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QUANTITATIVE AND QUALITATIVE ANALYSIS OF STEVIOL GLYCOSIDE IN STEVIA BY VARIOUS ANALYTICAL METHODS: A REVIEW

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ABSTRACT

Stevia rebaudiana, a perennial herb in the family Asteraceae, is a popular natural sweetener with sensory and functional attributes that outperform those of conventional high-potency sweeteners. Stevia rebaudiana leaves contain sweet glycosides that have biological effects. The various glycosides included in extracts must be measured since, in addition to the major glycosides, they may also contribute to activity. The isocratic HPLC method was validated for the identification of dulcoside A, steviolbioside, rebaudioside C, and rebaudioside B. The development and validation of the HPTLC technique for the simultaneous measurement of three steviol glycosides in Stevia rebaudiana leaves—steviolbioside, stevioside, and rebaudioside-A—also employed the ICH (International Conferences on Harmonisation) guidelines.

Near-infrared reflectance spectroscopy (NIRS) was used to assess the steviol glycosides (SGs) content (w/w) dry basis in Stevia rebaudiana leaves using high-pressure liquid chromatography (HPLC) as the reference technique. The noncariogenic and caloric sweeteners known as steviol-glycosides, which are present in the leaves of Stevia rebaudiana, may have had positive effects on human health.

Despite the fact that steviol-glycosides are generally regarded to be safe, investigations on animals have shown that the aglycone steviol can have negative consequences. 2D NMR methods and ultra-high performance liquid chromatography with electrospray ionisation mass spectrometry (UHPLC-MS) were used to discover steviol glycosides. The steviol glycosides were then quantified by NMR using anthracene as an internal standard.

KEYWORDS: ***Steviol Glycoside, HPLC, HPTLC, Stevioside.***

INTRODUCTION

Stevia rebaudiana is a little perennial with sessile, oppositely oriented leaves that can reach heights of 65 to 80 cm. A semi-humid subtropical plant, it is simple to cultivate in a kitchen garden. A Japanese collaboration established for the goal of commercializing stevioside and stevia extracts gave stevioside, the main sweetener found in the leaf and stem tissues of stevia, its first serious consideration as a sugar alternative in the early 1970s. Stevia leaves are a key source of natural sweetener for the expanding food market because they offer sensory and functional qualities that are superior to many other high-potency sweeteners. Flavonoids, alkaloids, chlorophylls, xanthophylls, hydroxycinnamic acids, oligosaccharides, free sugars, amino acids, lipids, and trace elements are all present in the dry extract of stevia leaves. One of the stevia glycosides, stevioside, is around 300 times sweeter than saccharose and has potential health benefits.

Sweetener extracts from S. rebaudiana have been associated to anti-hypertensive, anti-hyperglycemic, and anti-human rotavirus action, among other benefits to human health. Liquid chromatography was used in combination with UV, MS, and ELS detection to analyze the sweet stevia glycosides; steviol being the prevalent aglycone backbone (Cacciola et al., 2011). Stevioside, one of the stevia glycosides, is nearly 300 times sweeter than saccharose and may be especially beneficial for those with diabetes, obesity, heart disease, and dental cavities. Fruit custard, jam, chikki, basen ladu, wheat ladu, biscuit, grape juice, bun, tea, and milk shake all included stevia. It was discovered to have a larger amount of the anti-nutritional component oxalic acid, which may impair calcium, iron, and other nutrient bioavailability.

The sweet taste sensation is produced by glycosides such as dulcoside A, rebaudiosides A-E, steviolbioside, and stevioside. The tastiest species, S. rebaudiana Bertoni, has all eight ent-kaurene glycosides, including stevioside. It also includes stigmasterol, b-sitosterol, and campesterol, as well as steviol, a plant-derived enzymatic hydroxylation product. Other compounds with no sweet flavour can also be detected in Stevia

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species, and some of them may even be harsh. Stevia contains two types of seeds: black and tan. Black seeds have better viability and germination potential, but tan seeds are nonviable since they are formed without fertilisation[1,2,3,4,5,6,7].

