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An Efficient Microwave-assisted Extraction Process of Stevioside and Rebaudioside-A from *Stevia rebaudiana* (Bertoni)†

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ABSTRACT:
Introduction – Stevioside and rebaudioside-A are major low-calorie diterpene steviol glycosides in the leaves of *Stevia rebaudiana*. They are widely used as natural sweeteners for diabetic patients, but the long extraction procedures required and the optimisation of product yield present challenging problems.
Objective – To develop a rapid and effective methodology for the extraction of stevioside and rebaudioside-A from *S. rebaudiana* leaves and to compare yields using different extraction techniques.
Methodology – Dried and powdered leaves of *S. rebaudiana* were extracted by conventional, ultrasound and microwave-assisted extraction techniques using methanol, ethanol and water as single solvents as well as in binary mixtures. Conventional cold extraction was performed at 25°C for 12 h while ultrasound extraction was carried out at temperature of 35 ± 5°C for 30 min. Microwave-assisted extraction (MAE) was carried out at a power level of 80 W for 1 min at 50°C.
Results – MAE yielded 8.64 and 2.34% of stevioside and rebaudioside-A, respectively, while conventional and ultrasound techniques yielded 6.54 and 1.20%, and 4.20 and 1.98% of stevioside and rebaudioside-A, respectively.
Conclusion – A rapid and efficient method has been developed for the extraction of stevioside and rebaudioside-A in optimum yields using MAE procedure. This method has the advantage of rapid extraction and fast screening of a large number of *S. rebaudiana* samples for assessment of planting material. MAE saves considerable time, energy and has implications in the quality assessment of stevioside and rebaudioside-A prior to their industrial production from the leaves of *S. rebaudiana*.
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Keywords: microwave-assisted extraction; stevioside; rebaudioside-A; *Stevia rebaudiana*; *Asteraceae*

Introduction

Stevia rebaudiana Bertoni is a herbaceous perennial plant of the family Asteraceae, and is indigenous to Paraguay and Brazil. It is cultivated in parts of Asia, Europe and Canada. The leaf extract of *S. rebaudiana* is used in Japan, Korea and South America to sweeten soft drinks, soju, soya sauce, yoghurt and other foods, whereas in the United States it is used as a dietary supplement. In Japan and Korea consumption of *S. rebaudiana* leaf extract is 200 and 115 tons/year respectively (Kinghorn *et al*., 2001). Leaf extract of this plant has been used traditionally for the treatment of diabetes. As a natural sweetener *S. rebaudiana* has beneficial effects on human health, including anti-hypertensive (Chan *et al*., 2000), anti-hyperglycemic (Jeppesen *et al*., 2002), antioxidant (Xi *et al*., 1998), non-cariogenic (Das *et al*., 1992) and anti-human rotavirus (Takahashi *et al*., 2001) activities. *S. rebaudiana* produces high-potency low calorie sweeteners in its leaf tissues (Brandle *et al*., 1998), and among these the major components are stevioside and rebaudioside-A, diterpene glycosides of the aglycone steviol (ent-13-hydroxy kaur-16-en-19-oic acid). These compounds exhibit characteristic organoleptic properties (Phillips, 1989) and have sweetness intensities more than 300 times that of sucrose (Crammer and Ikan, 1986).

Stevioside has a 20% market share of low-calorie sweeteners in Japan (Kikuchi, 1985). According to some estimates, the market potential of these sweeteners ranges between 4 to 8% in Japan, whereas in Eastern countries the market is valued at US$1200 million. Many patents on the processing of *S. rebaudiana* leaves for the production of stevioside and rebaudioside-A are available including 150 Japanese patents on this subject. The process steps are essentially extraction, pre-treatment, separation, purification and refining. Most of the reported processes use coagulating agents and organic solvents. Some of the selected processes utilise chromatographic separation (Matsushita and Ikushige, 1979) and chelating agents followed by solvent extraction (Kumar, 1986). A process involving pre-treatment of the extract with lime and the use of ion exchange columns has been reported (Giovaneto, 1990). Tan *et al*. (1988) holds a Japanese patent for the production of *Stevia* glycosides by supercritical fluid extraction with carbon dioxide and co-solvents such as methanol, ethanol, and acetone. Kienle (1992) holds a similar US patent. Pól *et al*. (2007a) reported a pressurised fluid extraction (PFE) method for the extraction of stevioside from *S. rebaudiana* using methanol

