

Extraction of Natural Sweetener from Stevia Leaves Using Pressurized Hot Water

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Abstract

Pressurized Hot Water Extraction (PHWE) is a green and efficient technique for recovering bio-active molecules from natural materials using subcritical compressed liquid water in the temperature range of 50-150°C. The demand for natural substitutes of sugar for diabetics is increasing sharply. Stevia is one of such products available in the market which is extracted from stevia (*Stevia rebaudiana*) leaves. The two major sweet components (chemically known as glycosides) in stevia are stevioside and rebaudioside. The objective of this work is to study the effects of various parameters on the extraction of glycosides from stevia leaves using the PHWE technique. Experiments are conducted by varying different parameters, such as temperature (30-135°C), pressure (1-20 atm), extraction time (30-120 min), water to feed ratio (2-100 ml/gm), number of stages (1-3), stirring rate (0-350 rpm), and nature of feed pretreatment. The High-Performance Liquid Chromatography (HPLC) is used to identify steviosides and rebaudiosides in the extracted aqueous solution. The concentration of glycosides is measured using the colorimetric method namely, the phenol sulfuric acid method. The performance of the PHWE process has been evaluated by calculating the yield. The optimum condition is found at 120°C and 5 atm where the maximum total yield of 7.6% of glycosides is obtained in two consecutive extractions.

Keywords: Bio-active; Sugar; Natural; Chromatography; Nutraceuticals

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Introduction

Various green extraction methods for recovering food polyphenols from vegetables are summarized in the author's previous article [1]. PHWE has been popular with the increasing appeal for green technology especially for extracting food additives or supplements. PHWE is proved as a potential green solvent technique replacing the conventional solvent extraction using ethanol for the extraction of gypenosides from *Gynostemma pentaphyllum* [2]. PHWE has been widely used for the extraction of aromatic compounds from herbs and other plants such as rosemary, marjoram, oregano, sage, and clove. Active components such as glycosides and kavalactones from kava root and berberine, baicalin and glycyrrhizin from medicinal plants [3-11] have been extracted using PHW. A list of applications (1999-2014) of PHWE for the extraction of polyphenols can be found in the literature [12]. Stevia (*Stevia rebaudiana* Bertoni) has recently seen greater attention with the rise in demand for low-carbohydrate, low-sugar food alternatives. Stevia which is

also called sweet leaf, or sugar leaf is native to subtropical and tropical South America and Central America (North to Mexico). The molecular structure of different sweet composites present in stevia leaves is shown (**Figure 1**).

Various composites are formed depending on the radicals R1, R2, and R3. The number of components present in stevia leaves and their sweetness is listed (**Table 1**) [13].

Two major sweet components were named steviosides and rebaudiosides. These compounds are 250-300 times sweeter than sucrose (ordinary table sugar). Stevia does not significantly alter blood glucose, and so is attractive as a sweetener to diabetics and others on carbohydrates controlled diets. They are thermally stable, pH stable, and do not ferment. There are several hypotheses in regard to the source of the bitter aftertaste of stevia glycosides. Phillips [14] described that the presence of essential oils, tannins, and flavonoids is responsible for the bitter aftertaste. Soejarto et al. believed that the sesquiterpene lactones are responsible for the bitter aftertaste [15]. Tsanava

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et al. suggested that caryophyllene and spathulenol contribute decisively to the aftertaste [16]. Nevertheless, as pointed out by Phillips, stevioside, and Rebaudioside-A are partially responsible for the aftertaste, even though the contribution of rebaudioside-A is significantly less than that of stevioside [14].

A brief overview of the process protocols or extraction methods for isolation and identification of several components from stevia leaves which are developed by other researchers is provided here. Kohda et al. obtained the first two of these, rebaudiosides A and B, from methanol extracts together with the major sweet substance stevioside and steviolbioside, a minor constituent which was first prepared from stevioside by alkaline hydrolysis [17,18]. Subsequently, it was suggested that rebaudioside B was an artifact formed from rebaudioside A during the isolation [19,20]. Stevioside has been converted by enzymatic and chemical procedures to rebaudioside A.

Pasquel et al. reported a procedure to develop a process to obtain stevia extract of better quality [21]. The proposed process included two steps:

- Pretreatment of the leaves by Supercritical Fluid (SCF)
- Extraction of the stevia glycosides by SCF such as CO₂ as solvent and water and/or ethanol as co-solvent

However, such type of processes involves very high pressure and yield of glycosides is very less. Extraction using SCF is not very economical, as capital investment is very high.

