Borneo International Journal of Biotechnology (BIJB)
Vol. 1 (December 2020), 77 – 87

e-ISSN 2716-697X

Research Article

SEPARATION OF STEVIOL GLYCOSIDES FROM *Stevia rebaudiana* USING DIFFERENT AQUEOUS EXTRACTION TECHNIQUES

Chong Saw Peng*, Mustapha bin Akil and Norellia binti Bahari

Agrotechnology and Bioscience Division, Malaysian Nuclear Agency (Nuclear Malaysia),
Ministry of Energy, Science, Technology, Environment and Climate Change (MESTECC),
Bangi, Selangor, Malaysia
*Corresponding author's email: sawpeng@nm.gov.my
Received date: 14 March 2018 | Accepted date: 16 December 2019

ABSTRACT

Stevia rebaudiana has recently gained the attention of the food industry as one of the natural sweeteners. The sweet flavour is contributed by the glycoside compounds, especially the rebaudioside A and stevioside, which are the stevia main chemical markers. The aim of the work reported here was to compare the different extraction techniques of stevia leaves using different technologies such as the high pressure and ultrasonic on the extraction of steviol glycosides. In this paper, the extraction techniques yielding the highest glycosides from the leaves of *Stevia rebaudiana* were determined using hot water extraction (HWE), pressurised liquid extraction (PLE) and ultrasound-assisted extraction (UAE). The steviol glycoside yields were quantified by two chemical markers, rebaudioside A and stevioside of *Stevia rebaudiana* using high-performance liquid chromatography (HPLC) analysis. The result showed that the HWE managed to obtain 1,110 mg of steviol glycosides. The PLE obtained 294 mg steviol glycosides and the UAE obtained 427.5 mg steviol glycosides. As a conclusion, the results suggested the most efficient technique for stevia extraction in this study was the HWE.

Keywords: hot water extraction, pressurised liquid extraction, ultrasound-assisted extraction, steviol glycosides, rebaudioside A, stevioside

INTRODUCTION

Stevia rebaudiana Bertoni, belonging to the Asteraceae family, is a natural non-caloric sweetener native to Paraguay. Stevia contains 11 main steviol glycosides, namely, stevioside, rebaudioside A to F, rubusoside, steviolmonoside, steviolbioside, and dulcoside A, which are responsible for the typical sweet taste (Geuns, 2003). Although stevioside and rebaudioside A have similar chemical structures, they have significant differences in sweetness and taste quality (Wheeler et al., 2008). Stevioside tastes about 150 – 300 times and rebaudioside A tastes about 200 – 400 times sweeter than sucrose (Geuns, 2003). Stevia products are considered healthy and natural foods and have gained attention in the international markets (Gasmalla, Yang, Musa, Hua, & Ye, 2017) with China, India, Brazil, Korea, Mexico, United States, Indonesia, Tanzania, and Canada as producers (Ahmad, Khan, Hayee, & Nazir, 2014).

In the previous study, many methods such as ultrasonic-assisted extraction, pressurized hot water extraction, and acidified water extraction have been reported to separate steviol glycosides from *S. rebaudiana* (Liu, Li, Xu, & Zhou, 2007; Kootstra & Huurman, 2017; Kovačević et al., 2018). Among all these methods, the aqueous extraction of the stevia leaves was the most indicated choice and presents the highest yields of rebaudoside A and stevioside in the crude extract (Abou-Arab, Abou-Arab, & Abu-Salem, 2010; Chhaya, Mondal, Majumdar, & De, 2012a, 2012b; Mondal & Chhaya, 2012; Rao, Prasad, Sridhar, & Ravikumar, 2012; Periche, Koutsidis, & Escriche, 2014; Sardhara, 2015). This is because water is a universal solvent that can dissolve most substances than any other liquid. This makes water the primary extraction solvent used in most of the extracts.

In this study, we will compare the different aqueous phase extraction methods for the separation of steviol glycosides from stevia. Three commonly used extraction methods like hot water extraction, pressurised extraction and sonication extraction will be studied individually to see their effect on the bioactive molecules, leading to the recovery of glycosides from plant matrices (Roohinejad et al., 2017). We will determine the highest steviol glycosides yield from the leaves of *S. rebaudiana* using hot water extraction (HWE), pressurised liquid extraction (PLE) and ultrasound-assisted extraction (UAE).

