MIXTURES OF *S.REBAUDIANA* LEAVES AND STEMS WASTE FOR *STEVIOSIDES* SYNTHESIS USING ETHANOL-WATER SOLVENTS

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ABSTRACT

*Mixtures of S.rebaudiana leaves and stems waste were applied for steviosides synthesis. The various ethanol-water as polarity solvents were employed in the ultrasound-assisted extraction (UAE). The HPLC (High Performance Liquid Chromatography) with Eurospher 100–5*NH_2 *column at 1mL/*m^3 *were used for steviosides analysis. The maximum yields were achieved by the time of 30 min., temperature of 40*^oC *and ethanol-water ratio of 80:20 (v/v). The second order kinetics model best fits the obtained data with* R^2 *and k of 0.992 and 0.02 min*$^{-1}$*. Utilization of wastes as an alternative feed stock provides a cost-effective and environmental friendly way for steviosides extraction.More extensive investigation needs to be performed to compare the various advantageous processing and technical feasibilty.*

KEYWORDS: *S.rebaudiana; Steviosides; Polarity solvents; Kinetics and UAE*

1.0 INTRODUCTION

The *Steviosides (Steviol g.)* are to be processed from *Stevia rebaudiana* (*S. rebaudiana*) plant as feedstocks. These have up to 300 times sweeter than sugar, are better in term of taste quality due to less bitter and more sweeter than *rebaudiosides A*. The plant of *S.rebaudiana bertoni* can be found initially in Brazil and Paraguay rain forest. Nowadays, it has been growing in Malaysia, especially in Pahang and Melaka. Among 150 *stevia* species, but the only one with significant sweet tasting properties is *S.rebaudiana*. Over the years, the number of countries in which *stevia* is available as a sweetener has been increasing (Brandle et al., 2007; Jaitak et al., 2008; Lemus-Mondaca et al., 2011). Actually, according to some researchers, *steviosides* can be separated from *S.*

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rebaudiana leaves, and it has own benefits, without discarding its stems. Therefore, various advantageous and economic methods of *steviosides* extraction are needed to find a highest and qualified yield. Moreover, effective methods, low cost solvents and process optimization are also required for better yield of steviol *glycosides*. This will increase the sales profit of *steviosides* from mixture of *S. rebaudiana* leaves and stems waste.

The steviol *glycosides* extraction from *S. rebaudiana* leaves is known as *steviosides*, which is done by using the different solvents and process mechanisms. A combined process mechanism involving a solid or liquid extraction step, followed by a liquid or liquid-purifying step, is traditionally used to extract the *glycosides* from stevia (Erkucuk et al., 2009; Puri et al., 2011).

However, the *glycosides* extract has a pronounced bitter after taste that is responsible for many of the restrictions on the use of stevia as a sweetener. The *steviosides* from the *S. rebaudiana* leaves have been also resulted using classical techniques: maceration or thermal extraction, either requiring long processing time and low efficiency, in case of maceration, or facing thermal degradation, in case of infusion and decoction. The most conventional extraction processes described in the literature follow a similar methodology. The *stevia* leaves are extracted with hot water or alcohols. In some cases, the leaves are pretreated with non polar solvents such as chloroform or hexane to remove the essential oils, lipids, chlorophyl, and other non polar substances. The extract is clarified by precipitation with salt or alkaline solutions. The extract is concentrated and redissolved in methanol for crystallization of the *glycosides*. The crystals are formed almost by pure *steviosides* (Dacome et al., 2005; Wang et al., 2006; Puri et al., 2011).

In order to increase the *steviosides* productivity, several intensification techniques like ultrasonic waves, supercritical fluids or microwaves were associated with extraction of plant's compounds. These techniques have been developing for the yield and quality of extracted products improvement (Wang et al., 2006; Mandal et al., 2007; Liazid et al., 2010; Wiyarno, et al., 2010). From these, ultrasound assisted and microwave extractions emerged as two promising techniques from an economical point of view, being inexpensive, simple and efficient. These procedures increase at least one of the major parameters governing extraction: the kinetic, through the partial mass transfer rates, the interfacial area or the driving force (Cravotto, et al., 2008; Jaitak, et al., 2009; Goula, 2012).

