Revista Brasileira de Farmacognosia 27 (2017) 510–518

Brazilian Journal of Pharmacognosy
REVISTA BRASILEIRA DE FARMACOGNOSIA
www.elsevier.com/locate/bjp

Original Article

Turbo-extraction of glycosides from *Stevia rebaudiana* using a fractional factorial design

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ARTICLE INFO

Article history:
Received 29 November 2016
Accepted 24 February 2017
Available online 1 April 2017

Keywords:
Design of experiments
Stevioside
Rebaudoside A
Hydroethanolic extract
Desirability

ABSTRACT

Stevia rebaudiana (Bertoni) Bertoni, Asteraceae, leaf extract has recently called the attention of food industry as a proposal for natural sweetener. The sweet flavor is attributed to the glycosides, in especial stevioside and rebaudioside A, which are the plant main chemical markers. The aim of the work reported here was to optimize the turbo-extraction of stevia leaves using water, ethanol 70% and 90% (w/w) as green solvents. A 2^{5-2} factorial design was applied to study the linear effects of the drug size, solvent to drug ratio, temperature, time and also the turbolysis speed on the extraction of glycosides. The glycosides exhaustive extraction showed that ethanol 70% gave better results and was used for turbo-extraction. The stevioside and rebaudioside A contents were quantified by a validated method by high performance liquid chromatographic with photodiode array detector. The contents of stevioside and rebaudioside A in fluid extract increased with the drug size, but decreased at high shearing speeds and solvent to drug ratio, while their yields decreased at higher temperature and were not affected by turbo speed. An increase in solvent to drug ratio reduced significantly the glycosides percent in dried extract. Optimal solution for *S. rebaudiana* leaves turbo-extraction was determined by desirability functions. The optimal extraction condition corresponded to drug size of 780 μm, solvent to drug ratio of 10, extraction time of 18 min; temperature of 23 °C and turbo speed of 20,000 rpm, resulting in yields of 4.98% and 2.70%, for stevioside and rebaudioside A, respectively. These yields are comparable to the ones recently published for dynamic maceration, but with the advantage of shorter extraction times. This work demonstrates that turbolysis is promising for *S. rebaudiana* glycosides extraction and stimulate new research on the purification of these extracts, which may become an interesting source of income for developing countries such as India and Brazil.

Introduction

The "estevia" is a shrub popularly known worldwide and belongs to the 261 members of genus in the family Asteraceae and has the botanical name of *Stevia rebaudiana* (Bertoni) Bertoni. It originates from the Valley of Amambay, which extends from northeast Paraguay to south Brazil and southern Argentina. The plant is a perennial shrub height up to 30 cm. Its leaves are sessile, 3–4 cm long, shaped as spatula or long blade with sawn edges. The adaxial surface is slightly glandular. The branches are fragile and the roots are rhizome type, slightly branched. The flowers are composite, surrounded by epicalyces housing, color wine, light and pentamerous. The fruit is striated (Madan et al., 2010).

Stevia leaves contain eight terpene glycosides, identified as stevioside, rebaudoside A, B, C, D and E, dulcoside A and C. The main glycosides are stevioside and rebaudoside A, summing up to 5–10% of the drug (Singh and Rao, 2005; Abou-Arab et al., 2010; Yadav et al., 2011) and give a pronounced sweet flavor. Stevia is described in the Brazilian Pharmacopoeia 5th Ed. (Farmacopeia Brasileira, 2010) indicating dried leaves as the part used and must contain at least 12% of total carbohydrates and 4% steviosides.

According to Madan et al. (2010), the species has been used for centuries by the Guarani Indians as a sweetener in some drinks, especially the mate tea, and the active component that has the highest sweetness index is rebaudoside A while stevioside is the main component and gives a post digestive bitter taste. The

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http://dx.doi.org/10.1016/j.bjp.2017.02.007

concentration of these diterpene glycosides may vary depending on cultivars, soil and climate, and agronomic techniques (Yadav et al., 2011).

The medicinal use of the species is described in the control of obesity, high blood pressure, digestive problems and contraceptive by indigenous peoples in many countries of Latin America (Sardhara, 2015; Madan et al., 2010). Preclinical and clinical studies have been conducted to evaluate the antioxidant activity (Sharma et al., 2012), the reproductive system, kidney function, blood pressure, the effect on blood glucose and anti-viral activity, as well as studies on pharmacokinetic (Madan et al., 2010), toxicity, side effects and allergenic (Urban et al., 2015).

In many countries stevia has its use regulated as a dietary supplement and thus is not used as a sweetener, such as in Europe. But in 2008, the FDA has categorized the species as Generally Recognized as Safe (GRAS) after publication by the Joint Committee of Experts on the FAO Food Additives/WHO – JECFA, which established the monograph of glycosides of steviol (WHO, 2004). The product approved for industrial and commercial use is a powder with minimum of 95% of steviol glycosides and the sum of stevioside and rebaudosideo A not less than 70%.