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and water as solvents. However, no report in the literature is available on the extraction of stevioside and rebaudioside-A using a microwave-assisted extraction (MAE) technique. MAE is gaining popularity because it allows faster extraction, reduced solvent use and higher recovery. In continuation of our work on this plant (Megeji *et al*., 2005; Kaul *et al*., 2006; Jaitak *et al*., 2008), we report here MAE methodology developed for the first time for the extraction of two major steviol glycosides from *S. rebaudiana*. This method is more effective in terms of yield, time and energy consumption in comparison to conventional and ultrasound techniques.

Experimental

Instrumentation. HPLC analysis data were acquired using a Waters (Milford, MA, USA) automated 717 system with autosampler, a 7725i Rheodyne injector, a 996 photodiode array detector (PAD), a 600 quaternary gradient pump, an AF online degasser and a model 600 controller. Chromatographic separation was performed at ambient temperature (25°C) using a Lichrosphere® amino column (250 × 4 mm i.d.; 5 μm particle size) from Merck (Darmstadt, Germany) connected to a Lichrosphere® amino guard column (4 × 4 mm i.d.; 5 μm particle size). The autosampler was maintained at ambient temperature. Data were recorded and analysed using Millennium software version 3.50.1. All samples and standards were filtered through 0.24 μm Millipore filters.

^{1}H- and ^{13}C-NMR spectra were recorded using a Bruker Avance-300 (Billerica, MA, USA) operating at 500 MHz (^{1}H) and 125 MHz (^{13}C). Spectra were recorded at 25°C in pyridine-d_5 with TMS as internal standard (0.00 ppm). Chemical shifts were recorded in δ (ppm) relative to TMS signal and coupling constants (*J*) are given in Hz. Mass spectra were recorded on a Waters Q-TOF-MS with electrospray ionisation (ESI) in both MS and MS-MS modes with the aid of Waters Masslynx software (version 4.0). Each sample was dissolved in acetonitrile:water (50:50, v/v) and directly injected into the ESI source at a flow rate of 1.5 μL/min.

MAE was performed using a CEM (Orsay, France) Discover™ focused microwave (2450 MHz; 300 W). Ultrasound assisted extraction was carried out in an ultrasonicator bath (Elma Ultrasonic, Singen, Germany).

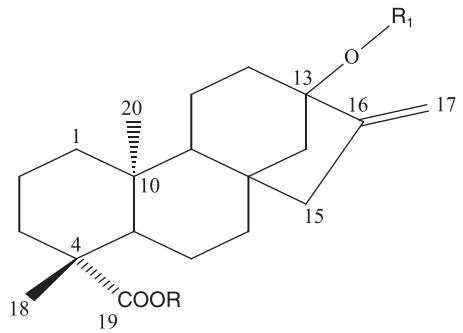
Chemicals and reagents. Stevioside and rebaudioside-A were isolated from the dried leaves of *S. rebaudiana*. Their purity was confirmed by HPLC and structures were elucidated by ^{1}H- and ^{13}C-NMR and by HPLC-MS/MS. HPLC-grade acetonitrile and water were obtained from JT Baker (Botavia, IL, USA). Methanol was purchased from S.D. Fine Chemicals (Mumbai, India). Ethanol was purchased from Bengal Chemicals, Kolkatta, India.

