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Steviol Quantification at the Picomole Level by High-Performance Liquid Chromatography

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A simple and highly sensitive reversed-phase high-performance liquid chromatographic method (RP-HPLC) has been developed for the determination of steviol (SV) using dihydroisosteviol (DHISV) as an internal standard (IS). SV and DHISV were derivatized by reaction of the acids with 4-(bromomethyl)-7-methoxycoumarin in an aprotic solvent (DMF or acetone). The resulting ester derivatives were separated on an ODS column (250 × 4.6 mm i.d., 5 μm particle size) using fluorescence detection with excitation at 321 nm and emission at 391 nm. The mobile phase consisted of acetonitrile/water (80:20 v/v) with a flow rate of 1 mL min^{-1}. A linear relationship was observed for concentrations between 0.5 and 50 μg/mL of SV, and the detection limit was 100 pg. For application of this method to samples of beer fortified with stevioside, a simple procedure for extraction of the beer with diethyl ether and derivatization in DMF was applied. Whereas beer samples spiked with SV gave a linear response over the range 0.1−15 μg/mL beer, no SV could be detected in beer samples enriched in stevioside that had been stored for over 3 years. The application of the method to plant samples involved preparation of an acid fraction containing the SV analyte, derivatization, and sample cleanup using small silica columns and thin-layer chromatography. A sensitive determination of 594 ng of steviol present in 100 mg of dry plant material was performed with high precision and accuracy.

KEYWORDS: ***Stevia rebaudiana*** **(Bertoni) Bertoni; steviol; dihydroisosteviol; 4-(bromomethyl)-7-methoxycoumarin; fluorescence detection; biological fluids; plants**

INTRODUCTION

Stevia rebaudiana (Bertoni) Bertoni is a perennial shrub of the Asteraceae (Compositae) family native to certain regions of South America (Paraguay and Brazil). The main sweet component in the leaves of *S. rebaudiana* Bertoni is stevioside. Its content varies between 4 and 20% of the dry weight of the leaves depending on the cultivar and growth conditions (*1*). Other compounds present in lower concentration are dulcoside A (±0.5%), steviolbioside (trace), and rebaudiosides A (±3%), B (trace), C (±1.5%), D, E, and F (traces). The presence of steviolbioside and rebaudioside B in extracts might be due to artifacts of the extraction procedure (*2*, *3*).

Stevioside is a diterpene glycoside. It is a high-intensity sweetener that is ∼300 times sweeter than sucrose (0.4% solution). In several countries it is used as a low-calorie sweetener in a wide range of food products and beverages. The plant, its extracts, and stevioside have been used for several years as a sweetener in Brazil, Korea, and Japan. In the United States it has been used as a dietary supplement since 1995 (*4*).

The human body does not take up oral stevioside (*5*−*7*), and none of the digestive enzymes from the gastrointestinal tract of different animals and man are able to degrade stevioside into steviol (SV), the aglycon of stevioside (*8*−*10*). Nevertheless, in feeding experiments with rats and hamsters stevioside was metabolized to SV by the bacterial flora of the cecum. SV was found in the blood of the animals with the maximum concentration occurring after 8 h (*10*, *11*). Although bacteria isolated from the human colon are able to transform stevioside into SV in vitro (*9*, *10*), it has never been proven that this is also the case in vivo nor that the SV possibly formed is taken up directly from the colon. Mutagenic effects of SV in a forward mutation assay under conditions of metabolic activation (*12*) and/or its metabolites were published (*12*−*18*). After metabolic activation, it was shown that so far unknown steviol metabolites caused mutations in *Salmonella typhimurium* TM677, that is, transitions, transversions, duplications, and deletions at the guanine phosphoribosyltransferase (*gpt*) gene (*15*). However, stevioside and steviol were inactive in various TA strains of *S. typhimurium*, *Escherichia coli* WP2 *uvrA*/pKM101, and the *rec*-assay using *Bacillus subtilis* even when activated microsomal fraction was present (*16*, *17*). The direct mutagenic activity of 15-oxosteviol was refuted by ref *19* but confirmed by ref *18*. The activity of