Stevia products are considered healthy and natural foods and have gained attention in international markets (Gasmalla et al., 2014) with China, India, Brazil, Korea, Mexico, United States, Indonesia, Tanzania and Canada as producers (Ahmad et al., 2014). Recently, the commercial giant Coca Cola Company has launched the "green" product "Coke Life" and is expected to highly increase the stevia world sales (Ausfood, 2015).

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). The aqueous extraction of the powdered leaves was the most indicated choice until recently and presents the highest yields of stevioside and rebaudosídeo A in crude extract (Abou-Arab et al., 2010; Chhaya et al., 2012a,b; Mondal and Chhaya, 2012; Rao et al., 2012; Periche et al., 2014; Sardhara, 2015).

The use of methanol was also proposed to give selective extraction and easy purification (Rajab et al., 2009), but the end product is no longer classified as GRAS. Because of the blooming interest in stevia products, there is a large number of publications and patents on the extraction and purification of stevioside using different methods (Periche et al., 2015; Jentzer et al., 2015), such as enzymatic extraction (Puri et al., 2012), bleaching agents and selective precipitation processes using metal ions (Kienle, 2010; Abou-Arab et al., 2010), selective extraction by supercritical fluid extraction and separation processes for adsorptive methods using chromatographic columns, chemical solvents and activated carbon (Rajab et al., 2009), ion exchange resins, micro, ultra and nanofiltration (Zhang et al., 2000; Reis et al., 2009; Rao et al., 2012; Chhaya et al., 2013).

Recently, the glycosides extraction from stevia using ethanol 70 and 90% was studied by dynamic maceration coupled with a multivariate approach and resulted in stimulating advantages over previous methods (Martins et al., 2016). Meanwhile, the turbo-extraction or turbolysis can be advantageous over dynamic maceration and is based on extraction with stirring and simultaneous reduction of particle size as a result of application of high shearing force, so that with the disruption of the cells there is a rapid dissolution of the active substances, resulting in extraction times of the order of minutes and almost completely exhausted the plant drug (Sonaglio et al., 2003).

Nevertheless, turbolysis of stevia has never been attempted (Martins et al., 2016) and together with the use of different grade ethanol as green and GRAS solvent and the application of experimental designs (Costa-Machado et al., 2013; Paulucci et al., 2013; Martins et al., 2013) this process could give a promising and optimized method for stevia extraction.

Thus, the aim of this work was to study the turbo-extraction of stevioside and rebaudosídeo A from *S. rebaudiana* dried and powdered leaves by applying a fractional factorial design that allow the evaluation of the main effects of drug powder size, weight ratio solvent to drug, temperature, agitation and time on the yield of these glycosides.

Material and methods

Stevia drug characterization

The leaves of *Stevia rebaudiana* (Bertoni) Bertoni, Asteraceae, came from a plantation located at latitude 22° 79′81.059″S and longitude 47° 11′56.138″W, grown by the CPQBA – Multidisciplinary Center for Chemical, Biological and Agricultural Sciences from State University of Campinas (Campinas – SP, Brazil), with a voucher deposited at the CPQBA herbarium with file number 273. The plant was supplied to CPQBA by Cenargen (Brasília, DF, Brazil) in 1998, according to Dr Illio Montanari Jr.

The pharmacognostic characterization of drug was performed according to the Brazilian Pharmacopoeia 5th Edition (Farmacopeia Brasileira, 2010) after drying at 45 ± 2 °C in a circulating air oven and milling. The physicochemical parameters determined were the moisture content, total ash, swelling index and solvent uptake.

Turbolysis extraction study

In order to determine the stevioside and rebaudioside A content in the drug, a preliminary exhaustive extraction study was performed by using water, alcohol 70% and 90% as solvents. Fine drug powder (5 g) was added to 50 ml of solvent and stirred at room temperature (25 °C) for 2 h. The drug powder as filtered and then the procedure was repeated three-fold with 50 ml of fresh solvent. The fourth and final extraction step was run for 12 h at same conditions (50 ml fresh solvent under stirring and 25 °C). The extraction runs were carried out in triplicate and the stevioside content (CEST), rebaudoside A content (CREB) and the total solids content (TSOL) were determined.

The turbo-extraction or turbolysis was performed using a high shear stirrer Ultraturrax T-25 (Ika Ltda, Sao Paulo Brazil). Grounded stevia leaves together with 50 ml of ethanol 70% as extracting solvent were poured into a 50 ml beaker and submitted to the high shear stirring according to temperature, velocity and time pre determined by the experimental design. The extracts were filtered on Whatman® qualitative filter paper grade 1 (pore size 11 μm) and stored at −8 °C for further chromatographic determination of TSOL and quantification of CEST and CREB by HPLC-PAD.