In the pressent investigation, mixtures of *S. rebaudiana* stems and leaves were employed for *steviosides* synthesis. The various ethanol-water as polarity solvents were added in the ultrasound-assisted extraction (UAE). The HPLC with Eurospher 100–5NH$_2$ column at 1mL/m^3 were used for *steviosides* analysis. The effect of process parameter on the *steviosides* yield was searched for. The kinetics of *steviosides* synthesis was also justified. The composition of steviol *glycosides* in those mixtures *S. rebaudiana* leaves and stems waste was also analyzed. Therefore, this investigation may benefit the Malaysian Stevia for *steviosides* formation in analyzing and optimizing their new breed.

2.0 MATERIALS AND METHODS

2.1 Chemicals and reagents

The *S. rebaudiana* was provided by Pahang's Agricultural Park, Kuantan, Pahang, Malaysia. The samples were first dried under the sun for moisture content reduction from the plant. Dried samples were ground in an electrical grinder to obtain fine powder and were stored in dark, closed container until analysis. The chemicals and reagents which are ethanol, acetonitrile and standard solution of *steviosides* were also purchased from Sigma Aldrich Sdn Bhd.

2.2 Extraction procedures

Prior to the extraction, the powder of *s.rebaudiana* stems and leaves were sieved up to 60 mesh sizes. The samples were extracted based on the various ethanol in water (80:20 (40:10), 60:40,40:60,20:80,100:0, v/v), temperatures (20, 30, 40, 50 and 60°C) and time (10, 20, 30, 40 and 50 min) in an ultrasonic-assisted extraction (UAE) with the power of 250 W. After the extraction step, the samples were filtered through microfiber filter disk using vacuum pump filter. The combined percolations were evaporated to dryness on a rotary evaporator at 55^{0}C and dissolved in water. Then, the samples were analyzed by High Performance Liquid Chromatography (HPLC) using Eurospher 100 – 5 NH$_2$ column at flowrate of 1mL/m^3 and temperature of 35°C.

2.3 Stock and standard solution preparation

The 2.5 mg of the standard solutions were prepared by diluting with 80% ethanol in water into volumetric flask respectively in prior to sonicate them for 15 minutes. Standard solutions were prepared before running the High Performance Liquid Chromatography (HPLC). Solutions were prepared by diluting 0.25 mg/mL of stock solution into a 10 ml volumetric flask for concentration of 0.001 to 0.1 mg/mL.

2.4 Identification and analysis of *steviosides* using HPLC

The *steviosiedes (steviol g.)* containing solution was identified and analyzed using HPLC method. The HPLC approved existency of the resulted steviol glicosides, and the sample would be stated as crude steviol glicosides. The crude *steviosides* were dissolved with distilled water before sonicating for 10 minutes until free-bubble. After that, the samples were sonicated for 10 minutes. Finally, 5 ml of samples were injected into vial through a syringe filter (0.45 μm) for futher analysis using HPLC.

3.0 RESULTS AND DISCUSSIONS

3.1 HPLC analysis

Figure 1 shows the determination of *steviosides* and rebaudiosides in the stevia samples based on the process parameters. The extraction time, temperature and percentage of ethanol in water are investigated at 30 minutes, 40^0C and 80:20 (v/v) or 40:10 (v/v). The signal UV wavelength is in the range of 205-215 nm, and the retention time for the *steviosides* compound in the sample is 3.07 min closes to the *steviosides* standard with the time of 3.28 min.

The most compounds of *steviosides* are found in the plant leaves, followed by rebaudiosides and other seven minor compound of steviol *glycosides* (Jaitak et al., 2008; Samah et al., 2012). Figure 2 reflects the calibration curve. The curve is used to determine the concentration of the *steviosides* and rebaudiosides in the sample. It has been shown that the result obey a simple linear regression as express in Equation (1).

$$y = 238.51x + 372.9 \tag{1}$$

where y = area

x = amount (ng/u1)

The obtained results of HPLC analysis are given in the Figure 1, it could be determined the concentration of *steviosides* as shown in Figure 2.