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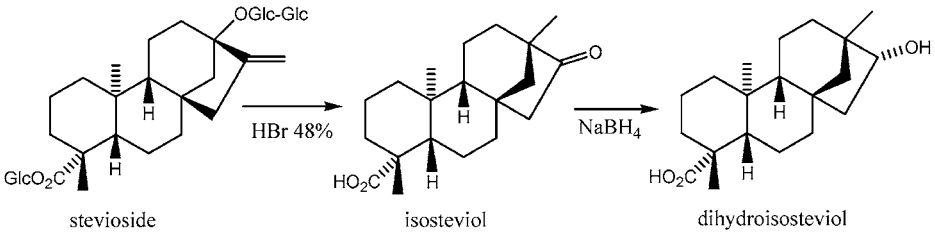

Figure 1. Synthesis of dihydroisosteviol.

steviol in *S. typhimurium* TM677 was very low and was only ~1/3000 that of 3,4-benzopyrene; that of steviol methyl ester 8,13 lactone was 1/24500 that of furylfuramide (*18*). More information is needed about the possible uptake of SV by the colon and its presence in the blood. Hence, the sensitive measurement of SV is of utmost importance. However, the measurement of low concentrations of free SV suffers from two main difficulties: the sensitivity and specificity of a UV detector are rather low, and losses due to adsorption of the free carboxylic acid to active surfaces may be high. Not every laboratory has easy access to LC-MS equipment, so we developed a method for derivatization of SV as a fluorescent ester, which allows for quantification of SV at the picomole level using dihydroisosteviol (DHISV) as an internal standard (IS).

MATERIALS AND METHODS

Chemicals and Solutions. Reagents, HPLC grade solvents, and chemicals were from Acros (water, acetonitrile, $CHCl_3$, sodium periodate, sodium sulfate, and *N*,*N*-diisopropylethylamine), BDH (MeOH, EtOH, and *N*,*N*-dimethylformamide), Fluka (hydrobromic acid, 4-(bromomethyl)-7-methoxycoumarin and silica gel 60 F254 TLC plates), Biosolve (acetone), and Applichem (sodium borohydride).

Stevioside was purchased at Stevita Co. Inc. (Arlington, TX) and purified by repeated crystallization from MeOH to >97% purity. The main remaining impurities were rebaudioside A and steviolbioside.

To avoid losses due to adsorption of SV to active glass surfaces, all manipulations were done in polypropylene tubes and pipet tips.

HPLC Instrumentation and Chromatographic Conditions. The Shimadzu SCL-6A HPLC system consisted of an SIL-6A autosampler, an LC-6A pump, and an RF-10AXL scanning fluorescence detector. Data acquisition and analysis were performed using a personal computer and Shimadzu Class-VP chromatography software. The analytical column was a reversed-phase ODS column (250 × 4.6 mm i.d., 5 μm particle size) obtained from Alltech Europe (Lokeren, Belgium). The analysis was performed using an isocratic solvent mixture of acetonitrile/water (80:20 v/v) at 1 mL min^{-1}. The column eluate was monitored fluorometrically (λ_{ex} = 321 nm, λ_{em} = 391 nm).

Preparation of SV. A solution of 2 g of pure stevioside and 3 g of sodium periodate in 150 mL of water was stirred for 20 h at room temperature to effect cleavage of C−C bonds of vicinal hydroxyls in the sugar moieties. Then KOH (15 g) was added and the solution refluxed for 2 h. The mixture was cooled in ice water for 30−60 min, then slowly acidified to pH 5.5 with glacial acetic acid, and extracted three times with 200 mL of diethyl ether. The organic layer was washed three times with water, dried over 3 g of sodium sulfate, filtered, and evaporated using a stream of N_2 (*20*). Crystallization from MeOH gave 294 mg of a white crystalline product, identical with authentic SV. The authenticity and purity of SV were confirmed by mass spectrometry (electrospray, negative mode), ^{13}C NMR, and ^1H NMR. SV had a melting point of 202−203 °C (*14*); $[\alpha]_D$ = − 96.4 (*c* 6.666 g L^{-1} $CHCl_3$/MeOH 1:2).

Preparation of DHISV, the Internal Standard. In a first step pure stevioside was hydrolyzed to yield isosteviol (ISV) according to the method of ref *21*. A solution of 1 g of the glycoside in 15 mL of 48% aqueous hydrobromic acid was stirred at room temperature for 20 h. The ISV formed crystallized rapidly from the solution. The crystals were collected by centrifugation and washed several times with water. Hexagonal ISV crystals (147 mg) with a melting point of 226−228 °C, $[\alpha]_D$ = +291.1 (*c* 10 g L^{-1} MeOH), were obtained from ether/hexane (*21*).