The effect of five turbolysis extraction factors were investigated by applying a 2^{5-2} fractional factorial design, as shown in Table 1 (Moura-Costa et al., 2012; Araújo et al., 2014; Martins et al., 2016). The extraction factors selected, according to Martins et al. (2016), were the solvent to drug mass ratio (S/D), average drug powder size ($D50$), high shear speed (SS), extraction time (t), and temperature (T). Their effect on the extract glycosides content CEST and CREB were quantified by liquid chromatography. To allow the elucidation of the effect of drug division level on the extraction, the drug was divided in two size fractions, called fine powder (150–212 μm) and coarse powder (710–850 μm), with average sizes of 181 μm and 780 μm, respectively.

Besides the glycosides content in fluid extracts CEST and CREB their extraction yields, YST and YREB, were calculated based on the weight of starting plant material, as defined in Eqs. (1) and (2). The values of TSOL, CEST and CREB were also used to

Table 1
Stevia rebaudiana turbo-extraction fractional factorial design 2^{5-2} presenting factors, their levels[a] and extraction results.

Run	*D*50 (μm)	*S*/*D* (g/g)	*t* (min)	*T* (°C)	SS (rpm)	CEST (mg/ml)	CREB (mg/ml)	YST (%)	YREB (%)	DST (%)	DREB (%)
1	−1	−1	−1	−1	+1	2.37	1.25	4.65	2.45	6.19	3.27
2	+1	−1	−1	+1	−1	2.83	1.52	4.26	2.28	6.24	3.35
3	−1	+1	−1	+1	−1	0.12	0.07	0.11	0.06	0.76	0.45
4	+1	+1	−1	−1	+1	0.17	0.10	0.15	0.09	1.19	0.70
5	−1	−1	+1	+1	+1	2.22	1.21	4.50	2.46	5.28	2.88
6	+1	−1	+1	−1	−1	2.75	1.49	5.22	2.83	6.63	3.59
7	−1	+1	+1	−1	−1	0.14	0.08	0.12	0.07	1.24	0.71
8	+1	+1	+1	+1	+1	0.21	0.12	0.15	0.09	1.52	0.87

[a] Actual values for each factor: drug mean size *D*50 (−1) 181 μm, *D*50 (+1) 780 μm; solvent to drug ratio *S*/*D* (−1) 10 g/g, *S*/*D* (+1) 30 g/g; extraction time *t* (−1) 3 min, *t* (+1) 18 min; extraction temperature *T* (−1) 23 °C, *T* (+1) 80 °C; turbo speed SS (−1) 5000 rpm, SS (+1) 20,000 rpm. Stevioside and rebaudioside contents in fluid extract CEST and CREB (mg/ml); Stevioside and rebaudioside yields YST and YREB (%). Stevioside and rebaudioside contents in dried extract, DST and DREB (%).

calculate the glycosides content in dried extracts, DST and DREB. The liquid extracts were dried in Erlenmeyer's under nitrogen flux of 10 ml/min at room temperature. The definitions used to calculate DST and DREB are given in Eqs. (3) and (4).

$$\text{YST}\ (\%) = \frac{\text{CEST} \times V}{Wd} \times 100 \quad (1)$$

$$\text{YREB}\ (\%) = \frac{\text{CREB} \times V}{Wd} \times 100 \quad (2)$$

$$\text{DST}\ (\%) = \frac{(\text{CEST} \times V)}{(\text{TSOL} \times V)} \times 100 \quad (3)$$

$$\text{DREB}\ (\%) = \frac{(\text{DREB} \times V)}{(\text{TSOL} \times V)} \times 100 \quad (4)$$

where *V*, volume of liquid extract collected; *Wd*, weight of drug used in extraction run.

Glycosides quantification by HPLC-PDA

The glycosides stevioside and rebaudoside A were quantified by high performance liquid chromatography, HPLC-PDA. The HPLC equipment used was an Ultimate 3000 (Thermo Fischer Scientific, Sunnyvale, CA, USA) with a chromatographic column Phenomenex C18 (5 μm, 250 × 4.6 mm) and a photodiode array detector set to 210 nm (Liu et al., 2010; Woelwer-Rieck et al., 2010; Aranda-Gonzalez et al., 2014; Jentzer et al., 2015). The software Chromeleon 7.1.2 Chromatographic Data System (Thermo Fischer Sci, Sunnyvale, CA, USA) was used for data acquisition and analysis. The oven was set to 50 °C and the acetonitrile/water mobile phase was operated in gradient mode varying from 10:90 to 90:10, v/v in 45 min at flow of 0.7 ml/min. The pure analytical standards of the glycosides (Sigma–Aldrich Co, São Paulo, Brazil) were used as reference standards to prepare the calibration curves. Solutions containing concentrations of 50, 100, 200, 400 and 600 μg/ml for rebaudoside A and 28, 84, 140, 224, 560 and 840 μg/ml for stevioside in methanol were prepared and injected in quintuplicate for the calibration curves construction. Depending on glycosides content of the extracts, the sample injection volume varied from 5 to 25 μl. Both extract samples and analytical standards were solubilized in methanol and filtered through 0.45 μm filter (Millipore Co., Billerica, MA, USA) prior to injection. All extracts samples were injected in triplicate. Method resolution was calculated based on selectivity (separation factor), efficiency and retention (capacity factor) by the software Chromeleon 7.1.2.

