

Large-scale profiling of diterpenoid glycosides from *Stevia rebaudiana* using ultrahigh performance liquid chromatography/tandem mass spectrometry

Behnaz Shafii · Ramin Vismeh · Randy Beaudry ·
Ryan Warner · A. Daniel Jones

Received: 8 February 2012 / Revised: 20 April 2012 / Accepted: 21 April 2012 / Published online: 12 May 2012
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Abstract The plant *Stevia rebaudiana* accumulates a suite of diterpenoid metabolites that are natural sweeteners finding increased use as sugar substitutes. To guide breeding of stevia plants that accumulate substances with desirable flavor in high yield, rapid and accurate methods are needed to profile these substances in plant populations. This report describes an 8-min ultrahigh performance liquid chromatography–tandem mass spectrometry method for separation and quantification of seven stevia glycosides including steviolbioside; stevioside; rebaudiosides A, B, and C; rubusoside; and dulcoside as well as aglycones steviol and isosteviol. This negative mode electrospray ionization/multiple reaction monitoring method yielded low limits of detection <1 ng/mL for steviol, 6 ng/mL for isosteviol, and <15 ng/mL for all stevia glycosides. Stevioside and Reb A, B, and C were quantified in more than 1,100 extracts from stevia leaves as part of a large-scale profiling exercise. Leaf tissue levels in this population spanned about two orders of magnitude for stevioside (2–125 mg/g dry weight), Reb A (2.5–164 mg/g), Reb B (0.5–50 mg/g), and Reb C (1.5–125 mg/g), but levels of individual metabolites exhibited independent variation. The wide spread of metabolite levels highlights the utility and

importance of performing targeted metabolic profiling for large plant populations.

Keywords Stevia glycosides · Diterpenoids · Ultrahigh performance liquid chromatography · Tandem mass spectrometry · Large-scale metabolite profiling · Natural products

Abbreviations

CID	Collision-induced dissociation
DW	Dry weight
HILIC	Hydrophilic interaction chromatography
LLOD	Low limit of detection
LLOQ	Low limit of quantification
MRM	Multiple reaction monitoring
Reb	Rebaudioside
UHPLC	Ultrahigh performance liquid chromatography

Introduction

Stevia rebaudiana Bertoni is a sweet herb from the family Compositae that is native to Brazil and Paraguay and is cultivated in countries from almost all continents. Natural non-caloric sweeteners called stevia glycosides are glycoconjugates of the diterpenoid aglycone steviol and can be extracted from the leaves of the stevia plant. These sweeteners have been commercially available in much of East Asia and are used to sweeten a variety of food products. Stevia leaf and extracts are also used as dietary supplements and for skin care in the USA [1].

Stevia glycosides consist of a diverse group of metabolites that differ in sugar composition, positions of substitution, and flavor properties. Structures of steviol, its isomer

B. Shafii · R. Vismeh · A. D. Jones
Department of Chemistry, Michigan State University,
East Lansing, MI 48824, USA

R. Beaudry · R. Warner
Department of Horticulture, Michigan State University,
East Lansing, MI 48824, USA

A. D. Jones (✉)
Department of Biochemistry and Molecular Biology,
Michigan State University,
603 Wilson Road, Biochemistry Room 212,
East Lansing, MI 48824, USA
e-mail: jonesar4@msu.edu

isosteviol, and important stevia glycosides are depicted in Fig. 1. Due to the variation in the structures of these diterpene glycosides, the quality of their flavors varies from one sweetener to another. For instance, rebaudioside A (simply termed Reb A from here on) imparts a sweet taste whereas stevioside exhibits a significant bitter aftertaste [2, 3]. This difference in biological activity is due to the presence of an extra glucose moiety in the Reb A structure.

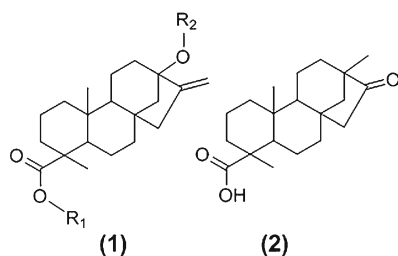
Anticipated worldwide growth in usage of stevia glycosides as natural sweeteners is driving a need for improved plant breeding to enhance yields of those specialized metabolites with desirable flavor qualities and decrease levels of less desirable phytochemicals. To achieve these goals, rapid analytical methods are needed for quantitative profiling of an assortment of stevia glycosides in large numbers (thousands) of stevia tissue extracts. Such analytical methodologies should be capable of distinguishing isomeric glycosides that exhibit similar physical properties. Liquid chromatography (LC) coupled to ultraviolet (UV) absorbance or mass spectrometry (MS) detection has been the prevailing approach used for analysis of stevia glycosides. However, since these substances lack UV absorbance except at short wavelengths, the long-standing approach has involved polar (often amino) columns with spectrophotometric detection at 210 nm [4, 5]. One limitation of this approach has been that minor stevia glycosides may be obscured by other phytochemicals including overlapping or tailing peaks from the major stevia constituents stevioside and rebaudioside A. As a consequence, the minor stevia glycosides have not been emphasized.

