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Optimization of Green Extraction Methods for the Recovery Of Stevia Glycosides

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In recent years, the rising rate of obesity and health problems associated with metabolic syndrome indicators (diabetes, cardiovascular, blood pressure) are turning consumers to exploring healthy, low-sugar and low-fat diets, as well as to using low-calorie sweeteners. In the current food market, sweeteners of synthetic origin, such as aspartame, sucralose, cyclamates etc. are available, which although they provide a sweet, calorie-free taste, they have often been accused of toxicity. This fact promotes the growing need for consumption of natural sweeteners, such as sweeteners from the plant *Stevia rebaudiana Bertoni*, which are 200-300 times sweeter than sugar and contain zero calories. The main objective of this work was to optimize the recovery of glycosides by dried stevia leaves. Dried samples were treated using green extraction techniques, namely ultrasound and microwave assisted extraction, using water as solvent, and extracts were compared with the conventional method. Several parameters, such as stevia / water ratio, time, temperature and power (microwave, ultrasound) were tested in order to optimize the glycoside recovery. The final extracts were evaluated for their total yield, antioxidant capacity and total phenolic content. The quantity and type of glycosides was also evaluated using HPLC. Based on the results, differences were observed between the extracts in terms of yield, antioxidant capacity and glycoside content. The optimum conditions were then used for the extraction of glycosides from samples that were dried and fertilized with different methods, to examine the effect of cultivation conditions on the quality and quantity of glycosides. Oven drying resulted in samples containing higher quantity of RebA and stevioside, while fertilization resulted in lower stevioside content.
Keywords: ultrasound and microwave assisted extraction, freeze drying, phenolics, antioxidant activity, RebA, stevioside

1. Introduction

In many industrialized countries around the world, the population consumes larger amounts of sugar than the recommended daily allowance, which can lead to serious health problems, such as diabetes and obesity (De Ruyter et al., 2012). In an environment of increasing sales of sweet products and snacks, the growing number of overweight or chronically obese people leads the global market to develop food products with little or no sugar or with low-calorie sugar substitutes (Shah et al., 2010), which provide the same sweet taste with a much lower caloric load (Liman et al., 2014).
In the current food market sweeteners of synthetic origin are available, such as aspartame, sucralose and cyclamates. However, despite their sweet taste and absence of calories, they are often accused of toxicity. This promotes the growing need of natural sweeteners consumption. Among the most important natural sweeteners are the glycosides from the plant *Stevia rebaudiana Bertoni*. Stevia is an endemic herb native to Brazil and Paraguay (Madan et al., 2010). The ingredients of Stevia that cause the intense, sweet taste are called steviol glycosides and are 200-300 times sweeter than sugar, containing zero calories. Due to its low

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glycemic index, Stevia is safe for use by both diabetics and hypoglycemics. Steviol glycosides have been used as table-top sweeteners, in soft drinks, dairy products and some confectionery products.
Stevia use as a natural sweetener (E960) has been approved by European legislation since 2011 (EC 1131/2011) and according to the European Food Safety Authority, the recommended daily dose of steviol glycosides is 4 mg/ kg body weight (Aidoo et al., 2015). However, the consumer response is modest, due to its unwanted metallic and bitter aftertaste. The origin of this unwanted aftertaste is presumably attributed to factors, such as the presence of residues (e.g. flavonoids) after extraction, the ratio of glycoside types in the extract, and the way of leaf cultivation (Kaushik et al., 2010).
The recovery of glycosides from Stevia leaves involves three basic processes: extraction with water or another solvent, ion exchange and precipitation, followed by crystallization and drying. Regarding extraction process, Soxhlet represents a simple, widely used and conventional method, but with various disadvantages, such as high time and solvent consumption, along with elevated temperatures that can cause thermal degradation of heat-sensitive components (Luque de Castro, Priego-Capote, 2010). The application of ultrasound and microwave assisted extractions (UAE, MAE) in food processing presents great interest as these methods enhance the extraction yields of important ingredients from plant materials (Vilkhu et al., 2008) reducing the extraction time by not altering the physical and chemical properties of the extract (Plaza et al., 2012).
The aim of the present study is the optimization of the recovery of glycosides from dried stevia leaves through conventional and green extraction techniques, namely Soxhlet and combination of ultrasound and microwave assisted extraction, respectively, using water as solvent. Several parameters, such as stevia / water ratio, time, temperature and power are tested. The extracts are evaluated in terms of extraction yield, antioxidant capacity, total phenolic content and glycoside concentration. The quantity and type of glycosides are evaluated using high performance liquid chromatography (HPLC). Afterwards, the optimum conditions are used for the extraction of glycosides from samples that are dried and fertilized with different methods, so that the effect of cultivation conditions on the quality and quantity of glycosides is examined.