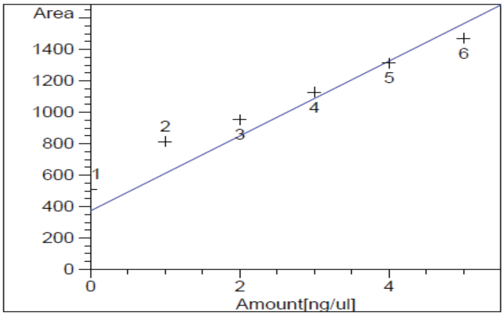

Figure 1. HPLC chromatogram of *steviosides* and *rebaudiosides* at t=30 min, T=40°C and Ethanol/H2O=80:20 (v/v) or (40:10)

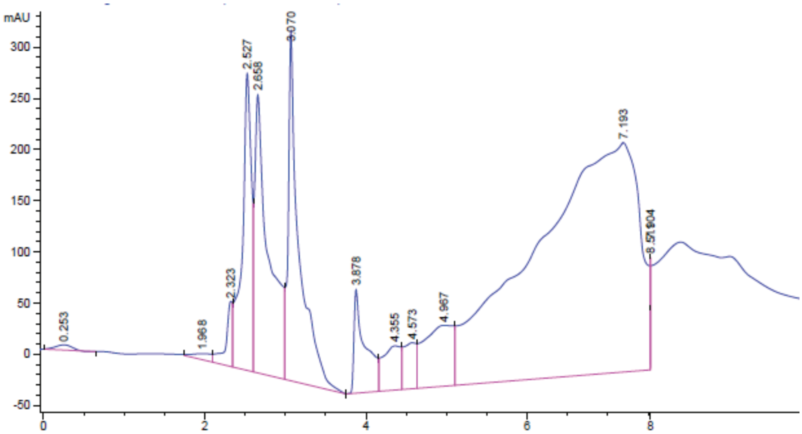

Figure 2. Calibration curve of standard solution

3.2 Effect of process parameters on the *steviosides* yield

3.2.1 Temperature effect on the steviosides yield

The extraction efficiency of Ultrasound-Assisted Extraction (UAE) is influenced by the various factors. Chemical constituents of the plants are mostly heat sensitive and that make the extraction temperature as the most important parameter. The solubility of solute and the diffusion coefficient rises as the increased temperature which favors extraction (Cravoto et al., 2008; Vetal, et al., 2012). Figure 3 reflects the effect of temperature on the yield of *steviosides*, and the other extraction conditions are fixed as follows: the extraction time, power and percentage of ethanol in water are 30 minutes, 250 W and 80:20 (v/v) or 40:10 (v/v).

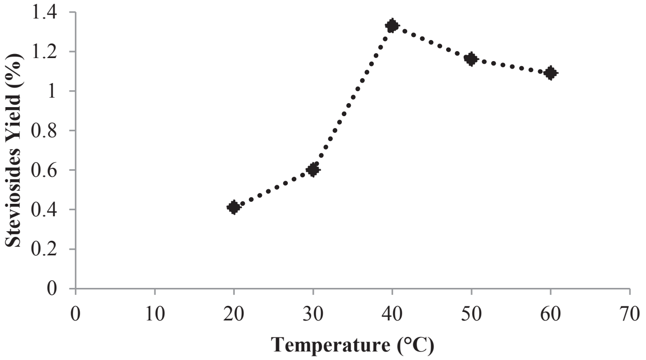

Figure 3. Effect of temperature on *steviosides* yield at t=30 min. and Ethanol/H_2O=80:20 (v/v) or (40:10) (v/v)

The extraction temperature displays a positive linear effect on the yield of *steviosides* with the temperature ranges from 20-40°C, and then the decreased yield as the increased temperature. These indications relates with the acoustic cavitation and diffusion through the cell walls which results the two main physical phenomenon in UAE. The yield of stevisides increases at the higher temperature. Unfortunately, the higher temperature causes the decreased surface tension, and the vapour pressure within micro buble increases, it would be affected by the damping of the ultrasonic wave (Cravotto, et al., 2008; Ying et al., 2010). Therefore, the yield of steviolsides becomes lower with the extraction temperature is over 40°C. Based on the curve and achieved results, the optimum extraction temperature is 40°C.

3.2.2 Time influence on the steviosides yield

The influence of extraction time on the yield of *steviosides* is shown in the Figure 4. The conducted experiments are guided by the various temperature, power and percentage of ethanol in water of 40°C, 250W and the percentage of ethanol in water of 80:20 (v/v) or 40:10 (v/v). The ultrasound-assisted extraction of *steviosides* gives the maximal yield on the time of 30 minutes. The effectivity of operated ultrasound is in the first 30 minutes, it takes the same time compared with another seeds oil extraction, like pomegranate seed oil (Goula, 2012).

