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Identification and quantitative estimation of stevioside in dry leaves of *Stevia rebaudiana* using conventional, spectrophotometric, and advanced extraction techniques

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Abstract
Stevioside and rebaudioside-A are diterpenoid glycosides found in *Stevia rebaudiana Bertoni* that provide intense sweetness with low caloric value, making them valuable alternatives to synthetic sweeteners. This study aimed to identify and quantify stevioside and rebaudioside in dry stevia leaves using a range of analytical methods, including Anthrone, 3,5-dinitrosalicylic acid (DNSA), thin-layer chromatography (TLC), and spectrophotometry, alongside extraction optimization through ultrasonic-assisted extraction (varying probe size, amplitude, and time), soaking, alcoholic extraction, and supercritical fluid extraction (SCFE). Experimental results demonstrated that ultrasonic treatment at 50 µm amplitude for 5 min (14 mm probe) yielded higher stevioside concentrations compared to lower amplitudes or shorter durations. Alcoholic extraction consistently produced higher stevioside yields than aqueous extraction. TLC confirmed the presence of stevioside and rebaudioside, while SCFE with cosolvents improved the quality of extracts by reducing bitter aftertaste. Results highlight the influence of extraction parameters on yield and purity, supporting process optimization for large-scale applications in the food and nutraceutical industry.

Keywords: Stevia, stevioside, rebaudioside-A, ultrasonic extraction, anthrone method, DNSA method, SCFE, TLC

Introduction
The natural sweetener *Stevia rebaudiana Bertoni* has gained increasing attention for its diterpene glycosides, particularly stevioside and rebaudioside-A, which are 200-300 times sweeter than sucrose. Unlike synthetic sweeteners, stevia extracts are non-cariogenic, non-caloric, and safe for diabetic populations. However, commercial utilization is limited by extraction efficiency, cost, and undesirable bitter aftertaste. Stevioside, the dominant glycoside, has slightly bitter notes, whereas rebaudioside-A is sweeter and less bitter, making their quantification crucial for improving product quality.
Several extraction and quantification methods have been reported, including conventional hot water/alcohol extraction, ultrasonic-assisted extraction, and advanced supercritical fluid extraction (SCFE). Spectrophotometric methods, including Anthrone and DNSA, remain widely used for total carbohydrate and glycoside quantification. This study integrates multiple methodologies to systematically analyze stevioside content under different extraction conditions, evaluate analytical approaches, and optimize parameters for improved yield and quality.

Materials and Methods
Plant Material
Dried leaves of *Stevia rebaudiana* were powdered and stored in airtight containers until further use.

Chemicals and Reagents
- Anthrone reagent (0.2% in concentrated H2SO4)
- DNSA reagent

- Standard stevioside/rebaudioside-A/D-glucose (97% Reb-A commercial sugar-free sample)
- Solvents: ethanol, methanol, butanol, ethyl acetate, chloroform, hexane
- Calcium hydroxide [Ca(OH)2] for purification

Sample Preparation

10 g of stevia powder was mixed with 100 mL distilled water and subjected to ultrasonic treatment using probes of 12 mm and 14 mm diameters at amplitudes of 10-50 µm for 1-10 min. Samples were labeled accordingly (e.g., S1, S2, S3, S4).

Anthrone Method

- Carbohydrate content was estimated by treating extracts with anthrone reagent and measuring absorbance at 620 nm using a spectrophotometer.
- Standard curve was prepared with known concentrations (0.1-0.9 mg/mL).
- Concentration of stevioside was calculated as: [C_{unknown} =]

DNSA Method

- Reducing sugars in alcoholic extracts were quantified using DNSA reagent.
- Absorbance was measured at 540 nm.

Thin Layer Chromatography (TLC)

- Performed on silica gel 60 F254 plates with ethyl acetate: methanol: water (75:15:10) as mobile phase.
- Spots visualized under iodine vapor chamber.
- Rf values compared to standard stevioside /rebaudioside.

Soaking Extraction

Leaf powder soaked in normal, cold, and warm water, and alcohol for 12 hrs; extracts were analyzed spectrophotometrically.

Alcoholic Extraction

Stevia leaves extracted in ethanol; absorbance measured at 620 nm to estimate stevioside concentration.

Supercritical Fluid Extraction (SCFE)

- Pretreatment with CO2 at 200 bar, 30°C.
- Glycoside extraction at 120-200 bar, 16-45°C with water/ethanol cosolvents.
- Extracts compared with conventional methods.

