JCBPS; Section B; November 2017 – January 2018, Vol. 8, No. 1 ; 089-097. E- ISSN: 2249 –1929

[DOI: 10.24214/jcbps.B.8.1.8997]

Journal of Chemical, Biological and Physical Sciences

An International Peer Review E-3 Journal of Sciences

Available online at www.jcbsc.org

Section B: Biological Sciences

CODEN (USA): JCBPAT Research Article

Assisted Ultrasonic Extraction of Glucosydes Present in Stevia (*Stevia Rebaudiana Bertoni*)

***Gabriel Ríos Cortés[1], Lluvia Iris Rodríguez Lechuga[1], Ada María Ríos Cortés[2], Roger Manuel Sales Chávez[1], Mara Angélica Ramírez Bello[1], Minerva Rosas Morales[2].**

[1]Departamento de Ingeniería Química y Bioquímica, Instituto Tecnológico de Orizaba, Avenida Oriente 9 Núm. 852, Colonia Emiliano Zapata. C.P. 94320, Veracruz., México.
[2]Centro de Investigaciones en Biotecnología Aplicada (CIBA-IPN), Tepetitla de Lardizábal, C.P. 90700, Tlaxcala., México

Received: 27 October 2017**; Revised:** 20 November 2017; **Accepted:** 03 December 2017

Abstract: The goal of the present research is to evaluate the ultrasonic efficiency in the extraction of Stevia glycosides, and compare its performance with the soxhlet traditional method. In this analysis, three extraction solvents were used: water, methanol and ethyl acetate. In addition, it was found that the developed extractions using water as a solvent presented the greatest performance for the soxhlet method, as well as for the ultrasonic method. In the case of soxhlet method using water as solvent, after seven hours extraction, a yield of 23.8% was obtained, with an extract weigh of 1.19 gr. On the other hand, the sample irradiated for 2 hours with ultrasound, showed a 24.6% yield, and 1.23 gr. of extract using water as solvent. Regarding the extracted glycosides, Rebaudioside A was the major compound, yielding 1.0 gr while Stevioside yield was 0.23 gr, both from 5 gr. of Stevia by ultrasonic extraction.

Keywords: Stevia, Glycosides, Sweetener, Extraction, Ultrasonic.

1. INTRODUCTION

The market competitiveness and the strongest need to protect the environment have generated the need to change traditional manufacturing processes like extraction through solvents or high hydrostatic pressure and countercurrent vapor distillation. These processes are characterized by the employment of high amount of solvents, long time extraction, low performances, and a great energy waste. Besides, environment damage can arise because of the use of innovative and polluting extractive methods.

Among these innovations, emerges the concept of "green extractions", which is defined as the invention, design, and application of chemical products and processes to reduce or eliminate the usage and generation of hazardous substances[1].

An applied definition to a green extraction process of natural components is: "Green Extraction is based on the discovery and design of extraction processes which will reduce energy consumption, allows the use of alternative solvents and renewable natural products, and ensure a safe and high quality extract/product [1].

In the last years, technology based on the use of ultrasonic has become a valuable tool in engineering food processes. Among the advantages found in ultrasonic extractions, the flexibility and easiness when operating the equipment, short periods in operation time, low temperatures, high performances and ecologically friendly characteristics are highlighted[2]. The ultrasonic application in extractive processes are convenient when the row material or active component stability is thermally unstable during extraction. One of the main benefits given by ultrasound in these systems, is the inducted mass transference increase produced by the cavitation process. Cavitation can be generally defined as the occurrence of formation, growth and the subsequent collapse of microbubbles or cavities that occur in an extremely small interval of time (milliseconds) releasing a great amount of energy. The method to generate efficient cavities production can be considered as the main criterion to differentiate the four types of cavitation: acoustic, hydrodynamic, optical and particle cavitation. From the previous classification, it can be said that acoustic and hydrodynamic cavitation are the result of predominant tensions in a liquid, while optical and particle cavitation are the consequence of a local deposition of energy. From the four types of cavitation, just the acoustic and the hydrodynamic ones can generate the intensity needed in the fluctuation of the mean pressure to develop chemical and physical processes. The maximum size reached by the cavity, determines the magnitude in the pulse pressure/temperature produced in the collapsing point. It is in the final phase of collapsing, that speed in the liquid reaches the sound speed, and the temperature and pressure in the cavity can be massive (3000 K-5000 K y >10000 bar), [3,4]. The forces that act in the cavitation phenomenon, are the ones that make possible the use of ultrasonic means in the extraction processes of natural products through an erosional effect on the vegetal material provoking a rupture in the cell wall, and consequently the extraction of the required particles in the eroded surface.