It used direct current (30 amp) through an aqueous extract from leaves (90-100°C) for 2 hrs via aluminum electrodes (small

amount of HCl is added in solution to make it more conductive) to remove impurities [22]. After filtration, the solution is passed through mixed resin amber lite MB-1, finally, dry powder is obtained using evaporation. Such a process where high ampere current and adsorption by resin are employed is not very efficient. In addition, such a process involves a high cost because of resin. Persons described the method for defatting leaves by chloroform and Ca(CO₃)₂. Then extraction and crystallization are performed by dioxane and methanol respectively. Such processes had a disadvantage of using hazardous and toxic organic solvents (methanol, dioxane, chloroform) and additional filtration was required to remove Ca (CO₃)₂ [23].

Morita et al. used water as an extraction medium and then crystallization by the addition of methanol [24]. Kumar Sampath used di or tri-carboxylic acid, calcium oxide and diatomaceous earth for removal of impurities from aqueous extract. Butanol is used for Liquid-liquid extraction [25]. However, this process involved multiple steps with many solvents and chemicals. The procedure for removal of impurities is longan and many hazardous chemicals are used.

Roger Giovanetto used Ca(OH)₂ to remove impurities. Acidic and alkali resin was used for further purification. ScCO₂ was used to remove taste impairing components by Kienle [26,27]. Commercial pectinase, Ca(OH)₂, bentonite, phosphoric acid were used to remove impurities from water extract by Abelyan et al [28]. Kotesch et al. used steam for extraction and Ca(OH)₂, alumina, resins for impurities removal [29]. Extraction, purification, analysis, and properties of sweet compounds from stevia were summarized in recent review articles [30-32].

The methods found in the prior art often used extraction that required a significant volume of organic solvents and were rather tedious. Hence, methods that are rapid require a low volume of organic solvent and have high extraction efficiency is attractive options. Authors have patented a novel process protocol that is economical, simple, efficient, cost-effective and easy to carry out. The process avoids hazardous, toxic reagents, unacceptable process solvents, and conditions. The detailed discussion of the process is out of the scope of this article [33].

The main objective of this study is to investigate the impacts of various parameters on the yield of glycosides from stevia (*Stevia rebaudiana*) leaves using the PHWE process. The factors affecting

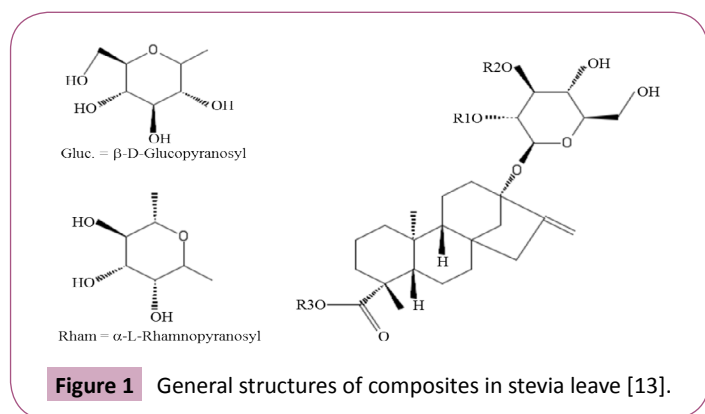


Figure 1 General structures of composites in stevia leaf [13].

Table 1. Structures and amount of major components in stevia leaves.

S. No.	Composites	Radicals			% present in leaves	Sweetness (sucrose=1)
		R1	R2	R3		
1	Stevioside	Gluc	H	Gluc	43595	100-270
2	Steviolbioside	Gluc	H	H	-	43753
3	Rebaudioside A	Gluc	Gluc	Gluc	43500	150-320
4	Rebaudioside B	Gluc	Gluc	H	-	43753
5	Rebaudioside C (Dulcoside B)	Rham	Gluc	Gluc	43467	40-60
6	Rebaudioside D	Gluc	Gluc	Gluc ² -Gluc ¹	-	200-250
7	Rebaudioside E	Gluc	H	Gluc ² -Gluc ¹	-	150-200
8	Dulcoside A	Rham	H	Gluc	½-1	40-60

PHWE such as temperature, pressure, stirring rate, extraction time, sample particle size, water to sample ratio, and the number of extraction stages are investigated.