The HWE is the most common method used for herbal extraction. In HWE, the stevia leaves are combined with water and are heated to 100°C. When the HWE process is complete, a highly concentrated solution combination of extracted stevia and water will be obtained. The PLE is innovated from HWE to reduce the energy consumption and processing time during extraction. Recently, PLE has become a popular green extraction method for different classes of compounds present in numerous kinds of matrices such as environmental, food and botanical samples. PLE is an extraction technique that uses water as an extraction solvent at temperatures above the atmospheric boiling point of water (100°C/273 K, 0.1 MPa), but below the critical point of water (374°C/647 K, 22.1 MPa) (Plaza & Turner, 2015).

The ultrasound range used in the UAE is from 20 kHz to 2000 kHz (Handa, Khanuja, Longo, & Rakesh, 2008). Ultrasound creates an effect of acoustic cavitation which will increase the contact between solvents and substrate and permeability of cell walls. During the ultrasound, the plant properties are altered and disrupt the plant cell wall. It helps to release the compounds and increase the mass transport of the solvents into the plant cells (Dhanani, Shah, Gajbhiye, & Kumar, 2013). The UAE's advantages are mainly that it saves time and reduces the use of solvents in extraction. However, when more than 20 kHz of energy used in ultrasound, it will form free radicals that may affect the active phytochemicals in the plant (Handa et al., 2008; Kaufmann & Christen, 2002).

The stevia variety S10A used in the study was produced under the mutation breeding program using gamma irradiation in Gamma Greenhouse at the Malaysian Nuclear Agency in Malaysia. The S10A stevia variety showed significantly increased biomass with shorter internode distance between the leaves compared to the control stevia variety obtained from the Malaysian Agricultural Research and Development Institute (MARDI). Moreover, the S10A variety is more adapts to the local climate compared to the control (Ahmad et al., 2018)

MATERIALS AND METHODS

Materials

Stevia rebaudiana variety S10A produced through mutation breeding using gamma irradiation in the Malaysian Nuclear Agency was planted by Duta Nusajaya Sdn. Bhd. in Penampang, Sabah, Malaysia. The fresh leaves were harvested, washed and dried at 40°C for 24 h then were powdered to 20 – 30 mm mesh size and stored at room temperature for further use (Figure 1).

Macroporous resin AB-8 was purchased from Bengbu Dong Li Chemical Co. Ltd (China). Their physical properties were listed in Table 1. They were pre-treated by dipping them in ethanol for 48 h, then washing with ultrapure water thoroughly to remove the monomers and porogenic agents trapped inside the pores during the synthesis process.

Figure 1 Stevia fresh leaves, dried leaves, and stevia leave powder sieved through 20 – 30 mm mesh size that were used in the study

Table 1 The physical properties of macroporous resin AB-8

Resin type	Functional group	Average pore (nm)	Particle diameter (mm)	Polarity
AB-8	Polystyrene	13 – 14	0.3 – 1.25	Low polarity

Ferrous sulphate and calcium oxide were obtained from Fisher Scientific (UK). Activated charcoal was obtained from Duchefa Biochemie (Netherland). Stevia standard, rebaudioside A and stevioside were purchased from Sigma-Aldrich (Germany).

Methods

Hot Water Extraction (HWE)

The hot water extraction was performed based on Anvari and Khayati (2016) method, with some modification using a heating mantle. Approximately 40 g of stevia leaves were powdered to 20 – 30 mm mesh size and were boiled in 4 L soft water for 1 h. The crude extract was collected after cooled down and the remaining stevia powder was boiled in another 4 L soft water for 1 h. The boiling process was repeated for 3 times, each time 4 L of soft water was added. After the boiling process, the crude extract was concentrated at 100°C to a final volume of 3 L (Anvari & Khayati, 2016). The crude extract was cooled down and pH was adjusted to 10 by adding sodium hydroxide. Approximately 2 g of ferrous sulphate and 1 g of calcium oxide were added to 100 mL of crude extract and mixed for 1 h for the chemical treatment process. The crude extract was allowed to stand for 4 h to fully precipitate the impurities. A yellowish solution was obtained after the flocculation. The solution was filtered through a press filter and adjusted to pH 7 by adding hydrochloric acid. During the decolourisation process, 10 g of activated charcoal was added to 1 L of crude extract and mixed for 1 h. A transparent solution was obtained by removing the activated charcoal through ultra-filtration (Davis, 2001).

