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Research paper

Green extraction of glycosides from *Stevia rebaudiana* (Bert.) with low solvent consumption: A desirability approach

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Abstract

The sweet flavor of *Stevia rebaudiana* (Bert.) leaf extract is well known and has raised the interest of huge food companies due to its natural bid. The extraction of their main glycosides stevioside and rebaudioside A is an important step on the preparation of final Stevia granules. The aim of the work reported here was to study and optimize the dynamic maceration of Stevia leaves using water and ethanol as green solvents. For instance, a fractional factorial design was chosen to evaluate the individual effects of the drug powder size, weight ratio of drug to solvent, temperature, agitation, and time on the yield of these glycosides. The glycosides were quantified by high pressure liquid chromatography. An exhaustive extraction by successive maceration steps showed that ethanol 70% was superior to water and ethanol 90% for stevioside and rebaudioside extraction. The liquid extract composition in dry basis and the yield of stevioside and rebaudioside A were significantly affected by the drug to solvent weight ratio, showing that larger volumes of solvent should be used. Furthermore, increasing solvent volume favors the extraction of the stevioside by a twofold factor as compared to rebaudioside A. Among the other factors, only drug powder size affected the yield of rebaudioside A significantly. The optimal solution for *S. rebaudiana* leaves dynamic extraction estimated by desirability functions methodology led to a condition which allows obtaining extraction yields of 2.31 and 1.24% for stevioside and rebaudioside A and their concentrations in dried extract corresponding to 8.38 and 4.51%, respectively. These high yields were obtained with drug to solvent ratio (1:10, w/w) much higher than previous works, thus resulting in a more sustainable and green process.

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Keywords: DOE; Stevioside; Rebaudioside; Factors; Optimal

1. Introduction

Stevia rebaudiana (Bert.) Bertoni is a shrub native from the Valley of Amambay, formed by an intersection region for Paraguay, Brazil and Argentina. The "estevia" is popularly known worldwide and belongs to the 154 members of genus in the family Asteraceae. The leaf contains eight terpene glycosides identified as stevioside, rebaudioside A, B, C, D and E, dulcoside A and C. However, its sweet flavor is well known and can be attributed mainly to the presence of stevioside and rebaudioside A, wherein the sum of both make up from 5 to 10% of the drug [1–3]. The active component that has the highest sweetness index is rebaudioside A. Notwithstanding the stevioside is the major component among the diterpene glycosides, a post digestive bitter taste is associated to its presence in high levels and causes certain rejection of Stevia by consumers.

Commercial interest and its use by the food and beverage industry have put the species in a prominent position in the international agricultural scene, as described [4] due to the social demand for healthy and natural foods. Since 1990, a large number of countries have been included as producers such as China, India, Brazil, Korea, Mexico, United States, Indonesia, Tanzania and Canada [5].

Because of this wide range of possibilities, there is a large number of publications and patents on the extraction and purification of stevioside using different methods [4,6–12]. The separation of the glycosides from the plant extract is hampered by several factors including impurities such as resins, proteins, organic acids and especially pigments (chlorophyll, carotene and xanthophyll). By now, the aqueous extraction of the powdered

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leaves presents the highest yields of stevioside and rebaudioside A in crude extract and were prepared by dynamic maceration, ultrasound and microwave extraction [2,8–12], while some authors also suggested the use of methanol and ethanol [13]. However, the use of ethanol is preferable because it is classified as GRAS [13] and is a green solvent [14,15] and already applied to pigments and antioxidants [16] or high quality foods [17]. The green extraction of natural products has become a hot topic in bioresource related areas and is based in six principles proposed by Chemat et al. [16]: plant variety selection and enhancement, alternative green solvents like water and agro-solvents, reduced energy consumption, no-waste but re-processed by-products, simplified but efficient process design and preclude process-generated contaminants.

The extractive studies from vegetable matrices shall be detailed in accordance with the purposes of extraction and type of chemical constituent to be obtained [4,6,17–24] and can be concentration equilibrium or exhaustive methods [25]. Among the so many extraction factors, the most important affecting the dynamic maceration are the degree of drug division, type and amount of solvent, temperature, agitation, pH and time [21,22,26–28]. Considering this, the concept of green extraction should also involve the use of optimization approach to find the best conditions to avoid excessive use of solvents and energy [14–16].