EXTRACTION METHOD

S. rebaudiana (Bertoni) leaves were ground and filtrated through a filter screen (ca.380m), then dried in an oven at 80°C until the weight remained constant. In an extraction container, five grammes of the powder were combined with extraction solvent. Samples were warmed for 5 minutes in a constant temperature water bath before being homogenized using High Speed Shear Homogenization. An FA25 system was used for extraction. The ethanol concentration was changed from 0% to 100%, the extraction period from 2 to 12 minutes, and the solid-to-liquid ratio from 1:10 to 1:30. Following the extraction, the mixture was vacuum filtered using medium-speed filter paper, and the filtrate was collected. The extraction solution was filtered through a 0.22 m membrane and the amounts of STV and RA were determined using HPLC. All extraction procedures were carried out three times.

The best conditions for SE extraction were 50% ethanol concentration, a solid-liquid ratio of 1:1, a 60-minute extraction period, a temperature of 60°C, and a one-time extraction.[8,10]

CHEMICAL COMPOSITION

Steviobioside, dulcosides A through E, and stevioside were discovered among the eight Stevia glucosides discovered. The leaves also included three esters of lupeol, the triterpene amyrin acetate, and the sterols stigmasterol, sitosterol, and campesterol.

Jhanol, austroinulin, 6-O-acetylaustroinulin, and 7-O-acetylaustroinulin, stevioside, and rebaudioside A have all been derived from stevia flowers.Six flavonoid glycosides, including apigenin-4'-O-glucoside, luteolin-7-O-glucoside, kaempferol-3-O-rhamnoside, quercetin, quercetin-3-O-glucoside, quercetin-3-O-arabinoside, and 5,7,3'-trihydroxy-3, 6,4'-d-galactopyranoside, have been extracted from aqueous Centauredin, a trimethoxy flavone.The essential oil included the sesquiterpenes -caryophyllene, trans--farnesene, humulene, candinene, caryophyllene oxide, and nerolidol, as well as the monoterpenes linalool, Terpinen-4-ol, and terpineol.

Two glucosyltransferases (GTases I and IIB) acting on steviol and steviol-glycosides were identified from S. rebaudiana I extracts. GTase IIA, a distinct transferase, was shown to be acting on steviol. Purified GTase I (subunit Mr 24,600) catalysed the glucose transfer from UDP-glucose to steviol and steviolmonoside (steviol-13-O-glucopyranoside), but not to other steviol-Glycosides. GTase IIB (subunit Mr 30,700) demonstrated broad substrate specificity by acting on steviol, steviolmonoside, steviolbioside (13-O--sophorosyl-Steviol), and stevioside.

Rebaudiosides A and C are the major stevioside components. Furthermore, the diterpene steviol (ent-13-hydroxykaur-16-en-19-oic acid) glycosides dulcoside A have different organoleptic properties.[5,6,7]

USES[20]

ANTI-DIABETIC PROPERTIES

Because stevia leaf extract has the capacity to promote insulin action on cell membrane, which in turn boosts insulin production and stabilises blood sugar level, it can reduce plasma glucose level and greatly improve glucose tolerance. For diabetic patients, stevia leaf is utilised as an additional dietary ingredient in the form of dried or powdered leaf, which boosts natural sweetness and revitalises the pancreatic glands. Stevioside improves insulin production in response to glucose, but it has little impact on fasting insulinemia.

BLOOD PRESSURE REGULATION.

Stevia is a cardiac tonic that controls heart rate, blood pressure, and other cardiopulmonary processes. Human systolic and diastolic blood pressure is decreased by stevia leaf extract dissolved in hot water. Regular consumption of the non-caloric sweeteners found in S. rebaudiana leaves (steviolglycosides) lowers blood cholesterol levels (Atteh et al. 2008) , improves cell regeneration and blood coagulation, inhibits the growth of cancer cells, and strengthens blood vessels (Barriocanal et al. 2008) , all of which have positive effects on human health. They reduce blood pressure by relaxing the arteries.

ANTI-OBESITY PROPERTY.

The natural sweetener ent-kaurene diterpene glycosides (stevioside and rebaudiosides), which is present in stevia leaves, has 300 times more sweetness than sucrose and has no calories. Stevia thus prevents persons from losing as much weight.

