounds displayed a consecutive loss of sugar moieties and the constant presence, at higher collision energy, of a product ion with $(m/z)^-$ 317, corresponding to the aglycone steviol. In Fig. 2, the fragmentation pattern of the SV at different collision energy (10–30 eV) is reported. Critically remarkable, for all steviol-glycosides an ion corresponding to $[M-162]^-$ was present even at lower cone voltage, which indicates that these compounds could easily lose a glucose moiety particularly in the presence of a pH below 4.5 (data not shown). Consequently, the cone voltage value and the pH of the buffer are key parameter to their correct identification.

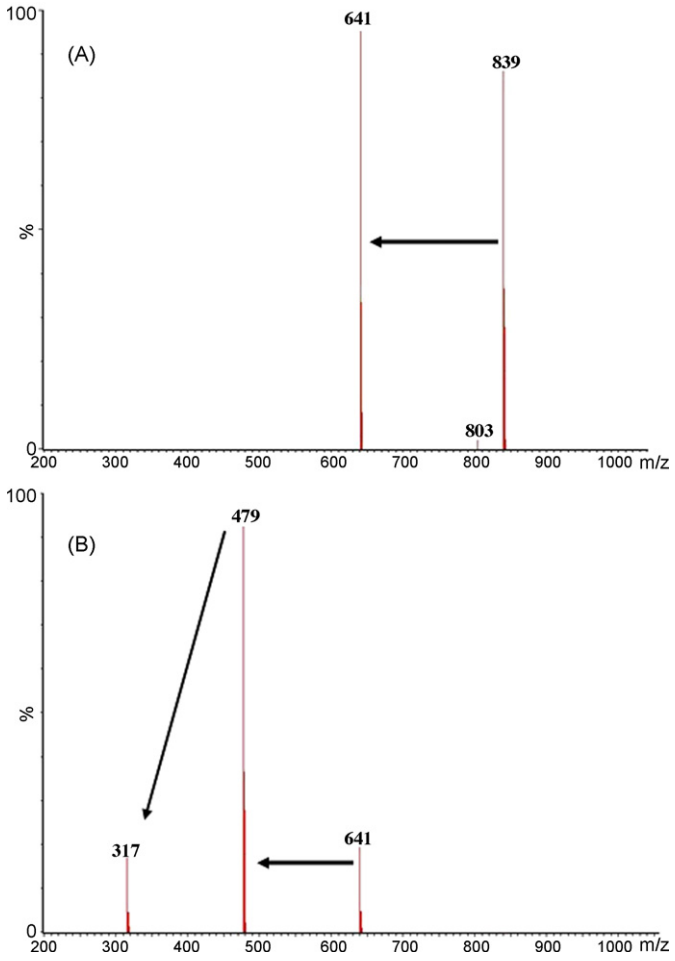

Fig. 2. Stevioside $(m/z)^-$ 839 $[M+Cl^{35}]^-$ fragmentation pattern at (A) lower and (B) higher collision energy. The product ion with $(m/z)^-$ 317 is the aglycone steviol.

3.4. Qualitative determination of ST-glycosides

Fig. 3 shows a typical UHPLC-SIR-MS chromatogram of a purified extract of Stevia leaves. The complete separation of all the steviol-glycosides was completed within only 3 min when using UHPLC column with a flow-rate of 0.5 ml min^{-1}, which is a balance between ionization and column performance.

The steviol-glycosides were previously identified on the basis of their UV spectrum, molecular ion (MS), related product ions (MS/MS) and, for SV, Ra and Sb, by co-chromatography with an authentic standard. Peaks **1** and **2**, the main components of Stevia leaves, were identified as Ra and SV, respectively. Peaks **6** and **7** were steviol-hexosides, probably steviol-19-O-glucoside and steviol-monoglucosyl ester [30], while peaks **3**, **4** and **5** were Rc, Du and Sb, respectively.

The CID-MS/MS of the peaks **A**, **B**, **C** and **D** gave a typical fragmentation pattern of ST-glycosides and at higher collision energy a product ion with $(m/z)^-$ 317, which correspond to steviol. Peak **B** has the same molecular weight of SV, thus it should be rebaudioside B (Rb). Regarding peak **A**, it has the same molecular weight of Sb, so it should be rubusoside.