2. Materials and Methods

Dry stevia leaves were delivered from Stevia Hellas, were ground and sieved on a 200 μm sieve. Water, methanol and HPLC grade acetonitrile were purchased from Fisher scientific (UK). Gallic acid and Folin-Ciocalteau reagent were purchased from Sigma–Aldrich.

2.1 Extraction Experiments

2.1.1 Ultrasound and Microwave assisted extraction (UMAE)

UMAE experiments were conducted in a XO-SM50 Ultrasonic Microwave Reaction System (Nanjing Xianou Instruments Manufacture co., Ltd., Nanjing City, China) using water as solvent and operating at 25 kHz frequency and 250 W microwave power. The studied parameters during extraction were: time: 15 and 30 min; temperature: 60 and 80 ^{o}C; stevia to solvent ratio: 1/10 and 1/20 g dry weight per mL solvent; ultrasound power: 50 and 250 W. After the extraction, the samples were centrifuged for 30 min at 3000 rpm.
The optimum UMAE conditions (15 min, 60 ^{o}C, 1/10 g dry weight/ mL solvent and 250 W ultrasound power) were then used for the recovery of glycosides from samples that were dried and fertilized with different methods (Table 1) to examine the effect of cultivation conditions on the quality and quantity of glycosides.

Table 1. Cultivation conditions.

Drying method	Fertilization
Drying under shadow	No
Oven drying	No
Solar drying for 1 day followed by Oven drying	No
Drying under shadow	Yes
Oven drying	Yes
Solar drying for 1 day followed by Oven drying	Yes

2.1.2 Soxhlet extraction (SE)

SE was performed using water as solvent. Two solid to solvent ratios were studied: 1/10 and 1/20 g dry weight per mL solvent. Extraction was completed when water was colourless with a total duration of 11 to 16 extraction cycles depending on the ratio. All the extraction experiments were conducted in duplicate.

2.2 Freeze Drying (FD)

FD was applied in order to remove the solvent and calculate the extraction yield. The samples were stored at −30°C for 48 h in a biomedical freezer (SANYO, MDF-236, Osaka, Japan) and afterwards were dehydrated for 5 h using a laboratory freeze-dryer (Leybold-Heraeus GT 2A, Koln, Germany) under high vacuum (3 mbar).

2.3 Extraction Yield (EY) calculation

After extraction, centrifugation and drying of the extracts, the EYs were calculated as follows:

EY= (g dry obtained extract / g dry Stevia leaves) x100 (1)

2.4 Determination of antioxidant activity (AA)

AA was evaluated with the use of stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) reagent (Stramarkou et al., 2017). The UV-M51 BEL Photonics UV-Vis spectrophotometer was used to measure the absorbance at 515 nm. The Radical Scavenging Activity (% RSA), which expresses the ability of the sample to inactivate free radicals, was calculated based on the Equation:

$$\%\mathrm{RSA} = \frac{(\mathrm{ABS_{DPPH}} - \mathrm{ABS_{mix}})}{\mathrm{ABS_{DPPH}}}\ 100\% \quad (2)$$

where ABS_{DPPH} is the absorbance of blank DPPH and ABS_{mix} is the absorbance at 20 min. AA was expressed through the IC50 index, which indicates the concentration of the sample required to inhibit DPPH radical by 50%. IC50 values were calculated graphically using a calibration curve constructed by plotting the extract concentration against RSA. Measurements were conducted in duplicates.