Experimental Procedure

Materials and chemicals

Dry stevia leaves were supplied by New Universal System in Mumbai, India, for this work. Commercial product of stevia leaves has also been supplied by New Universal System in Mumbai, India. Milli-Q water purified on a Milli-Q® Ultrapure Water Purification Systems has been used for extraction. CO₂ gas was supplied by Siggil Industrial Gases, India with 99.99% purity. Sucrose with 99.94% purity from Sisco research laboratories Pvt. Ltd., 98.04% concentrated H₂SO₄ from Ranbaxy fine chemicals limited, phenol with 99.97% purity from Ranbaxy Laboratories Limited and methanol with 99.5% purity from Spectrochem Pvt Ltd were used without further purification or treatment.

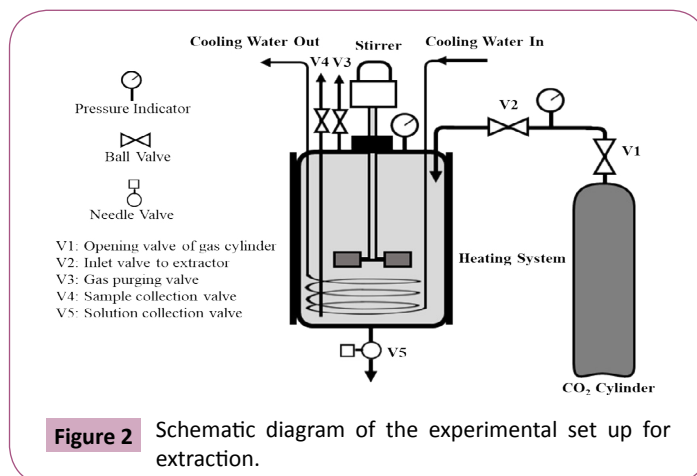
Pretreatment

Dry leaves are triturated in a grinder (Philips, super silent) into 400 mesh size. Optional pretreatment with supercritical carbon dioxide (at 300 atm, 45°C and at 200 atm, 45°C) is performed in a small laboratory scale ScCO₂ extractor to remove wax type materials. A known amount of ground leaves is charged in extractor and CO₂ is fed to the extractor maintaining its operating temperature, pressure and flow rate at the desired conditions. CO₂ flow rate is maintained from 0.7-1.0 kg/hrs for 3 hrs. A separator is kept in ice bath to get extracted materials in solid or liquid phase i.e. to avoid any losses. After 3hr operation, the unit is depressurized to atmospheric pressure and extractor is opened to collect leaves. This ScCO₂ along with its extract is depressurized by an expansion valve located before the separator to release the extracted materials by decreasing the pressure which reduces the solubility. Pure and clean CO₂ passes through the wet gas flow meter.

Water extraction

The PHWE experiments have been performed in two consecutive extractions at a temperature ranging from 30 to 120°C and a pressure ranging from 1 atm to 20 atm in batch mode. The experimental set up consists of 1-liter autoclave made of SS 316. This can be operated at a pressure of up to 300 bars and a temperature up to 250°C. A schematic diagram of the experimental set up is shown in **Figure 2**.

A stirrer is connected with the electrical motor. A controller is also attached to the system for controlling temperature and stirring rate. The autoclave is well insulated. One thermo-couple is immersed inside the autoclave to measure the temperature. Water cooling coils are provided to cool the liquid in the extractor. There is a drain line with a valve at the bottom of the autoclave to collect the extracted liquid. On top of the autoclave, one line with a valve is provided for pressurizing the system with the help of a CO₂ gas cylinder and another line is used for purging the gas to maintain the inside pressure at a fixed value



and for depressurization. A gas cylinder is used to pressurize the system and a heater is used for preheating the gas coming to the autoclave to maintain the temperature of the gas. A pressure indicator having a range from 0 to 450 bars is attached online to measure the pressure. To obtain a pressure higher than the cylinder pressure, an optional pneumatic pump can be attached on line to raise the system pressure.

Two types of feed are used here namely pretreated with supercritical CO₂ and untreated leaves. Continuous stirring at 350 rpm is maintained during one-hour extraction and CO₂ is used to pressurize the system. The mixture is filtered by cloth filter with a pore size of 5 μm and residue is again extracted with water at same operating conditions. The filtration set up used for this purpose consists of a vacuum pump, separator and filter hold up vessel. The system can be easily dismantled to take out the cloth filter for cleaning and changing. A vacuum is applied for efficient and quick filtration. The extracted solution is charged in the holdup vessel and the cloth is set tightly in bottom of vessel. After second extraction, mixture is filtered and residues are discarded.

Characterization by high-performance liquid chromatography (HPLC)

There are many methods like HPLC, TLC, and Colorimetric method to analyze stevioside in the aqueous extract. HPLC method is a very sophisticated method for analysis and the colorimetric method using a UV spectra photometer is used for quick estimation of the active ingredient.