In a second step the ketone function of ISV was reduced to form DHISV (4α,8β,13β,16α)-16-hydroxy-13-methyl-17-norkauran-18-oic acid, further called dihydroisosteviol (DHISV), after ref *18* (modified). A solution of 1 g of ISV in 50 mL of ethanol was cooled to 0 °C. Then 5 g of sodium borohydride ($NaBH_4$) was added with stirring at room temperature for ~2 h. The reduction of ISV was monitored by thin-layer chromatography (TLC). Silica gel 60 F254 plates were loaded with samples of the reaction mixture and developed with $CHCl_3$/MeOH (90:10). Detection was effected by spraying with 50% H_2SO_4 in EtOH and heating at 150 °C for 5 min. The R_f values for ISV and DHISV were 0.58 and 0.37, respectively. When reduction was completed, the solution was acidified with 6 N HCl and the pellet of NaCl was collected by centrifugation. DHISV was precipitated by adding water to the supernatant; the precipitate was collected by centrifugation and purified by crystallization from MeOH. The identity and purity of DHISV were confirmed by electrospray mass spectrometry (negative ion mode: $[M - H]^-$ at *m*/*z* 319) and by ^{13}C NMR and ^1H NMR spectrometry. DHISV had a melting point of 181−183 °C; $[\alpha]_D$ = +315.8 (*c* = 10 g L^{-1} MeOH). An outline for the conversion of SV into DHISV is shown in **Figure 1**.

Calibration Curves. Calibration samples corresponding to nine different concentrations of SV (end concentrations between 0.5 and 50 ng μL^{-1} DMF) were obtained by diluting a stock solution of SV with acetone and adding 4 μg of internal standard to each sample. The acetone was evaporated using a stream of N_2. To enable sensitive fluorometric analysis, SV and DHISV were derivatized by esterification of the free carboxyl group with the alkylating reagent 4-(bromomethyl)-7-methoxycoumarin [UPAC name: 4-(bromomethyl-7-*2H*-chromen-2-one] (**Figure 2**). This reaction was carried out in the aprotic solvent *N*,*N*-dimethylformamide (DMF) in the presence of 1 μL of *N*,*N*-diisopropylethylamine. No traces of water should be present. To ensure a quantitative derivatization, a 5-fold excess of reagent was used, for example, 250 μg of reagent was added to 50 μg of SV. When the amount of SV was lower than 10 μg, a larger excess of 50 μg of reagent and 1 μL of *N*,*N*-diisopropylethylamine in 200 μL of DMF was added to ensure quantitative derivatization. The reaction mixture was heated at 70 °C for 20 min, and 1 μL of the resulting sample solution was used for HPLC analysis. Standard curves were run in triplicate. To measure intraday variation, a sample was derivatized three times a day with an interval of 3 h. Interday variation was registrated on three random days of one week.

Extraction of Beer. Stepa beer (pH 4.5; Stepaja, Ghent, Belgium, lot 1999-01) containing stevioside was stored for >3 years in the dark at 16 °C. After the beer had been degassed, 4 μg of internal standard dissolved in acetone was added to 1 mL beer samples. These samples were extracted three times with equal volumes of peroxide-free diethyl ether. The combined ether fractions were evaporated to dryness under a stream of N_2. No water should be present, as this would interfere with the derivatization that must be executed under aprotic conditions. Derivatization of the water-free residues and HPLC analysis of the resulting ester compounds was carried out as described above.