Relatively few reports have coupled liquid chromatography with mass spectrometry for detection of these natural sweeteners. Analyses using LC with single-stage mass

spectrometry were described in a 2008 report [6], but in this case, spectrophotometric detection was used for quantitative analysis, with mass spectrometry employed to verify metabolite identities, perhaps because ion signals were distributed among different cationized forms. A more recent report by Gardana and co-workers in 2010 [7] reported levels of several stevia glycosides in leaf extract using an extensive cleanup procedure based on solid phase extraction, centrifugation, and filtering, followed by ultrahigh performance liquid chromatography coupled to single-stage mass spectrometry (UHPLC–MS). Stevia glycosides were detected using selected ion monitoring of chloride adduct ions in negative ion mode. To facilitate formation of these adduct ions, 0.1 % dichloromethane was added to the organic mobile phase component. Although the method is rapid, the sample preparation protocol is lengthy and would probably require substantial modification for large-scale analyses that have a short timeline. In addition, two different LC gradients were employed to quantify stevia glycosides and the aglycone steviol. To our knowledge, one earlier report described LC–MS–MS for elucidating structures of metabolites of stevia glycosides [8], employing MS–MS to confirm expected fragment ion masses. The LC protocol required 43 min per sample, but it was not clear that mass spectrometric detection was employed for quantitative analysis.

Several recent alternative approaches for analyses of stevia glycosides have also been reported. Cooks et al. employed another rapid approach for analysis of stevia sweeteners, desorption electrospray ionization mass spectrometry. This approach yields direct analysis of stevia leaves without need for metabolite extraction [9]. While these analyses were indeed rapid, the lack of a separation method capable of distinguishing isomers such as stevioside and Reb B limits the utility of this approach for comprehensive profiling of the desirable phytochemicals. To address resolution of isomeric stevia glycosides, Tuulia et al. used a 50-min two-dimensional (LC × LC) separation and electrospray ionization (ESI) time-of-flight mass spectrometry to separate nine stevia glycosides [10]. Another two-dimensional LC attempt for analysis of stevia glycosides was made recently by Cacciola et al. [11]. In this work, a polyamine column was coupled to a UHPLC C18 column and UV absorbance detection for two-dimensional separations that resolved five stevia glycosides and several flavonol glycosides. Although the method clearly resolved several stevia glycosides, the long separation times (~60 min) are not optimal for large-scale profiling of hundreds or thousands of tissues.

Despite these recent advances in stevia glycoside analyses, more traditional liquid chromatographic methods still play a workhorse role in these analyses. Stability of stevioside and Reb A in soft drinks was investigated using HPLC with a HILIC column and UV absorbance detection, and the



Compound	R ₁	R ₂	[M-H] ⁻ (m/z)
Steviol	H	H	317
Isosteviol	Structure # 2		317
Steviolbioside	H	β-Glc-β-Glc(2-1)	641
Rubusoside	β-Glc	β-Glc	641
Dulcoside	β-Glc	β-Glc-α-Rha(2-1)	787
Stevioside	β-Glc	β-Glc-β-Glc(2-1)	803
Reb B	H	β-Glc-β-Glc(2-1) β-Glc(3-1)	803
Reb F	β-Glc	β-Glc-β-Xyl(2-1) β-Glc(3-1)	935
Reb C	β-Glc	β-Glc-α-Rha(2-1) β-Glc(3-1)	949
Reb A	β-Glc	β-Glc-β-Glc(2-1) β-Glc(3-1)	965
Reb D	β-Glc-β-Glc(2-1)	β-Glc-β-Glc(2-1) β-Glc(3-1)	1127

Fig. 1 Structures of (1) steviol metabolites including glycosides and (2) isosteviol, showing nominal masses of [M-H]⁻ ions observed in negative mode electrospray ionization mass spectra. *Glc* glucose, *Rha* rhamnose, *Xyl* xylose

identities of degradation products were confirmed using LC–ESI–MSⁿ [12]. Improved extraction efficiencies were reported for stevioside and Reb A using rapid microwave-assisted extraction, using an amino column for HPLC separation and photodiode array detection [13].