Plant material. Leaf material from cultivated plants of *S. rebaudiana* was collected during September 2007 from the experimental farm of the Institute of Himalayan Bioresource Technology located at 1475 m above sea level at a latitude of 32°06′40″N and a longitude of 76°33′46″E in the mid hills of western Himalaya. Leaf samples from 9-month-old plants were collected during the flowering stage. Specimens were identified by taxonomist Dr. Brij Lal and a voucher sample was deposited in the herbarium section of our institute (no. IHBT-PLP-12609). The harvested leaves were shade-dried and powdered to 60 mesh size.

Isolation of steviol glycosides. Dried plant material (1 kg) was extracted with methanol:water 80:20 (v/v) for 12 h at room temperature (cold extraction). The hydro-methanolic percolation was repeated three times and the extracted material was filtered through analytical-grade filter paper. The combined percolations were evaporated to dryness (504.0 g) on a rotary evaporator at 60°C and dissolved in water. The resulting solution was fractionated with hexane, chloroform, ethyl acetate and butanol. All fractions were dried over anhydrous sodium sulphate and concentrated under reduced pressure at 50 ± 5°C yielding hexane (30.0 g), chloroform (10.0 g), ethyl acetate (10.5 g) and butanol (150.2 g) fractions, respectively. The butanol fraction was subjected to column chromatography over silica gel (60–120 mesh) by gradient elution with chloroform:methanol mixtures containing increasing proportions of methanol (i.e. 5, 10, 20 and 30%) to give four fractions (i–iv). Fraction iv (25.0 g) was re-chromatographed over silica gel using gradient elution with 5–30% methanol in chloroform yielding pure (**1**) stevioside (8 g), m.p. 196–198°C, and (**2**) rebaudioside-A (400 mg), m.p. 242–244°C. ^{1}H- and ^{13}C-NMR spectra of **1** and **2** were recorded in pyridine-d_5 and their structures were confirmed by comparison with the spectral data reported earlier in the literature (Kohda *et al*., 1976; Yamasaki *et al*., 1976). Compounds **1** and **2** were used as reference standards during the course of present work (Fig. 1).

Sample preparation. Leaves of *S. rebaudiana* were dried in open shade, milled to 60 mesh powder using a mortar (selected by sieve) and then stored at ambient temperature. Methanol, ethanol, water and their binary mixtures were used for the extraction of leaf powder by conventional, ultrasound and microwave-assisted extraction techniques. After extraction, the supernatant was filtered and dried under reduced pressure in a rotary evaporator at 50°C. Dried extracts were defatted with hexane and the resulting supernatants were discarded. The residual matter was dried *in vacuo*, dissolved in 10 mL of acetonitrile:water (80:20, v/v), and filtered through 0.45 μm filters prior to HPLC analysis. In the present work, the extraction percentages of stevioside and rebaudioside-A are defined as:

(mass of stevioside or rebaudioside-A in the extracted solution/ the mass of material (*Stevia* leaf sample) × 100



Sample no.	Diterpene glycosides	R	R_1
1.	Stevioside	β-Glc‴	β-Glc′-β-Glc″
2.	Rebaudioside-A	β-Glc⁗	β-Glc′-β-Glc″ (β-Glc‴)

Glc, glucose.

Figure 1. Structure of steviol glycosides.

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Conventional extraction method. Conventional extraction was performed with 100 mg of *S. rebaudiana* leaf sample, by adding 10 mL of different extracting solvents [methanol, ethanol, water, methanol:water (80:20, v/v) and ethanol:water (80:20, v/v)] in 25 mL flasks and maintaining at room temperature for 12 h (cold extraction). Extracts were filtered and concentrated *in vacuo* to dryness at 50°C. Further processing of the samples prior to HPLC analysis was as described above.

Ultrasound-assisted extraction. *S. rebaudiana* leaf powder (500 mg) was sonicated with 50 mL of different solvents [methanol, ethanol, water, methanol:water (80:20, v/v) and ethanol:water (80:20, v/v)] in an ultrasonicator bath at a temperature of 35 ± 5°C for 30 min. Extracts were filtered and concentrated to dryness in *vacuo* at 50°C and further processed as described above.