The use of ultrasonic in the extraction processes has been applied to develop the extraction of glycosides present in the Stevia leaves. Stevia, botanically recognized as *Stevia rebaudiana* (Bertoni), is one of the 154 members in the stevia genus, and it is one of the two species able to produce steviol glycosides. The habitat for the Stevia plant, ranges from the Southwest in the United States to the high lands in Brazil [5]. Its leaves contain a complex mixture of eight sweet diterpene glycosides, stevioside, steviolbioside, rebaudioside (A, B, D, E, F), and dulcoside. **Table 1** shows the sweetener potential and the percentage average of glycosides occurring in the Stevia leaf.

Table1. Sweetener potential of Stevia glycosides and their distribution in the leaf ([6]Jaworska *et al* 2012; [7]Goyal *et al* 2010)

Compound	Sweetener potential	% Present in the leaf
Stevioside	300	4-13
Steviolbioside	100-125	0.00059
Rebaudioside A	250-450	2-4
Rebaudioside B	300-350	<1
Rebaudioside C	50-120	1-2
Rebaudioside D	250-450	<1
Rebaudioside E	150-300	<1
Dulcoside	50-120	0.4-0.17

The Stevia therapeutic value is diverse; [8] it is known that the Stevia plant stimulates insulin secretion in the pancreas in the diabetes treatment and some other issues in carbohydrates metabolisms. Besides, it has been found that some extracts from the inferior part of the Stevia plant have antiviral properties, and it has a positive effect in the treatment of some other illnesses like neuralgia, anemia, lumbago, rheumatism, eczema, and dermatitis.

Even though there is a great interest in consuming these compounds, there is not an available product in the market that offers the 100% of glycoside content. The main reasons are ought to the difficulty in obtaining a no color extract, and because is not easy to separate the Stevioside from the Rebaudioside A, it is not possible to obtain a sweetener full of a wonderful flavor. This interest in the vast potential of steviosides, has generated the need to establish some methods in the extraction and purification of Stevia extracts. These methods include the conventional ones using aqueous and alcoholic solutions or organic solvents, followed by precipitation, coagulation, and crystallization.

2. EXPERIMENTALS

2.1. Materials

Grinding and sifting: To provide a better performance to the extraction, the Stevia leaf was submitted to a grinding process. This was performed in a porcelain mortar. The grinded Stevia was kept in clean and dry jars avoiding any humidity to the samples. Then, the powdery sample was sifted with a number 70 net.

Soxhlet Extraction: Several extractions were developed using Soxhlet equipment with three different solvents (methanol, water, and ethyl acetate, 50 ml each one). Stevia powder (5 gr) was weighted and submitted to a backflow during 7 hours until the extract was obtained and the volume was reduced in a rotary evaporator.

Lixiviation Extraction: The extraction was performed using 2 different solvents (methanol and water, 50 mL each one) in a beaker. Then Stevia powder (5 gr) was weighted and magnetically stirred, heated to reach ebullition during one hour until complete extraction and concentrated in a rotary evaporator.

Ultrasonic extraction: Three different extraction conditions were developed in the ultrasonic bath during 2 hours using three different solvents (methanol, water, and ethyl acetate, 50 mL each one). Stevia leaf powder (5 gr) was weighted in a heart shaped flask to full extraction. The final extract was concentrated in a rotary evaporator and two phases were observed. The supernatant was separated using a pipette and the bottom layer was left in the heart shaped flask which was filtrated and washed using distilled water. The aqueous layer from the washing was kept in a separating funnel and diethyl ether was added. This last step eliminated the undesirable coloring from the water. Finaly the aqueous phase was separated and filtered through an average pore glass sintered funnel. The filtrate was concentrated in the rotary evaporator until a crystalline brown powder was obtained.