Pressurised Liquid Extraction (PLE)

The pressurised liquid extraction was performed based on Plaza and Turner (2015), with some modification using steriliser HVE-50 (Hirayama). Approximately 40 g stevia leaves were powdered to 20 – 30 mm mesh size. The crude extract was extracted in aqueous solution (1:20) adjusted pH 3 and heated at 60°C with agitation for 6 h. After that, the crude extract was subjected to the pressurised liquid extractor at pressure 100 kPa and temperature 110°C for 10 min (Plaza & Turner, 2015). The crude extract was cooled down and filtered to collect the supernatant. The crude extract was adjusted to pH 10 by adding sodium hydroxide and heated to 60°C for 1 h. Approximately 2 g of ferrous sulphate and 1 g of calcium oxide were added to 100 mL of cooled crude extract and mixed for 1 h for the chemical treatment process. The crude extract was allowed to stand for 4 h to fully precipitate the impurities. A yellowish solution was obtained by removing the precipitates through a press filtration process. The solution was adjusted to pH 7 by adding hydrochloric acid. During the decolorisation process, 10 g of activated charcoal was added to 1 L of crude extract and mixed for 1 h. A transparent solution contained steviol glycosides were obtained by removing the activated charcoal through ultra-filtration (Davis, 2001).

Ultrasound-Assisted Extraction (UAE)

The ultrasound-assisted extraction was performed based on Tang-Bin, Wang, Gan, and Ling (2011), with some modification using Sonicator 4000 (Misomix). Approximately 40 g stevia leaves were powdered to 20 – 30 mm mesh size. The crude extract was extracted in aqueous solution (1:20) adjusted pH 3 and heated at 60°C with agitation for 6 h. After that, the crude extract was sonicated at 68°C, with sonic power (60 W) for 32 min (Tang-Bin et al., 2011). The crude extract was cooled down and filtered to collect the supernatant. The crude extract was adjusted to pH 10 by adding sodium hydroxide and heated to 60°C for 1 h. Approximately 2 g of ferrous sulphate and 1 g of calcium oxide were added to 100 mL of cooled crude extract and mixed for 1 h for the chemical treatment process. The crude extract was allowed to stand for 4 h to fully precipitate the impurities. A yellowish solution was obtained by removing the precipitates through the press filtration process. The solution was adjusted to pH 7 by adding hydrochloric acid. During the decolourization process, 10 g of activated charcoal was added to 1 L of crude extract and mixed for 1 h. A transparent solution contained glycosides was obtained by removing the activated charcoal through ultra-filtration (Davis, 2001).

High-Performance Liquid Chromatography (HPLC)

The transparent solution extracted from each method that contained steviol glycosides was analyzed using HPLC by comparing it to the standard rebaudioside A and stevioside. Rebaudioside A and stevioside were used as the marker compounds in this study. This is because rebaudioside A and stevioside are the major compounds among the 11 steviol glycosides, both of the compounds added up to more than 75% of the total steviol glycosides in stevia extract. Moreover, rebaudioside A has the best quality for sweetness amongst the other steviol glycosides, close to that of glucose (Chatsudthipong & Muanprasat, 2009). The HPLC conditions used in the study is shown in Table 2.

Table 2 HPLC conditions used for the steviol glycosides analysis.

Column	Agilent ZORBAX Carbohydrate Column 4.6 × 250 mm, 5 µm
Mobile Phase A	Water 20%
Mobile Phase B	Acetonitrile 80%
Injection	5 µL volume
Flow rate	1.0 mL/min (isocratic analysis)
Column temperature	30°C temperature
Detector	DAD Detection @ 205 nm, 4 nm BW; Ref: No; PW > 0.25 s (20 Hz)
Equipment	Agilent Technologies Infinity 1260 Quat pump, DAD, TCC, Autosampler.

