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Efficiency of Ethanol concentration and Heating methods assisted Extraction Process of Stevioside from *Stevia rebaudiana*

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Abstract

Original Article

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Introduction: Stevioside, a high-intensity non-nutritive sweetener, is extracted from the leaves of *Stevia rebaudiana Bertoni*. This sweet plant native to northeastern Paraguay and is a white, crystalline and odorless powder and approximately 300 times sweeter than sucrose. Procedures for isolation of stevioside from *S. rebaudiana* leaves on a pilot scale mostly involve liquid extraction with such solvents as chloroform-methane, glycerol, and propylene glycol, followed by refinement involving extraction into a polar organic solvent, decolorization, coagulation, ion-exchange chromatography, and crystallization.
Methods: In this study, the effect of ethanol concentration (25, 50 and 75 percent) and heating techniques (Hotplate, Ohmic, and Microwave) on the extraction rate of Stevia leaves Were studied. Stevioside was determined by HPLC analysis.
Results: Results indicated that heating methods do not effective on the extraction efficiency and the highest concentration of extraction obtained in 50 % of ethanol.
Conclusion: There is no significant difference between the extractions rates of different heating methods can be stated that this species efficiency of extraction does not relate with microwave radiation and it depending on the microwave heat.
Keywords: *Stevioside, Heating methods, extraction methods, Stevia rebaudiana, Ethanol*

Introduction

Increasing rate of diseases such as diabetes and obesity, which is associated with eating habits as well as getting more energy than needs so that causes people to tend to diet and low-calorie sweeteners. Many of these sweeteners have complications for consumers like phenylketonuria patients and carcinogenesis[1]. Hence the selection of sweetener is important in production process. Stevia Sweeteners as a non-calorie natural sweetener has been welcomed by industry and consumers all around the world[2,3]. stevioside has good stability during different processing and storage conditions. It has no interaction with most of food ingredients[3]. Furthermore, it is a nontoxic and anticancer compound[1,3-7].
Stevioside, a high-intensity non-nutritive sweetener, is extracted from the leaves of *Stevia rebaudiana* Bertoni, a sweet plant native to northeastern Paraguay and is a white, crystalline and odorless powder and approximately 300 times sweeter than sucrose. Structurally, stevioside is a glycoside with a glucosyl and a sophorosyl residue attached to the aglycone steviol, which has a cyclopentanonhydrophenanthrene skeleton. Stevioside and extracts of *Stevia rebaudiana* leaves are commercially available and used in many countries including Japan and several South American countries as a sweetener for a variety of food and beverages[5,8].
Procedures for isolation of stevioside from *S. rebaudiana* leaves on a pilot scale mostly involve liquid extraction with such solvents as chloroform-methane, glycerol, and propylene glycol, followed by refinement involving extraction into a polar organic solvent, decolorization, coagulation, ion-exchange chromatography, and crystallization[9].
In 2008, Vikas Jaitak and his co-workers had studied the efficient of Microwave-assisted Extraction Process of Stevioside and Rebaudioside-A from *Stevia rebaudiana*. The research showed that the microwave power in 80 watts for 1-minute increases extraction efficiency and the optimum extraction temperature is 50° c. But Vikas Jaitak had applied only one concentration of water and alcohol (ethanol: water (80:20, v/v)[10] in his research.
The plan of this research is intended to answer ahead questions:

- Do microwaves ray Increase increased the efficiency of the Extraction or the temperature caused by the microwave radiation increase it?
- What is the effect of different concentrations of Ethanol on the extraction efficiency?