Two additional peaks (**C** and **D**) were found by the use of sub-2 μm column. Peak **C** showed the same molecular weight of Ra, so it should be rebaudioside E, whereas peak **D** resulted to be an isomer of Du. Note that peak **D** can easily lose a rhamnose moiety at lower cone voltage thus producing an ion with $(m/z)^-$ 677, corresponding to $[M-146+Cl^{35}]^-$, that can be confounded with rubusoside or steviolbioside.

Table 2
Amount of steviol and its glycosides in Stevia leaves from southern Italy (Apulia) and commercial preparation (Truvia®).

Sample	SV	Ra	Rc	% Du	Sb	6	7	Total (%)	ST (mg kg^{-1})
1	6.0	0.7	0.2	1.0	0.3	0.3	0.1	8.6	5.3
2	4.8	2.0	0.4	0.5	0.1	0.1	N.D.	7.9	4.1
3	4.8	0.6	0.4	0.2	0.3	0.2	N.D.	6.5	4.6
4	4.1	1.3	3.4	1.2	0.3	0.1	N.D.	10.5	4.0
5	6.1	4.4	0.7	0.7	0.2	0.2	N.D.	12.3	2.7
6	4.8	1.7	3.8	1.1	0.4	0.4	0.2	12.6	3.8
7	6.7	3.4	0.6	0.5	0.3	0.1	N.D.	11.6	8.8
8	8.2	1.9	0.2	0.3	0.2	0.2	N.D.	11.0	6.1
9	7.4	1.2	2.2	0.9	0.2	0.2	0.1	12.3	13.2
10	5.1	0.8	1.1	0.6	0.3	0.1	N.D.	8.1	6.3
Media	5.8	1.8	1.3	0.7	0.3	0.2		10.1	5.9
SD	1.3	1.2	1.4	0.3	0.1	0.1		2.2	3.1
Media[a]	6.0	2.1	1.4	0.5	0.2	0.1		10.3	
SD[a]	1.6	1.4	1.6	0.4	0.2	0.1		2.8	
A, media	0.009	0.84	0.004	0.00002	0.0005	0.0001	N.D.	0.85	N.D.
A, SD	0.002	0.03	0.001	0.00001	0.0001	0.0001		0.03	
A, media[a]	N.Q.	0.85	N.Q.	N.D.	N.D.	N.D.	N.D.	0.85	
A, SD[a]		0.04						0.04	

N.D. not detected; N.Q. not quantifiable; A: Truvia®.
[a] Comparative data evaluated by the method of assay reported in the JECFA.[4]

Carefully investigating RT and the chemical structure of steviol-glycosides, it seems that when the carboxyl function is not esterified or it has a lower degree of esterification, RT is higher than its conjugated form. In fact, the RT of Rb, Sb and Ra is higher than SV, Ru and Re, respectively. If this consideration is correct, peak **D**, **6** and **7** correspond to ST-C_{13}-O-glc-glc-rha, ST-COO-glc and ST-C_{13}-O-glc, respectively.

3.5. Quantitative analysis of ST-glycosides

Stevioside, Ra and Rc represent the most abundant sweeteners found in Stevia leaves (Table 2). The contents of SV and Ra in different samples of Stevia were determined using calibration curves obtained by authentic standards. Dulcoside A, and the two steviol-hexosides were assayed using SV calibration curves, while Rc was evaluated by Ra calibration curve. Their amounts were then normalized by molecular mass ratios.

Consider that SV and Ra calibration curves showed highly different slopes (see Section 3.2.1); consequently, Rc percentage in Stevia leaves can differ significantly according to the curve used. In Table 2 the content of steviol-glycosides found in Stevia leaves is reported together the data obtained by the method proposed by JECFA [4]. In samples 4 and 6, Rc was more abundant than Ra. Regarding Du, Sb and compound 6, their amount was similar in all the analyzed Stevia leaves, while compound 7 was detected only in samples 1, 6 and 9. On the whole, both the single analytes and the total ST-glycosides content in Stevia leaves were highly variable. This may be related to different factors such as the harvest time, the stage of development of the plant and the type of field. In addition, a wide variability was found in the ratio ST/Ra which ranges from 1.4 to