Quantification of glycosides present in the crystalized product: Stevioside (TCI America) and Rebaudioside (TCI America) HPLC analysis was carried out in a HP HPLC (Separation module 1100) with a controller system (Agilent OpenLab Software intelligent). The samples were eluted in a 100-RP 18, using an Agilent Lichrosphere column (250 x 4 mm, 5 microns) with a flow rate of de 0.7 ml and a binary gradient mobile phase (0 to 35% acetonitrile in water) in a 15 min run time.

RESULTS

Glycosides extraction using the soxhlet method: The extraction solvents were methanol, water, and ethyl acetate with an extraction process time of 7 hours each . The quantification of Rebaudioside A and Stevioside and present in the extract was developed through HPLC. The chromatograms corresponding to Rebaudioside A (**1A**) and Stevioside (**1B**) are shown **Figure 1**, with retention time of 4.952 min and 6.238 min respectively.

A mixture of the standards was prepared and analyzed by HPLC **(Figure 1C)** to ratify the retention time of these two components in the mentioned mixture. Rebaudioside A (highest peak) was observed at 5.713 min retention time while Stevioside appears at 6.03 min. The water extraction sample produced 23.83% yield (1.19 g of extract).

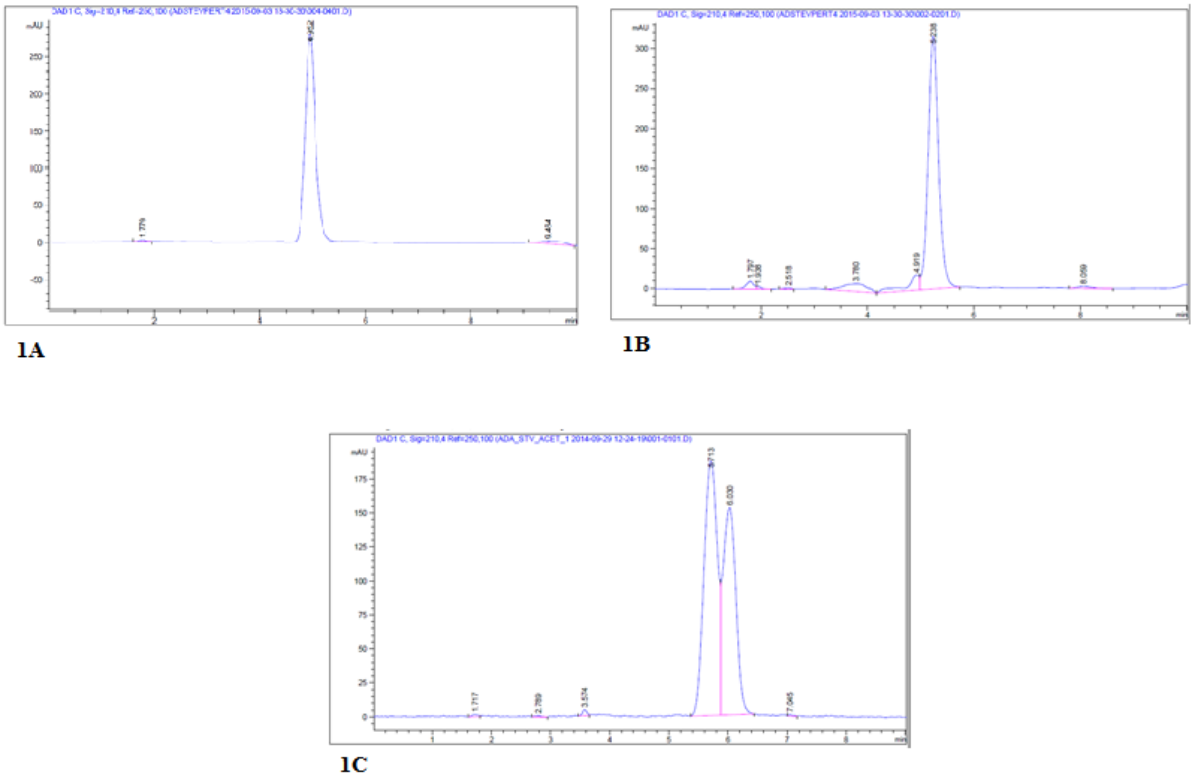

Figure 1. Chromatographs of the standards of Stevioside (1A), Rebaudioside A (1B) and mixtures of them. (1C).