In the present work, purification of the extract from stevia leaves involves different steps like electrocoagulation (EC), supercritical CO₂ treatment, etc. Quantification is done after each step by a colorimetric method (phenol-sulfuric acid method) but identification of the desired components needs analysis of HPLC. The HPLC analysis is used also to show that desired components are present in the solution after each step of purification.

The HPLC analysis is followed by the method suggested by Adduci et al. [22]. The instrument consists of HPLC apparatus (waters associates) with a UV detector (waters 2487, dual An absorbance

detector) at a wavelength of 210 nm and solvent pump (binary HPLC pump, waters 1525), using a catalytic column (4.6 mm × 15 cm, waters Symmetry® C18.5 μm). Acetonitrile and methanol (80:20 v/v) (Merck, HPLC grade) are used as the mobile phase at a flow rate of 1 ml/min. 20 μl is injected by syringe. The sample is sufficiently diluted to 10-50 ppm for injection in HPLC. **Figure 3** given below is obtained from the HPLC analysis of stevia extract powder in aqueous medium after sufficient dilution. The third peak at 3.2 min is noted as corresponding to stevioside.

Quantification by colorimetric method

Quantification of glycosides is done by the colorimetric method followed by a UV measurement as described by Saha AK et al. [34]. The colorimetric method used here is known as the phenol sulfuric acid method. Glycosides amount less than 200 μg/ml present in the sample is suitable for this method. The sample is prepared by diluting (1:100 to 1:500) aqueous extract with distilled water. Then 2 ml of sample is taken in a 25 ml test tube and 2 ml of 5% (wt/wt) phenol is added. Then 10 ml 98% concentrated H₂SO₄, is added directly into the solution within 3-4 seconds by means of burette. The mixture is then vortexed and allowed to stand for 30 min at room temperature. Readings are taken at 490 nm against a blank solution prepared by distilled water as the sample. A helios alpha UV-V is spectrophotometer model (Thermo electron corporation) is used for the absorbance measurements at 490 nm. A calibration curve is prepared from samples of sucrose of different concentrations ranging from 10 to 50 μg/ml and their absorbance at 490 nm with concentration as plotted in **Figure 4**.

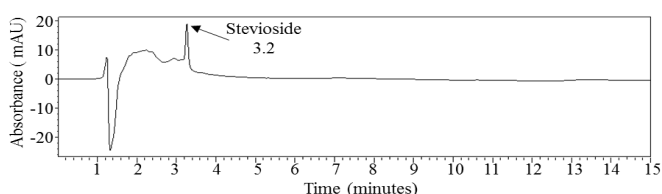


Figure 3 HPLC graph of standard stevia products.

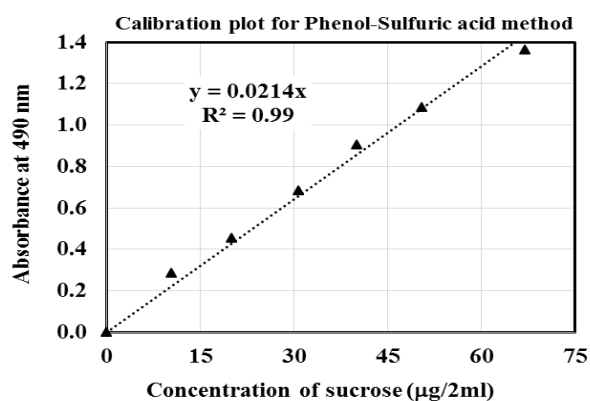


Figure 4 Calibration plot of sucrose according to phenol sulfuric acid method.

The calibration curve is well fitted in a straight line which agrees with Beer-Lambert Law. The error of measurement and calculation of yield is 3.5% maximum.

Results and Discussion

Processing protocol involving pretreatment, extraction, and purification has been evolved for each of these systems. Systematic parametric studies have been carried out to optimize the process conditions for higher yield and easier downstream processing for purification. Yield is calculated based on the amount of desired ingredients in the final product per unit amount of feed used. After each step of operation, amount of glycosides is calculated by phenol sulfuric acid method and HPLC analysis is done for identification. HPLC result of the aqueous extract is shown in **Figure 5**.

Three distinct peaks are observed at 1.4, 3.2 and 4.5 min respectively. Comparing with the standard plot, it is found that 2nd peak (3.5 min) is stevioside and 3rd peak (at 4.5 min) is rebaudioside A. First peak can be considered as an impurity (like pigment) in the solution.