Thus, the aim of this work was to study the green and optimized extraction of stevioside and rebaudosídeo A from *S. rebaudiana* leaves by dynamic maceration using a fractional factorial design 2^{5-2} allowing the evaluation of the effects of drug powder size D50, weight ratio of drug to solvent S/D, temperature T, agitation rpm, and time t, on the yield of these glycosides.

2. Material and methods

2.1. Stevia drug characterization

The leaf samples were supplied by the CPQBA – Multidisciplinary Center for Chemical, Biological and Agricultural Sciences of the UNICAMP (State University of Campinas, Campinas – SP, Brazil), where the plants were grown in the coordinates latitude 22 ° 79'81.059" S and longitude 47 ° 11'56.138" W. A voucher specimen of the plant was deposited in the herbarium with file number 273. The plant material was dried in a forced-circulation air oven at 45 ± 2 °C, and grounded in a stainless steel knife mill. The moisture content was determined in an MB45 (Ohaus Co, Parsippany-NJ, USA). The total ash and solvent uptake per weight of drug was determined in triplicate according to Brazilian Pharmacopoeia [29].

2.2. Exhaustive extraction by maceration

Exhaustive extraction studies were performed using water, alcohol 70 and 90% as solvents by 4 step macerations. 5g of plant drug was added to 50 ml of solvent and allowed to stand at room temperature (25 ± 2 °C). Solvent renewal (50 mL) after filtration was carried out three-times at intervals of 2 hours but a fourth final extraction step was run for 12 h. Assays were performed in triplicate. The stevioside and rebaudioside A were quantified by high-performance liquid chromatography. The statistical analysis on glycosides quantification and total solids content were conducted by ANOVA test ($p < 0.05$) followed by Dunnet test.

2.3. Dynamic maceration study

The dynamic maceration was performed using a multi point magnetic stirrer Mag-Multi 15 (Marte Ltd, Sao Paulo Brazil) using 5 g of powder leaves, 50 ml of 70% ethanol in a 125 ml Erlenmeyer and using a 20 mm cylindrical magnetic bar. The extracts were filtered on Whatman® qualitative filter. The extracts were stored at –8 °C for further chromatographic quantification of stevioside and rebaudioside A glycosides. The effects of five dynamic maceration extraction factors were investigated by applying a 2^{5-2} fractional factorial design, as shown in Table 1 [30,31]. The purpose of the factorial design was to assess the effect of the selected factors on the main extract composition parameters such as the yields of stevioside – YST and rebaudioside A – YREB and their contents in dried extracts, CST and CREB, respectively. The drug division size fractions were fine powder and coarse powder, with D50 of 181 and 780 μm, respectively.

2.4. Glycosides quantification

The quantification of stevioside and rebaudioside A were performed by HPLC, in a Dionex Ultimate 3000 (Thermo Fischer Scientific, Sunnyvale, CA, USA) with a diode array detector and a Phenomenex C18 (5 μm, 250 × 4.6 mm) column. The mobile

Table 1
S. rebaudiana dynamic maceration extraction fractional factorial design 2^{5-2} presenting the actual and coded values of the factors and their levels.

Run	D50 (μm)	D/S (g/mL)	t (h)	T (°C)	SS (rpm)	$C_{ST}E$ (mg/ml)	$C_{REB}E$ (mg/ml)	YST (%)	YREB (%)	CST (%)	CREB (%)
1	(−1) 181	(−1) 1:10	(−1) 1	(−1)25	(+1)600	2.23	1.20	1.49	0.78	6.22	3.28
2	(+1) 780	(−1) 1:10	(−1) 1	(+1)50	(−1)100	2.36	1.32	1.24	0.68	5.47	3.02
3	(−1) 181	(+1) 1:30	(−1) 1	(+1)50	(−1)100	0.18	0.10	2.36	1.25	6.89	3.66
4	(+1) 780	(+1) 1:30	(−1) 1	(−1)25	(+1)600	0.19	0.10	0.47	0.26	1.47	0.80
5	(−1) 181	(−1) 1:10	(+13	(+1)50	(+1)600	2.12	1.14	1.56	0.84	5.50	2.97
6	(+1) 780	(−1) 1:10	(+1)3	(−1)25	(−1)100	2.89	1.48	1.51	0.76	7.58	3.82
7	(−1) 181	(+1) 1:30	(+1)3	(−1)25	(−1)100	0.16	0.09	0.43	0.23	1.36	0.73
8	(+1) 780	(+1) 1:30	(+1)3	(+1)50	(+1)600	0.22	0.12	0.52	0.28	1.43	0.79