Although several methods have been developed for identification and quantification of stevia glycosides, our need to profile more than 1,100 plant tissues within a few months' time required development of a reproducible and rapid method for large-scale screening. It was desired to quantify both the abundant stevia metabolites as well as several additional stevia glycosides expected to be present in tissues at much lower levels. Here, an 8-min LC gradient is described that uses a C18 column packed with fused core particles for separation of nine known stevia glycosides including steviol; isosteviol; steviolbioside; stevioside; Reb A, B, and C; rubusoside; and dulcoside A. This protocol employs minimal sample processing that is limited to extraction, centrifugation, and filtering. To improve selectivity of detection and minimize effects of potential chemical interferences, multiple reaction monitoring (MRM) tandem mass spectrometry was optimized and performed. This method has been applied for analysis of more than 1,100 extracts from stevia plants to select plant lines with desirable biochemical phenotypes.

Experimental

Plant growth conditions

S. rebaudiana “Eirete” and “Criolla” plants (77 and 59 plants, respectively) were grown in a climate-controlled greenhouse. Dormant crowns were planted into 13-cm square plastic containers (1.1-L volume) filled with a commercial soilless medium (Suremix Perlite blend, Michigan Grower Products, Galesburg, MI) and grown under a constant temperature of 20 °C. Photoperiod was maintained at 16 h by supplementing ambient irradiance with ca. 60 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation provided by high-pressure sodium lamps from 0600 to 2200 hours daily. Plants were irrigated as needed with reverse osmosis-treated well water supplemented with nutrients at a rate of 125 mg L⁻¹ N, 12 mg L⁻¹ P, 100 mg L⁻¹ K, 65 mg L⁻¹ Ca, 12 mg L⁻¹ Mg, 1 mg L⁻¹ Fe and Cu, 0.5 mg L⁻¹ Mn and Zn, 0.3 mg L⁻¹ B, and 0.1 mg L⁻¹ Mo (MSU RO Water Special; GreenCare Fertilizers, Kankakee, IL). Three replicate samples were harvested from each plant at weekly intervals. A separate population of 984 stevia plants derived from an improved seed lot was grown outdoors at the Michigan State University Horticulture Teaching and Research Center, and single leaf tissue collections were performed for these plants.

Extraction

When outdoor-grown plants reached 15 cm in height, the second youngest fully expanded leaf was harvested for diterpene glycoside profiling. For greenhouse-grown plants, the two most apical, fully expanded leaves were collected. Extraction procedures were evaluated by performing extractions, at various ethanol/water proportions, of a stevia leaf tissue pool that had been previously dried and ground. Differences in analyte levels were minimal (<20 %) between extraction conditions, and the solvent that gave highest yield (60 % ethanol/40 % water) was employed in subsequent extractions. Leaf tissues were dried and pulverized, and 5–10 mg of the dried powder was extracted in a 1.5-mL vial with 1.0 mL of ethanol/water (60/40 %) containing 1 μM of digitoxin as an internal standard. The vial was vortexed for 30 min and centrifuged (8,000 $\times g$) for 3 min to pelletize cellular debris. Extract supernatant (225 μL) was diluted 6-fold and filtered using Millipore deep well filter plates (0.45 μm) by centrifugation (1,000 $\times g$) for 10 min into deep well 96-well receiving plates (VWR Scientific), which were stored at –20 °C until analyzed.

LC–MS–MS instrumentation

Analyses were conducted using a QTRAP 3200 mass spectrometer (AB/Sciex) equipped with binary LC-20AD pumps (Shimadzu), a SIL-HTc autosampler, and column oven. All mass spectrometric analyses, including data processing, were performed using Analyst v. 1.4.2 software (AB/Sciex).