RENAL FUNCTION

Stevioside from the leaves of S. rebaudiana was examined by Melis (1992) for its impact on renal function. It operates as a typical systemic vasodilator, causing hypotension, diuresis, and natriuresis. In polycystic kidney disease, steviol and its analogue are utilised**.**

OTHER USES

Antibacterial and antifungal activities are present in stevia. Tea made from stevia leaves can calm troubled stomachs. Foods are sweetened with the stevia leaf. Additionally, it contains anti-mutagenic, anti-proliferative, and antioxidant qualities**.**

ANALYTICAL METHODS

HPLC :

The primary drug, any reaction impurities, all possible synthetic intermediates, and any degrades are separated, quantified, and quantified using HPLC. It is the most precise analytical approach

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for quantitative and qualitative pharmacological product analysis. It has high resolution, quick analysis, greater mobile phase pressure, regulated flow rate, small sample size, moderate analytical conditions, and facile fractionation. The sample is injected at high pressure through a porous column, and the separation mechanism is based on solute adsorption on the stationary phase based on its affinity for the stationary phase[11,12].

HPLC OF STEVIA

The study used commercial samples containing varying concentrations of steviol glycosides, dried leaves of S. rebaudiana Bertoni from Paraguay, and dried at room temperature to a moisture level of 5% to 6%, as determined by a halogen lamp moisture balance model XM-120T (Cobos, Barcelona, Spain) at 105 °C.When the moisture loss was less than 0.1% in 180 seconds, the samples were regarded to have attained a constant mass[11].

HPLC CONDITION

An Agilent 100 HPLC system with a UV-vis detector tuned to 210 nm was used for liquid chromatography under isocratic conditions. Separation was performed on a Luna C18(2). At a constant flow rate of 1 mL/min, the mobile phase was composed of acetonitrile and (10mmol/l) sodium phosphate buffer (32:68). Clarity software, version 2.7.3.498 (2009), was used to conduct the chromatographic analysis. The injection volume of the sample was 20 L.

STEVIA REBAUDINIA EXTRACT

The leaves of two S. rebaudiana varieties, Morita and Criolla II, were harvested and dried in the dark in Mexico. To make the extracts, 0.5 g of powdered leaves were weighed and extracted three times in 30 minutes with water at 100°C. To separate the aqueous phases of the leaves, the extracts were centrifuged for 10 minutes (2500g, 10°C). Before HPLC analysis, the aqueous phases were transferred to a 25mL volumetric flask and filtered through a 0.45 m membrane filter**.** (Wöelwer-Rieck et al., 2010)[12].

ACCURACY

The triple recovery experiment with spiked samples produced an excellent recovery rate of 92.2-104.4%. Although no studies have been conducted to compare the accuracy of measuring minority glycosides using the same method, the recovery rates for dulcoside A, rebaudioside C, rebaudioside B, and steviolbioside are comparable to rebaudioside A (93-108%)(Chester et al., 2012; Jaworska et al.,2012; Wöelwer-Rieck et al., 2010) or stevioside (95.7-106%)(Chester et al., 2012; Ni et al., 2007; Wöelwer-Rieck et al., 2010)[11,12].

INTRADAY PRECISION

HPLC was used to analyse three glycoside concentrations, with RSD values ranging from 2.13 to 7.98%. The retention time was also used to assess intraday accuracy. The average retention time for rebaudioside C was 9.5 minutes, 10.28 minutes for dulcoside A, 20.73 minutes for rebaudioside B, and 22.29 minutes for steviolbioside.

INTERDAY PRECISION

There was intermediate or interday precision, with a 2.5% RSD for retention duration and a 9% RSD for peak area. Peak area RSD is slightly larger than for rebaudioside A and stevioside, however RSD 10% is often regarded enough. The circumstances given allow the identification of rebaudioside D, stevioside, and rebaudioside A(Tada et al., 2013)[11].

CONCLUSION

The proposed HPLC method simplifies the quantification of primary steviol glycosides while preserving good selectivity, sensitivity, and accuracy. The following extraction conditions are recommended: Bake for 30 minutes at 100°C without agitating or crushing the leaves. Clarification of the extracts through micro and ultrafiltration allows a suitable percentage of the main steviol glycosides to be eliminated. In a quick and simple procedure, the proposed technique is adequate for demonstrating that the main steviol glycosides (stevioside and rebaudioside A) are safer for health and have a smaller environmental impact than previous approaches published in the literature.