$C_{ST}E$ – stevioside content in fluid extract; $C_{REB}E$ – rebaudioside content in fluid extract; YST – yield of stevioside in relation to drug; YREB – yield of rebaudioside in relation to drug; CST – stevioside content in dried extract; CREB – rebaudioside content in dried extract.

phase was flow 0.7 ml/min of acetonitrile/water in gradient mode varying from 10:90 to 90:10 v/v, injection volume of the samples varied from 5 to 25 µL. The UV detector was set at 210 nm [7,32–34]. Stevioside and rebaudioside A standards used were supplied by Sigma-Aldrich Co (São Paulo, Brazil).

Calibration curves were prepared from solutions containing concentrations of 50 to 600 µg/ml for rebaudioside A and 28 to 840 µg/ml for stevioside. The samples and standards were solubilized in acetonitrile/water 1:1 and filtered through 0.45 µm filter (Millipore Co., Billerica, MA, USA) prior to injection. Samples were injected in triplicate.

2.5. Statistical analysis

The effects of factors studied on the YST, YREB, CEST and CREB in fractional factorial design were analyzed by ANOVA using Minitab 14 (Minitab, State College, USA), considering the level of significance of 5%. A model was fitted to the data considering the linear an interaction terms of the factors, according to Eq. (2), but only to the significant factors in the ANOVA analysis. The models were used to find optimal extraction condition by desirability functions approach, using "Response Optimizer" (Minitab v 14, State College, USA).

3. Results and discussion

The preliminary pharmacognostic analysis to characterize the *S. rebaudiana* leaves drug, resulted in the water content of 8.62 ± 0.20 % and total ash content of 8.16 ± 1.08%, in an acceptable range Brazilian Pharmacopoeia [29]. The swelling index for the three solvents were 1.67, 0.33, and 0.67 for water, ethanol 70% and ethanol 90%, respectively. The solvent uptake, i.e., the weight of solvent retained by drug, were 2.45, 1.77 and 1.49 g/g for water, ethanol 70% and ethanol 90%, respectively [22]. Exhaustive extractions of *S. rebaudiana* leaves were carried out by 4 step maceration using water, ethanol 70% and ethanol 90%. As can be seen in Fig. 1, % of rebaudioside A contents in fluid extracts are higher when using ethanol 70%, followed by ethanol 90%. The data show that when using ethanol 70% the two glycosides could be completely extracted from the drug after 4 h and two steps of dynamic maceration. Ethanol 70% resulted in final stevioside and rebaudioside A contents of 2.81 and 1.65 mg/ml, respectively. This corresponds to 2.15% of stevioside and 1.27% of rebaudoside A in the dry matter used. The results in Fig. 1 agree with the tests conducted by authors [4,35], which can be explained by the polarity of the ethanol 70%, favorable to the extraction of Stevia components like the glycoside terpenes, flavonoids, alkaloids, chlorophyll, xanthophyll, organic acids, oligosaccharides, amino acids and lipids [35]. Higher yields of rebaudioside A from *S. rebaudiana* were observed with ethanol 30% [4] and justified by the swelling and increasing the contact surface, however, the results herein indicate that ethanol 70% is adequate and has the advantage of easy concentration by evaporation.