Parametric study

The factors affecting pressurized hot water extraction are temperature, pressure, extraction time, particle size, pretreatment, water to sample ratio and number of stages. The effects of these factors on the yield have been analyzed on the systems under study and the results are enumerated in this section. Two types of feed have been investigated for glycosides namely dry leaves without grinding and ground leaves. **Table 2** presents the details of various experiments to extract glycosides from stevia leaves.

Effect of temperature

Figure 6 shows the effect of temperature on the yield of glycosides from stevia leaves. The change in yield of glycosides is significant when the temperature is varied from 30°C to 60°C but at a temperature above 60°C, it has less effect. The yield of glycosides in the second extract is almost constant with temperature. The yield was 6.7% at 120°C, which can be considered as optimum temperature because yield didn't increase substantially at 135°C.

As temperature increases, properties of water like viscosity, density, surface tension, polarity, etc. decrease that lead to a decrease in resistance of mass transfer. Thus, more glycoside is extracted from leaves at higher temperatures. The cell structure

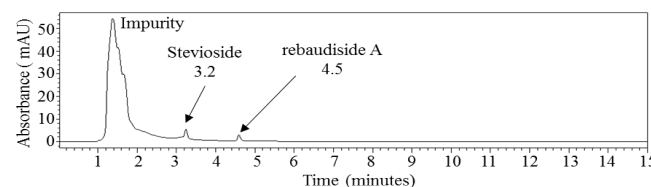
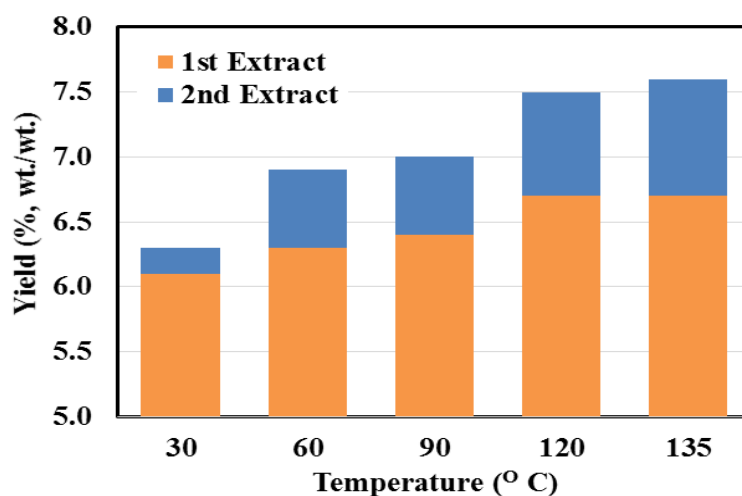
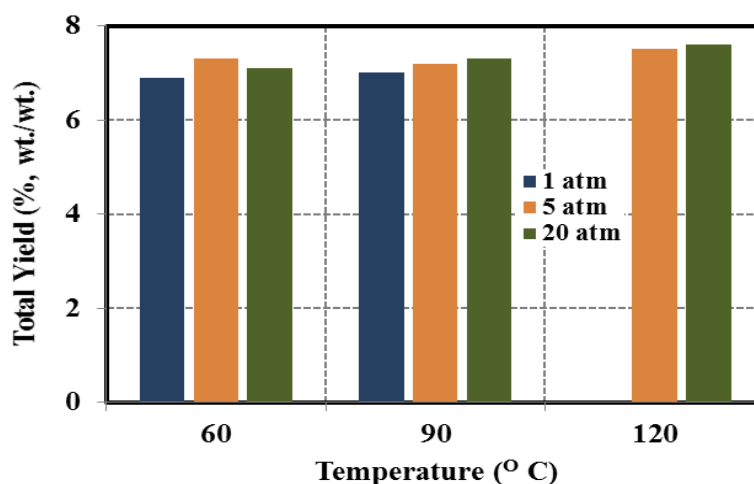


Figure 5 HPLC graph of aqueous extract.

Table 2. Various experiments with stevia leaves for extraction of glycosides.

Experiment No.	Feed Condition	Water:Feed (ml/gm)	Temperature (°C)	Pressure (atm)
1, 2, 3	Ground, pretreated with ScCO ₂	40	30, 60, 90	1
4, 5, 6, 7	Ground, pretreated with ScCO ₂	40	60, 90, 120, 135	5
8, 9, 10	Ground, pretreated with ScCO ₂	40	60, 90, 120	20
11, 12, 13	Ground, pretreated with ScCO ₂	60, 80, 100	60	1
14, 15, 16	Ground	40	30, 60, 90	1
17	Ground	40	120	5
18	Not Ground (Whole Leaves)	40	60	1

**Figure 6** Yield of glycosides from stevia leaves at different temperature.**Figure 7** Effect of pressure on yield of glycosides.

of leaves may be ruptured at higher temperature which leads to higher mass transfer to solvent.