Materials and Methods

Materials

Leaf material from cultivated plants of *S. rebaudiana* was collected from the experimental farm of the Institute of North Golsaran.
Ethanol used in the experiments made Ghadir ethanol industry in Iran and is rated Analytical.
Acetonitrile used for HPLC was purchased from Merck KGaA (purity (GC) 99.9%, filtered 0.2 mμ) and consumed water is obtained MILLIPORE device made in France

Method

For this study, the experimental method is used in the laboratory. All the leaves are powdered and mixed by a domestic mill then pass mash 40 sieve. The defined variables are included in the heating method and the concentration of ethanol.
The heat was applied in three ways: Hotplate, Ohmic, and microwave. Ethanol at concentrations of 25%, 5%, and 75% was used. Extractions were repeated three times. In any extraction immediately after the heating period, extract had filtered by paper filters and smooth solutions were transferred to a closed dark glass. In the next process, they were tested by HPLC. In the measurement of Steviol Glycosides, C18 column build by the Macherey-Nagel company in Germany was used. The mobile phase consisted of acetonitrile/water (60/40) and the flow rate was 1 ml/min. All mobile phase solutions were degassed for 15 min in ultrasound bath. The column temperature was kept constant at 28° C and UV detector at a wavelength of 254nm was used. The sample volumes were injected into the system was 20 µl per time and filtration (0.45 µm) was applied before any injection.

Instruments

The two hundredth and one-thousandth of a gram scale with accuracy Sartorius balance were used to measure weight.
Microwave oven used in this study is the household LG brand that built in South Korea.
An ohmic instrument that used in this study belongs to Shiraz University and is entirely handmade, capable to control voltage, amp transmits (up to 0.1 mA) and process temperature.
HPLC instrument that used in this study is made by KNAUER in Germany.

Methods of statistical analysis

Results were compared and analyzed by PASW Statistics 18 Software using Duncan and Tukey comparison tests. Graphs were plotted by Microsoft Office excel 2007.

Introducing treatments

Extraction by microwave heating: Extraction by microwave heating is shown in table 1.

Extraction by ohmic heating: Extraction by ohmic heating is shown in table 2.

Extraction by heating hot plate: Extraction by heating Hot Plate shown in table 3.

Results

Heating methods effects on the extraction rate

By comparing the data, there is no significant difference between ohmic heating and microwave heating besides that the chart does not show much difference between the heating methods in the extraction rate (table 4).

Effect of alcohol concentration on the stevioside extraction rate

Results indicate that there is a significant difference in extraction rate associated with alcohol concentration. Difference specified between 75% and 5% ethanol is shown in table 5. Figure 1 was shown the comparison of the effect of heating method on stevioside extraction. Figure 2 was shown the effect of ethanol concentration on the extraction rate.