Microwave-assisted extraction. Each sample (100 mg) of *S. rebaudiana* leaf powder was placed in a 50 mL quartz tube topped by a vapour condenser and was suspended in 10 mL of different solvents and binary mixtures [methanol, ethanol, water, methanol:water (80:20, v/v), ethanol:water (80:20, v/v)], filtered, concentrated to dryness and then processed as described above. Extraction was carried out at different power levels ranging from 20 to 160 W with extraction time range between 30 s to 5 min with a temperature range of 10–90°C.

HPLC analysis. Samples (20 μL each) were injected into the column and eluted at a flow-rate of 0.8 mL/min using an isocratic solvent system of acetonitrile:water (80:20, v/v). The column temperature was 25°C throughout the experiment. Stevioside and rebaudioside-A were detected at 205 nm by PAD and were identified by comparison of their retention times with those of authentic standards (Fig. 2).

Results and Discussion

Structural analysis of steviol glycosides

Stevioside and rebaudioside-A were isolated from the leaves of *S. rebaudiana*. Preliminary identification was carried out by melting point and TLC [stevioside, m.p. 196–198°C; R_f 0.60, ethyl

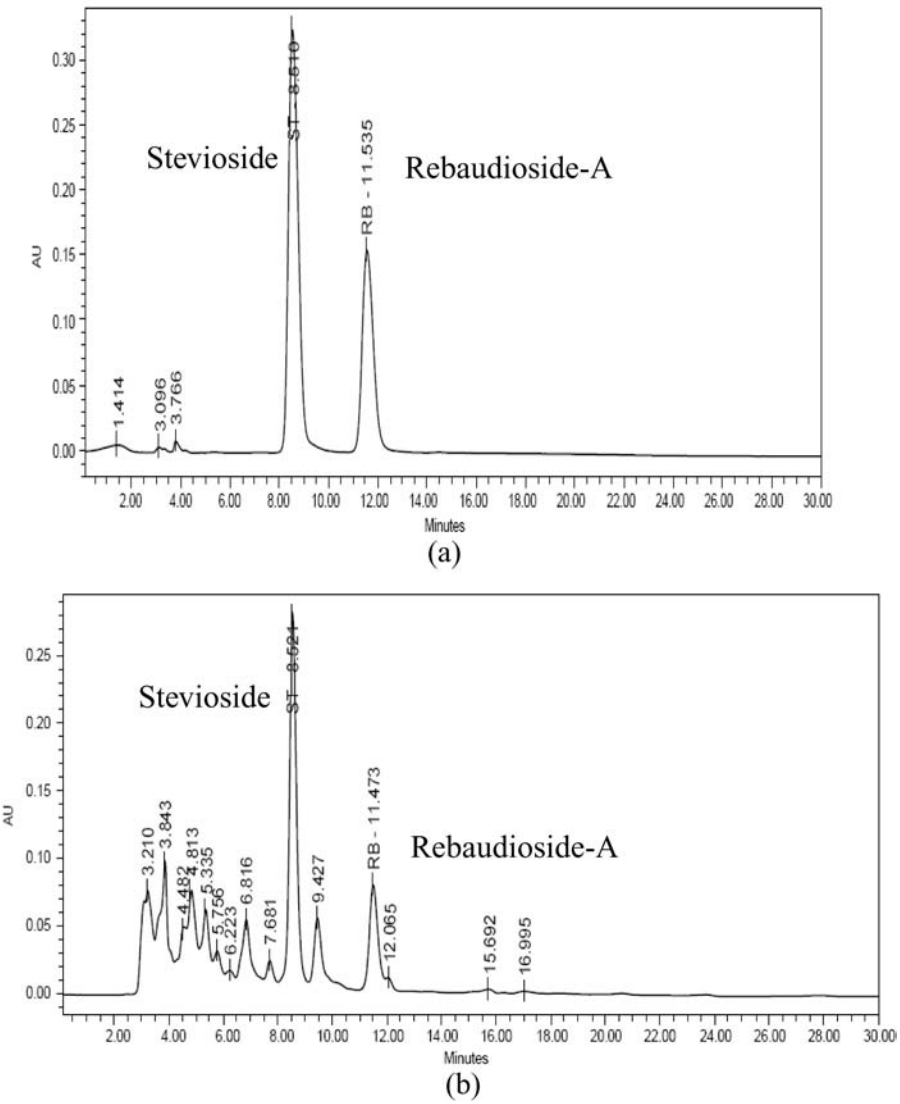

Figure 2. HPLC chromatogram of standards of (a) stevioside, rebaudioside-A and (b) *S. rebaudiana* extract from microwave extraction.