RESULTS AND DISCUSSION

Water is a universal solvent that can dissolve most of the compounds than other liquid. Besides, water is a polar solvent and their molecules are attracted to other polar molecules, such as those of glycosides. This explains why stevia compounds have such a high solubility in water. In this study, aqueous-based extraction was chosen to investigate the difference between 3 types of extraction technique used for stevia. The results in Figure 2 showed that steviol glycosides were successfully extracted from all 3 aqueous extraction techniques; HWE, PLE, and UAE (Table 3). With 40 g stevia leaves as the extraction material, a total of 1110 mg steviol glycosides containing 887.5 mg rebaudioside A and 222.5 mg stevioside were extracted using the HWE technique (Figure 3). In PLE, 294 mg steviol glycosides contained 247.5 mg rebaudioside A and 46.5 mg stevioside were extracted under pressurised conditions (Figure 4). In UAE, 427.5 mg steviol glycosides containing 380 mg rebaudioside A and 47.5 mg stevioside were extracted under ultrasonic conditions (Figure 5). HWE technique is known as the conventional technique that uses water as the solvent and is carried out generally at atmospheric pressure. In HWE, stevia leaves were packed in a still compartment and water was added in a sufficient amount and then brought to boil. Hot water acted as the main influential factor in free glycoside compounds of plant tissue (Azmir et al., 2013). During the HWE process, the water moved into the solid plant material and solubilised the glycoside compounds with similar polarity then extracted it from the plant (Amita & Shalini, 2014).

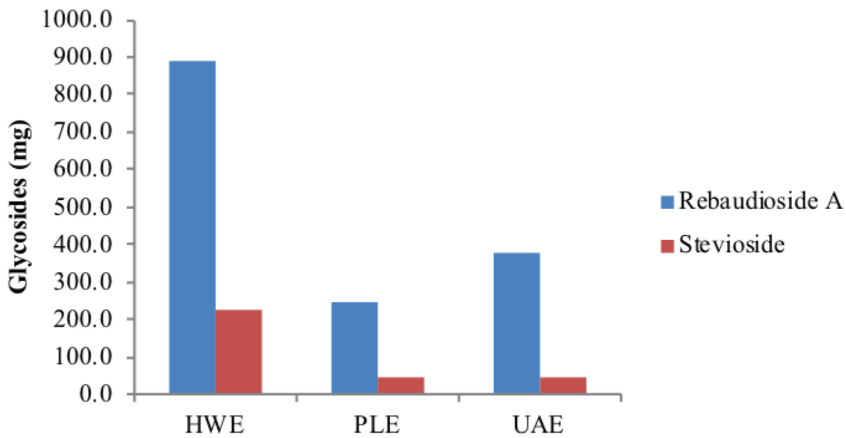

Figure 2 Glycosides yields of *S. rebaudiana* using different extraction techniques

Table 3 Analysis of means and standard deviations of rebaudioside A and stevioside using different extraction techniques

	Rebaudioside A (mg)			Mean	Standard deviation	Stevioside (mg)			Mean	Standard deviation
	1	2	3			1	2	3		
HWE	865.5	910.0	887.0	887.5	22.25	254.0	210.5	203.0	222.5	27.54
PLE	297.0	215.5	230.0	247.5	43.48	45.0	41.0	53.5	46.5	6.38
UAE	352.5	375.5	412.0	380.0	30.00	42.0	48.5	52.0	47.5	5.07

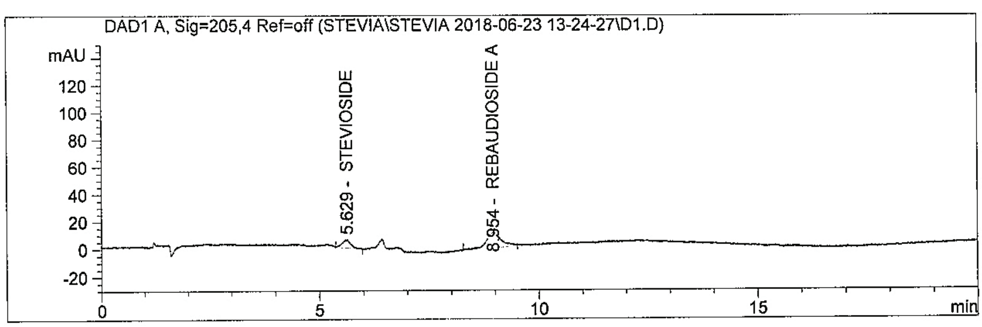

Figure 3 HPLC chromatogram of the rebaudioside A and stevioside extracted using HWE technique