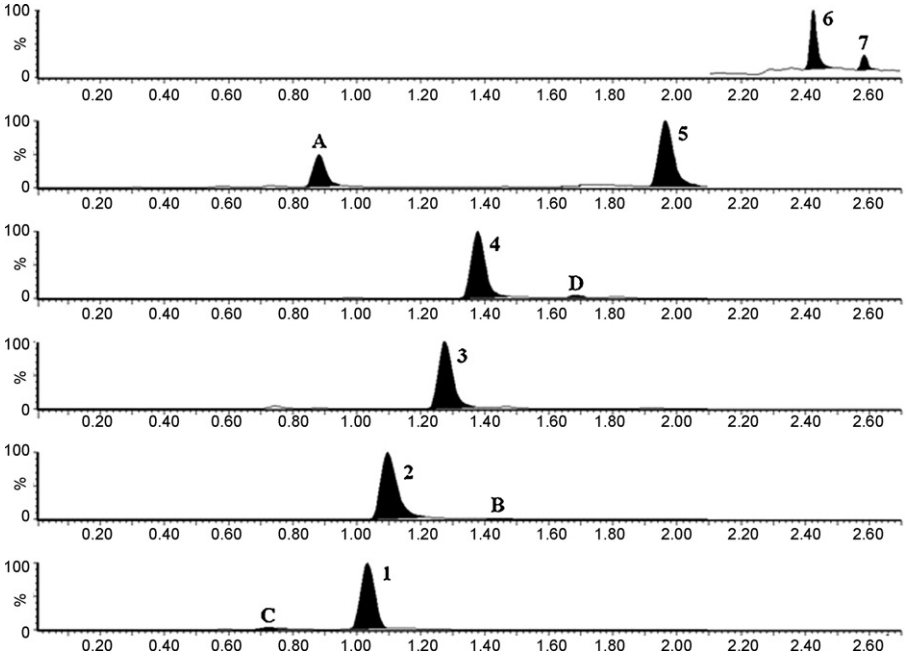

Fig. 3. Typical UHPLC-MS chromatogram (SIR mode) of Stevia leaf extract. 1 (Ra) and C (Re) $(m/z)^-$ 1001; 2 (SV) and B (Rb) $(m/z)^-$ 839; 3 (Rc) $(m/z)^-$ 985; 4 (Du) and D (Du isomer) $(m/z)^-$ 823; 5 (Sb) and A (Ru) $(m/z)^-$ 677; 6 and 7 (ST-glucosides) $(m/z)^-$ 515. The extracted ions correspond to $[M+Cl^{35}]^-$.

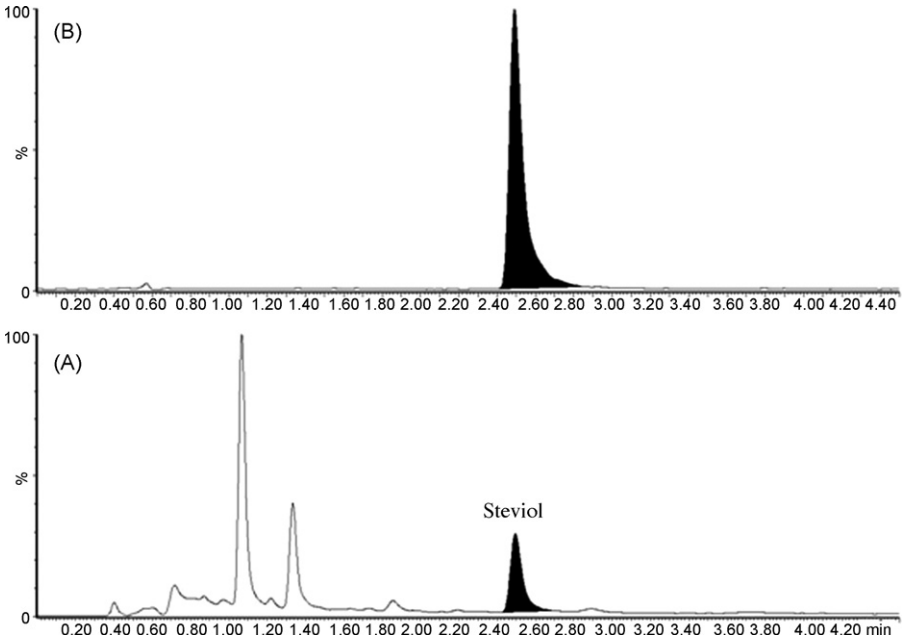

Fig. 4. Typical UHPLC-MS chromatogram of Stevia leaf extract before (A) and after SPE (B). Steviol: $(m/z)^-$ 317. Column: 1.8 μm HSS C_{18} (100 mm × 2.1 mm). Flow-rate: 0.6 ml min^{-1}. Eluent: 5 mmol l^{-1} ammonium acetate pH 6: CH_3CN (45:55, v/v).