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acetate:ethanol:water (8:2:1.2, v/v/v); rebaudioside-A, m.p. 210–212°C; R_f 0.45, ethyl acetate:ethanol: water (8:2:1.2, v/v/v)]. Identities were further confirmed by NMR and HPLC-MS/MS, respectively. Earlier Pól *et al*. (2007b) reported 2D HPLC × HPLC-MS for the analysis and quantification of steviol glycosides. In the present investigation an HPLC system linked to a PAD detector at a wavelength of 205 nm was employed for the quantification of stevioside and rebaudioside-A. The R_t values of stevioside and rebaudioside-A were found to be 8.651 and 11.473 min, respectively. ^{1}H- and ^{13}C-NMR (500 MHz, 125 MHz, pyridine-d_5) of stevioside: anomeric protons of δ_H 5.07 (1H, d, J = 7.1), 5.20 (1H, d, J = 7.3), 6.01 (1H, d, J = 7.5) and δ_C 40.6, 19.3, 38.3, 42.6, 57.2, 22.1, 41.6, 43.9, 53.8, 39.7, 20.6, 36.6, 85.9, 44.4, 47.5, 154.4, 104.5, 29.2, 177.1, 15.4; Glc′, 97.9, 84.6, 78.0, 71.2, 77.8, 62.4; Glc″, 106.7, 77.0, 71.3, 78.6, 62.7; Glc‴, 95.8, 73.8, 78.9, 70.8, 79.2, 61.8. ^{1}H- and ^{13}C-NMR (500 MHz, 125 MHz, pyridine-d_5) of rebaudioside-A: anomeric protons δ_H 5.03 (1H, d, J = 7.2), 5.24 (1H, d, J = 7.6), 5.31 (1H, d, J = 7.3), 5.97 (1H, d, J = 7.4). δ_C 41.9, 19.4, 38.3, 43.6, 57.0, 22.2, 41.8, 42.4, 54.0, 39.8, 20.7, 37.3, 86.6, 44.5, 47.9, 153.9, 104.5, 28.3, 177.0, 15.5; Glc′, 97.9, 80.5, 87.6, 70.6, 78.3, 62.3; Glc″, 104.5, 75.8, 78.4, 62.5; Glc‴, 104.5, 74.7, 77.7, 71.4, 77.6, 61.8; Glc⁗, 95.4, 73.6, 78.9, 70.4, 79.0, 61.6.

Percentage yields of stevioside and rebaudioside-A with different extraction methods