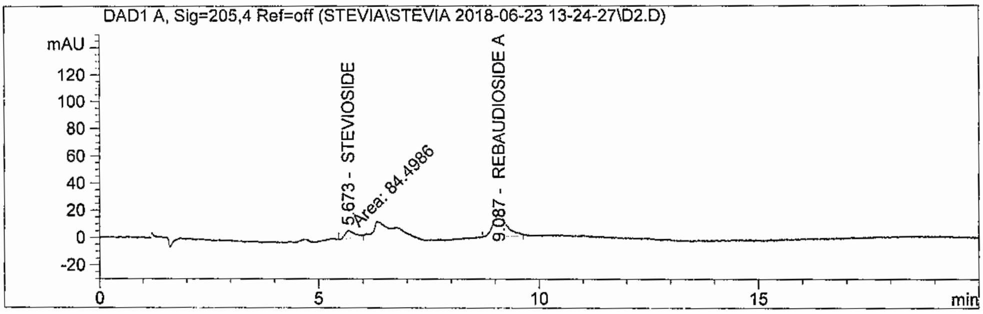

Figure 4 HPLC chromatogram of the rebaudioside A and stevioside extracted using PLE technique

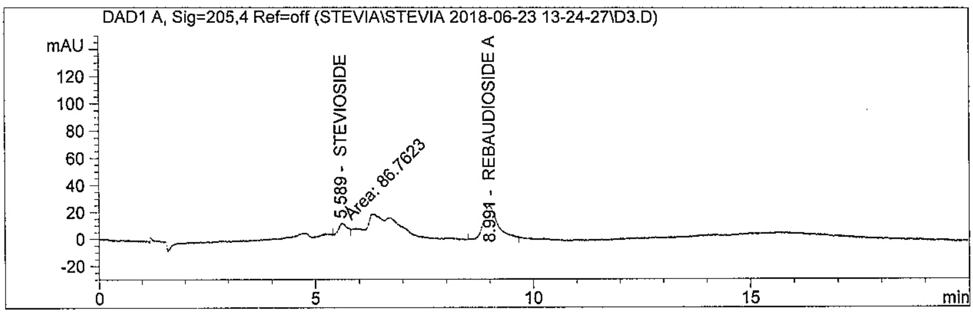

Figure 5 HPLC chromatogram of the rebaudioside A and stevioside extracted using UAE technique

New extraction techniques have been invented due to the need for novel methods of extraction enabling accelerating and shortening extraction times, efficient extraction, automation, and reduction of organic solvent consumption (Rafiee, Jafari, Alami, & Khomeiri, 2011). Two types of new extraction techniques, PLE and UAE were studied and compared to HWE in this study. In the PLE study, water was used as the solvent at elevated temperatures and pressures. During the PLE process, the water condition inside the PLE cell approached the supercritical region which resulted in more efficient extraction. The elevated temperature (110°C) allowed the stevia powder to become more soluble and achieve a higher diffusion rate by increasing the kinetics of the extraction process while the elevated pressure (100 kPa) kept the solvent below its boiling point to maintain the solvent in the liquid state. At elevated pressures and temperatures, water can penetrate stevia powder more efficiently (Raut et al., 2015). UAE is an extraction technique using ultrasound which is attributed to the effect of acoustic cavitation produced in the water by the passage of an ultrasound wave. In this study, ultrasound exerted a mechanical effect through ultrasonic waves that caused the physical and chemical properties of the material subjected to ultrasound were altered and disrupted the plant cell wall. It allowed greater penetration of water into the tissue and facilitated the release of compounds by increasing the contact surface area between the compounds and water. It enabled the compounds to diffuse quickly from the solid phase to the water (Zhang, Chen, Shi, & He, 1998; Dhanani et al., 2013).

Previous studies always asserted higher yields by PLE and UAE than HWE for bioactive compounds extraction from a plant (Huang & Ning, 2010; Li, Wang, Wang, Walid, & Zhang, 2012). However, our study demonstrated that PLE and UAE can potentially produce lower steviol glycosides yield than HWE. Among the 3 aqueous extraction techniques, HWE showed the highest amount in extracted steviol glycosides (1,110 mg) followed by UAE (427.5 mg) and PLE (247.5 mg) (Figure 2). Although the PLE and UAE techniques have advantages in extracting the compounds at elevated pressure and temperature or altered and disrupted the plant cell wall, it did not show a significant increase in steviol glycosides yield. The HWE with the continuous boiling effect at 100°C had yielded remarkably different on steviol glycosides content compared to PLE and UAE. These phenomena suggested that PLE and UAE likely resulted in the degradation of certain glycosides in stevia. PLE and UAE might degrade these steviol glycosides and thereby change their bioactivities, which deserves further investigation. In other words, the elevated pressure and temperature of PLE and powerful energy input of UAE might collectively contribute to the differences between PLE and UAE-obtained steviol glycosides and those obtained by HWE.