The yield of *steviosides* increases from time of 10 to 30 minutes, and the decreased yield with the increase of time extraction. In the ultrasonic extraction process, the ultrasonic wave is able to break the cell wall faster up to particular time (Wiyarno, et al., 2010; Ying et al., 2011). In this case, the increase of extraction time up to 30 minutes relects the increased yield, and it would be stable at 30 minutes.

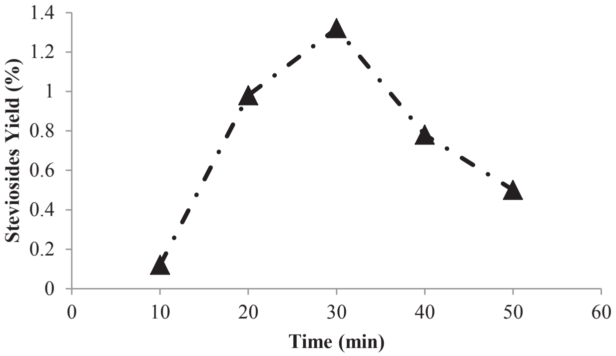

Figure 4. Effect of time on *steviosides* yield at T=40°C and Ethanol/H_2O=80:20 (v/v) or (40:10) (v/v)

The time extension after 30 min has no effect on the yield of *steviosides*. This appearance proves the fact that the extraction process presents two stages which are involved the penetration of the solvent into the cellular structure followed by the dissolution of soluble constituents in the solvent, it is called the characterization by a rapid rate, and the second one involves the external diffusion of soluble constituents through the porous structure of the residual solids and its transfer from the solution in contact with the particles to the bulk solution (Goula, 2012). Next, the used time for maximum *steviosides* synthesis using ultrasound system is 30 min. This time matchs the ultrasound system operation. The ultrasound duration should not exceed more than 30 min for ultrasound degradation control (Wiyarno, et al., 2010; Zou, et al., 2010).

3.2.3 Impact of of ethanol in water on the steviosides yield

The preferred solvents are the most important steps on the extraction yields. Ethanol as solvent can afford high extraction yield compared to another solvents (Jaitak, et al., 2009).

Figure 5 reflects the effect of the percentage of ethanol in water to the *steviosides* yield.

From the graph is shown the percentage of ethanol in water are 80:20 (40:10) (v/v), 60:40,40:60,20:8, the *steviosides* yield significantly increases, after that the yield decreases. These phenomenon approves the polarity solvents are active up to the higher yield of *steviosides*. The maximal yield of *steviosides* could be resulted by the percentage of ethanol in water of 80:20 (v/v) or (40:10) (v/v).

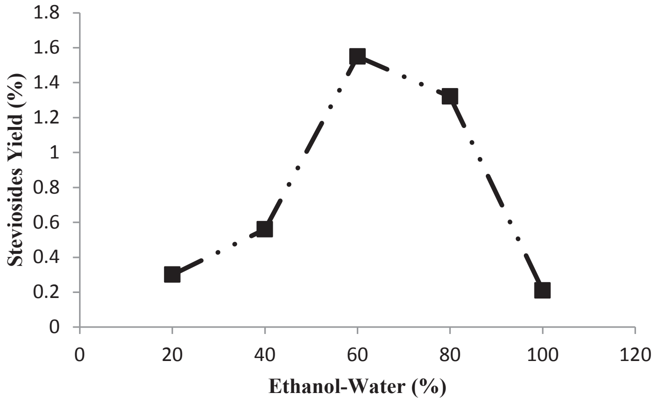

Figure 5. Effect of Eth/H_2O on *steviosides* yield at t=30 min and T=40^{0}C

3.3 Kinetics of *steviosides* synthesis using UAE

Kinetics of *steviosides* were determined based on the extraction time on the *steviosides* yield. The kinetics followed the pseudo first and second order for solid-liquid extraction model. The model would be taken into account the diffusion-dependent solid-liquid extraction. The equation would be interpreted by applying the boundary condition and given the linear form. The linear form would be found for solid-liquid extraction model.

The kinetics of *steviosides* synthesis using UAE were justified by the first and second-order. The first-order rate equation was given as:

$$\log\ (C_e - C_t) = \log C_e - \frac{k_{obs}}{2.303} t \qquad (2)$$

, where C_t is the concentration of the component in the liquid phase at time *t*, C_e is its concentration in the liquid phase at equilibrium, k_{obs} is an observed first order rate constant and *t* is the extraction time, as shown in Figure 6.

