STUDY ON THE EFFECT OF PROCESSING PARAMETERS ON THE EXTRACTION OF STEVIOSIDE USING ULTRASONIC EXTRACTOR

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ABSTRACT

Stevia rebaudiana is a sweet, green leaves that contain high amount of sweetener but has no calories. Inside *stevia rebaudiana* leaves, there are many sweet components includes stevioside, rebaudioside A, B, D, E and dulcoside. Among these sweet components, stevioside is the most abundant component. Stevioside is a natural sweetener and 300 times sweeter than sucrose. It is an alternative to sugar, that suitable for people who suffer from diabetes and hypertension because it has a negligible effect on blood glucose. In this present study, stevioside was extracted using an ultrasonic extraction method. They were 3 parameters that have been studied. The screening of the best extraction solvents (distilled water, ethanol 95% and 70% ethanol aqueous) were firstly investigated before designing the experiment by using one-factor-at-one time method. The 3 parameters involved included the ratio of leaves/solvent, extraction time and the ultrasonic frequency. Based on the result obtained, distilled water is the best extraction solvent with the ratio of leaves to solvent at 4:30 (w/v), extraction time of 20 minutes and the sonicator frequency at 20 kHz. It can be concluded that by using suitable types of solvent ratio, extraction time and sonicator frequency, the highest yield of stevioside have been obtained.

ABSTRAK

Stevia rebaudiana adalah sejenis daun manis berwarna hijau yang mengandungi kandungan manisan yang tinggi, tetapi tidak mengandungi kalori. Stevia ialah pemanis semulajadi dan kandungan gulanya adalah 300 kali ganda lebih banyak berbanding sukrosa. Stevia adalah sejenis gula alternatif yang sesuai untuk pesakit kencing manis dan mereka yang menderita sakit darah tinggi kerana kandungan gula di dalam stevia tidak memberi kesan terhadap paras glukosa di dalam darah. Di dalam stevia terdapat banyak komponen-komponen pemanis termasuk Stevioside, Rebaudioside A, B, D, E dan Dulcoside. Di antara kompononen-komponen pemanis ini, stevioside adalah komponen terbanyak di dalam daun stevia. Stevioside di ekstrak dengan menggunakan kaedah ultrasonik. Terdapat tiga parameter yang telah di kaji. Saringan terhadap pelarut paling baik (air suling, ethanol 95% dan ethanol 70%) di lakukan dahulu sebelum meneruskan ekperimen dengan menggunakan kaedah satu-faktor-pada-satu- masa. Tiga parameter terlibat adalah nisbah daun kepada pelarut, masa ekstrak dan frekuensi ultrasonik. Berdasarkan keputusan yang diperoleh, air suling adalah pelarut terbaik dengan nisbah daun kepada air suling 4:30 (w/v), masa pengektrakan 20 minit dan frekuensi sonicator 20 kHz. Kesimpulannya, dengan mengunakan nisbah pelarut, masa pengekstrakan dan frekuensi sonicator yang bersesuaian,penghasilan stevioside tertinggi telah diperolehi.

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LIST OF ABBREVIATIONS

UV- Vis	Ultra- violet visible spectroscopy
HPLC	High Performance Liquid Chromatography
HSPiP	Hansen solubility parameter value
OFAT	One factor at one time
OD	Optical density
RSM	Research Surface Methodology

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND OF STUDY

Stevia is the generic term used for food ingredients derived from the herb *Stevia rebaudiana*, commonly known as sweetleaf, honeyleaf or sugarleaf due to the high amount of sweetener in its leaf. It is a small perennial shrub plant with green leaves that belongs to the aster (Asteraceae) or chrysanthemum family of plants (Rita Elkins, 1997). Diterpene glycosides present in the *stevia rebaudiana* leaves are responsible for sweet taste of this leaf. The glycosides are stevioside, rebaudioside A, B, D, E, dulcoside A and B (Leung and Foster, 1996). According to Shi *et al*. (1999), among of these sugar molecules, stevioside is the most abundant ingredient in *stevia rebaudiana* leaves. Stevioside has no calorie in it contain, have been used as sweetener all over the world. It is a natural sweetener that is 300 times sweeter than sucrose (Allam *et al*., 2001). *Stevia rebaudiana* leaves contain a lot of other nutrition such as protein, fibers, carbohydrates, phosphorus, iron, calcium, potassium, sodium, magnesium, iron, zinc, vitamin C and vitamin A (Abou-Arab *et al*., 2010). Stevioside is an alternative sugar for anyone who has suffered from the blood sugar disorder or diabetes, obesity and hypertension who needs to limit their calorie, sugar and carbohydrate intake because it has a negligible effect on blood glucose, even enhancing glucose tolerance.