Extraction and Derivatization of SV from Plant Material. To 100 mg of freeze-dried powdered plant material was added 4 μg of internal standard in acetone solution. After evaporation of the acetone, the residues were extracted three times with 0.5 mL of 2% KOH in MeOH in Eppendorf conical flasks. During extraction the tubes were kept at 4 °C in the dark and vortexed continuously. After centrifugation,

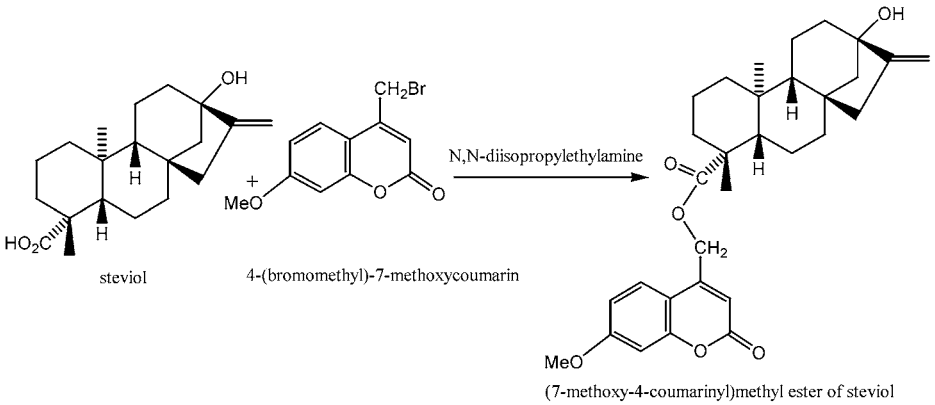

Figure 2. Reaction scheme for the derivatization of steviol.

the extracts were collected and 5 mL of water was added to the combined extracts. The lipids were removed by three extractions with 5 mL of diethyl ether. The water phase was acidified to pH 6 by the addition of 6 N acetic acid. Acidification with HCl to lower pH values (pH ≈3) was avoided to prevent the conversion of SV into ISV. The SV was extracted three times with equal volumes of peroxide-free diethyl ether. The combined ether fractions were evaporated to dryness under a stream of N_2, and the water-free residue was derivatized in dry acetone as described above.

After derivatization, the acetone was evaporated under a stream of N_2 and the residue dissolved in 1 mL of $CHCl_3$. Chromatographic sample cleanup was carried out using small columns of 250 mg silica gel applied to 1 mL pipet tips containing a plug of glass wool. The solution containing the (7-methoxy-4-coumarinyl)methyl ester derivatives of SV and DHISV was placed on the silica gel column. The column was rinsed first with 2 mL of $CHCl_3$ and then with 1 mL of $CHCl_3$/MeOH (98:2), and finally the mixture of ester derivatives was eluted with 1 mL of $CHCl_3$/MeOH (80:20). The eluate could be directly injected onto the HPLC column or further concentrated under a stream of N_2 for further sample cleanup by TLC.

TLC purification of the derivatives was carried out on silica gel 60 F254 plates using $CHCl_3$/MeOH (98:2) as the developing solvent. The coincident (7-methoxy-4-coumarinyl)methyl esters of SV and DHISV (R_f = 0.37) were localized under UV (366 nm) by their blue fluorescence. The silica gel was scraped off and eluted with $CHCl_3$/MeOH (80:20). After evaporation of the solvent under a stream of N_2, the residue was dissolved in 200 μL of acetone; 5 μL of this solution were injected onto the HPLC column.

RESULTS AND DISCUSSION

The present method was developed to determine the concentrations of SV in diverse biological fluids (urine and blood) and in feces, as well as in different foods using an HPLC method with fluorometric detection. DHISV, a structural analogue of SV, was chosen as internal standard as it should possess similar properties with respect to extraction, TLC, and fluorescence. An even closer analogue, that is, dihydrosteviol, could be prepared by hydrogenation of the alkenic linkage of SV using a palladium on carbon catalyst in methanol; however, the resulting product invariably was contaminated by isosteviol, the ketone rearrangement product of SV. Therefore, we preferred to use DHISV as an internal standard, prepared via reduction of isosteviol by $NaBH_4$; attack of this reagent proceeds with high diastereoselectivity from the exo face of the ketone group to form exclusively the endo alcohol product (*22*). Separation of the (7-methoxy-4-coumarinyl)methyl esters of SV and internal standard was performed on a reversed-phase ODS column (250 × 4.6 mm i.d., 5 μm particle size) from Alltech using an isocratic mobile phase mixture of acetonitrile/water (80:20). Under these conditions the retention times of the (7-methoxy-4-coumarinyl)methyl esters of SV and the internal standard were 13.5 and 21 min, respectively.