Optimization of instrumental parameters

Mass spectrometric analysis parameters were optimized by performing flow injection analyses of standard stevia glycosides (ChromaDex, Irvine, CA, USA) using isocratic elution of 50 % solvent A (10 mM aqueous ammonium acetate) and 50 % solvent B (acetonitrile) with a total flow rate of 250 $\mu\text{L}/\text{min}$. Enhanced product ion (EPI) scans were generated for the $[\text{M}-\text{H}]^-$ ions for each metabolite using electrospray ionization in negative ion mode to identify abundant product ions. Precursor ions were the deprotonated molecular species for each stevia glycoside using the following initial conditions to establish the masses of product ions used for parameter optimization: curtain gas, 10 (arbitrary units); ion spray voltage, 4,500 V; temperature, 500 °C; gas 1, 25 (arbitrary units); gas 2, 25 (arbitrary units); declustering potential (DP), 20 V; and entrance potential, 10 V. EPI spectra were initially generated using three different collision cell potentials (30, 50, and 70 V) to adequately survey a range of product ions. These parameters were used for selecting suitable product ions for MRM transitions. Source and gas parameters were optimized for all the standards

including the internal standard, digitoxin. Parameters that were optimized for individual compounds and their optimal values are presented in Table 1.

LC–MS–MS analyses

Analytes were separated using an Ascentis Express C18 column (5 cm×2.1 mm; 2.7 μm particles) using a reversed phase binary gradient. Solvent A was 10 mM aqueous ammonium acetate and solvent B was acetonitrile. Total solvent flow was maintained at 0.4 mL/min, and gradient elution was performed using the following solvent compositions: initial, 7 % B, held for 0.2 min; linear gradient to 20 % B at 0.3 min and then to 48 % B at 5 min; sudden increase to 99 % B at 5.01 min and to 100 % B at 7 min; followed by a linear gradient to initial condition at 7.01 min and a final hold at this composition until 8 min. Injection volume and column temperature were 5 μL and 40 °C, respectively. Instrumental conditions were optimized for MRM detection of each stevia glycoside ion as described below, and the optimized MRM conditions are presented in Table 1. For each set of 50 plant extracts that were analyzed, three technical replicates were analyzed for quality control purposes, and a set of calibration standards was analyzed at least three times for each set of two 96-well plates.

Standard calibration curves and quantification

Standard solutions of steviol, isosteviol, and the other stevia glycosides described in this report (ChromaDex) were prepared at 0.1, 1, 5, 10, and 20 μM in 50:50 ethanol/water. A cocktail of all standards was prepared for each concentration to reduce the analysis time. Digitoxin was added to each

standard solution at 1 μM to match the concentration in the plant tissue extracts. Standards were analyzed in triplicate ($n=3$) at the beginning of each batch of samples, halfway through the batch, and at the end of the batch. Leaf extracts were analyzed in random order using Microsoft Excel to randomize the order. Technical replicate injections of three to five plant tissue extracts were analyzed in each batch to assess instrument performance and method reproducibility.

Calibration curves were generated by integrating MRM chromatogram peaks for each target analyte and plotting the peak area ratio ($A_{\text{analyte}}/A_{\text{internal standard}}$) as a function of analyte concentration. Linear regressions were performed using $1/X$ weighting and by forcing the calibration line through the origin.

Limits of detection and quantification

The instrumental LLOD was defined as the lowest concentration that yielded a signal-to-noise ratio equal to 3 ($S/N=3$) for analysis of calibration standard solutions. Instrumental low limit of quantification (LLOQ) was defined as the level giving a signal-to-noise ratio equal to 10 ($S/N=10$) for each using authentic standards. Background regions of each chromatogram used for S/N calculations were defined manually.

Results and discussion

Ionization and collision-induced dissociation of stevia glycosides

All of the stevia glycosides, as well as steviol and isosteviol, yielded abundant $[M-H]^-$ ions in preliminary investigations

Table 1 MRM transitions and optimized instrumental parameters used for LC–MS–MS measurements of steviol, isosteviol, and stevia glycosides

Compound	MRM transition	Rt	DP	EP	CE	LLOD	LLOQ	R^2	Slope
Steviol	317>317	5.6	−65	−5	−30	0.7	2.3	0.992	28.4
Isosteviol	317>273	6.4	−20	−10	−50	6.0	20.0	0.993	0.176
Steviolbioside	641>479	3.7	−60	−10	−40	13.0	43.3	0.990	2.47
Rubusoside	641>479	3.9	−40	−7	−35	2.7	9.2	0.990	14.1
Digitoxin (IS)	763>633	5.3	−50	−10	−50	ND	ND	ND	ND
Dulcoside	787>625	3.7	−70	−10	−40	3.7	12.5	0.989	2.78
Stevioside	803>641	3.4	−45	−7	−40	5.7	19.1	0.988	3.27
Reb B	803>641	3.6	−55	−5	−50	14.3	47.6	0.988	1.79
Reb C	949>787	3.6	−60	−10	−70	7.5	24.8	0.991	0.997
Reb A	965>803	3.3	−70	−10	−65	5.0	16.8	0.995	2.12