Results

Calibration Curves

Standard curves were successfully constructed for glucose, stevioside, and rebaudioside. Calibration demonstrated linearity ($R^2 > 0.98$).

Figures

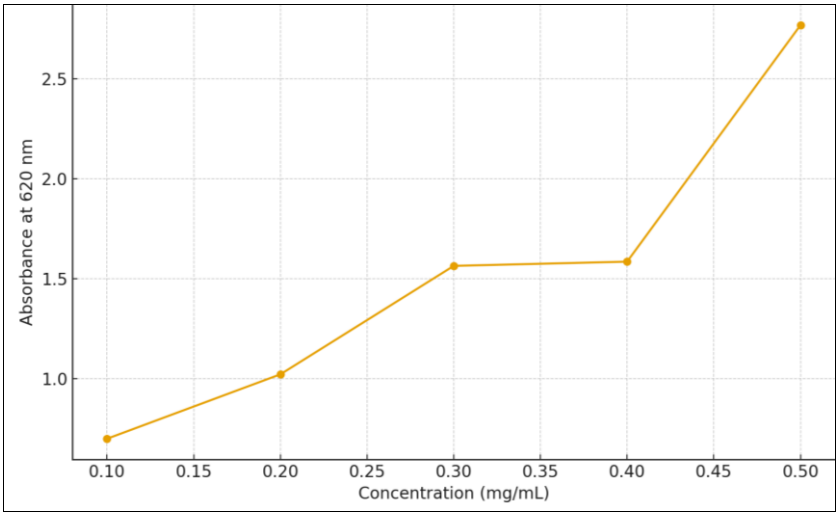

Fig 1: Standard curve prepared using Anthrone method showing linear relationship between concentration and absorbance.

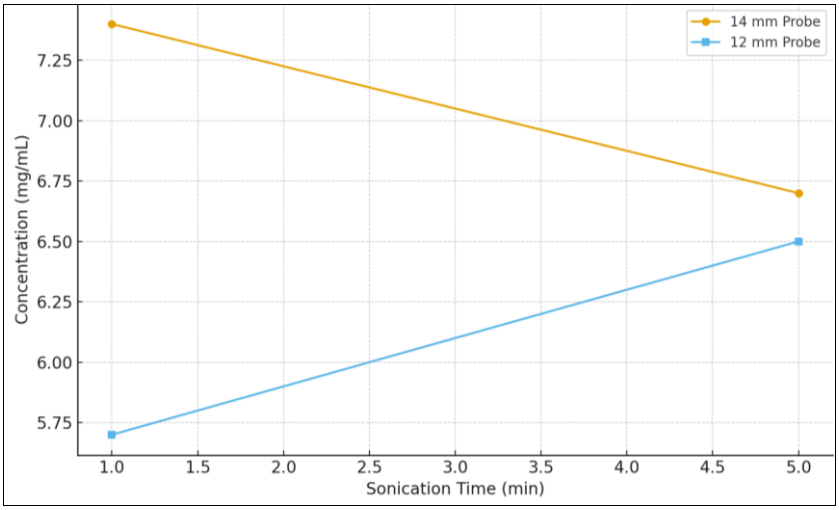

Fig 2: Effect of ultrasonic probe size and sonication time on stevioside yield.

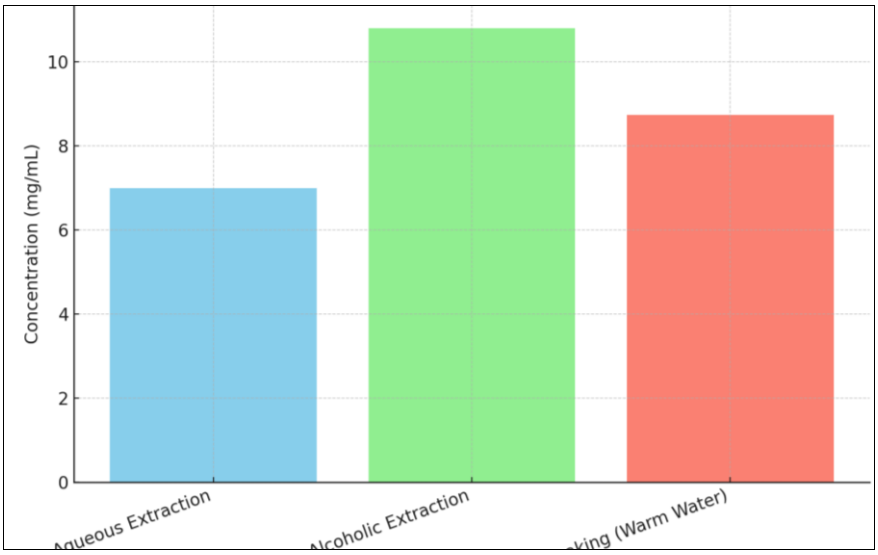

Fig 3: Comparative stevioside concentration obtained from different extraction methods.