Table 1. Comparison of the Ratio of Peak Areas of SV/IS of Standard Samples That Are Directly Derivatized and of Standard Samples Mixed with Beer and Derivatized after Extraction and Sample Cleanup (Mean ± SE)[a]

amount of SV (μg)	standard solution ratio SV/IS	beer ratio SV/IS	peak area SV as % of standards	peak area IS as % of standards
0.1	0.061 ± 0.008	0.089 ± 0.007	41.09 ± 0.08	34.56 ± 0.01
1	0.470 ± 0.016	0.462 ± 0.003	37.88 ± 0.02	38.56 ± 0.01
6	2.699 ± 0.025	2.825 ± 0.140	34.24 ± 0.05	34.21 ± 0.01
mean			37.73 ± 0.03	35.74 ± 0.03

[a] The peak areas of SV and internal standard (IS) of beer samples are given as a percentage of the areas of corresponding standard samples that are directly derivatized.

Using these fluorescent ester derivatives of SV and DHISV enabled a very sensitive measurement of SV. Whereas the detection limit for SV by UV (210 nm) is ∼50 ng per injection (*23*), a 500 times lower quantification limit of 100 pg per injection was achieved when a fluorescence detector was used. This detection limit was found to depend on the chemical noise caused by impurities present in the solvents and chemicals used. During method development, the quality of the solvents and chemicals used proved to be very critical for avoiding chemical noise and, hence, blanks should always be run.

The calibration curve $y = 0.348x$ was linear over the range of 0.5−50 ng injected with a correlation coefficient of 0.9992 (n = 3). The intraday (n = 9) and interday (n = 9) variations were 0.64 and 0.88%, respectively. The limit of quantitation was 100 pg per injection (peak height 5 times the noise level). To determine the losses of SV and internal standard during sample manipulation and cleanup, we compared three samples of SV (0.1, 1, and 6 μg), each containing 4 μg of internal standard. One series of samples was directly derivatized, the other one after mixing with 1 mL of beer followed by extraction and sample cleanup. The results are summarized in **Table 1**. For both SV and internal standard, peak areas after sample cleanup were ∼60% smaller than those of samples derivatized without sample cleanup. However, the ratio of SV/IS did not change significantly as a result of sample cleanup, implying that losses of SV were adequately corrected for by the internal standard. These results demonstrate the functionality and usefulness of DHISV as internal standard.

Analysis of beer samples containing stevioside that had been stored for >3 years in the dark at 16 °C did not reveal any trace of SV. Therefore, the methodology was checked by spiking

beer samples with known amounts of SV (between 0.1 and 15 μg/mL beer) and internal standard (4 μg/mL beer). Extraction and derivatization were performed as described above. Plotting the ratio of the peak areas of SV over internal standard versus added SV concentration produced a linear curve $y = 0.359x$ with a correlation coefficient of 0.9997. This result demonstrates that the recovery of both low and high amounts of added SV was excellent, implying that the failure to detect SV in the beer samples was not due to an inadequate methodology but was a consequence of the solution stability of stevioside, which was not degraded into SV after 3 years of shelf life.

After extraction of samples of freeze-dried powdered plant material from *S. rebaudiana*, traces of SV (5.9 ± 0.8 μg g^{-1} plant material) were detected. The identity of the SV peak was checked by co-injection of the sample with authentic (7-methoxy-4-coumarinyl)methyl ester of SV.

This low amount of SV in dried Stevia leaves presumably does not pose any health problems when dried leaves are consumed as a sweetener. To substitute for all sugar added to our food (~130 g/day), ~4.3 g of dried *Stevia* leaves would be required (10% sweetener content), implying an intake of ~25 μg of SV. This is a negligible amount of SV directly available for uptake by the intestines (*7*, *8*), as up to 250 mg of SV/kg of body weight fed to hamsters was without harmful effects (*24*).

The derivatization of crude plant extracts followed by sample cleanup lowers potential losses due to adsorption of free carboxylic acids to active surfaces. Following derivatization a very simple chromatographic purification using small silica columns removes 95% of the reagent and ~75% of free fatty acids that are possibly present (the latter are equally derivatized, results not shown), as well as more polar hydroxylated carboxylic acids. Further sample cleanup without large losses can be achieved efficiently by preparative TLC using $CHCl_3$/MeOH (98:2) as a solvent; the coeluting SV and DHISV derivatives are isolated by elution with $CHCl_3$/MeOH (80:20). The (7-methoxy-4-coumarinyl)methyl ester derivatives can be localized by their blue fluorescence (UV 336 nm). The detection limit for SV is ~100 pg per injection, which corresponds to the sensitivity of LC-MS as used by Koyama et al. (*7*).