Effect of pressure

The effect of pressure on the yield of glycosides is shown in **Figure**

7 where the yields of total glycosides obtained in two consecutive extracts at different pressure are plotted.

It is observed that the yield is almost constant with pressure; the variation of pressure has a negligible effect on the yield. However, higher pressure is maintained in the extractor to keep water in liquid form and it also reduces the evaporation loss.

Effect of pretreatment with ScCO₂

The yields of glycosides from ground leaves pretreated with/without supercritical carbon dioxide (ScCO₂) from temperature 30 to 120°C are shown in **Table 3**.

2-3% oily material which is insoluble in water at temperature up to 100°C is obtained during pretreatment with ScCO₂ (at 300 atm and 200 atm, 45°C). A comparison of yields is shown in **Figure 8**.

As seen in **Table 3 and Figure 8**, the effect of pretreatment with CO₂ is minimal. The yield of glycosides is slightly high with pretreated leaves than that of untreated leaves at temperature up to 90°C. After ScCO₂ pre-treatment, internal pores in the leaves may be opened up and more surface area is accessible for extraction.

Effect of grinding

PHWE experiments have been performed using leaves without grinding. Described as experiment number 18 in **Table 2**, 40 ml/gm water at 60°C is used. The first extraction gives 3.41% yield and the second one gives 0.82% yield as shown in **Figure 9**.

On the contrary, the yields of 6.1% at 60°C in the first extraction and 0.6% in the second extraction with 40 ml/gm water each are obtained from ground leaves (experiment number 15). PHW extraction of leaves with ScCO₂ pre-treatment gives 6.9% yield at 60°C (experiment number 2). Therefore, it is evident that the

grinding has a positive impact on the yield because of the access to more surface area for mass transfer.

Effect of extraction time

The effect of extraction time on the yield of glycosides by PHWE is shown in **Figure 10**. The yield reaches a nearly steady value at 5.9% after 40 minutes in the first extraction.

Initially, the yield increases sharply because of the higher initial concentration gradient. Water gets saturated with time and the yield reaches a fixed value at 40 minutes.

Effect of water to feed ratio

PHWE experiments have been performed with 5 gm ground leaves at 60°C at 1 atm for 1 hr. with different water to feed ratio. The effect of the water to feed the ratio on the yield of the first extract is shown in **Figure 11**.

As the water to feed ratio for PHW extraction increases the yield increases. However, the yield does not significantly change if the water to feed ratio exceeds 80 ml/gm. Mass transfer of glycoside is higher in dilute water for the same amount of feed due to the higher concentration gradient. Yield is generally higher with more water because more glycoside will be dissolved for the same concentration. However, other factors such as diffusion from pore space to the surface of the leaves also play a major role in extraction. When water to feed ratio exceeds 80 ml/gm, the

Table 3. Effect of pretreatment with ScCO₂ on yield of glycosides from ground leaves.

Feed Type	Exp. No	Temperature (°C)	Pressure (atm)	1 st Extract (%)	2 nd Extract (%)	Total (%)
Pretreated (ScCO ₂)	1	30	1	6.1	0.2	6.3
	2	60	1	6.3	0.6	6.9
	3	90	1	6.4	0.6	7
	6	120	5	6.7	0.8	7.5
Not Pretreated	14	30	1	5.1	1	6.1
	15	60	1	6.1	0.6	6.7
	16	90	1	6.3	0.5	6.8
	17	120	5	6.9	1.2	8.1