CONCLUSION

The study showed that the most efficient technique for stevia extraction is the HWE compared to PLE and UAE. The conventional HWE technique has been practised for decades with satisfying results and once again proved its efficiency in this study. Moreover, water is a cheap, safe and abundant solvent. Therefore, water is the universal solvent used mostly in bioactive plant compounds extraction.

ACKNOWLEDGEMENTS

This research was partially supported by research fund (Technofund TF0614D122) granted by the Ministry of Energy, Science, Technology, Environment and Climate Change (MESTECC), Malaysia. We thank Norazlina Noordin from Malaysian Nuclear Agency and our industry collaborator, Duta Nusajaya Sdn. Bhd. provided stevia raw material for the experiment.

REFERENCES

Abou-Arab, A. E., Abou-Arab, A. A., & Abu-Salem, M. F. (2010). Physico-chemical assessment of natural sweeteners steviosides produced from Stevia rebaudiana Bertoni plant. *African Journal of Food Science*, *4* (5), 269 – 281.

Ahmad, F., Ahmad, Z., Hassan, A. A., Ariffin, S., Noordin, N., Salleh, S., ... & Rahim, K. A. (2018). A Review on Gamma Greenhouse as a Chronic Gamma Irradiation Facility for Plant Breeding and Improvement Program. *Jurnal Sains Nuklear Malaysia*, *30* (1), 8 – 18.

Ahmad, S., Khan, F. A., Hayee, A., & Nazir, M. S. (2014). A Review on potential toxicity of artificial sweetners vs safety of stevia: A natural bio-sweetner. *Journal of Biology, Agriculture and Healthcare*, *4* (15), 1 – 12.

Amita, P., & Shalini, T. (2014). Concept of standardization, extraction and pre phytochemical screening strategies for herbal drug. *Journal of Pharmacognosy and Phytochemistry*, *2* (5), 115 – 119.

Anvari, M., & Khayati, G. (2016). Separation and purification of rebaudioside A from extract of *Stevia Rebaudiana* leaves by macroporous adsorption resins. *Polish Journal of Chemical Technology*, *18* (1), 127 – 132.

Azmir, J., Zaidul, I. S. M., Rahman, M. M., Sharif, K. M., Mohamed, A., Sahena, F., ... & Omar, A. K. M. (2013). Techniques for extraction of bioactive compounds from plant materials: A review. *Journal of Food Engineering*, *117* (4), 426 – 436.

Chatsudthipong, V., & Muanprasat, C. (2009). Stevioside and related compounds: therapeutic benefits beyond sweetness. *Pharmacology* & *Therapeutics*, *21*, 41 – 54.

Chhaya, C. S., Mondal, S., Majumdar, G. C., & De, S. (2012a). Clarification of Stevia extract by ultrafiltration: selection criteria of the membrane and effects of operating conditions. *Food and Bioproducts Processing*, *90* (3), 525 – 532.

Chhaya, C. S., Mondal, S., Majumdar, G. C., & De, S. (2012b). Clarification of stevia extract using cross flow ultrafiltration and concentration by nanofiltration. *Separation and Purification Technology*, *89*, 125 – 134.

Davis, S. B. (2001). The chemistry of colour removal: a processing perspective. *Proceedings* of the *South African Sugar Technologists' Association* 75, pp. 328 – 336.

Dhanani, T., Shah, S., Gajbhiye, N. A., & Kumar, S. (2017). Effect of extraction methods on yield, phytochemical constituents and antioxidant activity of Withania somnifera. *Arabian Journal of Chemistry*, *10*, S1193 – S1199.

Gasmalla, M. A. A., Yang, R., Musa, A., Hua, X., & Ye, F. (2017). 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*, *10* (5), 726 – 731.

Geuns, J.M.C. (2003). Molecules of interest stevioside. Phytochemistry 64, 913–921.

Handa, S. S., Khanuja, S. P. S., Longo, G., & Rakesh, D. D. (Eds.). (2008). *Extraction technologies for medicinal and aromatic plants*. Trieste, Italy: ICS-UNIDO.