The chromatograms of the analysis of the samples extracted with water (**2A**) and methanol (**2B**) are shown in **Figure 2**. In both samples, Rebaudioside A is higher amount than Stevioside.

Figure 2A shows the peaks at retention time 5.677 and 5.981 min corresponding to Rebaudioside and Stevioside respectively extracted through lixiviation with methanol **(2A)** and water **(2B)**. From the integrated areas, it was found a composition of 0.967gr in Rebaudioside A, and 0.223gr for Stevoside from the extract obtained using water as extraction solvent. On the other hand, for the methanolic extract produced 11.58% yield (0.579gr extract). The Rebaudioside A and Stevioside´s retention time are 5.68 and 5.99 min respectively, as shown in **Figure 2B.** The amount of Rebaudioside A obtained after the extraction was calculated to be 0.472g, while the Stevioside amount was 0.107 g. The method using ethyl acetate as solvent yielded 4.074% extract (0.203 g), with a composition in Rebaudioside A of 0.165g and 0.038g of Stevioside.

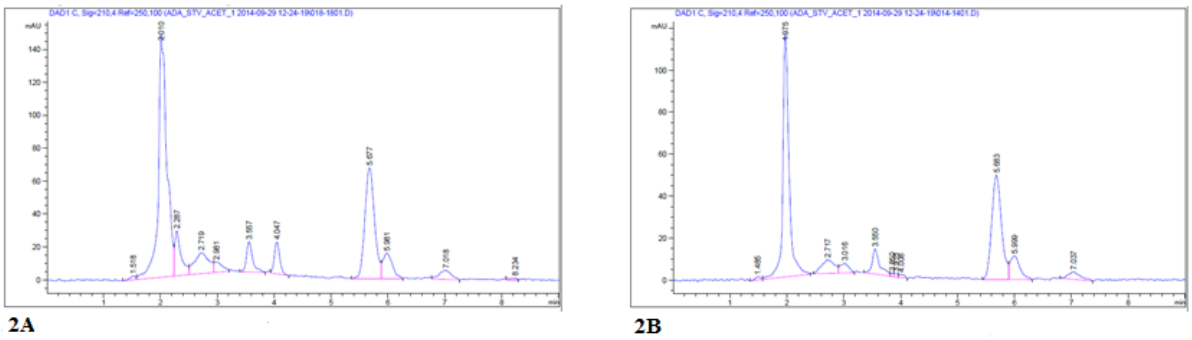

Figure 2. Chromatographs of stevioside and Rebaudioside A extracted through the soxhlet method using water (2A) and methanol (2B).

Glycosides extraction using lixiviation: In the lixiviation extraction, only two solvents were used: water and methanol. In the case of water, the obtained yield was 18.34%, the analysis through liquid chromatograph shows in **Figure 3A** showing Rebaudioside A and Stevioside retention time 5.671 and 5.9 respectively, with a composition of 0.762 g (Rebaudioside A) and 0.154 g (Stevioside). Methanol was also used for this extraction method, **Figure 3B** shows the retention time 5.620 and 5.931min for Rebaudioside A and Stevioside respectively. The calculated composition was 0.419 g for Rebaudioside A and 0.102 g for Stevioside.