Extraction of *S. rebaudiana* leaf powder was carried out using conventional, ultrasound and MAE methods in order to understand the variability in extraction of two major steviol glycosides, stevioside and rebaudioside-A. From the results shown in Table 1, it could be concluded that MAE afforded higher yields of stevioside (8.64%) and rebaudioside-A (2.34%), followed by conventional extraction (stevioside 6.54% and rebaudioside-A 1.20%) and the ultrasound method (stevioside 4.20% and rebaudioside-A 1.98%). The repeatability of MAE was expressed as % RSD (relative standard deviation for n = 6) and the values for stevioside and rebaudioside-A were 1.23 and 2.22%, respectively. The effect of different solvents and their binary mixtures was studied on the extraction yield of the major steviol glycosides under different extraction methods. The results indicated that the use of binary solvents, i.e. methanol:water (80:20, v/v) in MAE afforded the two steviol glycosides in optimum yields. Variable parameters under MAE, including irradiation power (20–160 W), temperature (10–90°C) and time of extraction (30–300 s), which varied the extraction efficiency of two glycosides, were selected in the present study. Other parameters such as sample quantity (100 mg) and solvent volume (10 mL) were kept constant.

Effect of microwave irradiation parameters on extraction

The efficiency of extraction of a sample inside a microwave oven is directly proportional to the irradiation power, the nature of the sample (roots, leaves, etc.) and the solvents used. Microwave energy is an electromagnetic radiation that causes molecular motion by migration of ions and rotation of dipoles, the latter referring to the alignment of molecules in both the solvent and the matrix. When extraction under MAE was carried out within the power range 20–160 W, initially the yields of stevioside and rebaudioside-A were enhanced and reached maximum at 80 W. Subsequently the yields decreased when the power level was increased up to 160 W (Fig. 3). Using a temperature range between 10 and 90°C, the yield of the two glycosides increased and reached maximum level at 50°C. On increasing the temperature up to 90°C, the yields of the two glycosides decreased substantially (Fig. 4). Fixing the power level at 80 W, the optimum yields of stevioside and rebaudioside-A were 8.64 and 2.34%, respectively, and fixing the temperature at 50°C gave yields of stevioside and rebaudioside-A of 8.58 and 2.36%, respectively. The ratio of stevioside and rebaudioside-A in both the conditions was found to be 3.6:1. It is likely that using less than 80 W power with temperatures below 50°C resulted in a lower availability of microwave energy to the sample. A power level of 80 W and a temperature of 50°C were clearly most suitable in breaking the analyte–matrix bonds and thus providing steviol glycosides in optimum yields. The decrease in yields of stevioside and rebaudioside-A with increase in power level beyond 80 W and an increase of temperature beyond 50°C may be explained in part by the adsorption of stevioside and rebaudioside-A on the powdered raw material surface.

Effect of solvent variability and time duration

In the present investigation extraction yields of two steviol glycosides were assessed using methanol, ethanol and water

Table 1. Percentage yields of stevioside and rebaudioside-A using different extraction methods

Sample no.	Method	Time	Stevioside (%)	Rebaudioside-A (%)	Total (%)
1.	Conventional	12 h	6.54	1.20	7.74
2.	Ultrasound	30 min	4.20	1.98	6.18
3.	Microwave	1 min	8.64	2.34	10.98

Sample no.	Diterpene glycosides	R	R_1
1.	Stevioside	*β*-Glc‴	*β*-Glc′-*β*-Glc″
2.	Rebaudioside-A	*β*-Glc⁗	*β*-Glc′-*β*-Glc″ *β*-Glc‴

Glc, glucose.

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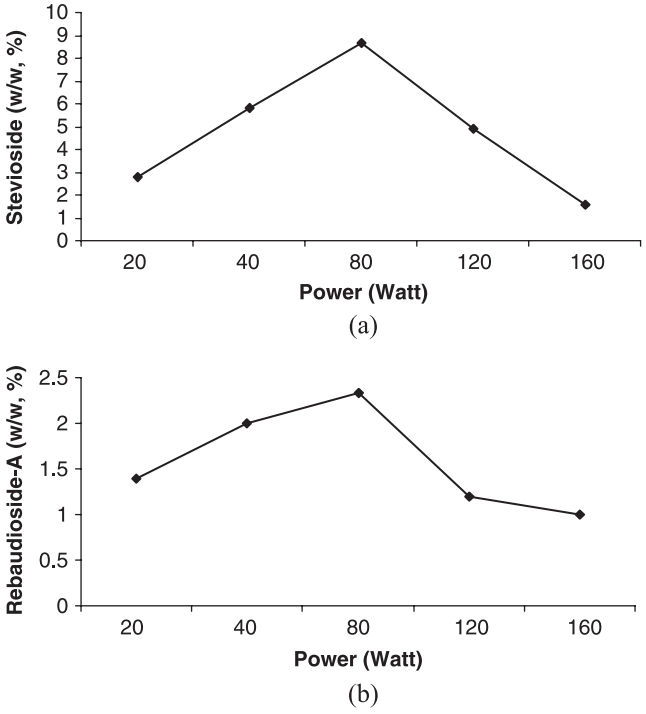