8.6 in Stevia leaves. This ratio should be as low as possible considering that Ra has better sweetening properties than SV and because a purified Ra from Stevia can be used as edulcorant.

Regarding SV, Rc, Du, Sb and St-glucosides, the amounts from our examination matched those given by the traditional LC-NH_2-UV, while for Ra they were slightly lower (Table 2).

Lacking chromophores, steviol-glycosides do not have a typical UV spectrum; consequently, their identification in LC-UV was only based on time retention, which could easily overestimate the actual amount of such compounds in Stevia leaves.

Truvia® contained mainly Ra, SV and Rc representing about 98, 1 and 0.5% of the total steviol-glycosides founded (Table 2).

3.6. Optimization of UHPLC-MS conditions for the evaluation of steviol

In a preliminary phase, different sub-2 μm and traditional HPLC columns were tested in order to optimize the condition of separation (see 3.3) and for steviol the best performance was achieved by a 100 mm × 2.1 mm HSS C_{18} column. The ST evaluation in Stevia leaf extracts was carried out in negative ion mode as the analyte signal resulted much higher than the one in positive ion mode. This could be due to the carboxylic moiety that loses a proton to form a negative molecular ion $[M-H]^-$. After testing both ESI and APCI sources, the ESI was adopted because it produced a stronger signal for the $[M-H]^-$ ion.

Regarding the composition of mobile phase, two ammonium buffers (acetate and formate) were investigated at different pH values in the range of 4–8. A strong MS response for steviol in negative ion mode was obtained using buffer 5 mmol l^{-1} ammonium acetate at pH 6. The MS/MS spectra of ST did not show the presence of typical product ions suggesting that it had broken down without producing major fragments. Thus, ST quantification was carried out in the SIR mode monitoring the ions with $(m/z)^-$ 317.

Steviol was analyzed in isocratic mode to enhance sensitivity, specificity and because some UHPLC-MS parameters such as injection volume, pH buffer, column, capillary voltage and Rf lens were different from those employed for the evaluation of steviol-glycosides. Regarding specificity, in gradient mode some unknown peaks have retention times similar to that of ST while in isocratic mode they elute earlier than steviol. For these reasons, we preferred to develop and validate two different UHPLC-MS methods to evaluate steviol and its glycosides in Stevia leaves and commercial products.

3.7. Quantitative determination of steviol

The purified Stevia leaf extracts were analyzed by UHPLC-C_{18}-MS in SIR mode and Fig. 4 reports a typical chromatogram of an extract before SPE (A) and after it (B). Steviol was detected in trace in all the extracts and its amounts were in range of 2.7–13.2 mg kg^{-1} (5.9 ± 3.1 mg kg^{-1}) of dried Stevia leaves. This result, even in the consideration of the limits due to a low quantity of samples ($n = 10$), is consistent with the data (about 5.9 mg kg^{-1}) reported by Minne et al. [27]. It is should be noted that these authors reported a LLOD of 100 pg injected, which makes our method 20 times more sensitive and able to detect about 2.5 μg ST kg^{-1} of dried leaves. Steviol was not detected in the commercial sweetener named Truvia® indicating that, if present, its amount was lower than 1 μg kg^{-1}.

4. Conclusion

The sub-2 μm columns offer a superior efficiency, a shorter analysis time, and a higher resolution than traditional C_{18} and amino columns used for the analysis of Stevia extract. Moreover, the UHPLC coupled with an MS detector makes the method more sensitive and specific. Solid-phase extraction resulted to be an adequate way to purify steviol and its-glycosides from hydrophilic interferences and to preserve the efficiency of the column.

The potential weakness of the described methods is the longer time of the sample preparation with respect to others published assays. This is mainly due to the purification steps by SPE and to the use of two distinct analytical methods for the quantification of steviol and its glycosides in Stevia leaves extract. On the other hand, the strength of the proposed UHPLC methods is the complete separation of all the peaks, the reduced times of analysis, the reproducible retention time and the specificity. On the whole, thanks to their shorter time of analysis and to the good performance, the val-

idated UHPLC methods could be used in QC laboratories for the routine analysis of Stevia leaves and their commercial extracts.