Multivariate analysis

The 2^{5-2} fractional factorial design, as shown in Table 1 (Moura-Costa et al., 2012; Araújo et al., 2014; Martins et al., 2016) was applied to investigate the linear effects of *S*/*D*, *D*50, SS, *t*, and *T* on the TSOL, CEST, CREB, YST, YREB, DST and DREB by response surface methodology, RSM, using Minitab 14.0 (Minitab, State College, USA), adopting the significance level of 5% ($p < 0.05$). A model considering linear terms of the factors was fitted to the data, according to Eq. (5).

$$Y_i = A_1 + A_2X_1 + A_3X_2 + A_4X_3 + A_5X_4 + A_6X_5 \quad (5)$$

The response surfaces fitted to Eq. (5) were used to find optimal extraction condition by applying the "Desirability Functions Method", using "Response Optimizer" (Minitab v 14, State College, USA).

Results and discussion

The *S. rebaudiana* leaves presented total moisture content of 8.62 ± 0.20%, ash content of 8.16 ± 1.08%, swelling index of 67% and solvent uptake of 149% in ethanol 90%, 33% and 177% in ethanol 70% and 167% and 245% in water. The drug moisture and ash contents are under the maximum limits established by the Brazilian Pharmacopoeia (Farmacopéia Brasileira, 2010) 13% and 9.5%, respectively, and can be attributed to adequate post-harvest processing, *e.g.* drying and storage, and also that the drug is acceptable for the forthcoming extraction study. Total ash represents a purity test for the presence of non-volatile organic substances by incineration, giving the sum of inorganic or intrinsic ash in *S. rebaudiana* and the contaminants from earthy origin. On the other hand, swelling index and solvent uptake by drug are used to estimate the volume of solvent that will be retained or absorbed by vegetal matter after extraction and the final extract volume recovered. This traditional pharmacognostic characterization is essential to guarantee a good phytopharmaceutical technology study of the plant. The *S. rebaudiana* swelling index and solvent uptake were, in descending order, water, ethanol 90% and ethanol 70%. In order to choose the best solvent for glycosides extraction from *S. rebaudiana* a preliminary exhaustive extraction by maceration was carried out.

The HPLC-PDA chromatogram showing stevioside and rebaudioside A peaks in extract injection can be seen in Fig. 1. Stevioside and rebaudioside A had elution times of 17.303 and 17.060 min, respectively. The calibration curves obtained and their correlation coefficients were $C = 0.0557A + 0.2918$ ($R^2 = 0.9996$) and $C = 0.0491A + 0.1154$ ($R^2 = 0.9999$), for stevioside and for rebaudioside A respectively. Furthermore, the resolutions calculated by the software Chromeleon 7.1.2 (Thermo Fischer Scientific, Sunnyvale, CA, USA) varied from 1.98 to 1.99 and 1.48 to 1.64 for stevioside and rebaudioside A, respectively.

The results of the exhaustive extraction can be seen in Table 2 and show that stevioside yields corresponded to 2.17, 2.80 and 2.41 mg/ml for water, ethanol 70% and 90%, respectively. Also, rebaudioside A yields were 1.39, 1.66 and 1.37 mg/ml, for water, ethanol 70% and 90%, respectively. The best result was found for ethanol 70% for both stevioside and rebaudioside A and their sum (4.46 mg/ml) since in all extractions the total volume of fluid extract