HPTLC

HPTLC has been shown to give good separation, qualitative and quantitative analysis of a variety of substances, including herbal and botanical nutritional supplements, nutraceuticals, conventional western medications, traditional Chinese medicines, and Ayurvedic (Indian) medicines. It outperforms other analytical techniques in terms of total cost and analysis time, and features include the ability to analyse multi-component crude samples, the application of a large number of samples and a series of standards using the spray-on technique, the choice of solvents for HPTLC development, the processing of standards and samples identically on the same plate, different and universal selective detection methods, and in situ spectra recording in seq.Furthermore, HPTLC can assist to limit the danger of harmful organic wastewater exposure and greatly reduce disposal issues, hence lowering environmental pollution.[13]

Isolation of steviol glycosides

S. rebaudiana air dried leaf powder (1 kilogramme) was extracted for 12 hours at room temperature with MeOH-H2O 80:20 (v/v). The percolations were dried and separated using hexane, chloroform, ethyl acetate, and butanol. All fractions were dried with anhydrous Na2SO4 and concentrated under reduced pressure at 50±5 °C to produce extracts in hexane (30.0 g), chloroform (10.0 g), ethyl acetate (10.5 g), and butanol (150.2 g). Butanol extract (150.2 g) was column chromatographed over silica gel using a gradient elution of CHCI3:MeOH with increasing proportions of MeOH (05%, 10%, 15%, 20%) in chloroform to yield four fractions (i-iv). Fraction (iii) was re-

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chromatographed over silica gel using gradient elution of 5- 20% MeOH in chloroform to yield pure steviolbioside (100 mg) having m.p.188–192° C, Fraction (iv) was re-chromatographed on silica gel using gradient elution of 5-30% MeOH in chloroform, producing pure stevioside (8 g) with a melting point of 196-198° C and rebaudioside-A (400mg) with a melting point of 242-244° C. .(Jaitak V and Gupta AP et al., 2008)[14]

HPTLC procedure

A Camag HPTLC system was utilised to analyse a 0.20mm layer thickness pre-coated silica gel HPTLC 60 F254 plate. For 30 minutes at room temperature and 50±5% relative humidity, ADC was pre-saturated with 20 ml mobile phase ethyl acetate-ethanol-water (80:20:12, v/v/v). Samples and standards were applied to the plate as 6mm broad bands with an automated TLC sampler (ATS4) under a N2 gas flow, 10mm from the bottom, 10mm from the side, 6mm of plate space between two bands, and application speed of 150 nm/s.

TLC plates were then dried in an air current using an air dryer in a wooden room with proper ventilation. Spraying acetic anhydride:sulphuric acid:ethanol (01:01:10, v/v/v) on bands was followed by heating on Camag HPTLC plate heater at 110° C for 2 minutes.

The plate was quantitatively analysed after 20 minutes in reflection-absorption mode at 510 nm, slit width 6mm x 0.3mm, scanning speed 20mm/s, and data resolution 100_m/step. The thin-layer chromatograms were photographed and preserved with the help of a Camag video documentation system and the Reprostar 3.(Jaitak V and Gupta AP et al., 2008)[14].

SENSITIVITY

The sensitivity of the technique was assessed using LOD and LOQ. The standard solutions were detected in the ranges of 1-6_g/spot for stevioside, and 0.5-3_g/spot for rebaudioside-A (n = 6) with a correlation value of 0.998-0.999. The detection and quantification limits were calculated using the calibration curve and experimentally confirmed in compliance with ICH guidelines. .(Jaitak V and Gupta AP et al., 2008)[14].

SPECIFICITY

The specificity of the procedure was evaluated by evaluating the standard and sample solutions. The bands of steviolbioside, stevioside, and rebaudioside-A in the samples were validated by comparing the RF values and overlaid spectra of the spotted bands with standards[14].