Based on results in Fig. 1 and the literature [21–24,31,35] and especially for the glycosides from *S. rebaudiana* [4,6–8,20,33,35], the dynamic maceration factors chosen for the fractional factorial study herein were the drug powder size – D50, temperature – T, time – t, agitation speed – SS and drug to solvent weight ratio – D/S. The results of these extraction factors on the stevioside and rebaudioside A contents in fluid extract, $C_{ST}E$ and $C_{REB}E$, on the yields of stevioside – YST and rebaudioside A – YREB and their contents in dried extracts, CST and CREB, respectively, are shown in Table 1. The stevioside and rebaudioside A contents in fluid extract varied from 0.18 to 2.89 mg/ml and 0.09 to 1.48 mg/ml, respectively. The stevioside and rebaudioside A yields in relation to drug

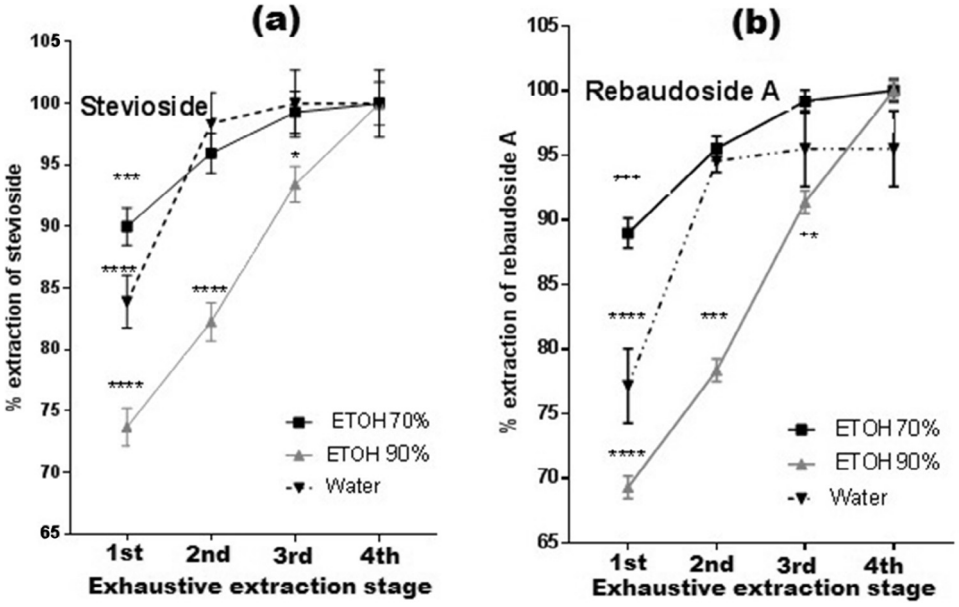

Fig. 1. Cumulative percent stevioside and rebaudioside A for extraction from leaf powder of *S. rebaudiana* as function of time for water, ethanol 70% and ethanol 90%. Symbols and bars represent mean and standard deviation, respectively. *Significantly different ($p < 0.05$) after 18 h extraction (ANOVA and Dunnet post test).