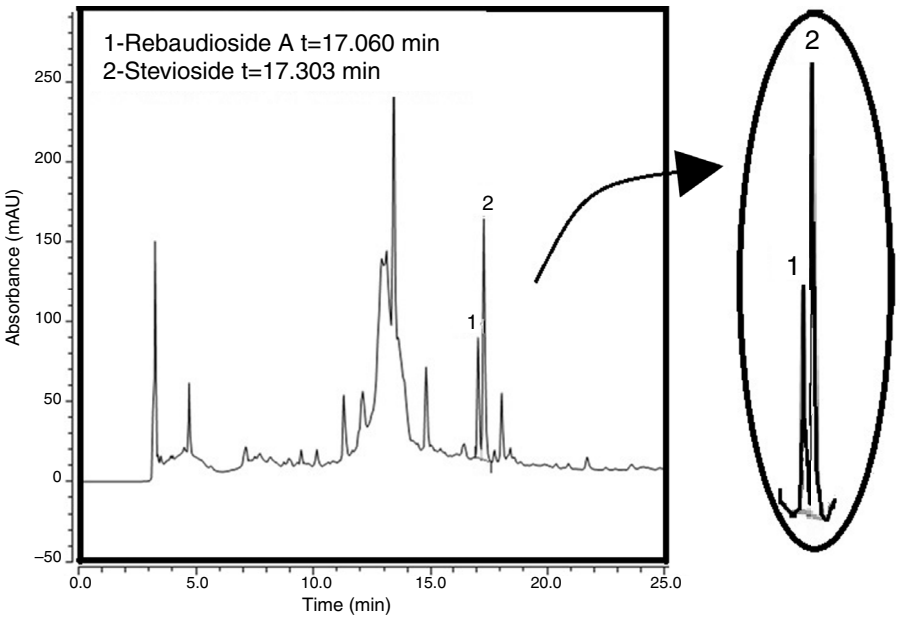

Fig. 1. HPLC-PDA chromatogram of *Stevia rebaudiana* leaf extract obtained by turbolysis using ethanol 70%, solvent to drug weight ratio 1:10, drug powder size 780 μm, temperature 50 °C, turbolysis speed 20,000 rpm and 1 h extraction. Peak 1 rebaudosíde A (17.060 min) and peak 2 estevioside (17.303 min).

Table 2
Cumulative stevioside, rebaudioside A and total solids contents in *Stevia rebaudiana* extracts obtained in a four-step exhaustive extraction by dynamic maceration.

Extraction time (h)	Solvent								
	Water			Ethanol 70%			Ethanol 90%		
	CEST (mg/ml)	CREB (mg/ml)	TSOL %	CEST (mg/ml)	CREB (mg/ml)	TSOL (%)	CEST (mg/ml)	CREB (mg/ml)	TSOL (%)
2	1.83	1.12	3.08	2.52	1.48	3.84	1.77	0.95	2.72
4	2.15	1.33	3.38	2.69	1.59	4.76	1.98	1.07	3.73
6	2.17	1.39	3.50	2.78[a]	1.65[a]	4.94[a]	2.25	1.25	4.01
18	2.17	1.39	3.67	2.80[a]	1.66[a]	5.20[a]	2.41	1.37	4.27
Final	2.17	1.39	3.67	2.80	1.66	5.20	2.41	1.37	4.27

[a] Stevioside and rebaudioside contents in fluid extract CEST and CREB (mg/ml); total solids content in fluid extract TSOL (%, w/w).

obtained was 100 ml. Rebaudoside A extraction from *S. rebaudiana* with different grades of ethanol up to 60% (Gasmalla et al., 2014) resulted in higher yields with ethanol 30%, justified by the swelling index, according to the authors. However, in other work (Martins et al., 2016) high swelling index and extraction yields for stevioside and rebaudioside A were observed for ethanol 70%, which is in agreement with this work and can be explained by the adequate polarity of this solvent for stevia components extraction, like organic acids, flavonoids, alkaloids, xanthophyll, oligosaccharides and glycosides (Muanda et al., 2011). Ethanol 70% has also giving excellent results in other natural products extraction, such as for curcuminoids from *Curcuma longa* (Martins et al., 2013), actives from of *Schinus terebenthifolius* shells (Moura et al., 2005) and in the turbolysis of popular plants from ancient tribes, using ethanol 38–56% (Moura-Costa et al., 2012). Based on literature for *S. rebaudiana* glycosides extraction (Liu et al., 2010; Gasmalla et al., 2014; Sardhara, 2015; Periche et al., 2015; Jentzer et al., 2015; Koubaa et al., 2015; Martins et al., 2016) and the results in Table 2 the solvent chosen for the turbolysis study was ethanol 70%. Besides, ethanol is classified as GRAS (Rajab et al., 2009) and also as a green solvent (Capello et al., 2007; Chemat et al., 2015) and so highly recommended for phytotherapics and nutraceuticals (Chemat et al., 2012; Torri et al., 2016).

The results of extraction by the fractional factorial design are shown in Table 1, which displays the values of CEST, CREB, YST, YREB, DST and DREB for all conditions studied. This data was analyzed by response surface methodology using Minitab 14.0 (Minitab, State College, USA) and resulted in linear models, according to Eq. (1), for each extract parameter as shown in Table 3, where the significant terms and respective coefficients for the model are indicated. The stevioside content in fluid extract, CEST (mg/ml) varied from 0.12 mg/ml to 2.83 mg/ml and was affected by the solvent to drug mass ratio – *S*/*D*, average drug powder size – *D*50 and high shear or turbolysis speed – SS, at the significance level of 5%, which was adopted for all analysis in the work herein. On the other hand, rebaudioside A content in fluid extract, CREB (mg/ml), ranged from 0.07 mg/ml to 1.52 mg/ml and was similarly affected by the same factors significant for stevioside, *S*/*D*, *D*50 and SS, which is expected by chemical similarity between these two glycosides from *S. rebaudiana*. All five factors studied in the fractional factorial design were chosen based on the "space of knowledge" as suggested by the up-to-date approach of the Quality by Design (Yan et al., 2014), since previous knowledge indicate these are the most important in turbo-extraction. However, the results show that only *S*/*D*, *D*50 and SS affected the extraction, while extraction time (*t*) and temperature (*T*), did not. The extraction time and temperature are usually very important factors in herbal drug extractive processes because they may alter the kinetics of active removal from plant matrix by equilibrium dislocation. However, turbolysis is a high energy extraction requiring reduced time and temperature. Probably these two factors were not significant due to the short extraction times of 3 and 18 min, as well as moderate temperatures of 23 and 80 °C.