All calibration curves were forced through zero with $1/X$ weighting

Rt retention time (in minutes), *DP* declustering potential (in volts), *EP* entrance potential (in volts), *CE* collision potential (in volts), *LLOD* low limit of detection (in nanograms per milliliter) for an injection of 5 μL, *LLOQ* low limit of quantification (in nanograms per milliliter) for an injection of 5 μL, *Slope* slope of the calibration curve, *IS* internal standard, *ND* not determined

of their ionization behavior. Such behavior was expected for the aglycones steviol and isosteviol as well as Reb B and steviolbioside, as these substances have carboxylic acid groups that readily undergo deprotonation. More surprising was the dominance of deprotonated ions for stevia glycosides with acylglucoside moieties, since their most acidic groups are the sugar hydroxyls. Since deprotonated ions were of such high abundance, addition of substances such as CH_2Cl_2 to form chloride adduct ions was deemed unnecessary [7]. The internal standard digitoxin, a steroidal glycoside, also yielded abundant $[\text{M}-\text{H}]^-$, and was judged to exhibit ionization behavior similar to the stevia glycosides.

Plant tissues often accumulate complex mixtures of specialized metabolites. To develop a selective method for quantification of stevia glycosides, tandem mass spectrometry, in MRM mode, was selected to minimize interference by other phytochemicals. In order to maximize the ion current for each MRM transition, collision-induced dissociation (CID) was performed on each $[\text{M}-\text{H}]^-$ ion using multiple collision potentials, and the potential that yielded the strongest signal was chosen for the MRM method. As was expected, losses of glucose units were the dominant fragmentations that occurred upon CID. Figure 2 presents CID-enhanced product ion spectra for $[\text{M}-\text{H}]^-$ from Reb A and isosteviol as representatives of all analytes of interest. For all stevia glycosides, transition from $[\text{M}-\text{H}]^-$ to the fragment corresponding to loss of one anhydroglucose was selected for MRM. Yields of fragment ions derived from $[\text{M}-\text{H}]^-$ of steviol were formed only at negligible abundances at collision cell potentials up to 70 V. Increasing the collision potential further yielded numerous product ions, all at low abundance. Owing to this behavior, the method incorporated a selected ion monitoring mode, transmitting the steviol $[\text{M}-\text{H}]^-$ (m/z 317) through both mass analyzers. Isosteviol, however, showed loss of 44 ($-\text{CO}_2$) upon CID, and this transition was used for the MRM method.

Rapid profiling of stevia metabolites using LC–MS–MS

Profiling of specialized metabolites in large numbers of complex biological samples has become more practical in

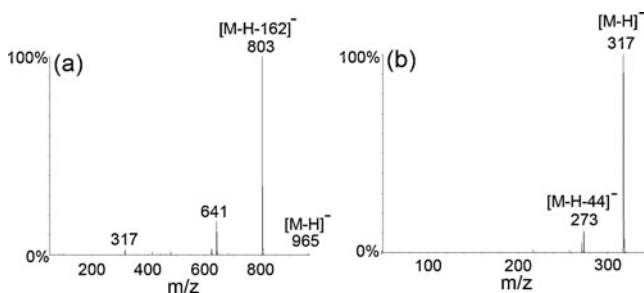


Fig. 2 EPI mass spectra for $[\text{M}-\text{H}]^-$ ion of (a) Reb A (m/z 965) and (b) isosteviol (m/z 317)

recent years following improvements in chromatographic and mass spectrometric technologies. Fast ultrahigh performance LC separations have been useful for exploring the biochemical diversity of plant introgression lines [14] and establishing unanticipated connections between levels of primary and specialized metabolites that have led to gene function discoveries [15]. In the current study, the effort has focused on profiling bioactive stevia metabolites extracted from leaves of more than 1,100 stevia plants to guide breeding of plants with desirable flavor and product yields.