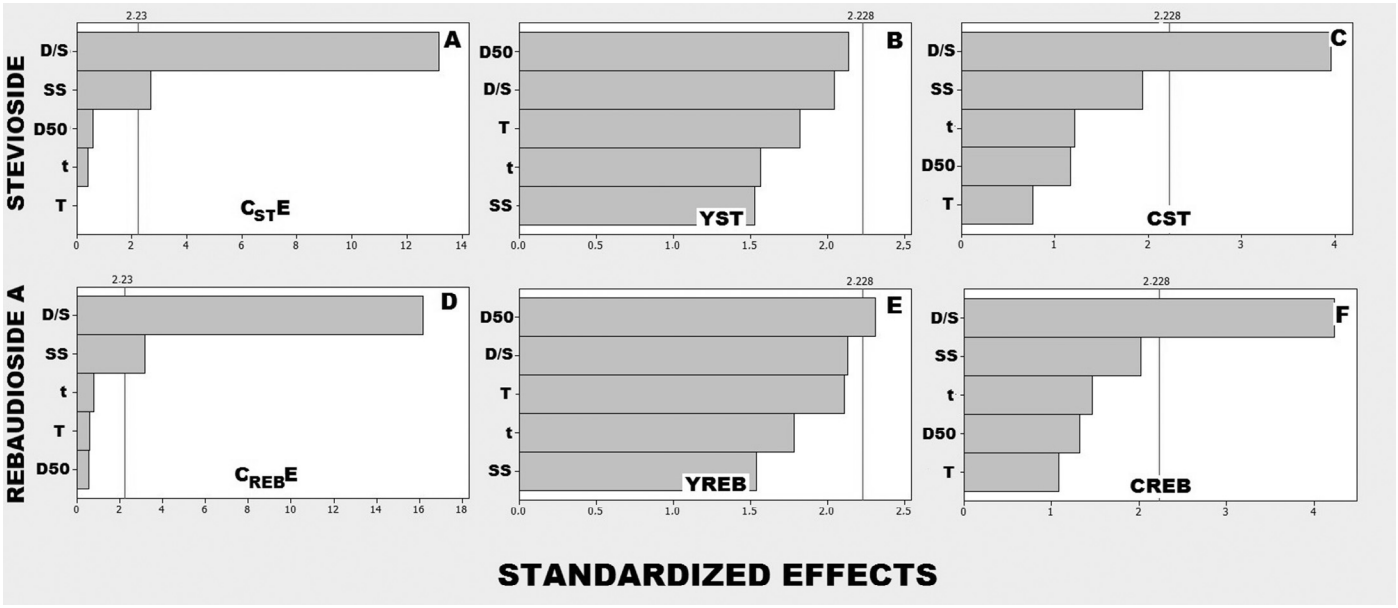

Fig. 2. Pareto plots showing the standardized effects of factors drug powder size – D50, temperature – T, time – t, agitation speed – SS and drug to solvent weight ratio – D/S, on the responses studied: stevioside content in fluid extract – CSTE, rebaudioside A content in fluid extract – CREBE, stevioside yield – YST, rebaudioside yield – YREB, stevioside content in dried extract – CST and rebaudioside content in dried extract – CREB.

weight used ranged from 0.43 to 2.36 % and 0.23 to 1.25%, respectively. The stevioside and rebaudioside A contents in dried extract varied from 1.36 to 6.89% and 0.73 to 3.82%, respectively.

The Pareto charts for the standardized effects can be seen in Fig. 2 and the result of ANOVA on the fractional factorial design is summarized in Table 2, where the significant effects are marked and the terms coefficients are shown. The stevioside and rebaudioside A contents in fluid extract were affected significantly by the drug to solvent weight ratio, D/S and the agitation speed, SS. The yield of stevioside-YST was not affected by any of the factors studied in the 5% level, while the rebaudioside A-YREB was affected by the drug powder size, D50. The stevoside and rebaudioside A contents in dried extracts, CST and CREB, were affected by the drug to solvent ratio, D/S, in the significance level of 5%. The results in Table 2 and Fig. 2 are in close agreement. The coefficients k_i indicated in Table 2 for the terms of significant factors are also an indicative of the most influential effects. The k_i for D/S and SS linear effects on stevioside content in fluid extract $C_{ST}E$ are −1.034 and −0.211, respectively, indicating that the effect of D/S is fivefold stronger than the effect of SS. The same trend is observed for the rebaudioside A content $C_{REB}E$, where the coefficients k_i for the effects of D/S and SS are −0.5450 and −0.1063, respectively. The negative signs of the k_i indicate that for both stevioside and rebaudioside A the increase in D/S and SS values causes a decrease in $C_{ST}E$ and $C_{REB}E$. The k_i for D/S effect on glycoside contents in dried extract CST and CREB corresponds to −1.701 and −0.8875 for stevioside and rebaudioside A, respectively. Again the effect of D/S on these glycoside contents is negative and it is twofold higher for rebaudioside than for stevioside.

The contour plots for extracts properties as functions of the significant factors are shown in Fig. 3. The rebaudioside A yield

Table 2
Summary of factorial ANOVA for the principal terms in *S. rebaudiana* dynamic maceration extraction fractional factorial design 2^{5-2}.