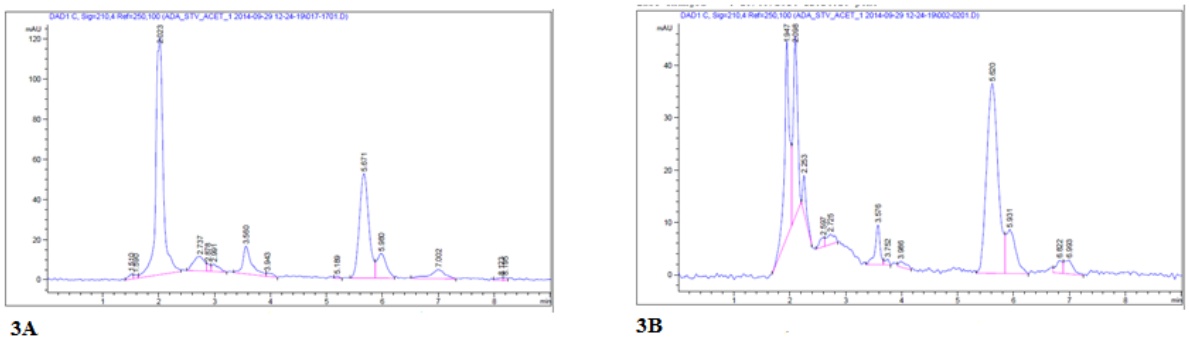

Figure 3. Chromatographs of stevioside and Rebaudioside A extracted through lixiviation with methanol (3A) and water (3B).

Glycoside extraction assisted through ultrasound: In the ultrasonic assisted extraction, water, methanol and ethyl acetate were used as solvents. The same relation 50 ml of solvent per each 5g of substrate was kept. The obtained results for water as extraction solvent were 1 gr of Rebaudioside A and 0.23 gr de Stevioside with a retention time of 5.636 y 5.946 min respectively, as shown in **Figure 4A**. The chromatogram of the sample obtained with ethyl acetate extraction is shown in **Figure 4B**, showing a retention time of 5.628 min for Rebaudioside A and 5.938 min for stevioside. This extraction produced a 16.99% yield, corresponding to 0.85 gr of extract. **Figure 4C** shows the chromatogram composition when water is used as extraction solvent producing 0.509 gr of Rebaudioside A and 0.114 gr of Stevioside.

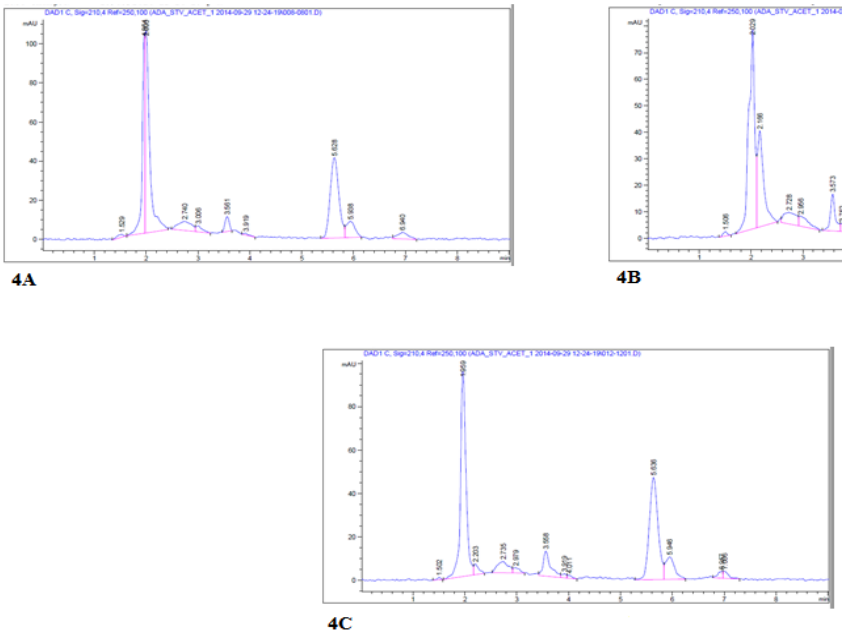

Figure 4. Chromatographs of Stevioside and Rebaudioside A extracted through ultrasonic method using methanol (4A), ethyl acetate (4B) ad water (4C).

In **table 2**, the obtained results from the different extraction methods and the different solvents are summarized.

Table 2. Amount (g) of Stevioside and Rebaudioside A obtained from the Stevia extracts through soxhlet, lixiviation y ultrasonic assisted extraction.