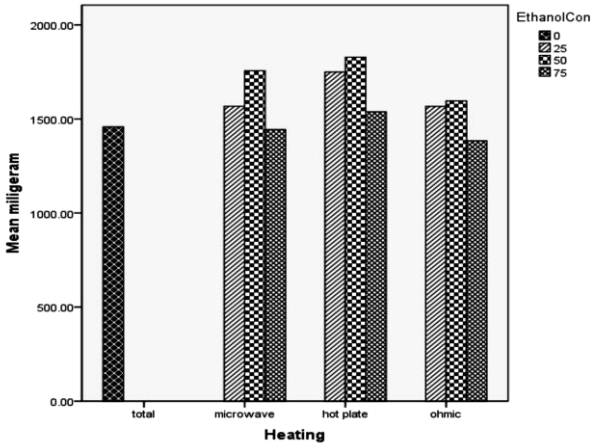

Figure 1. Comparison of the effect of heating method on stevioside extraction

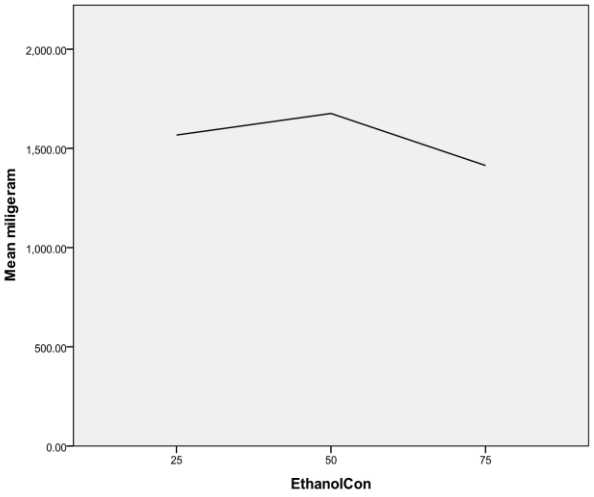

Figure 2. The effect of ethanol concentration on the extraction rate

Table 1. Extracted by Microwave Heating

Heating method	Power (W)	Weight of leaf (gr)	Volume of solvent (ml)	Ethanol concentration	Initial temperature °C	Final temperature° C	Heating time	Treatments name
Microwave	180	10	200	25%	24	49	3':30"	a1
Microwave	180	10	200	25%	24	51	4':00"	a2
Microwave	180	10	200	25%	25	50	4':00"	a3
Microwave	180	10	200	5%	25	54	4':00"	a4
Microwave	180	10	200	5%	25	51	4':00"	a5
Microwave	180	10	200	50%	26	53	4':00"	a6
Microwave	180	10	200	75%	26	57	4':00"	a7
Microwave	180	10	200	75%	26	54	3':30"	a8
Microwave	180	10	200	75%	26	51	3':00"	a9

Table 2. Extracted by Ohmic Heating

Heating method	Voltage	Amp	Weight of leaf (gr)	NaCl added (gr)	Volume of solvent (ml)	Ethanol concentration	Initial temperature °C	Final temperature °C	Heating time	Treatments name
Ohmic	270	0.4	10	1	200	25%	15	50	3':40"	O1
Ohmic	270	0.5	10	1	200	25%	15	50	3':54"	O2
Ohmic	270	0.5	10	1	200	25%	15	50	3':40"	O3
Ohmic	270	0.4	10	1	200	50%	71	50	4':38"	O4
Ohmic	270	0.3	10	1	200	50%	18	50	4':56"	O5
Ohmic	270	0.2	10	1	200	50%	18	50	4':59"	O6
Ohmic	270	0.0	10	1	200	75%	18	50	7':31"	O7
Ohmic	270	0.0	10	1	200	75%	19	50	7':40"	O8
Ohmic	270	0.0	10	1	200	75%	19	50	9':41"	O9

Table 3. Extracted by Heating Hotplate Te

Heating method	Weight of leaf (gr)	Volume of solvent (ml)	Ethanol concentration	Initial temperature °C	Final temperature° C	Heating time	Agitator	Treatments name
Hot Plate	10	200	25%	29	55	8’	2	B1
Hot Plate	10	200	25%	28	50	6’	2	B2
Hot Plate	10	200	25%	29	50	6’	2	B3
Hot Plate	10	200	75%	32	5۹	6’	2	B4
Hot Plate	10	200	75%	27	53	5’	2	B5
Hot Plate	10	200	75%	29	50	2’	2	B6
Hot Plate	10	200	5%	27	50	2’	2	B7
Hot Plate	10	200	5%	28	50	2’	2	B8
Hot Plate	10	200	5%	32	51	4’	2	B9

Table 4. Compare Means of Heating Methods Data ANOVA Milligram

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	221745.415	3	73915.138	2.862	.056
Within Groups	671440.974	26	25824.653		
Total	893186.389	29			

milligram

		Heating	N	Subset for alpha = 0.05	
				1	2
Tukey HSD[a,b]	dimension1	Total	3	1458.1395	
		ohmic	9	1515.3844	
		microwave	9	1589.2477	
		Hotplate	9	1705.2371	
		Sig.		.059	
Duncan[a,b]	dimension1	Total	3	1458.1395	
		ohmic	9	1515.3844	1515.3844
		microwave	9	1589.2477	1589.2477
		Hotplate	9		1705.2371
		Sig.		.193	.063

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 6.000.
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Table 5. Analysis Of Ethanol Concentration Data ANOVA Milligram

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	339918.395	2	169959.197	8.557	.002
Within Groups	476686.545	24	19861.939		
Total	816604.939	26			

Milligram

	Ethanol Con		N	Subset for alpha = 0.05	
				1	2
Tukey HSD[a]	dimension1	75	9	1455.2170	
		25	9		1627.9276
		50	9		1726.7246
		Sig.		1.000	.315
Duncan[a]	dimension1	75	9	1455.2170	
		25	9		1627.9276
		50	9		1726.7246
		Sig.		1.000	.150

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 9.000.