The influence of the significant factors, *S*/*D*, *D*50 and SS, on the contents of stevioside and rebaudioside A in fluid extracts can be

Table 3
Summary of factorial ANOVA for the principal terms in *Stevia rebaudiana* turbo-extraction fractional factorial design 2^{5-2}.

Factor	Responses					
	CEST (mg/ml)	CREB (mg/ml)	YST (%)	YREB (%)	DST (%)	DREB (%)
*D*50 (μm)	$p = 0.000$[a] $k_i = 1.345$	$p = 0.000$[a] $k_i = 0.080$	$p = 0.302$	$p = 0.283$	$p = 0.106$	$p = 0.016$[a] $k_i = 0.059$
S/*D* (g/g)	$p = 0.000$[a] $k_i = -1.185$	$p = 0.000$[a] $k_i = -0.633$	$p = 0.000$[a] $k_i = -2.24$	$p = 0.000$[a] $k_i = -1.205$	$p = 0.000$[a] $k_i = -2.668$	$p = 0.000$[a] $k_i = -1.415$
t (min)	$p = 0.092$	$p = 0.467$	$p = 0.09$	$p = 0.047$[a] $k_i = 0.071$	$p = 0.860$	$p = 0.223$
T (°C)	$p = 0.473$	$p = 0.724$	$p = 0.022$[a] $k_i = -0.15$	$p = 0.046$[a] $k_i = -0.071$	$p = 0.767$	$p = 0.280$
SS (rpm)	$p = 0.000$[a] $k_i = -0.114$	$p = 0.000$[a] $k_i = -0.061$	$p = -0.04$	$p = 0.442$	$p = 0.513$	$p = 0.493$
R^2_{adj}	99.8%	99.9%	99.4%	99.3%	99.6%	99.8%

*D*50, drug mean size; *S*/*D*, solvent to drug ratio; *t*, extraction time; *T*, extraction temperature; SS, turbo speed. Stevioside and rebaudioside contents in fluid extract CEST (mg/ml) and CREB (mg/ml); Stevioside and rebaudioside yields YST (%) and YREB (%). Stevioside and rebaudioside contents in dried extract, DST (%) and DREB (%).
[a] Significant at 5% level ($p < 0.05$); k_i, coefficients for the model, R^2_{adj}, adjusted *R* squared.

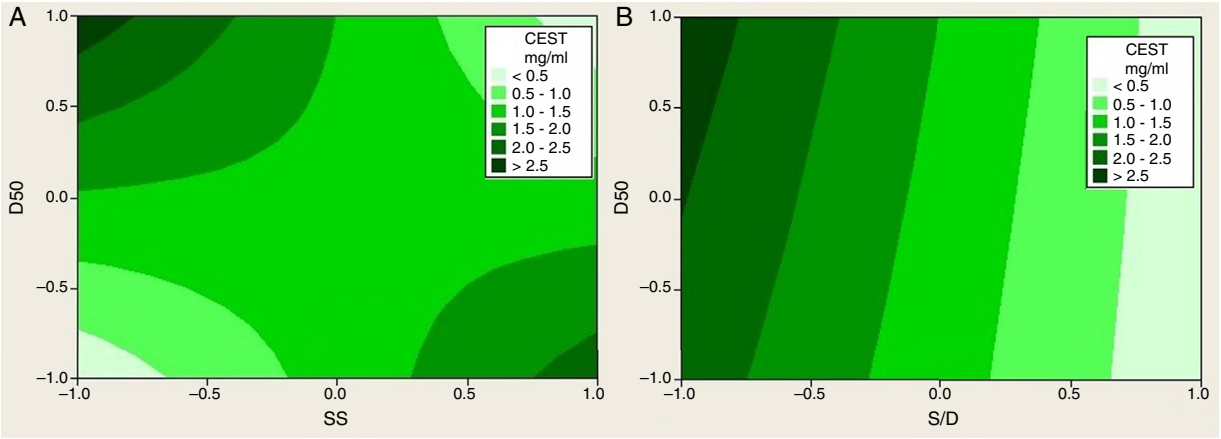

Fig. 2. Contour plots for stevioside content (CEST, mg/ml) in fluid extract as functions of: (A) drug granulometry (*D*50) and high shear speed (SS); and (B) drug granulometry (*D*50) and solvent to drug ratio (*S*/*D*).