ACCURACY

The recovery test was used to assess the analytical procedure's accuracy. This entailed adding known amounts of the reference standard components from stock solution to one of the pre-analyzed samples. The known standards were diluted on the basis of on the proportion of three glycosides contained in the pre-analyzed sample. There were three concentration levels examined (low, medium, and high). Samples were produced in triplicate at each level and analysed using the previously described process. The degree of accuracy was given as a percentage (observed concentration/theoretical concentration x 100).

CONCLUSION OF HPTLC

In this work, an HPTLC technique was devised to quantify steviolbioside, stevioside, and rebaudioside-A in the leaves of S. rebaudiana plants, which are typically used as sugar substitutes. In comparison to existing analytical approaches, the method is straightforward, cost effective, environmentally friendly, and easily adaptable for simultaneous screening and quantitative measurement of steviol glycosides. Within the defined parameters, the approach was verified and shown to be selective, linear, repeatable, and accurate. The approach will be appropriate for quality control of steviol glycoside synthesis in S. rebaudiana leaves.

UHPLC-MS

CHEMICALS

Stevioside, rebaudioside A, steviolbioside and steviol their purity was higher than 98%. Ammonium acetate, ammonium formate and dichloromethane. Methanol and acetonitrile LC-MS grade. Water

EXTRACTION AND PURIFICATION BY SPE

Two hundred grams of dried Stevia leaves (105 °C, 2 h) were finely grinded into a fine powder and passed through a 500m (35 mesh) filter. To optimize the extraction conditions, the powder was divided into different quantities and sonicated with 20 ml of methanol for 10 min. The mixture was centrifuged at 1500g for 5min and the supernatant transferred into a 25ml flask. The volume obtained for every extract was set up by methanol, diluted with methanol, and centrifuged at 4000g for 1min. The alcoholic extract was diluted with water (2 ml), loaded on a 3 ml HLB Oasys 100mg SPE cartridge pre-activated with methanol (3 ml) and then washed with water (5 ml).

The steviol-glycosides were eluted from the cartridge using 3ml of 70% methanol in water and the volume then adjusted to 10 ml by methanol. The solution obtained was serially The resulting solution was serially diluted and centrifuged at 4000g for 1 minute before introducing 2:l into the UHPLC-MS. After sonicating 1 g of dry Truvia® powder with 15 ml of water for 10 minutes, the volume was increased to 20 ml by water. Diluting the solution, filtering it through a 0.22µmfilter, and injecting 2:1 into the UHPLC-MS system.(Gardana C and Scaglianti M et al., 2010)[17]

SPECIFICITY

Co-chromatography using an authentic standard validated the peak identities of SV, Ra, Sb, and ST, while molecular weight analysis indicated Rc, Du, and ST-glucosides.

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Following the ions corresponding to [M+Cl35] and [M+Cl37], quantitative analysis of steviol-glycosides was done.

PRECISION

The assay's intra- and inter-day precision was validated by analysing spiked samples three times in five days. UHPLC-MS(MS) validated peak purity and identification. The precision was validated by calculating the standard deviations of the quantities and the retention duration.

ROBUSTNESS AND TOUGHNESS

Several chromatographic settings, including flow rate of± 0.1 ml min-1, column temperature of± 3° C, organic strength and pH± 10%, cone voltage of ±2 eV, and capillary voltage of ±0.2 kV, were used to assess robustness. The Wilcoxon test was used to analyse the data, with a significant threshold of $p > 0.05$ considered. (Gardana C and Scaglianti M et al., 2010)[17].

QUANTITATIVE ANALYSIS OF STEVIOL GLYCOSIDES

The quantitative analysis of steviol-glycosides by UHPLC-MS was carried out using a chromatographic system coupled to a Quattromicro triple quadrupole mass spectrometer. The analyses were carried out in gradient mode by a 1.8m c18 hss column maintained AT 80°C and the flow-rate was 0.5 ml min1. The eluents were 2mmoll1 ammonium acetate pH 6.5, 0.1% CH2Cl2 in CH3CN, 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+Cl35] and [M+Cl37]. The capillary voltage was set to 3.0 kV while the cone voltage was specific for each compound. The source temperature was 130 degrees Celsius, while the desolvating temperature was 380 degrees Celsius.