Discussion

The sweet herb *S. rebaudiana* (Bertoni) has a valuable future and is extensively used in various areas of the world. Stevia and its metabolites have commercial value in number of countries as sugar substitutes in foods, beverages, and medicines[11].

Some studies reported Stevioside effects on blood pressure regulation[12-14], cancers because of anti-tumorous and cytotoxic properties[15], kidney disease[16], obesity[17], inflammatory bowel disease (IBD), dental caries[11], also known as tooth decay.

According to above the efficiency of the extraction method is important. In 2008, Vikas Jaitak and his co-workers had studied the efficient of Microwave-assisted Extraction Process of Stevioside and Rebaudioside-A from *Stevia rebaudiana*. The research showed that the microwave power in 80 watts for 1-minute increases extraction efficiency and the optimum extraction temperature is 50° c. But Vikas Jaitak had applied only one concentration of water and alcohol (ethanol: water (80:20, v/v)[10] in his research.

In this study we compared three different heating methods for Stevioside extraction including Microwave, Ohmic, and Hot Plate; also, we compared different Ethanol concentration as a solvent. In three methods room temperature considered as initial temperature and according to the references final temperature should set at 50°C. Stevioside extraction by three procedures was done on different days, different initial temperatures and different Ethanol concentration.

Table 1 shows power, the weight of leaf, the volume of solvent, initial temperature, final temperature, Ethanol concentration which exert for microwave and heating times as results of these conditions.

Table 2 shows voltage, ampere, the amount of NaCl, the weight of leaf, the volume of solvent, initial temperature, final temperature, Ethanol concentration which exert for ohmic and heating times as results of these conditions. In this method, NaCl plays electrically conductive rule because ethanol isn't conductive.

Table 3 shows the weight of leaf, the volume of solvent, the weight of leaf, the volume of solvent, initial temperature, final temperature, Ethanol concentration which exert for Hot Plate and heating times as results of these conditions.

We used SPSS software version 24.0 for ANOVA test. Table 4 shows ANOVA results for compare means extracted Stevioside of heating methods data. There is no significant difference between the extractions rates of Stevioside by different heating methods (p=0.056). In addition, we used Tukey and Duncan tests as supplementary tests for analyzing the results. Not only ANOVA analysis showed no significant difference but also Tukey and Duncan results demonstrated that there are no differences between heating methods which represent in table 4.

In this study, we used different Ethanol concentration to evaluate the effects of ethanol concentration on efficiency of Stevioside extraction. Table 5 shows analysis of Ethanol concentration data, ANOVA, Tukey and Duncan analysis demonstrated maximum Stevioside on 5% Ethanol concentration. Despite of Stevioside organic nature, our results showed maximum amount in presence of 5% Ethanol concentration. On of probable reasons for these results is presence of other water-soluble compounds in the leaves which can facilitate the extraction process by its polar properties.

Conclusion

In conclusion, since there is no significant difference between the extractions rates of different heating methods can be stated that this species efficiency of extraction does not relate with microwave radiation and it depending on the microwave heat.

The lack of significant differences between the extractions rates of different heating methods can be stated that this species efficiency of extraction does not relate to microwave radiation. Hence it can be concluded that is just related to the heat that is made by microwave itself, so in this case, any method that can Increase the temperature to the desired point (50°c) will lead to increase in the extraction rate study on the effect of ethanol concentration on the extraction by using of the Duncan test and comparative chart was illustrated that extraction efficiency enhanced by an increase in the concentration of ethanol up to 5% and in higher concentration (75%) extraction rate is significantly decreased.

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