2.5 Total Phenolic Content (TPC) determination

TPC was determined according to the spectrometric method of Folin-Ciocalteu (Stramarkou et al., 2021). The absorbance was measured at 765 nm. Results are expressed as mg of gallic acid equivalents (GAE)/ mL extract. Measurements were conducted in duplicates.

2.6 Identification and quantification of glycosides

Identification and quantification of glycosides were based on their chromatographic behaviour on HPLC using an HPLC Shimandzu HP 1100 Series (USA) equipped with a diode array detector and an automatic Agilent 1200 Series injector. Glycosides were analysed with Luna 5 μm C18 (Phenomenex) analytical column (250 x 4.6 mm I.D.) at ambient temperature. HPLC grade acetonitrile: water (80:20) was used as eluent solvent at a flow rate of 1 mL / min. Detection of glycosides was accomplished using a diode array system at a wavelength of 200 nm. The identification and quantification of peaks was performed by comparison to external standards using solutions of RebA and stevioside diluted to different concentrations (Stramarkou et al., 2017).

2.7 Statistical Analysis

One-way and factorial analyses of variance (ANOVA) were applied in order to analyse the results. All statistical tests were performed with STATISTICA software (version 10, StatSoft®Inc., Palo Alto, USA).

3. Results and Discussion

Figure 1 presents the total extraction yield of the studied processes, whereas Figures 2 and 3 show the antioxidant activity and the total phenolic content of the obtained extracts, respectively.

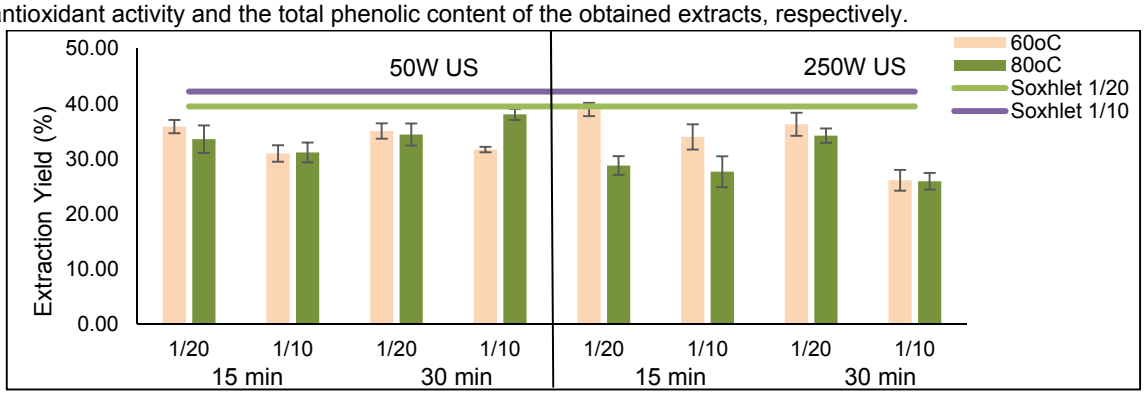

Figure 1. Extraction Yield (EY) (expressed in %) of selected extract. Number of replicates (N) = 2, error bars show the standard deviation between the replicates