The chemical complexity of plant tissues presents a substantial challenge to the goal of rapid metabolite profiling in large numbers of tissue samples. While it is often the case that the selectivity of tandem mass spectrometry avoids problems associated with interference by other compounds, LC–MS–MS analyses still encounter situations where non-target metabolites interfere with measurements of target compounds. In the case of stevia glycosides, this issue arises owing to in-source fragmentation of metabolites containing acylglucoside groups. For example, mass spectra of the isomeric compounds stevioside (Fig. 3a) and Reb B (Fig. 3b) show different ionization chemistry, with the acylglucoside isomer (stevioside) showing extensive neutral loss of the sugar moiety from the ester, whereas Reb B, which lacks acylglucoside groups, underwent minimal fragmentation before mass analysis. The mass spectra shown in Fig. 3 were generated before parameters such as the declustering potential were optimized. All of the acylglucosides examined in this study underwent in-source fragmentation even after parameter

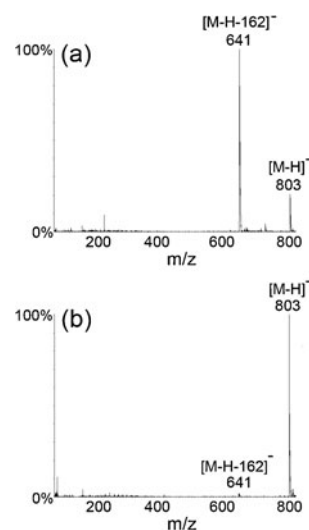


Fig. 3 Negative mode electrospray ionization enhanced mass spectra of (a) stevioside and (b) its isomer Reb B showing different amounts of in-source fragmentation, as evidenced by the abundance of the ion at m/z 641. Source parameters used to generate these spectra differ from optimized source parameters used for multiple reaction monitoring analyses

optimization. Because the group of stevia glycosides includes numerous members with acylglucoside groups, formation of fragments in the ion source has the potential to form fragment ions that have the same masses as $[M-H]^-$ ions of less glycosylated metabolites. This behavior may not pose a serious problem for targeted LC/MRM analyses, but should be taken into account during efforts to identify stevia glycosides present in plant extracts.

The structural similarity of stevia glycosides, coupled with the wide range of concentrations at which they occur in extracts of plant tissues, presents a special set of considerations during efforts for large-scale metabolite screening, in which speed and reproducibility from sample to sample are desirable. As in the study described below, plant breeding can be directed to identify individual plants that have desirable metabolic traits, but such efforts usually require analyses of hundreds to thousands of extracts in a single study. For this reason, short analysis times are essential. Because the mass spectrometer does a good job of discriminating compounds of different masses, the need for chromatographic separation largely arises to distinguish isomers and compounds that yield fragment ions at the same masses as other target metabolites. After extensive evaluation of various chromatographic parameters, a protocol was settled upon based on an 8-min gradient UHPLC separation that resolved isomer peaks (Fig. 4) with chromatographic resolution (R_s) of 1.4 where two isomers were detected (Fig. 4e). Chromatographic peaks for all stevia glycosides were symmetrical, with widths at half height of approximately 4 s.

Profiling of diterpenoid glycosides in stevia leaves

Owing to high concentrations of the most abundant stevia glycosides, tissue extracts were diluted from 5- to 10-fold before LC-MS-MS analyses. To achieve quantification of low-level glycosides, extracts were not diluted sufficiently to keep all measurements within the calibrated range of concentrations that gave a demonstrated linear response. After dilution, LLOQ values for all of the stevia glycosides corresponded to less than 1 $\mu\text{g/g}$ dry weight, or more than two orders of magnitude below the observed levels. Extraction of smaller quantities of plant tissue would have involved greater uncertainties in weighing the tissue, and it was decided to weigh about 10 mg for each plant tissue sample. Standard stevia glycoside concentrations of 20 μM , corresponding to approximately 10 mg/g dry weight, fell into the linear range for all glycoside metabolites. As a result, it is anticipated that measured levels above 50 mg/g dry weight may slightly underestimate tissue levels owing to nonlinear electrospray ionization responses at high concentrations. About half of the Reb A measurements exceed this level, and these measurements may underestimate Reb A quantities. Since only about 1 % of measurements entailed such high concentrations, these outlier samples, which are often displaying a desirable trait, were readily recognized from the LC-MS-MS results.