Figure 3. Effect of power on the extraction of (a) stevioside and (b) rebaudioside-A.

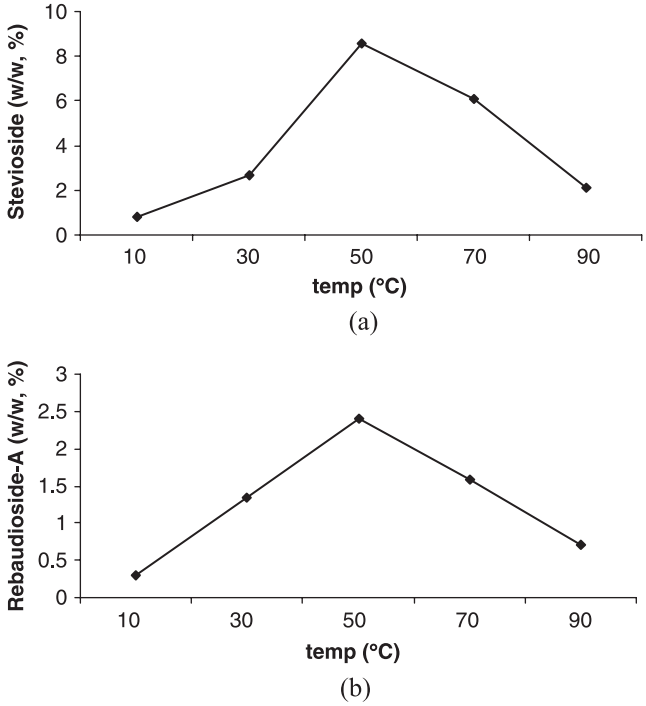

Figure 4. Effect of temperature (*T*, °C) on the extraction of (a) stevioside and (b) rebaudioside-A.

independently and also in binary mixtures. The results indicated that use of single solvents in MAE afforded two steviol glycosides in low yields. This is also in agreement with earlier studies reported by Guo *et al*. (2001). Using methanol: water at 80:20 (v/v) ratio afforded optimum yields of the two steviol glycosides owing to the enhancement in polarity of the binary mixtures.

The capability of solvent molecules to absorb microwave energy and to transfer it in the form of heat to other molecules partly depends on the dissipation factor (tan δ) defined as ratio of the dielectric loss (ε'') to the dielectric constant (ε') of the solvent (Kingston and Jassie, 1988). Using binary mixtures (80:20) of methanol:water, with variable dielectric constants, enhanced the polarity and afforded optimum yields of the two steviol glycosides. In the presence of polar molecules, MAE offers rapid availability of energy within the total volume of solution and subsequent rapid heating due to collision with surrounding molecules. For a fixed irradiation condition of 1 min at 80 W power, the effect of different solvents and binary mixtures was studied for optimum extraction of stevioside and rebaudioside-A. The maximum extractions of stevioside (8.64%, w/w) and rebaudioside-A (2.34%, w/w) were obtained in methanol:water (80:20, v/v), as shown in Fig. 5(a, b). The extraction was carried out at different time intervals and optimum yields of both the glycosides were obtained in 1 min [Fig. 6(a, b)].