Either DMF or acetone was used as aprotic solvent for derivatization of the SV and DHISV carboxylic acids. The low volatility of DMF is advantageous when no sample cleanup is necessary, thus enabling the direct use of the reaction vials in the HPLC injector. Acetone was used for samples that require extensive sample cleanup (plant material, blood samples) as acetone is easily evaporated following the esterification reaction.

In conclusion, the present RP-HPLC procedure for fluorometric quantification of SV is highly sensitive and specific, simple, precise, accurate, and reproducible, thus permitting direct analysis of diverse biological fluids with a high sample throughput. The procedure is also suitable for the analysis of plant material after chromatographic sample cleanup using small silica columns and TLC. Only minute amounts of biological samples are required. The assay described was used for the determination of SV in blood plasma, feces, and urine samples from volunteers administered stevioside during clinical studies (unpublished results). The analysis procedure presented here is the first one able to quantify SV in diverse biological samples using DHISV as internal standard.

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LITERATURE CITED

(1) Geuns, J. M. C. Safety of *Stevia* and stevioside. *Recent Res. Dev. Phytochem.* **2000**, *4*, 75−88.

(2) Kennelly, E. J. Sweet and non-sweet constituents of *Stevia rebaudiana* (Bertoni) Bertoni. In *Stevia, the Genus Stevia. Medicinal and Aromatic Plants−Industrial Profiles*; Kinghorn, A. D., Ed.; Taylor and Francis: London, U.K., 2002; Vol. 19, pp 68−85.

(3) Starratt, A. N.; Kirby, C. W.; Pocs, R.; Brandle, J. E. Rebaudioside F, a diterpene glycoside from *Stevia rebaudiana*. *Phytochemistry* **2002**, *59*, 367−370.

(4) Kinghorn, A. D. Overview. In *Stevia, the Genus Stevia. Medicinal and Aromatic Plants−Industrial Profiles*; Kinghorn, A. D., Ed.; Taylor and Francis: London, U.K., 2002; Vol. 19, pp 1−17.

(5) Yamamoto, N. S.; Bracht, K.; Ishii, A. M.; Kemmelmeier, E. L.; Alvarez, F. S.; Bracht, A. Effect of steviol and its structural analogues on glucose production and oxygen uptake in rat renal tubules. *Experientia* **1985**, *41*, 55−57.

(6) Bracht, A. M.; Kemmelmeier, F. S.; Ishii, E. L.; Alvarez, M.; Bracht, A. Effect of *Stevia rebaudiana* natural products on cellular and sub-cellular metabolism. *Arq. Biol. Tecnol.* **1985**, *28*, 431−455.

(7) Koyama, E.; Sakai, N.; Ohori, Y.; Kitazawa, K.; Izawa, O.; Kakegawa, K.; Fujino, A.; Ui, M. Absorption and metabolism of the glycosidic sweeteners, *Stevia* related compounds in human and rat. *Food Chem. Toxicol.* **2003**, *41*, 875−883.

(8) Geuns, J. M. C.; Augustijns, P.; Mols, R.; Buyse, J. G.; Driessen, B. Metabolism of stevioside in pigs and intestinal absorption characteristics of stevioside, rebaudioside A and steviol. *Food Chem. Toxicol.* **2003**, *41*, 1599−1607.

(9) Hutapea, A. M.; Toskulkao, C.; Buddhasukh, D.; Wilairat, P.; Glinsukon, T. Digestion of stevioside, a natural sweetener, by various digestive enzymes. *J. Clin. Biochem. Nutr.* **1997**, *23*, 177−186.

(10) Koyama, E.; Kitazawa, K.; Ohori, Y.; Izawa, O.; Kakegawa, K.; Fujino, A.; Ui, M. In vitro metabolism of the glycosidic sweeteners, stevia mixture and enzymatically modified stevia in human intestinal microflora. *Food Chem. Toxicol.* **2003**, *41*, 359−374.

(11) Nakayama, K.; Kasahara, D.; Yamamoto, F. Absorption, distribution, metabolism and excretion of stevioside in rats. *J. Food Hyg. Soc. Jpn.* **1986**, *27*, 1−8.