References

[1] E.J. Kennelly, in: D. Kinghorm (Ed.), Stevia—The Genus *Stevia*, Taylor & Francis, 2002, ISBN 0-415-26830-3, p. 68.
[2] J.M.C. Geuns, Recent Res. Dev. Phytochem. 4 (2000) 75.
[3] J.M.C. Geuns, J. Buyse, A. Vankeirsbilck, L. Temme, in: J.M.C. Geuns, J. Buyse (Eds.), Proceedings of the First Symposium on the Safety of Stevioside, Euprint Edition, KULeuven, 2004, p. 75 (ISBN: 9074253024).
[4] JECFA, Joint FAO/WHO Expert Committee on Food Additives Monographs 5, Compendium of Food Additive Specifications from 69th JEFCA Meeting, Rome, June 17–26, 2008.
[5] V. Chatsudthipong, C. Muanprasat, Pharmacol. Ther. 121 (2009) 41.
[6] D.J. Brusick, J. Food Chem. Toxicol. 46 (2008) S83.
[7] A. Roberts, A.G. Renwick, Food Chem. Toxicol. 46 (2008) S31–S39.
[8] M.C. Carakostas, L.L. Curry, A.C. Boileau, D.J. Brusick, Food Chem. Toxicol. 46 (2008) S1–S10.
[9] A.I. Nikiforov, A.K. Eapes, Int. J. Toxicol. 27 (2008) 65.
[10] Y. Aze, K. Toyoda, K. Imaida, Y. Hayashi, et al., Eisei Shikenjo Hokoku Bull. Natl. Inst. Hyg. Sci. 109 (1991) 48.
[11] C. Toskulkao, L. Chaturat, P. Temcharoen, T. Glinsukon, Drug Chem. Toxicol. 20 (1997) 31.
[12] A.P.M. Nunes, S.C. Ferreira-Machado, R.M. Nunes, F.J.S. Dantas, et al., Food Chem. Toxicol. 45 (2007) 662.
[13] C. Gardana, P. Simonetti, E. Canzi, R. Zanchi, et al., J. Agric. Food Chem. 51 (2003) 6618.
[14] H. Mizukami, K. Shiiba, H. Ohashi, Phytochemistry 121 (1982) 1927.
[15] M. Sakaguchi, M. Kan, Ciencia Cult. 34 (1982) 235.
[16] F. Fullas, J. Kim, C.M. Compadre, A.D. Kinghorn, J. Chromatogr. 464 (1989) 213.
[17] A.A. Dacom, C.C. da Silva, C.E.M. da Costa, J.D. Fontana, J. Adelman, Process Biochem. 40 (2005) 3587.
[18] Y. Hashimoto, M. Moriyasu, S. Nakamura, S. Ishiguro, M. Komuro, J. Chromatogr. 161 (1978) 403.
[19] M.S. Ahmed, R.H. Dobberstein, J. Chromatogr. 245 (1982) 373.
[20] H.C. Makapugay, N.P.O. Nanayakkara, A.D. Kinghorn, J. Chromatogr. 283 (1984) 390.
[21] N. Kolb, J.L. Herrera, D.J. Ferreyra, R.F. Uliana, J. Agric. Food Chem. 49 (2001) 4538.
[22] P. Mauri, G. Catalano, C. Gardana, P.G. Pietta, Electrophoresis 17 (1991) 367.
[23] J. Pól, B. Hohnová, T. Hyötyläinen, J. Chromatogr. A 1150 (2007) 85.
[24] V. Jaitak, A.P. Gupta, V.K. Kaul, P.S. Ahuja, J. Pharm. Biomed. Anal. 47 (2008) 790.
[25] L.K. Hearn, P.P. Subedi, J. Food Comp. Anal. 22 (2009) 165.
[26] A.U. Jackson, A. Tata, C. Wu, R.H. Perry, et al., Analyst 134 (2009) 867.
[27] V.J. Minne, F. Compernolle, S. Topped, J.M.C. Geuns, J. Agric. Food Chem. 52 (2004) 2445.
[28] V.P. Shah, K.K. Midha, J.W. Findlay, H.H. Hill, et al., Pharm. Res. 17 (2000) 1551.
[29] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 75 (2003) 3019.
[30] B.H. de Oliveira, J.F. Packer, M. Chimelli, D.A. de Jesus, J. Biotechnol. 131 (2007) 92.