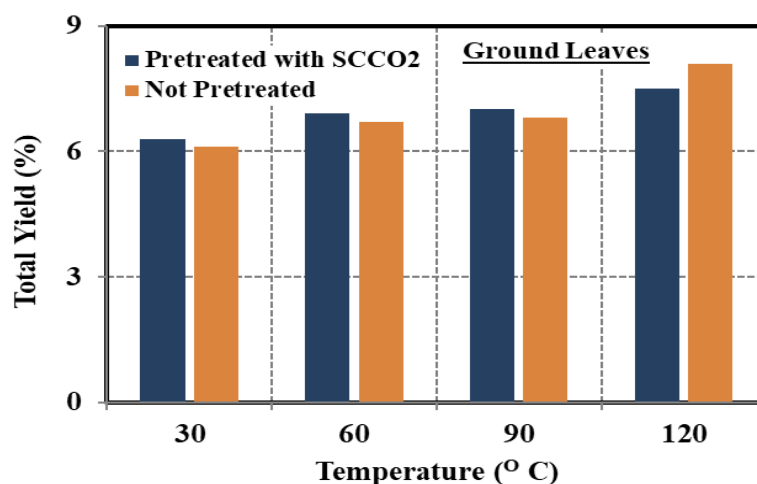


Figure 8 Effect of pretreatment with ScCO₂ on yield of glycosides from ground leaves.

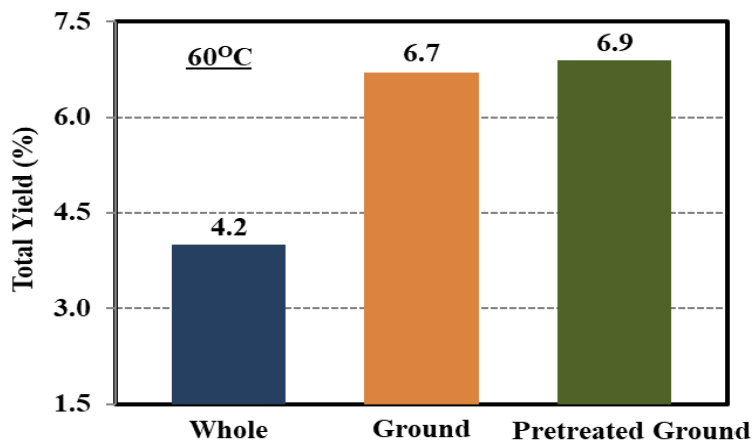


Figure 9 Effect of pretreatment on yield of glycosides in aqueous extract.

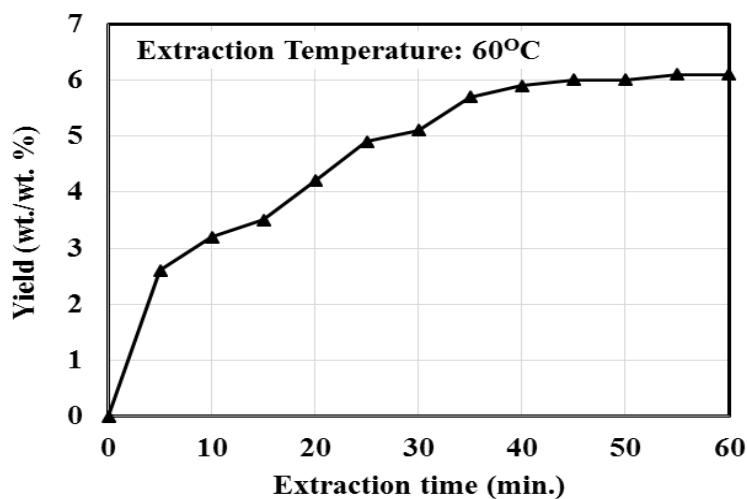


Figure 10 Effect of extraction time on the yield of glycosides in aqueous extract.

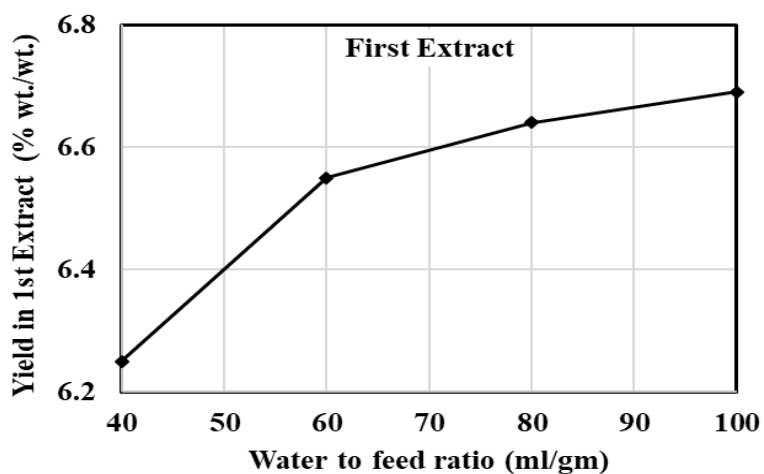


Figure 11 Effect of solvent to feed ratio.