Ultrasonic Extraction Results

- Higher amplitude (50 µm) and optimized sonication time (5 min) improved stevioside yield.
- At 14 mm probe, 5 min sonication yielded 6.7-7.4 mg/mL, whereas prolonged sonication (>10 min) led to reduced efficiency.

Table 1: Stevioside concentration at varying probe sizes and amplitudes, Ultrasonic extraction table:

Probe	Amplitude	Time (min)	Conc. (mg/mL)
14 mm	50 µm	5	6.7
14 mm	50 µm	1	7.4
12 mm	50 µm	5	6.5
12 mm	50 µm	1	5.7

Alcoholic Extraction

Ethanol extracts yielded higher stevioside concentration (10.8 mg/mL per 100 g leaves) compared to aqueous extraction (6.5-7.9 mg/mL).

Soaking Method

Warm water soaking resulted in 8.74 mg/mL concentration, slightly higher than cold and normal water extractions.

TLC Analysis

Rf values of experimental samples matched those of stevioside and rebaudioside standards, confirming compound identity.

DNSA Analysis

- **Gujarat samples:** 0.837 mg/mL (85.71% single sugar fraction)
- **Vanshree variety:** 1.02 mg/mL (88.10% single sugar fraction)f

SCFE Results

- **Pretreatment yield:** 3.0%
- **Extraction yield (120 bar, 16°C, 9.5% water):** 3.4%
- Extracts had improved sweetness profile with reduced bitterness compared to conventional methods.

Discussion

Results demonstrate that extraction parameters significantly affect stevioside yield and purity. Ultrasonic-assisted extraction provided rapid and efficient glycoside recovery, with optimal performance at intermediate sonication times and higher amplitudes. Prolonged sonication, however, decreased yield, likely due to degradation of glycosides. Alcoholic extraction consistently provided higher stevioside concentrations, indicating that ethanol enhances solubilization of glycosides. Soaking in warm water proved effective but required extended time.

TLC confirmed the presence of stevioside and rebaudioside, while DNSA analysis validated reducing sugar fractions in the extracts. SCFE offered superior extract quality, reducing bitterness and providing sweeter fractions, though yield was comparatively low. These findings suggest combining ultrasonic pretreatment with SCFE could enhance both yield and sensory properties.

Conclusion

This study highlights the impact of extraction methods on stevioside and rebaudioside yields from stevia leaves. Ultrasonic-assisted extraction optimized at 50 µm amplitude for 5 min (14 mm probe) provided significant improvement in yield. Alcoholic extraction outperformed aqueous methods, while SCFE improved extract quality by reducing bitterness. Integrating optimized sonication with advanced SCFE could offer a scalable solution for industrial stevia extract production.

References

1. Cramer B, Ikan R. Sweet glycosides from the stevia plant. Chemistry in Britain. 1987;23:915-917.
2. Phillips K. Stevia extract: patents and applications. European Patent Office. 1987.
3. Soejarto DD, *et al*. Potential sweetening agents of plant origin. Journal of Natural Products. 1983;46:590-599.
4. Tan NH, *et al*. Japanese patent on SCFE extraction of stevia glycosides. 1988.
5. Pasquel A, *et al*. Supercritical fluid extraction of stevia leaves. Journal of Food Engineering. 1999;40:111-117.
6. Alvarez M, Couto MA. Extraction and purification of stevia glycosides. Journal of Agricultural and Food Chemistry. 1984;32:113-117.
7. Nikolova-Damyanova B, *et al*. HPLC analysis of stevia glycosides. Journal of Chromatography A. 1994;673:107-113.
8. Rodrigues CEC, *et al*. Modeling of supercritical fluid extraction curves. Brazilian Journal of Chemical Engineering. 2000;17:159-173.