Huang, S., & Ning, Z. (2010). Extraction of polysaccharide from *Ganoderma lucidum* and its immune enhancement activity. *International Journal of Biological Macromolecules*, *47*, 336 – 341.

Kaufmann, B., & Christen, P. (2002). Recent extraction techniques for natural products: microwave-assisted extraction and pressurized solvent extraction. *Phytochemical Analysis*, *13*, 105 – 113.

Kootstra, A. M. J., & Huurman, S. (2017). *Extraction of steviol glycosides from fresh Stevia using acidified water; comparison to hot water extraction, including purification* (Pagv-nr.; No. 722). Lelystad: Wageningen UR, PPO/Acrres.

Kovačević, D. B., Barba, F. J., Granato, D., Galanakis, C. M., Herceg, Z., Dragović-Uzelac, V., & Putnik, P. (2018). Pressurized hot water extraction (PHWE) for the green recovery of bioactive compounds and steviol glycosides from Stevia rebaudiana Bertoni leaves. *Food Chemistry*, *254*, 150 – 157.

Li, X., Wang, Z., Wang, L., Walid, E., & Zhang, H. (2012). Ultrasonic-assisted extraction of polysaccharides from Hohenbuehelia serotina by response surface methodology. *International Journal of Biological Macromolecules*, *51* (4), 523 – 530.

Liu, C., Li, L. S., Xu, L. L., & Zhou, Z. M. (2007). Separation and identification of stevioside and Rebaudiodside A in Stevia by HPLC. *Fenxi Shiyanshi (Chinese Journal of Analysis Laboratory)*, *26* (7), 23 – 26.

Mondal, S., & De, S. (2012). Prediction of ultrafiltration performance during clarification of stevia extract. *Journal of Membrane Science*, *396*, 138 – 148.

Periche, A., Koutsidis, G., & Escriche, I. (2014). Composition of antioxidants and amino acids in Stevia leaf infusions. *Plant Foods for Human Nutrition*, *69* (1), 1 – 7.

Plaza, M., & Turner, C. (2015). Pressurized hot water extraction of bioactives. *TrAC Trends in Analytical Chemistry*, *71*, 39 – 54.

Rafiee, Z., Jafari, S. M., Alami, M., & Khomeiri, M. (2011). Microwave-assisted extraction of phenolic compounds from olive leaves; a comparison with maceration. *Journal of Animal & Plant Sciences*, *21* (4), 738 – 745.

Rao, A. B., Prasad, E., Sridhar, G. R. S., & Ravikumar, Y. V. L. (2012). Simple extraction and membrane purification process in isolation of steviosides with improved organoleptic activity. *Advances in Bioscience and Biotechnology*, *3*, 327 – 335.

Raut, P., Bhosle, D., Janghel, A., Deo, S., Verma, C., Kumar, S. S., ... & Tripathi, D. K. (2015). Emerging Pressurized Liquid Extraction (PLE) techniques as an innovative green technologies for the effective extraction of the active phytopharmaceuticals. *Research Journal of Pharmacy and Technology*, *8* (6), 800 – 810.

Roohinejad, S., Nikmaram, N., Brahim, M., Koubaa, M., Khelfa, A., & Greiner, R. (2017). Potential of novel technologies for aqueous extraction of plant bioactives. In H. D. Gonzalez & M. J. G. Munoz (Eds.), *Water extraction of bioactive compounds: From Plants to drug development* (pp. 399 – 419). Amsterdam, Netherlands: Elsevier.

Sardhara, A. M. (2015). *Extraction, purification and formulation of extract from from natural product*. Mumbai, India: Institute of Chemical Technology. pp. 90.

Tang-Bin, Z., Wang, M., Gan, R. Y., & Ling, W. H. (2011). Optimization of ultrasound assisted extraction of anthocyanins from mulberry using response surface methodology. *International Journal of Molecular Sciences*, *12* (2), 3006 – 3017.

Wheeler, A., Boileau, A. C., Winkler, P. C., Compton, J. C., Prakash, I., Jiang, X., & Mandarino, D. A. (2008). Pharmacokinetics of rebaudioside A and stevioside after single oral doses in healthy men. *Food and Chemical Toxicology*, *46* (7), S54 – S60.

Zhang, Y., Chen, T. H., Shi, Z. Q., & He, B. L. (1998). Studies on the separation of Rebaudioside A by recrystallization. *Ion Exchange. Adsorption*, *14*, 515 – 520.