Stevioside has been used as a food and medicine (Kinghorn and Soejarto, 2002) for many years in Japan and Paraguay. It is also consumed as a dietary supplement, food product and beverage product in United Stated and Europe, such as in soft drinks, ice cream, yogurt, cake, cereals, jam, beverages, biscuit, chewing gums, candies and bakery product (Chatsudthipong and Muanprasat, 2008). Nowadays, stevioside has been commercialized widely in Japan and Europe with 200 metric tons of stevioside being extracted for the market. The vast majority of *stevia rebaudiana* leaves in the Japanese market is cultivated in China especially in Fujian, Zhejiang and Guangdong Provinces.

Sweeteners are divided into three categories which are sugar with natural carbohydrate, sugar alcohol and low-calorie sweetener. Sugars with natural carbohydrate increase the blood glucose level, for example cane sugar. Sugar alcohol contains half calories of carbohydrate sugar, for example sorbitol and xylitol. Lastly low-calorie sweetener can be further divided into two categories; i) from plant origin, and ii) artificial sweetener, which contains no calorie and does not rise up the sugar level in our blood.

According to Saedesai and Waldshan (1991), artificial sweetener may induce insulin secretion and rise in appetite and the long term effect of weight gain. There are many side effect of Aspartame which are brain tumors, chronic fatigue syndrome, parkinson’s disease, alzeimer’s. mental retardation, birth defects and diabetes (Mercola, 2011).

Figure 1.1: *Stevia Rebaudiana*

Figure 1.2: Dried stevia leaves

Figure 1.3: Ultrasonic extractor

1.2 PROBLEM STATEMENT

Conventionally, stevioside is extracted from *stevia rebaudiana* leaves by dissolving the leaves into solvent for certain time with certain temperature without using any assisted equipment. Some of the researchers use conventional equipment such as soxhlet extractor, but this equipment has disadvantages such as use longer extraction time and high solvent volume. This conventional method of stevioside extraction has low efficiency and yield. Alternatively an efficient and improved method is needed in order to minimize the extraction time and solvent volume so that higher yield of stevioside can be obtained. Ultrasonic extraction method used low extraction time and solvent volume, simple procedures and use simple apparatus. These experiments can also benefits to large scale industries that involved in stevioside production. By using suitable parameters, the operational cost and time can be reduced.

1.3 RESEARCH OBJECTIVES

The objective of this experiment is to study the effect of processing parameters (solid-solvent ratio, extraction time and ultrasonic frequency) on the extraction of stevioside from *Stevia rebaudiana* by using ultrasonic extraction method.

1.4 SCOPES OF STUDY

In order to achieve the objective, four scope of study are presented as follow:

a. To study the best extraction solvent among distilled water, ethanol 95% and ethanol 75%.
b. To investigate the effect of solid-solvent ratio between range of 1:30 to 4:30.
c. To evaluate the effect of extraction time between range of 20 to 60 minutes.
d. To examine the effect of ultrasonic frequency between range of 20 to 60 kHz.

1.5 RATIONALE AND SIGNIFICANT OF STUDY

The increasing number of Malaysian people having diabetes and other disease related to high sugar consumed must not be ignored. This study can be significant for people having these diseases by substituting sucrose consumed with sweetener. Other than that, this study can be beneficial to large scale industries that process the *stevia rebaudiana* leaves into sweetener. Other than that, identifying the suitable extraction parameters on the extraction of stevioside from *stevia rebaudiana* leaves would definitely be beneficial in the large-scale industries in terms of saving on the production cost and time.

CHAPTER 2

LITERATURE REVIEW

2.1 STEVIOSIDE

In *stevia rebaudiana* leaves, stevioside, rebaudioside A-F and dulcoside are the components responsible for the sweet taste. Among these components, stevioside is the predominant sweetener, which represent (5-10 % w/w) of dried leaves. Rebaudioside A is the second most abundant sweet component (2-4 % w/w), followed by rebaudioside C (1-2 % w/w) and dulcoside A (0.4-0.7 % w/w) (Kinghorn and Soejarto, 1985). Stevioside content varies between 4 and 20% of the dry weight of the leaves depending on the growing condition (Geuns, 2003). According to Simon (2002), stevioside (ent-13-hydroxykaur-16-en-18-oic acid) is a glycoside of the diterpene derivative that is used as a natural sweetener, which substitutes the artificial sweetener such as saccharin, aspartame, thaumatin and sucralose. Artificial sweetener can be describe as any chemical compound which is sweet to taste, but does not contain any sucrose.