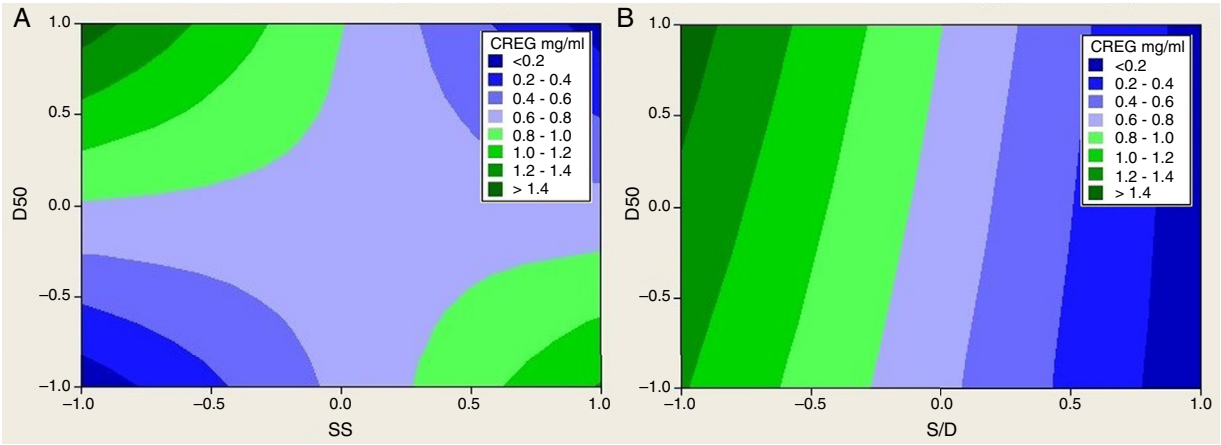

Fig. 3. Contour plots for rebaudioside A content (CREB, mg/ml) in fluid extract as functions of: (A) drug granulometry (*D*50) and high shear speed (SS); and (B) drug granulometry (*D*50) and solvent to drug ratio (*S*/*D*).

seen in Figs. 2 and 3, respectively. The contour plot of stevioside content, CEST (mg/ml), as a function of *D*50 and SS is shown in Fig. 2A, while CEST values as a function of *D*50 and *S*/*D* is shown in Fig. 2B. Fig. 2A shows that CEST values are higher in the corners of the contour plot, with combinations of large *D*50 and low SS or the opposite. This result is interesting because it shows that coarse powders need more solvent for the extraction, but at the same time fine powder can give good extractive result with less solvent. The contour plot in Fig. 2B shows that *S*/*D* exerts a strong effect on CEST, and smaller solvent to drug ratios means higher stevioside extraction. This effect is expected for turbolysis, since the extraction times are only few minutes and larger solvent amounts means higher diffusion rates, or faster actives removal from drug matrix. Fig. 3A and B show the rebaudioside A content in fluid extract, CREB (mg/ml), as function of *D*50 vs SS, and *D*50 vs *S*/*D*, respectively. It is easy to realize the strong similarity between the contour plots

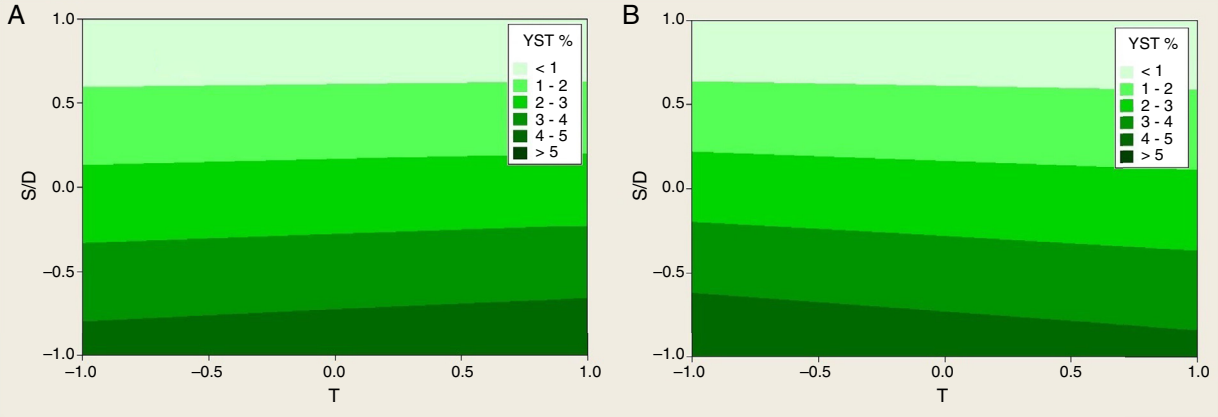

Fig. 4. Contour plots for stevioside extraction percent yield (YST, %) from *Stevia rebaudiana* leaves as functions of: (A) solvent/drug ratio (*S*/*D*) and temperature (*t*); and (B) solvent to drug ratio (*S*/*D*) and extraction temperature (*T*).