The targeted LC/MRM method detected signals for nine target metabolites plus digitoxin, a steroidal glycoside used as internal standard. The primary aim of this effort was to compare levels of the desirable sweeteners stevioside and

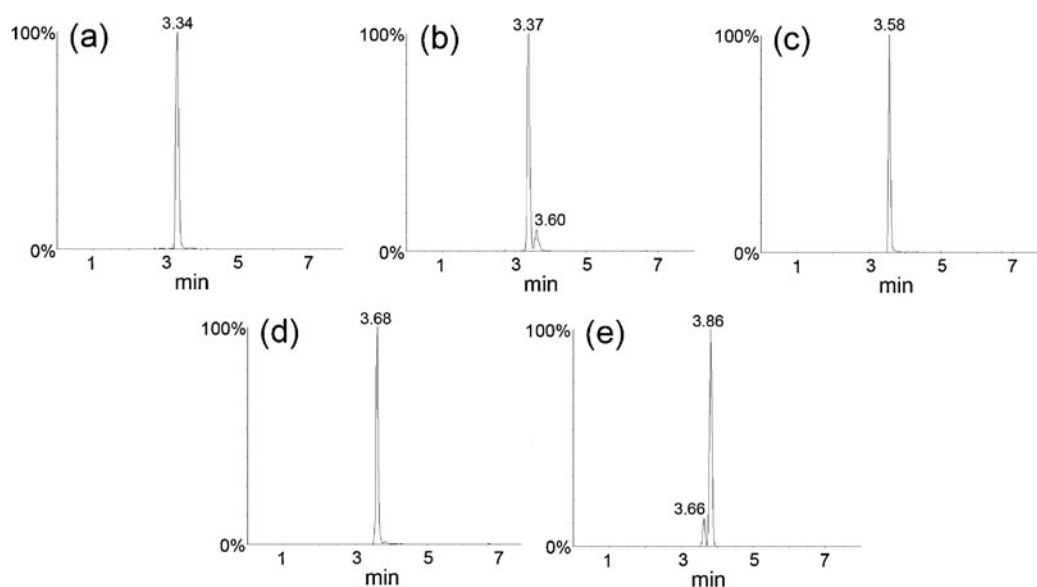


Fig. 4 Extracted ion LC-MS-MS chromatograms generated from a mixture of stevia glycoside standards, showing **a** Reb A, **b** stevioside and Reb B, **c** Reb C, **d** dulcoside A, and **e** steviolbioside and rubusoside. Additional standards are not shown because they were not measured as part of the targeted LC/MRM method

Reb A, B, and C in more than 1,100 stevia leaf extracts and use this information to guide subsequent plant breeding efforts. Reproducibility of the method was assessed using technical replicate injections of leaf extracts, and these analyses yielded relative standard deviations <5 % for all target analytes. The diversity of the content of the four metabolites of interest across this large set of tissue samples is depicted in the histograms displayed in Fig. 5. Our findings demonstrate that stevia glycoside abundances range over about two orders of magnitude as follows (normalized to dry weights): stevioside (2–125 mg/g), Reb A (2.5–164 mg/g), Reb B (0.5–50 mg/g), and Reb C (1.5–125 mg/g). A few plant extracts had high contents of specific stevia glycosides. For example, 12 tissue extracts (approximately 1 % of the population) contained more than 40 mg/g dry weight of Reb C, with two tissue extracts exceeding 100 mg/g DW.

Separation of stevia glycoside isomers present in stevia leaves

The LC/MRM method resolved chromatographic peaks for the target glycosides, based on characteristic chromatographic retention times and MRM mass transitions. However, additional peaks were frequently observed, and these did not correspond to signals that could be attributed to any of the stevia glycosides available as standards. These features are illustrated in Fig. 6. In the first instance (Fig. 6a) that reports results for a single stevia leaf extract, at least six chromatographic peaks are evident for the MRM transition of m/z 641>479, which targets detection of steviolbioside (peak a2) and rubusoside (peak a3). For this particular extract, the greatest signal (peak a1) does not correspond to any metabolite for which standards were available. Figure 6b displays signal for an additional channel that was added for this specific analysis, targeting detection of Reb F using MRM transition m/z 935>773. The surprising evidence in this chromatogram suggests that the Reb F channel detects one major and one minor

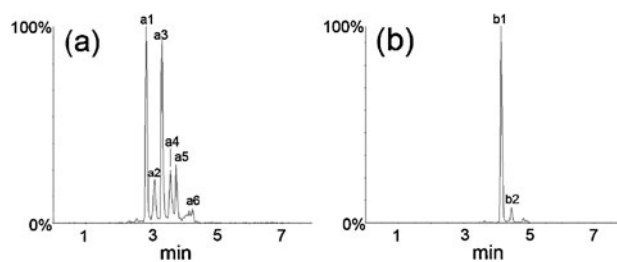


Fig. 6 Extracted ion UHPLC–MS–MS chromatograms of stevia leaf extracts showing two MRM channels on stevia leaf extract sample. (a) m/z 641>479 (steviolbioside and isomers) and (b) m/z 935>773 (Reb F and isomer)

isomer. To our knowledge, this extent of diversity in isomers among the stevia glycosides has not previously been demonstrated, though a recent report of a UHPLC–MS method reported a second isomer of dulcoside A [7]. Such findings highlight the dilemma faced in large-scale metabolite profiling, in that increased throughput usually comes at a cost of decreased resolution. The 8-min gradient described in this study, when coupled to MRM detection, provides a robust compromise between resolution and throughput.