Stevioside appear as a white, crystalline and odourless powder. Unlike many low-calorie sweeteners, stevioside is stable at high (100°C) temperatures and over a range of pH values (pH 3-9) (Kinghorn & Soejarto, 1985). It contains no calories, and does not darken upon cooking (Crammer & Ikan, 1986). The sweetness of these glycosides compared to sucrose is dulcoside A (50–120), rebaudioside A (250–450), rebaudioside B (300–350), rebaudioside C (50–120), rebaudioside D (250–450), rebaudioside E (150–300), steviobioside (100–125), and stevioside (300) (Crammer & Ikan, 1986).

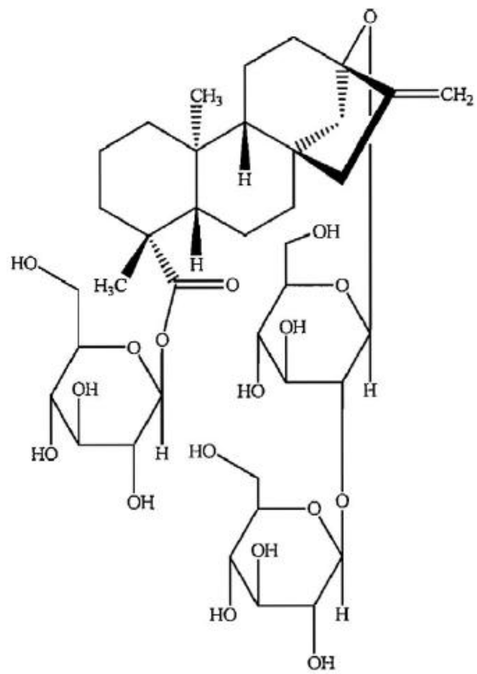

Figure 2.1: Chemical structure of stevioside. Stevioside molecular formula is $C_{38}H_{60}O_{18}$ (Carakostas *et al*, 2008)

Figure 2.2: Stevioside powder

Table 2.1: Properties of stevioside

Matrix	**Properties of Stevioside**
Synonyms	13-[(2-o-beta-D-glucopyranosyl-beta-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid beta-D glucopyranosyl ester
Physical form	Oily liquid
Color	Almost colorless
Odor	Odorless
Specific refractive	1.434-1.437
Melting point	196-198ºC
Molecular weight	804.88
Molecular Formula	$C_{38}H_{60}O_{18}$
Solubility	Soluble in water and alcohol

Solubility in water	0.13%
Stable pH (100% concentration)`	4.5-6.5
Density	0.840-0.890
Storage	Store at 4ºC, in dark place
Testing method	HPLC, Uv-Vis
Toxicity	Not toxic
Optical activity	25 - 39.3
Hazard indentification	Not classified as dangerous

Sources: Yussof and Sarmidi (2003)

2.2 STEVIOSIDE EXTRACTION

Conventionally, stevia leaves were dried and used as tea by Paraguayan. Some of them chew the leaves and treat them as medicine (Sharma, 2007). Nowadays, many methods of stevia extraction have been improved. This is because; the stevia leaves would leave an unpleasant aroma and unclear, green color into drink or food. People nowadays prefer to consume a simple, readymade and crystalline powder of stevia extract in daily life. Basically, the extraction of stevioside from stevia leaves start by drying the leaves and mesh them into small particles, then dissolving into selected solvent. After that, the mixtures are filtered and the remaining solvent-leaves extract mixture is crystallized or dried to make it powder. Ethanol, methanol, water and ethanol aqueous are common solvents used in stevia extraction.

According to Abou-Arab *et al.* (2010), stevia leaves contain 61.93% dry weight of carbohydrate. The extraction of stevioside was carried out at different ratio of leaves/solvent by using three solvents which were hot water (65°C), methanol and ethanol aqueous. For the hot water extraction, the highest yield of stevioside was at 1:35 ratio of leaves/water. Both methanol and ethanol aqueous showed the optimum ratio of leaves/solvent which yields maximum stevioside content was at ratio 4:1.

2.3 ULTRASONIC EXTRACTION METHOD

According to Allinger (1975), ultrasonic can be defined as cell destruction or cell disintegration. Ultrasonic have the wave measurement from 20 kHz until 10MHz. Cell integration occurs when protein or enzyme released from cells or organelles inside the cell from sonication process. To obtain the product desired, cell membrane must be disrupted. When the cell membrane disrupted, solvent used in the sonicator would penetrate into cellular material. The mechanical activity of sonicator improves the solvent mass transfer. The high intensity of waves of from ultrasonic can generate the growth and collapse of bubbles inside liquids, a phenomenon known as cavitation. There are several factors that affect the mass transfer which are the heating of the medium, the microstirring at interfaces and several structural effects and the asymmetric implosions of the cavitation bubbles close to a solid surface.