Microwave-assisted extraction method is precise, saves considerable time and energy, and is suitable for the quality control of *S. rebaudiana* plant material used on commercial scale by industry.

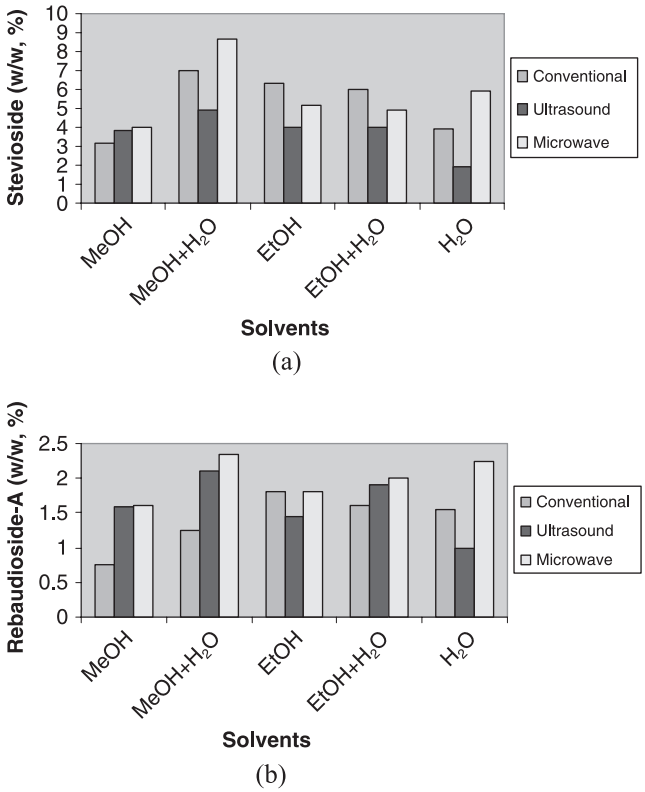

Figure 5. Comparison of effect of solvents [MeOH, MeOH + H_2O (80:20), EtOH, EtOH + H_2O (80:20), H_2O] in conventional, ultrasound and microwave-assisted extraction of (a) stevioside and (b) rebaudioside-A.

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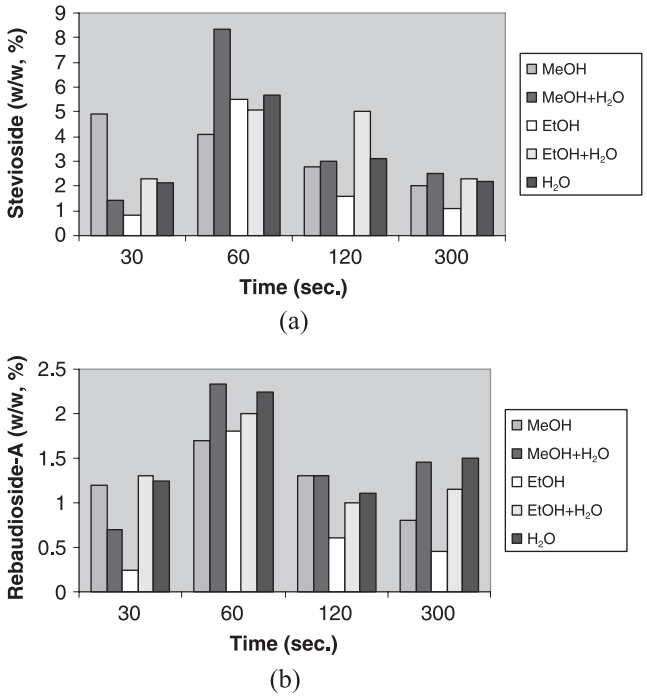

Figure 6. Effect of time on the extraction of (a) stevioside and (b) rebaudioside-A in different solvents.

during the course of this work. The authors are also thankful to Mrs Vijaylata Pathania for HPLC analysis and to Sh. Ramesh Kumar Sharma for technical assistance.

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