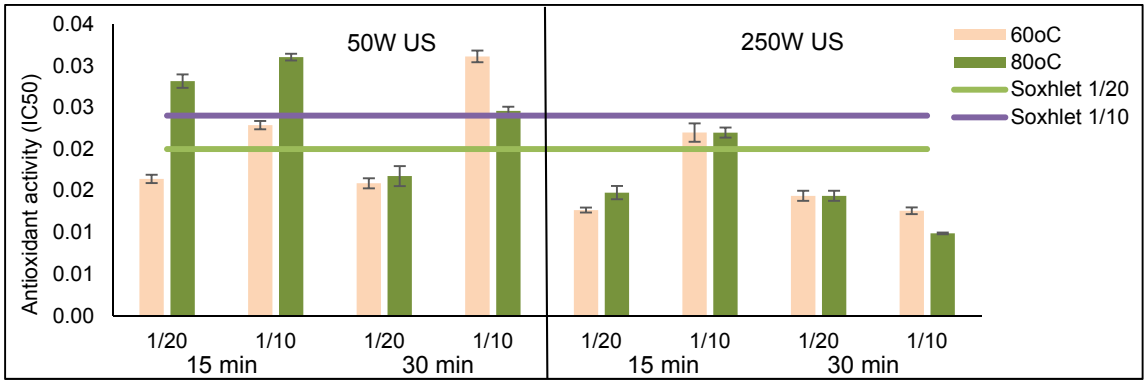

Figure 2. Antioxidant activity (expressed in IC50) of selected extracts. Number of replicates (N) = 2, error bars show the standard deviation between the replicates

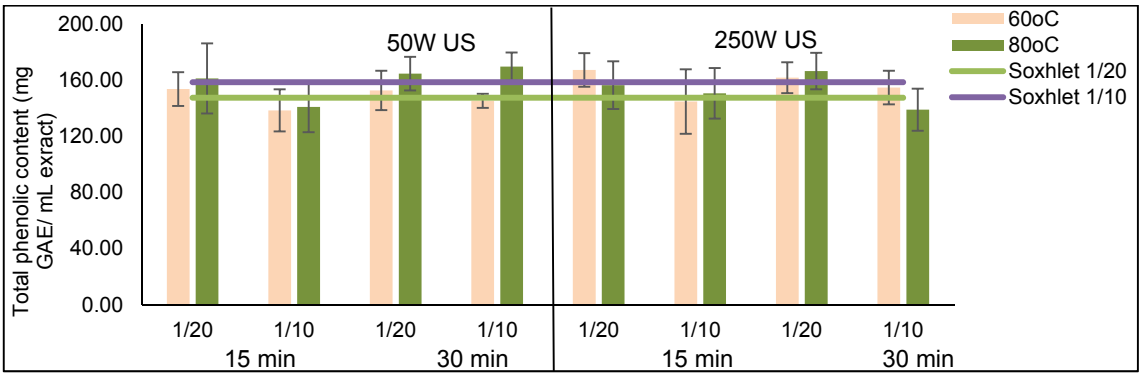

Figure 3. Total phenolic content (expressed in mg GAE/ mL) of selected extracts. Number of replicates (N) = 2, error bars show the standard deviation between the replicates