Factor	Responses					
	$C_{ST}E$ (mg/ml)	$C_{REB}E$ (mg/ml)	YST (%)	YREB (%)	CST (%)	CREB (%)
D50 (μm)	p = 0.058	p = 0.590	p = 0.058	p = 0.048* k_i= −0,1419	p = 0.271	p = 0.215
D/S (g/g)	p = 0.000* k_i= −1.034	p = 0.000* k_i= −0.5450	p = 0.068	p = 0.059	p = 0.003* k_i= −1.701	p = 0.002* k_i= −0.8875
T (min)	p = 0.699	p = 0.454	p = 0.148	p = 0.109	p = 0.252	p = 0.173
T (oC)	p = 1.000	p = 0.566	p = 0.099	p = 0.061	p = 0.461	p = 0.303
SS (rpm)	p = 0.023* k_i= −0.211	p = 0.010* k_i= −0.1063	p = 0.158	p = 0.156	p = 0.082	p = 0.071

* Significant at 5% level ($p < 0.05$); k_i – coefficients for the model.

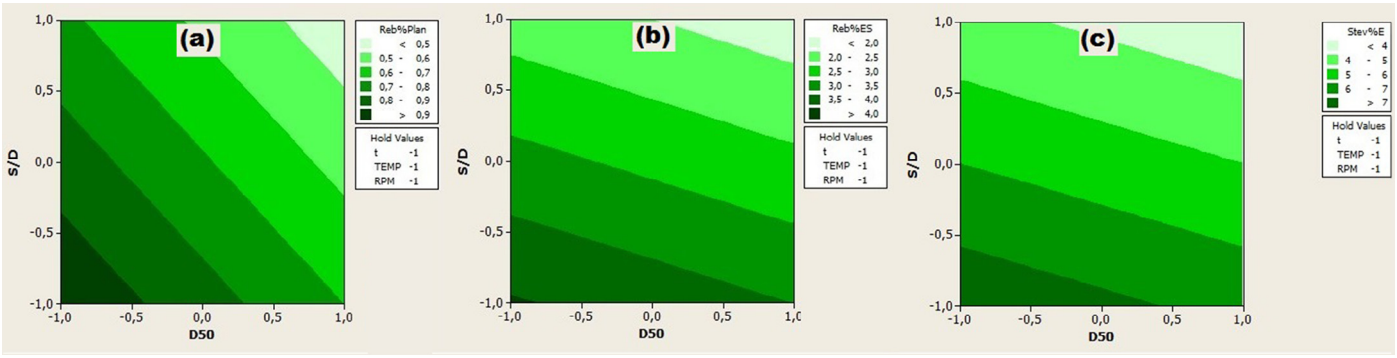

Fig. 3. Contour plot for: a) rebaudioside A yield, b) rebaudioside A and c) stevioside content in dried extract as function of drug granulometry (D50) and ratio of drug to solvent (D/S) at 25°C, 100 rpm and 1 h extraction.

as function of drug size (D50) and ratio of drug to solvent (D/S) at 25°C, 100 rpm and 1 h extraction is presented in the contour in Fig. 3a. According to the plot, an increase in D50 decreases the YREB, which is in agreement with Ref. [25] since smaller drug powder have higher surface area consequently increasing the contact area and the rate of mass transfer between powder and solvent. The contour plot for rebaudioside A content in dried extract as function of drug size (D50) and ratio of drug to solvent (D/S) at 25°C, 100 rpm and 1 h extraction is shown in Fig. 3b. The contour plot shows that the content of this glycoside in dried extract decreases with an increase in the drug to solvent weight ratio, meaning that for higher extraction outputs one must use high volumes of solvent. The stevioside content in dried extract as function of drug size (D50) and ratio of drug to solvent (D/S) is displayed in the contour plot of Fig. 3c, which shows CST at 25°C, 100 rpm and 1 h extraction. Following the trend in other contour plots, CST decreases with increasing D/S. This result shows that using larger volumes of solvent favors the stevioside and rebaudioside A extraction in detriment of other extractable from *S rebaudiana*, which is of great interest. However, the use of large amounts of solvents may incur in higher costs. The use of larger volumes of solvent for plant material extraction was reported before, and the best results found herein with drug to solvent weight ratio of 1:10 has been already adopted and recommended in previous works for other plants [22,30,31,36], however majority of authors pointed much higher ratios for *S rebaudiana*, from 1:35 to 1:50 [2,4,20].