Two explanations are considered for the apparent detection of additional stevia glycoside isomers. Since some enzymes involved in specialized metabolite biosynthesis exhibit substrate promiscuity, more than one isomer may be formed from a specific substrate. In such cases, numerous products, often including isomers, may form from common biosynthetic catalysts [16]. We cannot rule out that some of these peaks arise from diversity in attached sugars or sites or orientations of attachment. An alternative explanation suggests that some of the extra peaks arise from metabolites that are more extensively glycosylated, and in-source fragmentation of acylglycosides could generate ions of the same masses as the target glycosides. Most of the peaks attributed to isomers elute later than the target metabolites, and it is anticipated that more glycosylation would usually shorten retention times on the C18 column. For these reasons, it is

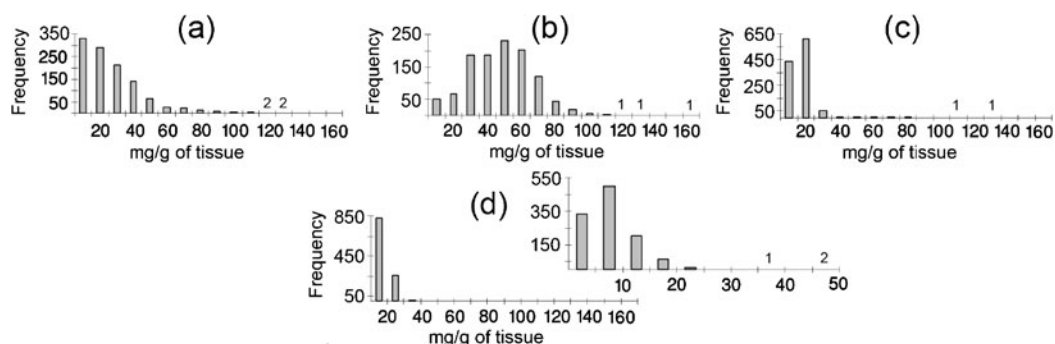


Fig. 5 Distribution of a stevioside, b Reb A, c Reb C, and d Reb B in more than 1,100 extracts from stevia leaves. The inset in d shows the magnified values for Reb B for lower levels

proposed that variable glycosylation may be more extensive than has previously been resolved using chromatographic methods.

Conclusions

Profiling of bioactive metabolites in large numbers of plant tissue samples invariably encounters the conflict between throughput and depth of coverage of the target metabolites. As chromatographic systems improve in resolution and selectivity, it is frequently the case that metabolites that had previously been missed are now resolved and detected, yet the throughput needed for large-scale profiling often mandates a sacrifice of analytical resolution. The LC–MS–MS method used in this study for quantifying stevia glycosides and aglycones achieved a functional compromise between these pressures and delivered analysis times of 8 min/sample, plus about 1 min interval for injection of the next extract. Limits of detection were far lower than needed for characterization of plant extracts, yielding low nanogram-per-milliliter limits of quantification. It is anticipated that this methodology should be readily adapted to measure stevia glycosides and their degradation products in beverages and other food products. This method was used for quantification of stevia glycosides in more than 1,100 extracts from stevia leaves from a large plant population, usually in batches of two 96-well plates at a time. The broad range of levels measured in this study serves to indicate the chemical diversity among stevia leaf tissues, and the small fraction of hyper-accumulating plants instructs us regarding the need to analyze large numbers of samples when developing plants with desirable biochemical traits.

Acknowledgments The authors are grateful for access to the QTRAP 3200 mass spectrometer housed in the RTSF Mass Spectrometry Facility at Michigan State University and technical support from Manshui Zhou, Lijun Chen, Beverly Chamberlin, Lynn Sage, and Patrick Abeli. Support for this project was provided by PureCircle Sdn Bhd and Michigan AgBioResearch.

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