Considering Figure1, the highest extraction yield was observed at lowest temperature (60^{o}C), shortest time (15 min) and lowest solid/ solvent ratio (1/20). Indicatively, the ratio of 1/20 and the lowest extraction time lead to optimal performance. The disruption of the plant tissue is effective during the 15 min extraction. Also, the temperature of 60^{o}C leads to maximum extraction efficiency, as the lower temperature leads to preservation of the ingredients. These results are in agreement with other studies, which have found that the yield increased sharply when the temperature increased from 40°C to 60°C and decreased slightly when temperature increased above 80^{o}C (Liu et al., 2010; Xu et al., 2019). Therefore, the most suitable temperature range for Stevia extraction is between 60 and 80^{o}C with 60^{o}C being considered as the lowest optimum temperature.
The extraction time positively affects the amount of phenolic compounds. Nevertheless, longer extraction times combined with extreme conditions of ultrasound power (250 W) and temperature (80 ^{o}C) result in poor recovery and degradation of phenolic compounds. In general, the increase in temperature enhances the extraction of phenolic compounds from stevia leaves.
However, when ultrasound power of 250 W, 30 min extraction and 1/10 extraction yield are applied, the elevated temperature of 80 ^{o}C damages the heat-sensitive phenolic compound, but increases the antioxidant activity. The explanation of this fact is linked to the combination of two phenomena: firstly, the increased formation and accumulation of Maillard-type antioxidants at high temperatures and, secondly, the generation of degradation products from phenolic compounds, which contribute to additional antioxidant properties (Que et al., 2008; Nakagawa et al., 2016). Finally, longer extraction time and 1/20 ratio lead to greater antioxidant activity. Table 2 presents the glycoside content of various extracts. Table 2 indicates that extractions performed at 250 W ultrasonic power yield, in general, richer glycoside extracts in both stevioside and rebaudioside A. In addition, the stevia to solvent ratio of 1/10 leads to higher stevioside and RebA contents than that of 1/20 and for this reason it is selected for further study. Comparing stevioside and RebA, they present the same trend when changing the different extraction parameters, with stevioside having a larger concentration in Stevia extracts than RebA. The maximum stevioside and RebA yields are obtained at solid/solvent ratio of 1/10, time of 30 min and ultrasound power of 50 W. These results are in agreement with the high extraction yield of this extract, presented in Figure1.

Table 2. Glycoside content (expressed in g/ 100 g dried leaves) of selected extracts. Number of replicates (N) = 2, +/- shows the standard deviation between the replicates. Values not sharing the same superscript (separately for stevioside and RebA) are significantly different (p<0.05)

Stevia/ solution ratio	Time (min)	Temperature (^{o}C)	US power (W)	g stevioside/100g dried leaves	g RebA/100 g dried leaves
1/20	15	60	50	$12.20^{a} \pm 0.24$	$9.02^{a,b} \pm 0.25$
1/10	15	60	50	$12.19^{a} \pm 0.22$	$8.74^{b,c} \pm 0.21$
1/10	15	80	50	$10.29^{a} \pm 0.24$	$7.52^{c,d} \pm 0.16$
1/20	30	60	50	$9.24^{b} \pm 0.20$	$6.77^{d} \pm 0.14$
1/10	30	60	50	$6.69^{c} \pm 0.16$	$4.85^{e} \pm 0.10$
1/20	30	80	50	$6.75^{c} \pm 0.14$	$4.83^{e} \pm 0.11$
1/10	30	80	50	$20.21^{d} \pm 0.56$	$13.48^{f} \pm 0.30$
1/20	15	60	250	$10.34^{a,b} \pm 0.25$	$7.25^{d} \pm 0.20$
1/10	15	60	250	$14.71^{e} \pm 0.31$	$10.25^{a} \pm 0.21$
1/20	15	80	250	$14.69^{e} \pm 0.30$	$10.27^{a} \pm 0.23$
Soxhlet				$14.27^{e} \pm 0.29$	$9.93^{a,b} \pm 0.20$

In general, the 1/10 ratio had a better performance with an average value of stevioside equal to 12.82 g/ 100 g dried Stevia leaves, whereas the respective value of stevioside at 1/20 ratio was 10.64 g/ 100 g. In the same context, the lower extraction time results in better yields since 15 min extractions recover an average value of 8.84 g RebA/ 100 g dried leaves, while 30 min extractions achieve an average value of 7.48 g/ 100 g.
Based on the above results, the optimum UMAE conditions were: 15 min, 60 ^{o}C, 1/10 g dry weight/ mL solvent and 250 W ultrasound power and were used for the extraction of Stevia dried and fertilized with different methods. Table 3 shows the quality determination of these extracts.