Sonicator will irritate the transfer of molecular between the solid and solvent, usually resulting in a greater yield of extraction in the application of ultrasonic method. There are two types of phenomenon occur in the solvent extraction which are diffusion and rinsing. The plant cell walls can be destroyed by sonication, but there are some cell walls that are not destroyed. Diffusion will occur to the unbroken cell wall. The rinsing phenomenon occurs at the broken cell wall, where cell content will be rinsed by solvent once the cell walls are broken.

Moulton *et al.* (1982) has reported that sonication increased the recovery of dispersed protein progressively as the solvent ratio changed from 1:10 to 1:30. Zhao & Shuna (2006) have studied on the effect of ultrasound on extraction yield of active compound from the herbal material by using ultrasonic extraction, resulted on the optimum parameter were at time of 30 minutes, temperature of 80°C, power of 24W, leaves/ethanol aqueous ratio of 1:25 and solvent concentration of 50%. Based on report by Celeghini *et al.* (2001), the optimum parameters of ultrasonic extraction of “guaco” leaves were 20 minutes of time, 1:10 of leaves/ethanol aqueous ratio at room temperature. According to Liu *et al.* (2010), the highest stevioside yield from ultrasonic assisted extraction were at temperature of 68°C, sonic power of 60W and 32 minutes of extraction time. By using 1:10 of leaves/distilled water ratio for 20 minutes of extraction at 25°C and output power of 100 W, Alupului *et al.* (2009) proved that these parameters were the optimum extraction condition for a fast and reliable ultrasound assisted extraction for stevia.

Table 2.2: Stevioside yield by using different parameters

Solvent	**Ratio (leaves;solvent)**	**Extraction time (minutes)**	**Frequency (kHz)**	**Yield(g stevioside/100g extratct)**	**References**
Distilled water	1:10	20	40	2.3	(Alupului& Lavric,2009)
Distilled water	1:5	20	20	2.26	(Alupului &Lavric, 2008)
55% ethanol aqueous	1:5	20	20	2.25	(Alupului &Lavric, 2008)
70% ethanol aqueous	1:5	20	20	2.23	(Alupului &Lavric, 2008)
Distilled water	1:10	32	20	7.4	(Liu *et al*, 2010)
Distilled water	1:4	45	25	highest stevioside yield	(Garcia-Noguera *et al*.,2009)
Methanol Distilled water	1:4 1:35	-	-	94.90% 80.21% stevioside recovery	(Abou-Arab *et al.*,2010)

2.4 BENEFITS OF ULTRASONIC EXTRACTOR

Ultrasonic was evaluated as a simpler and more effective alternative to conventional extraction methods for the effective isolation of important substances from plant tissues, because of the contact surface area between solid and liquid phase is much greater, due to particle disruption taking place (Xiao and Hu, 2002). The utilization of ultrasonic cavitations for extraction and food preservation is a new powerful processing technology that can not only be applied safely and environmentally friendly but also efficiently and economically (Allinger, 1975). It is expected that the use of ultrasonic extractor for sample preparation purposes in environmental analytical chemistry will become more widespread, owing to its simplicity, ease of use, speed, and enhanced safety when compared with other, more traditional sample preparation procedures (Ashley, 1998).

Ultrasonic extraction has proven to be equally or more efficient than soxhlet extraction. Soxhlet extraction is a time consuming method, involved large volume of solvent and exposed to the hazard of boiling solvents. Compared with soxhlet, ultrasonic technique usually provide a relatively low cost method, low extraction time, using small volume of organic solvent and simplicity of the apparatus and extraction procedure.

2.5 EFFECT OF ULTRASONIC FREQUENCY

Ultrasonic irradiation has proven to have almost no harmful intermediates of concern to humans (Kitajima *et al.,* 2006). The important property of ultrasonic oscillation is their resonant frequency. It is conditioned that effectiveness of technologies processes depends from oscillation amplitude and maximal level of amplitude obtained when ultrasonic oscillation system excites at resonant frequency (Khmelev, 2007).In general, an increase in ultrasonic intensity (Watts/cm^2) leads to an increase in cavitations effects. In ultrasonic extraction, under the same ultrasonic intensity, an increase of ultrasonic frequency reduces the production and intensity of cavitations in liquid. The lowest energy required to achieve cavitations, increases with the increase of surface tension, the viscosity of liquid or the hydrostatic pressure, and with the decrease of temperature.