We have (m/z) 551 and 553 for steviolmonoglucosides, (m/z) 677 and 679 for steviolbiosides, and (m/z) 823 for steviolbiosides. (m/z) 839 and 841 for stevioside, (m/z) 985 and 987 for rebaudioside C, and (m/z) 1001 and 1003 for rebaudioside A. (Gardana C and Scaglianti M et al., 2010)[17].

QUANTITATIVE ANALYSIS OF STEVIOLS

The chromatographic system consisted of a UHPLC-MS equipped with a triple quadrupole mass spectrometer mod. Quattromicro. A 1.8m HSS C18 column was used for separation at a flow-rate of 0.6 ml min1. The column was maintained at 60 °C and the isocratic separation was performed using a solution containing 5mmoll1 ammonium acetate pH 6:CH3CN. The 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. The source and desolvating temperature was 120 and 350 °C, respectively. (Gardana C and Scaglianti M et al., 2010)[17].

SENSITIVITY

The calibration curve was linear in the range of 0.05–10_gml−1 for SV, 0.1–10_gml−1 forRa, 0.025–10_gml−1 for Sb, and 5–100 ng ml−1 for ST. The LLOD for SV, Ra, Sb andST was 15, 50, 10 and 1 ngml−1, respectively.

PRECISION

The intra- and inter-day precision (n = 5) was determined by analysing the spiked samples in triplicate; the repeatability for SV-glycosides and ST was found to be in the range of 1.8-3.5% and less than 4.2%, respectively. In terms of inter-day precision, the%RSD ranges from 2.2 to 3.8%, with ST-glycosides and ST showing lower than 5.7%, respectively. The retention time %RSD was less than 0.5%. Linearity was investigated for SV and Ra in the range of 40-180mgg1 dry leaves[17].

NMR

CARBON NMR SPECTROSCOPY

Carbon NMR spectroscopy has recently been applied to investigations of human cerebral metabolism. Studies of glucose and amino acid metabolism have been performed on normal subjects using both direct carbon NMR15 and proton detection of the carbon nucleus. These dynamic NMR spectroscopic methods have allowed measurement of glucose transport, oxidation of glucose, glutamate turnover, and glutamine synthesis in the human brain. NMR spectroscopy can demonstrate alterations in cerebral metabolism in epileptogenic regions of the human brain, but further studies are needed to correlate these findings with MRI, quantitative relaxation and volumetric MRI studies, histological and molecular studies of human tissue, and clinical outcomes of the subjects studied. NMR spectroscopy will undoubtedly play an important role in both the diagnosis of subjects with focal epilepsy who are surgical candidates and in increasing our understanding of the pathophysiology of human epilepsy.[18]

PREPARATION OF THE SAMPLE

SR-1 through SR-8 samples were carefully weighed, dissolved in 700 µL of NMR solvent, and vortexed for 1 minute. Freeze-dried liquid extracts (SR-9 to SR-15). The resultant powder was carefully weighed and dissolved in the NMR solvent in ten milligrams. The NMR solvent was insoluble in sample SR-15, thus it had to be dissolved in deuterium oxide. To remove insoluble elements, samples SR-9 and SR-10 were centrifuged at 10000 rpm for 5 minutes. The resultant solutions were transported in 600µ L sections to the NMR tubes.(Pieri V and Belancic A et al., 2011)[19]

QUANTITATIVE ANALYSIS BY NMR

Preliminary research was carried out to determine the best solvent for NMR analysis of steviol glycosides in pure S. rebaudiana extracts (SR-1 to SR-8). Deuterium oxide, DMSO-d6, methanol-

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d4, acetonitrile-d3_deuterium oxide (80:20), and pyridine-d5 were among the solvents or solvent mixes that allowed complete dissolution of the samples. The acquired 1H NMR spectra were distinguished by three separate groupings of signals, which corresponded to the backbone protons of the steviol glycosides, non-anomeric protons of the sugar moieties, and anomeric protons in combination with the olefinic protons at C-17. Pyridine-d5 separated important anomeric resonances fairly well, and since most NMR literature data of steviol glycosides has been recorded using pyridine-d5,11,12 direct comparison of 13C resonances for analyte identification purposes was possible.(Pieri V and Belancic A et al., 2011)[19]

MASS SPECTROSCOPY

Mass spectrometry (MS) is usually recognized as an instrumental technique for separation of electrically charged species in the gas phase . The ion source is where the charged species (ions) are created. In some circumstances, the ion source further facilitates the transport of solid-Analytes that are in the gas phase or liquid phase. The mass analyzer is then filled with the gas-phase ions.