Table 3. Extraction yields, antioxidant activity, total phenolic content and glycoside content of UMAE extracts from Stevia leaves dried and fertilized with different methods. Number of replicates (N) = 2, +/- shows the standard deviation between the replicates. Values not sharing the same superscript (separately for each measurement) are significantly different (p<0.05)

Drying method	Fertilization	Extraction Yield (%)	Antioxidant Activity (IC50 10^{-2})	Total Phenolic Content (mg/ mL)	g stevioside/ 100 g dried leaves	g RebA/ 100 g dried leaves
Drying under shadow	No	$39.6^{a} \pm 0.89$	$1.23^{a} \pm 0.02$	$221.23^{a} \pm 3.85$	$18.05^{a} \pm 0.37$	$6.96^{a} \pm 0.17$
Oven drying	No	$34.3^{b} \pm 0.73$	$1.11^{a} \pm 0.02$	$184.93^{b} \pm 3.65$	$19.89^{a} \pm 0.45$	$6.08^{b} \pm 0.13$
Solar drying for 1 day followed by oven drying	No	$26.8^{c} \pm 0.59$	$1.05^{a} \pm 0.02$	$174.83^{b} \pm 3.19$	$10.07^{b} \pm 0.20$	$4.52^{c} \pm 0.13$
Drying under shadow	Yes	$22.2^{c} \pm 0.85$	$1.20^{a} \pm 0.03$	$174.00^{b} \pm 4.05$	$8.71^{b} \pm 0.22$	$2.50^{d} \pm 0.06$
Oven drying	Yes	$39.9^{a} \pm 1.03$	$1.50^{b} \pm 0.03$	$189.67^{b} \pm 4.03$	$13.53^{c} \pm 0.34$	$4.89^{c} \pm 0.10$
Solar drying for 1 day followed by oven drying	Yes	$32.4^{b} \pm 0.80$	$2.27^{c} \pm 0.01$	$178.37^{b} \pm 4.85$	$13.16^{c} \pm 0.27$	$4.68^{c} \pm 0.10$

Regarding the extraction yields, oven drying with fertilization, as well as drying under shadow without fertilization were the optimum drying conditions. The fertilization lowered the extraction yield in the case of drying under shadow, but this trend was not followed in the cases of oven drying and solar drying, where the fertilization acts positively. The response of Stevia to fertilization is also reported by another study, which has found that fertilizer requirement for Stevia is moderate and varies according to the environment (Benhmimou *et al.*, 2018). The elevated temperatures and intense light during oven and solar drying respectively, possibly favour the extraction of Stevia cultivated with nutrients, whereas the absence of fertilization is favoured in milder drying conditions like those of drying under shadow (room temperature and no light).
The lower the value of IC50, the higher the antioxidant capacity of the extracts. Therefore, the extracts of the leaves dried with solar drying for 1 day followed by oven drying without fertilization have the optimum antioxidant activity. This is expected because of the high temperatures during solar drying that degrade various components of Stevia leaves, which contribute to the total antiradical capacity. On the other hand, fertilization plays a negative role. Oven drying without fertilization results in samples containing higher quantity of stevioside, while drying under shadow without fertilization leads to higher RebA concentration. In general, fertilization results in lower stevioside and RebA content. Overall, oven drying and drying under shadow without fertilization have the optimum performance regarding all the studied parameters of extraction yield, antioxidant activity, phenolic compounds and glycoside content.

4. Conclusions

Various process conditions significantly affect the quality characteristics of the examined extracts. The temperature of 60^{o}C led to maximum extraction efficiency, as the lower temperature leads to preservation of the ingredients. The highest extraction efficiency, also similar to Soxhlet extraction (39%) was obtained at 60^{o}C, 1/20 stevia to solvent ratio, 15 min and 250 W. The increase in extraction time increased the antioxidant activity and the total phenolic content of the extracts. The glycoside content of various extracts ranged from 11 to 33 g/ 100 g dried leaves. Cultivation conditions also had significant impact on quality and quantity of steviol glycosides with drying under shadow without fertilization being, in general, the optimum condition, especially for RebA recovery (6.96 g/ 100 g dried leaves).