		Soxhlet	Lexiviation	Ultrasonic
Water	Extract (gr)	1.19	0.917	1.23
	Rebaudioside A (gr)	0.967	0.762	1.0
	Stevioside (gr)	0.223	0.154	0.23
Methanol	Extract (gr)	0.579	0.522	0.625
	Rebaudioside A (gr)	0.472	0.419	0.518
	Stevioside (gr)	0.107	0.102	0.106
Ethyl acetate	Extract (gr)	0.203	-	0.850
	Rebaudioside A (gr)	0.165	-	0.509
	Stevioside (gr)	0.038	-	0.114

In **Table 3,** the Glycosides extracts yields obtained through the ultrasonic process using water, methanol, and ethyl acetate as solvents are show.

Table 3. Glycoside extract performances obtained using the ultrasonic method.

	Yield %		
Solvent	Extract	Rebaudioside A	Stevioside
Water	24.6	81.3	18.7
Methanol	12.5	83	16.96
Ethyl acetate	17	59.88	13.41

DISCUSSION

When applying the ultrasonic assisted extraction no source of heat besides the one generated by the cavitation method was used. This feature allowed the study of the direct effect of the ultrasonic extraction of glycosides in the Stevia. As it is observed in Table 2, the soxhlet and ultrasonic extraction methods using water as a solvent, presented a higher extract amount than the lixiviation method. The extraction yield through Soxhlet was of 23.8%, and the Rebaudioside A and Stevioside yields were 81.26 and 18.73 respectively. This can be compared with the extract obtained by the ultrasonic assisted process, which was of 24.6g with Rebaudioside A and Stevioside yields of 81.3% 18.7% each one. The extract and glycosides yields are closer, but also it should be considered difference in the extraction time, which was of 7 h for the soxhlet method and 2 h by the ultrasonic assisted extraction.

One of the most important variables in each extractionmethod is the temperature. When using the ultrasonic assisted process, higher yields with low extraction temperatures can be obtained compared with other extractive processes. The temperature applied in the soxhlet method was of 88°C compared with the reached temperature in the ultrasonic bath 45°C. The cavitation effect in a lower temperature, favored the glycosides extraction, the rupture of the cell wall caused by the cavitation produces the release of a higher concentration of glycosides, differing to the dragging effect in the soxhlet method.

In the assisted extraction using ultrasound, d three solvents (water, methanol and ethyl acetate) were teste. As it is shown in Table 3, when water was used in the ultrasonic process, it was obtained 1.23 gr of extract, which results in a 24.6% yield, corresponding to 1 g of Rebaudioside A and 0.23 gr of Stevioside. This means a higher yield if compared with the methanol extract (0.625 g) showing a 12.5%yield, which corresponds to 0.518 g of Rebaudioside A and 0.106 g of Stevioside. Regarding the ethyl acetate extract, the obtained yield was of 17%, producing 0.850 g of extract with a composition of 0.509 g of Rebaudioside A and 0.114 g of Stevioside. Even though the extract yield in ethyl acetate was higher than the methanol one, the glycosides amount was lower. This issue can be explained due to the polarity of the extraction solvent favoring the chlorophylls extraction and dragging flavonoid a phenolic compounds too. The lower mass of the extracted glycosides can be attributed to their poor solubility in ethyl acetate if compared to water or methanol. Žlabur *et al* .[2] found that when using ethanol as a solvent in a conventional extraction 82.68 mg g^{-1} can be obtained while using water as extraction solvent produces 74.72 mg g^{-1} of extract, being the Stevioside the major compound followed by the Rebaudioside A.Gasmalla *et al.*[8] developed assisted extractions by the ultrasonic process of Stevia glycosides using different dissolutions of ethanol and isopropanol as extraction solvent at a 30ºC temperature. The report shows 30g $100g^{-1}$ when using 30% ethanol, and 30% isopropanol solution produces 35g $100g^{-1}$. In the present research, the higher extraction yield was obtained using water as a extraction solvent, and in all the three cases, the major extracted glycoside was the Rebaudioside A being the one with the greatest market interest. The difference can be attributed to the type of ultrasonic equipment that were used in these two works. [2]Žlabur *et al.*[2] used an ultrasonic immersion equipment, which presents higher power and the cavitation acts directly on the vegetal substrate causing a more violent cell rupture and at the same time homogenizing the extraction mixture, resulting in the mixing of glycoside crystals the chlorophylls, flavonoids and phenol compounds present in the Stevia leaves. On the other hand, the ultrasonic bath causes the cell rupture, releasing the flavonoids, phenols, chlorophylls and glycosides of interest, without provoking any homogenization, but promoting the formation of two phases, allowing purer extracts and a higher yield in the extraction.