The mass Analyzer sorts the ions—in space or time—according to the mass to charge ratios (m/z). The separated ions are detected by an ion detector in the space or time domain. Mass spectra are created by processing the electrical signals the ion detector produces. In fact, mass spectra can be viewed as histograms, which provide information on the number of ions at different m/z values. The original molecules, their fragments, or other species created during the ionisation process may match the detected ions. MS enables direct identification of molecules based on the mass-to-charge ratio as well as fragmentation patterns. As a result, it serves as a qualitative analytical method with high selectivity.[15,16]

LCMS

SAMPLE, STANDARDS AND REAGENTS

Leaves from different varieties of Stevia rebaudiana cultivated in the South of Spain in laboratory (7 varieties named from LAB1 to LAB7), greenhouse (7 varieties from GH1 to GH7) and field (7 varieties from FIELD1 to FIELD7) were provided by Vitrosur Lab.S.L.U. (Los Palacios y Villafranca, Sevilla, Spain). Sixty days after the plant was transplanted, samples were taken from it.In order to assess how the sampling procedure affected the amount of steviol glycosides, samples of leaves and branches from the types grown in the field were also taken. Extra Synthese (Genay, France) provided the following compounds: stevioside, rebaudioside A, B, C, and D, steviolbioside, rubusoside, and dulcoside A. All of the chemicals were analytical-grade or better. .(Molina CM and Medina VS et al., 2016)[16].

APPARATUS AND INSTRUMENTS

A Jet Stream Technology-equipped 6460 Triple Quad LC-MS detector on an Agilent Technologies 1200 Series LC system. The extracts were analysed using an electrospray ion source, also from Agilent. Deionized water with 0.1% formic acid (A) and methanol with 0.1% formic acid (B) were the mobile phases utilised in the separation along with a Mediterranea Sea C18 analytical column (5 m, 15 0.46 cm) from Teknokroma. The gradient went as follows: 60% B at the beginning, 100% B after 15 minutes, and 100% B for another 5 minutes.

To balance the column between analyses, a post-run of 5 minutes was programmed. The injection volume was 10 L, and the flow rate was constant at 0.7 mL/min. The temperature in the column was set to 40 °C.

In the triple quadrupole MS, colliding gas was made of high-purity nitrogen (99.999%). The chemicals were identified using ESI-MS/MS in SRM mode. The nebulizer gas temperature was set at 325 °C, and the sheath gas flow and temperature were both set at 12 mL/min and 350 °C, respectively. The nebulizer was configured for negative ionisation with the nozzle voltage at 1000 V, capillary voltage at 6000 V, and pressure at 45 psi. The dwell duration was 50 ms.(Molina CM and Medina VS et al., 2016)[16].

Extraction step

By macerating Stevia leaves or branches for two hours with 25 mL of an ethanol-water (35:65 v/v) solution as the extractant, steviol glycosides were extracted. Re-extraction of the solid residue was done in order to ensure quantitative extraction, and the concentration was less than 1% of the initial extract**.** .(Molina CM and Medina VS et al., 2016)

CONCLUSION

A perennial herb in the Asteraceae family known as stevia rebaudiana is prized for its sweetness and steviol glycosides (SGs). The secondary metabolites, or SGs, in stevia are what give it its sweetness.

The SG biosynthesis pathway in the leaves is responsible for their production. The bulk of the genes in this pathway that code for enzymes have been cloned.

The product's defining characteristic is stevia. Stevioside and rebaudioside A are among of the major metabolites of several SGs. Stevioside can also be produced by enzymes and microbiological organisms, according to research. These have no discernible negative effects when taken and are non-mutagenic, non-toxic, antibacterial, and non-mutagenic.