There are many and various models of equation can be used to describe the solid-liquid extraction process (Zou, et al., 2010; Garkal, et al., 2012; Goula, 2012). One of the used models for solid-liquid extraction description is first order (Garkal, et al., 2012; Vetal, et al., 2012). The extraction data illustrated a straight line pseudo-first order graph and the value of first order rate constant, k_{obs} cauld be computed. This model equation is employed to describe the extraction process with the equilibrium-dependent solid-liquid extraction model, and it takes into account the diffusion-dependent solid-liquid extraction process.

The kinetic data are further analyzed using second order kinetic model. The developed model based on the assumption that the extraction follows the second order law, and it could be expressed as:

$$\frac{dC_t}{dt} = k(C_e - C_t) \tag{3}$$

Integrating the above equation by applying the boundary condition gives the linear form equation as below:

$$\frac{t}{C_t} = \frac{1}{h} + \frac{1}{C_e}t \tag{4}$$

where *h* is the initial extraction rate (g/L min), and *t* approaches 0,

$$h = kC_e^{\ 2} \tag{5}$$

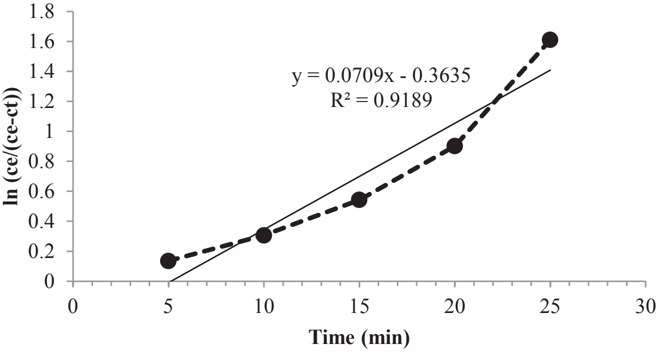

Figure 6. Pseudo-first-order kinetics of *steviosides* synthesis

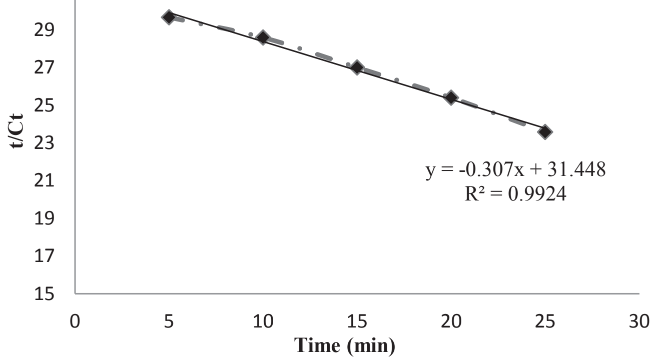

Figure 7. Pseudo-second-order kinetics of *steviosides* synthesis

The graph of $\frac{t}{C_t}$ versus t should give a straight line if this kinetic model could be established. In addition, $\boldsymbol{k}$ and $\boldsymbol{h}$ are determined from the slope and the intercept of the graph. A model based on a second-order kinetics is justified (Figure 7). It shows the second-order kinetics are most suitable model for solid-liquid extraction process of *steviosides*. It has been also approved by other extractions of solid-liquid, like eugenol from Ocimum. s leaves (Zoe et al., 2010; Garkal, et al., 2012).

4.0 CONCLUSIONS

The mixtures of s.rebaudiana leaves and stems waste can be used as raw materials for *steviosides* (steviol g.) synthesis. The synthesis of *steviosides* using ultrasound-assisted extraction (UAE) and ethanol-water as polarity solvents depends on the process parameters. The various of process parameters affects the *steviosides* yields from s.rebaudiana leaves and stems mixtures. The maximum yields are resulted by the time of 30 min, temperature of 40^{o}C and ratio of ethanol-water of 80:20 (v/v) or 40:10 (v/v). The kinetics of *steviosides* separation are approved by the second-order with R^2 and k of 0.992 and 0.02 min^{-1}. Combination of various solvents and processing can be developed for *steviosides* yield improvement. The resulted *steviosides* from S.rebaudiana leaves and stems waste as an alternative feed stock offers an economic and ecological consideration.

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