(12) Pezzuto, J. M.; Compadre, C. M.; Swanson, S. M.; Nanayakkara, N. P. D.; Kinghorn, A. D. Metabolically activated steviol, the aglycone of stevioside, is mutagenic. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 2478−2482.

(13) Temcharoen, P.; Pimbua, J.; Glinsukon, T.; Rojanapo, W.; Apibal, S. Mutagenic activity of steviol to *Salmonella typhimurium* TM677: Comparison of the activity of S9 liver fractions from five laboratory animal species. *Bull. Health Sci. Technol.* **1998**, *1*, 38−45.

(14) Temcharoen, P.; Klongpanichpak, S.; Glinsukon, T.; Suwannatrai, M.; Apibal, S.; Toskulkao, C. Evaluation of the effect of steviol on chromosomal damage using micronucleus test in three laboratory animals species. *J. Med. Assoc. Thailand* **2000**, *83*, 101−107.

(15) Matsui, M.; Matsui, K.; Kawasaki, Y.; Oda, Y.; Noguchi, T.; Kitagawa, Y.; Sawada, M.; Hayashi, M.; Nohmi, T.; Yoshihira, K.; Ishidate, M.; Sofuni, T. Evaluation of the genotoxicity of stevioside and steviol using six *in vitro* and one *in vivo* mutagenicity assays. *Mutagenesis* **1996**, *11*, 573−579.

(16) Matsui, M.; Sofuni, T.; Nohmi, T. Regionally-targeted mutagenesis by metabolically-activated steviol: DNA sequence analysis of steviol-induced mutants of guanine phosphoribosyltransferase (*gpt*) gene of *Salmonella typhimurium* TM677. *Mutagenesis* **1996**, *11*, 565−572.

(17) Klongpanichpak, S.; Toskulkao, C.; Temcharoen, P.; Apibal, S.; Glinsukon, T. Lack of mutagenicity of stevioside and steviol in *Salmonella typhimurium* TA98 and TA100. *J. Med. Assoc. Thailand* **1997**, *80*, 121−128.

(18) Terai, T.; Ren, H.; Mori, G.; Yamaguchi, Y.; Hayashi, T. Mutagenicity of steviol and its oxidative derivatives in *Salmonella typhimurium* TM677. *Chem. Pharm. Bull.* **2002**, *50*, 1007−1010.

(19) Procinska, E.; Bridges, B. A.; Hanson, J. R. Interpretation of results with 8-azaguanine resistance system in *Salmonella typhimurium*: no evidence for direct acting mutagenesis by 15-oxosteviol, a possible metabolite of steviol. *Mutagenesis* **1991**, *6*, 165−167.

(20) Ogawa, T.; Nozaki, M.; Matsui, M. Total synthesis of stevioside. *Tetrahedron* **1980**, *36*, 2641−2648.

(21) Mosettig, E.; Beglinger, U.; Dolder, F.; Lichti, H.; Quitt, P.; Waters, J. A. The absolute configuration of steviol. *J. Am. Chem. Soc.* **1963**, *85*, 2305−2309.

(22) Al'fonsov, V. A.; Bakaleinik, G. A.; Gubaidullin, A. T.; Kataev, V. E.; Kovylyaeva, G. I.; Konovalov, A. I.; Litvinov, I. A.; Strobykina, I. Y.; Strobykin, S. I.; Andreeva, O. V.; Korochkina, M. G. Chemistry and structure of diterpenes of the kaurane series: II. Reduction of isosteviol. *Russ. J. Gen. Chem. (Engl. Transl.)* **2000**, *70*, 953−960.

(23) Geuns, J. M. C.; Malheiros, R. D.; Moraes, V. M. B.; Decuypere, E. M.-P.; Compernolle, F.; Buyse, J. G. Metabolism of stevioside by chickens. *J. Agric. Food Chem.* **2003**, *51*, 1095−1101.

(24) Wasuntarawat, C.; Temcharoen, P.; Toskulkao, C.; Mungkornkarn, P.; Suttajit, M.; Glinsukon, T. Developmental toxicity of steviol, a metabolite of stevioside, in the hamster. *Drug Chem. Toxicol.* **1998**, *21*, 207−222.

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