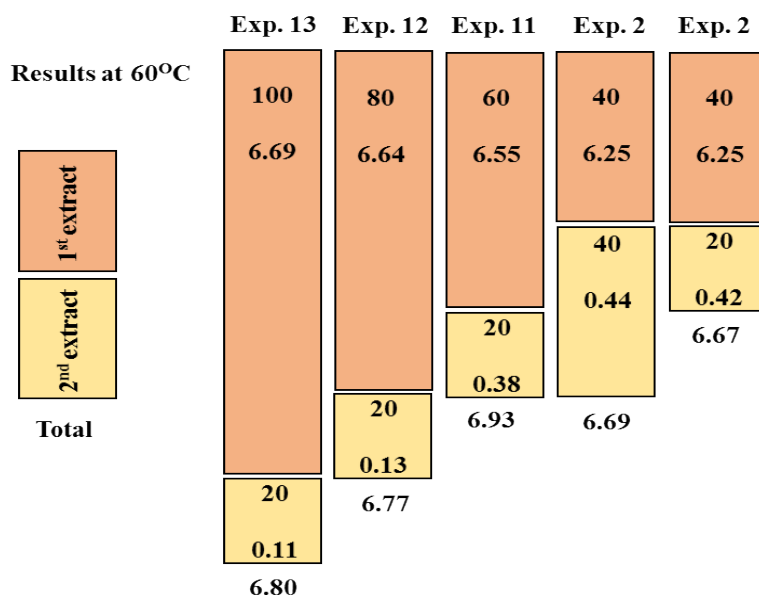


Figure 12 Comparison of yield with different combinations of water to feed ratio.

overall mass transfer is not controlled by the bulk water phase, instead it is controlled by the pore to surface mass transfer. Because of the fact that same ground leaves are used for all experiments, the pore structure is assumed to be same and mass transfer rate from inside pore to surface of leaf is also same. Therefore, increasing water amount up to certain value doesn't improve the yield anymore.

Effect of number of stages

Theoretically, it is well established that two to three stages of extraction are better than single-stage extraction with the same total amount of solvent. The effect of a number of stages is illustrated in **Figure 12**. Results obtained at 60°C are compared here. Third stage extractions were also conducted but no significant amounts of active components are found.

In the case of total 60 ml water to 1 gm feed ratio, total yield was 6.67% from two stages extraction with 40 ml/gm (first extraction) and 20 ml/gm (2nd extraction). On the other hand, total yield was 6.55% from single extraction with 60ml/gm water to feed ratio.

Similarly for 80 ml/gm, total yield was 6.64% in single stage compared to two-stage total yield of 6.7% (for 40+40 split) and 6.93% (60 ml/gm+20 ml/gm split). For 100 ml/gm case, total yield was 6.69% in single-stage and total yield was 6.77% for two-stage (80 ml/gm+20 ml/gm split).

In all cases, the total yield is higher in the case of two-stage extraction compared to single-stage extraction. Maximum yield (6.93%) is achieved by the combination of 60 ml/gm in the first extraction and 20 ml/gm in the 2nd extraction

Effect of stirring

One PHWE experiment has been performed with ground leaves without stirring at 60°C and it gives 4.31% yield whereas the PHW extraction with stirring at 60°C gives a 6.25% yield. Ground leaves of Stevia tend to settle down and accumulate on the bottom of the PHW extractor; therefore the amount of exposed leaves presents in the upper portion of the extractor becomes very low. Extraction without stirring is very inefficient as most of the materials are not accessible to PHWE.

Conclusion

Pressurized Hot Water Extraction (PHWE) has been used in this work for the recovery of stevioside from stevia leaves (*Stevia rebaudiana*). The major advantages of PHWE include a relatively low operating pressure and environmental friendliness of water. Experiments have been conducted with and without pretreatment of natural materials and the process, parameters have been optimized. A systematic parametric study has been undertaken by varying the parameters like temperature (in the range of 30 to 135°C), pressure (in the range of 1-20 atm), extraction time (in the range of 30 to 60 min), volume of water (in the range of 40-100 ml for 1 gm of feed), particle size (un-ground and ground), number of stages (1 to 3), stirring rate (0-350 rpm), and nature of feed pretreatment. The phenol sulfuric acid colorimetric method using a UV spectrophotometer is used to analyze the active ingredient like glycosides. It has been observed that temperature of PHWE plays an important role in the recovery of the bio-active compounds, though pressure does not have much effect. The maximum yield of 7.6% is achieved at 120°C and 5 atm with 40 ml water for 1 gm of ground leaves in a two-stage operation.

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