One of the advantages of a multivariate experimental approach as the one adopted herein is the possibility of applying an optimization method to find the best suitable condition among the several combinations of the factors studied [37]. Considering this, the results shown in Table 1 and 2 were optimized by the desirability functions technique [37]. The optimization was performed using Minitab 14 (Minitab, State College, USA) and settings for the simultaneous maximization of YST, YREB, CST and CREB. All factors weights and importance were set to 1.0.

The results of desirability study can be seen in Table 3. YST, CST and CREB individual desirabilities were 1.00 while YREB was 0.997, resulting in a high global desirability of 0.9924. The best set of extraction conditions was found to be D50 = 181 μm, D/S = 1:10 g/g; t = 1 h; T = 50°C; SS= 100 rpm. Under this dynamic maceration condition the yields of stevioside and rebaudioside A are 2.30% and 1.24% and the stevioside and rebaudioside A contents in dried extract are 8.38% and 4.51%, respectively. These values are very close to the ones found for 18 h exhaustive extraction of *S. rebaudiana* leaves, as shown in Figs 1 and 2, demonstrating that dynamic maceration using ethanol 70% is very effective for stevioside and rebaudioside A extraction within a short time, moderate temperature and low solvent consumption. The majority of previous extraction studies for Stevia found best yields with drug to solvent ratio from 1:35 to 1:50 [2,4,20], while the data reported herein support equivalent yields with ratio of 1:10. Literatures for other plants support our findings [22,30,31,36]. Recently, an innovative green extraction of *S. rebaudiana* by microwave assisted water steam extraction [17] was developed to obtain crude Stevia extract and evaluated as chocolate sweetener. The authors used a

Table 3
Summary of desirability functions optimization.

Settings for optimization						D	Optimal value
RESP	Goal	Target	Upper	Weight	Importance		
YST (%)	Maximize	2.20	2.20	1	1	1.000	2.314
YREB (%)	Maximize	1.25	1.25	1	1	0.997	1.242
CST (%)	Maximize	8.00	8.00	1	1	1.000	8.377
CREB (%)	Maximize	4.00	4.00	1	1	1.000	4.507

Global desirability: 0.99241.
Optimum set of conditions: D50 = 181 μm, D/S = 1:10 g/g; t = 1 h; T = 50°C; SS= 100 rpm.

drug to solvent ratio of about 1:2, but yielded only 1.4 g of extract powder from 500 g of Stevia leaves [17], while in this work 2.6 g of extract powder was obtained from 5 g of Stevia leaves.

Furthermore, the ethanol extracts obtained herein can be easily concentrated because of ethanol low vapor pressure. The latent heat of water and ethanol vaporization are 540 and 204 cal/g, respectively [38], meaning that each gram of ethanol 70% consumes 30.6% and 43.6% less energy to be evaporated than equivalent weight of ethanol 30% and water, respectively The process reported herein indicates all requirements for an optimal green extraction process. Also, these values of stevioside and rebaudioside A concentrations in extract may facilitate further purification steps.

4. Conclusions

The exhaustive extraction of *S. rebaudiana* leaves by a multistage dynamic maceration showed that ethanol 70% gives better results than water and ethanol 90%. The multivariate experimental approach to dynamic maceration of *S. rebaudiana* leaves by a 2^{5-2} fractional factorial design demonstrated that the drug to solvent weight ratio affected the yield and composition of the extract in dry basis and that larger volume of the ethanol 70% should be used. However, the effect of increasing solvent volume is twofold higher for stevioside than for rebaudioside A in fluid extract, favoring the extraction of the former. Drug powder size affected only the yield of rebaudioside A. The optimal solution for *S. rebaudiana* leaves dynamic extraction under the conditions studied herein are 181 μm drug powder size, 1:10 g/g ratio of drug to solvent; 1 h extraction time; 50°C temperature and 100 rpm stirring speed. This study resulted in Stevia glycoside extraction using an agro-renewable-solvent combined with process conditions leading to low solvent, time and energy consumption but with adequate yields, committed to the basic principles of green extraction.

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