CONCLUSIONS

The ultrasonic assisted extraction has proved to be an efficient technique in the glycosides extraction from the Stevia leaf. The use of water as extraction solvent and the ultrasonic process allows a higher yield extraction (24.6%) and more importantly, it is environment-friendly.

The use of ultrasound in glycosides extraction produces two phases, allowing the formation of glycosides crystals with little presence of chlorophylls produced color. This is a great advantage in the glycosides purification since typical solvents for color removing such as diethyl ether can be avoided.

The assisted ultrasonic extraction using water as solvent, allows the increase of the Rebaudioside A yield, which is the glycoside with greater market interest. The conditions to have an optimal extraction of Rebaudioside A using the assisted ultrasonic extraction can be summarized as 5 ml of water per 5 gr of Stevia leaf and 2 sonication hours' time.

ACKNOWLEDGEMENTS

Thanks to the analytical and applied chemistry laboratory of the department of chemical and biochemical engineering at the Technological Institute of Orizaba and National Polytechnic Institute for their support in the achievement of this research.

REFERENCES

1. Chemat F., Abert M., Cravotto G. (2012) Green extraction of natural products: Concept and principles.Int. J. Mol. Sci. 13, 8615 – 862.
2. Žlabur, Ŝ. J., Vocca, S., Dobriĉevia, N., Braĉia, M. (2015). Optimization of ultrasound assited extraction of funtional ingredients from Stevia rebaudiana Bertoni leaves. International Agrophysics.
3. Riesz, P. Berdahl, D. Christman, C, L. 1985. Free radical generation by ultrasound in aqueous and noaqueosus solutions. Environ. Health Perspect, 64, 233-252.
4. Esclapez, M. D., García-Pérez, V. J. (2011). Ultrasound-Assisted Extraction of Natural Products. Food Eng Rev, 3, 108-120.
5. Afandi, A.; Sarijan, S.; and Shaha, R.K. (2013) Optimization of rebaudioside A extraction from *Stevia rebaudiana* (Bertoni) and quantificaction by high performance liquid chromatograpy analysis. J. Trop. Resour. Ssustain. Sci., (1), 62-70.
6. Jaworska, K., Krynitsky. A. J., Raden. J. I. (2012). Simultaneous Analysis of steviol and steviol glycosides by liquid chromatography with ultraviolet detection on a mixed mode column: Application to stevia plant material and stevia containing dietary supplerments. Journal of AOAC International. Vol. 95, No. 6.
7. Goyal, S. K., Goyal, R.K. (2016). Stevia (Stevia rebaudiana) a bio-sweetener: a review. International Journal of food sicences and nutrition. February; 6(1):1-10.
8. Adesh, A. B., B. Gopalakrishna, B., Kusum, S. A. & OP, Tiwari. (2012). An overview on Stevia: A natural calorie free sweetener. International Journal Of advances in Pharmacy, Biology and Chemistry, 1(3), 362-368.

9. Mohammed AbdalbasitA. Gasmall, Ruijin Yang, Abubakr Muja, Xiao Hua, Fayin Ye. (2014). Influence of sonication process parameters to the state of liquid concentration of extracted rebaudioside A from Stevia (Stevia rebaudiana bertoni) leaves. Arabian Journal of Chemistry.

Corresponding author: Gabriel Ríos Cortés

Departamento de Ingeniería Química y Bioquímica, Instituto Tecnológico de Orizaba, Avenida Oriente 9 Núm. 852, Colonia Emiliano Zapata. C.P. 94320, Veracruz., México.

Online publication Date: 03.12.2017