The production of cavitation bubbles are difficult to obtain at high ultrasonic frequency than at low ultrasonic frequencies. In order to increase the cavitation bubbles at high ultrasonic frequency, intensity of the applied sound must be increased, to ensure that the cohesive forces of the liquid media are overcome and voids are created.

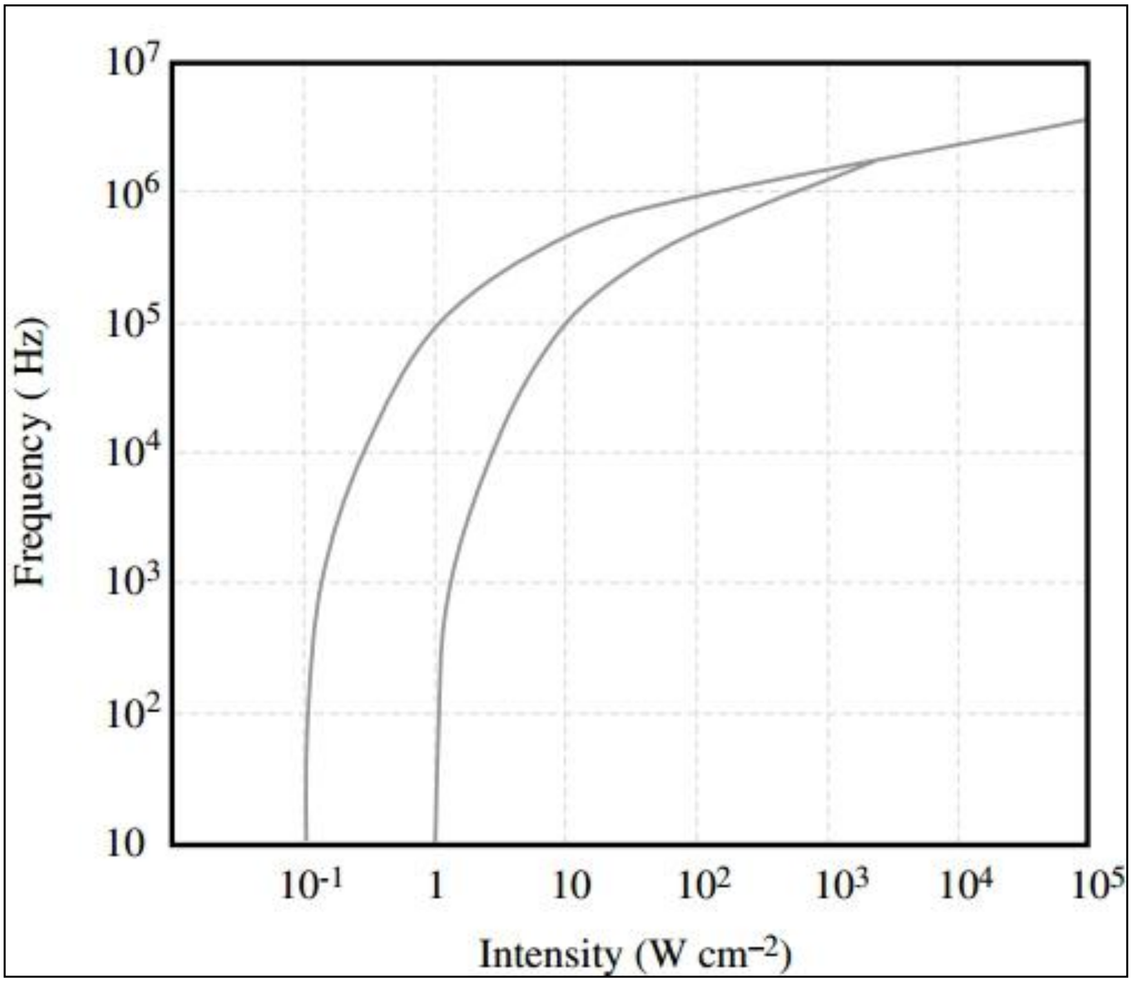

Figure 2.3: Variation of intensity of sonication versus the threshold frequency for aerated water and air-free water. (Mason and Lorimer, 1989)

Based on Figure 2.3, it can be seen that 400 kHz needed 10 times power to induce cavitation in water, compared with 10 kHz. At very high frequencies, the cycle of compression and decompression caused by the ultrasonic waves becomes so short that the molecules of the liquid cannot be separated to form a void and, thus, cavitation is no longer obtained (Santos *et al*, .2009).