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References

Aidoo R. P., Afoakwa E. O., Dewettinck K., 2015, Rheological properties, melting behaviours and physical quality characteristics of sugar-free chocolates processed using inulin/polydextrose bulking mixtures sweetened with stevia and thaumatin extracts, LWT - Food Science and Technology, 62, 592-597.

Benhmimou A. Ibiriz M., Douaik A., 2018, Effect of NPK Fertilization on the Growth, Yield, Quality and Mineral Nutrition of New Sweet Plant in Morocco (Stevia rebaudiana Bertoni), American Journal of Biology and Life Sciences, 6(3), 36–43.

Kaushik R. Narayanan P., Vasudevan V., 2010, Nutrient composition of cultivated stevia leaves and the influence of polyphenols and plant pigments on sensory and antioxidant properties of leaf extracts, Journal of Food Science and Technology, 47(1), 27-33..

Liman E. R., Zhang Y. V., Montell C., 2014, Peripheral coding of taste, Neuron, 81(5), 984–1000.

Liu J., Li J. W., Tang J., 2010, Ultrasonically assisted extraction of total carbohydrates from Stevia rebaudiana Bertoni and identification of extracts, Food and Bioproducts Processing, 88, 215-221.

Luque de Castro M. D., Priego-Capote F., 2010, Soxhlet extraction: Past and present panacea, Journal of Chromatography A, 1217, 2383-2389.

Madan S., Ahmad S., Singh G.N., 2010, Stevia rebaudiana (Bert.) Bertoni - A Review, Indian Journal of Natural Products and Resources, 1 (3), 267-286.

Stramarkou M., Papadaki S., Kyriakopoulou K., 2017, Effect of drying and extraction conditions on the recovery of bioactive compounds from Chlorella vulgaris, Journal of Applied Phycology, 29, 2947–2960.

Nakagawa K., Ritcharoen W., Puchong S.U., 2016, Antioxidant properties of convective-air-dried Spirulina maxima: Evaluation of phycocyanin retention by a simple mathematical model of air-drying, Food and Bioproducts Processing, 100, 292–302.

Plaza M., Santoyo S., Jaime L., 2012, Comprehensive characterization of the functional activities of pressurized liquid and ultrasound-assisted extracts from Chlorella vulgaris, LWT - Food Science and Technology, 46(1), 245–253.

Que, F., Linchun M., Xuehua F., 2008, Comparison of hot air-drying and freeze-drying on the physicochemical properties and antioxidant activities of pumpkin (Cucurbita moschata Duch.) flours, International Journal of Food Science and Technology, 43(7), 1195–1201.

De Ruyter, J. C. *et al.* 2012, Effect of sugar-sweetened beverages on body weight in children: Design and baseline characteristics of the Double-blind, Randomized INtervention study in Kids, Contemporary Clinical Trials, 33 (1), 247-257.

Shah, A. B., Jones, G. P., Vasiljevic, T., 2010, Sucrose-free chocolate sweetened with Stevia rebaudiana extract and containing different bulking agents - effects on physicochemical and sensory properties, International Journal of Food Science and Technology, 45(7), 1426-1435.

Stramarkou, M. *et al.* ,2021, Comparative Analysis of Different Drying Techniques Based on the Qualitative Characteristics of Spirulina platensis Biomass, Journal of Aquatic Food Product Technology. 0, 1–19.

Vilkhu, K., Raymond M., Lloyd S., 2008, Applications and opportunities for ultrasound assisted extraction in the food industry - A review, Innovative Food Science and Emerging Technologies, 9, 161-169.

Xu, S. *et al.* 2019, Extraction of steviol glycosides from Stevia rebaudiana (Bertoni) leaves by high-speed shear homogenization extraction, Journal of Food Processing and Preservation, 43 (12), 14250.