A validated analytical technique guarantees that the results it produces are accurate, trustworthy, and consistent. Therefore, these techniques are necessary to guarantee that items of the highest quality are placed onto the market.

The quantitative analysis of steviol glycosides in S. rebaudiana leaves collected from various locations was found to be repeatable using these methods, and they will act as a quality

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control indicator to watch over the commercial production of stevioside and its related molecules throughout various stages of its processing.

REFERENCES

1. *Yadav SK and Guleria P, "Steviol Glycosides from Stevia:Biosynthesis Pathway Review and their Application in Foods and Medicine." 2012, 52, 988-998.*
2. *Goyal SK and Goyal RK, "Stevia (Stevia rebaudiana) a bio-sweetener: a review." Int J Food Sci Nutr. 2010, 61(1), 1-10.*
3. *Yadav AK and Singh S, "A review on the improvement of stevia [Stevia rebaudiana (Bertoni)]." Can. J. Plant Sci. 2011, 91, 1-27.*
4. *Gupta E and Purwar S," Nutritional and therapeutic values of Stevia rebaudiana: A Review." J. Med. Plants Res. 2013, 7(46), 3343-3353.*
5. *Muanda FN and Soulimani R, "Study on chemical composition and biological activities of essential oil and extracts from Stevia rebaudiana Bertoni leaves." 2011, 44(9), 1865-1872.*
6. *Lemus MR and Vega GA, "Stevia rebaudiana Bertoni, source of a high-potency natural sweetener: A comprehensive review on the biochemical, nutritional and functional aspects." 2012, 132(3), 1121-1132.*
7. *Savita SM and Sheela K, "Stevia rebaudiana–A functional component for food industry." J. Hum. Ecol. 2004, 15(4), 261-264.*
8. *Castro MR and Díaz ME, "Membrane separation processes for the extraction and purification of steviol glycosides: an overview." 2020, 1-23.*
9. *Narula P and Pal B, "A comprehensive review of method development by HPLC." Wor. J. of Pharm. Res. 2021, 10(6), 1839-1858.*
10. *Xu S and Wang G, "Extraction of steviol glycosides from Stevia rebaudiana (Bertoni) leaves by high-speed shear homogenization extraction." J. of Food Process. and Preser. 2019, 43(12), 1-12.*
11. *Aranda GI and Moguel OY, "Validation of HPLC-UV method for determination of minor glycosides contained in Stevia rebaudiana Bertoni leaves." Biomed. Chromatogr. 2015, 29(5), 733-738.*
12. *Lorenzo C and Serrano DJ, "Fast methodology of analysing major steviol glycosides from Stevia rebaudiana leaves." 2014, 157, 518-523.*
13. *Rashmin P and Mrunali P, "HPTLC method development and validation: strategy to minimize methodological failures." J. of food and drug anal. 2012 , 20(4), 794- 804*
14. *Jaitak V and Gupta AP, "Validated high-performance thin-layer chromatography method for steviol glycosides in Stevia rebaudiana." J. of pharm. and bio. Anal. 2008, 47(4-5), 790-794.*
15. *Urban PL,"Quantitative mass spectrometry: an overview. Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Sciences." 2016, 374(2079).*
16. *Molina CM and Medina VS, "Development and application of a quantitative method based on LC–QqQ MS/MS for determination of steviol glycosides in Stevia leaves." 2016, 154, 263-269.*
17. *Gardana C and Scaglianti M, "Evaluation of steviol and its glycosides in Stevia rebaudiana leaves and commercial sweetener by ultra-high-performance liquid chromatography-mass spectrometry." J. of chromate. A. 2010, 1217(9), 1463-1470.*
18. *Novotny EJ, "Overview-the role of NMR spectroscopy in epilepsy. Magnetic resonance imaging." 1995, 13(8), 1171-1173.*
19. *Pieri V and Belancic A, "Identification and quantification of major steviol glycosides in Stevia rebaudiana purified extracts by 1H NMR spectroscopy." J. of agri. and food chem. 2011, 59(9), 4378-4384.*
20. *Chatsudthipong V, Muanprasat C (2009). Stevioside and related compounds: therapeutic benefits beyond sweetness. Pharmacol. Ther. 121:41-54.*