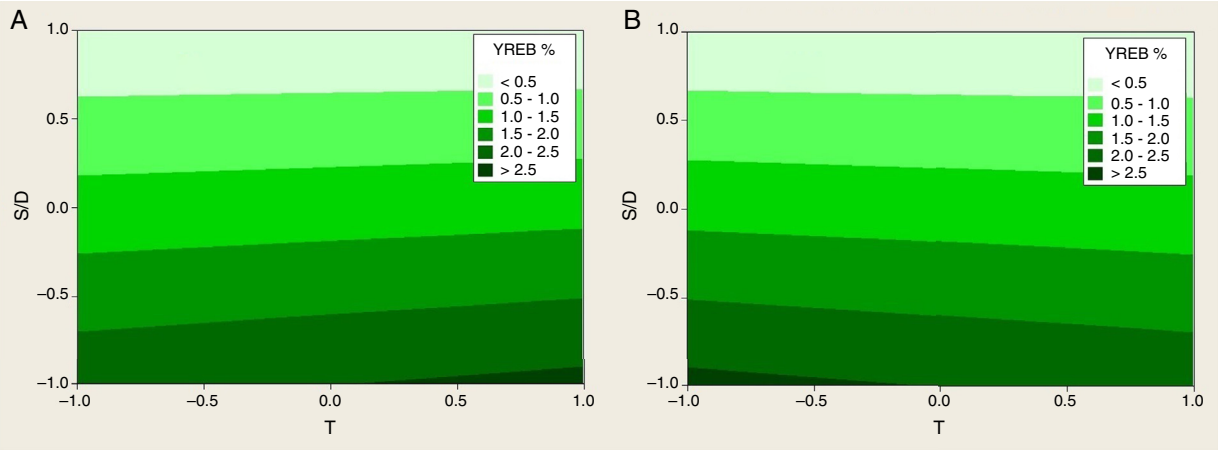

Fig. 5. Contour plots for rebaudioside A extraction percent yield (YREB, %) from *Stevia rebaudiana* leaves as functions of: (A) solvent/drug ratio (*S*/*D*) and temperature (*t*); and (B) solvent to drug ratio (*S*/*D*) and extraction temperature (*T*).

in Figs. 2A-3A and 2B-3B. As a matter of fact, the significant factors were the same for stevioside and rebaudioside A contents in fluid extract, which could be explained by the chemical similarity between these two glycosides. Due to this one may also conclude that finer powder need less solvent but larger amounts of solvent gives better extraction. Again for rebaudioside A the factors *t* and *T* did not affect extraction significantly, due to characteristics of the turbolysis technique. It is important to reinforce that the determination coefficients, or the adjusted *R* squared, given in Table 3 show very good fits of the models being 99.8% and 99.9% for CEST and CREB, respectively. The fitted equations for the multiple linear CEST and CREB dependency on the three significant factors, *D*50, SS and *S*/*D*, are show in Eqs. (6) and (7):

$$\text{CEST}\ \ (\text{mg/ml}) = 1.345 + 0.145\left(\frac{D50 - 480.5}{299.5}\right) - 1.185\left(\frac{SD - 20}{10}\right) - 0.114\left(\frac{SS - 12,500}{7500}\right) \quad (6)$$

$$\text{CREB}\ \ (\text{mg/ml}) = 0.725 + 0.080\left(\frac{D50 - 480.5}{299.5}\right) - 0.633\left(\frac{SD - 20}{10}\right) - 0.061\left(\frac{SD - 20}{7500}\right) \quad (7)$$

The stevioside and rebaudioside A yields are represented as percentages of the drug weight used in the experimental runs. The values are shown in Table 1 and correspond to 0.11% to 5.22% for stevioside and from 0.06%to 2.83% for rebaudioside A. The contour plot showing the response of stevioside yield, YST, as function of *S*/*D* and *t* is shown in Fig. 4A, while its response as function of *S*/*D* and *T* is shown in Fig. 4B. Fig. 4A shows higher YST decreases with *S*/*D* increase, e.g. decreases for higher amounts of solvent. Although the effect of *t* resulted significant in RSM analysis, the contour lines show that *S*/*D* influence is much stronger. The contour plot in Fig. 4B shows that *S*/*D* strongly affects YST as well, and lower solvent amounts gave higher stevioside yields. The effect of *T*, shown in contour lines of Fig. 4B is also less important than the *S*/*D*. These results are confirmed by the coefficients in Eq. (8), since coefficient for *S*/*D* is more than ten-fold higher. The rebaudioside A yields are shown in Fig. 5A and B. The YREB values are higher for coarse particles and low temperatures, as shown in the contour plot in Fig. 5A. The topographic view of YREB responses as function of *S*/*D* and *t* can be seen in Fig. 5B, which shows a strong effect of *S*/*D*. The data of YST and YREB were fitted to the model from Eq. (5), and resulted in Eqs. (8) and (9), with adjusted *R* squared of 99.1% and 99.0%, respectively (Fig. 6).

$$\text{YST}\ \ (\%) = 2.382 - 2.24\left(\frac{SD - 20}{10}\right) - 0.15\left(\frac{T - 51.5}{28.5}\right) \quad (8)$$

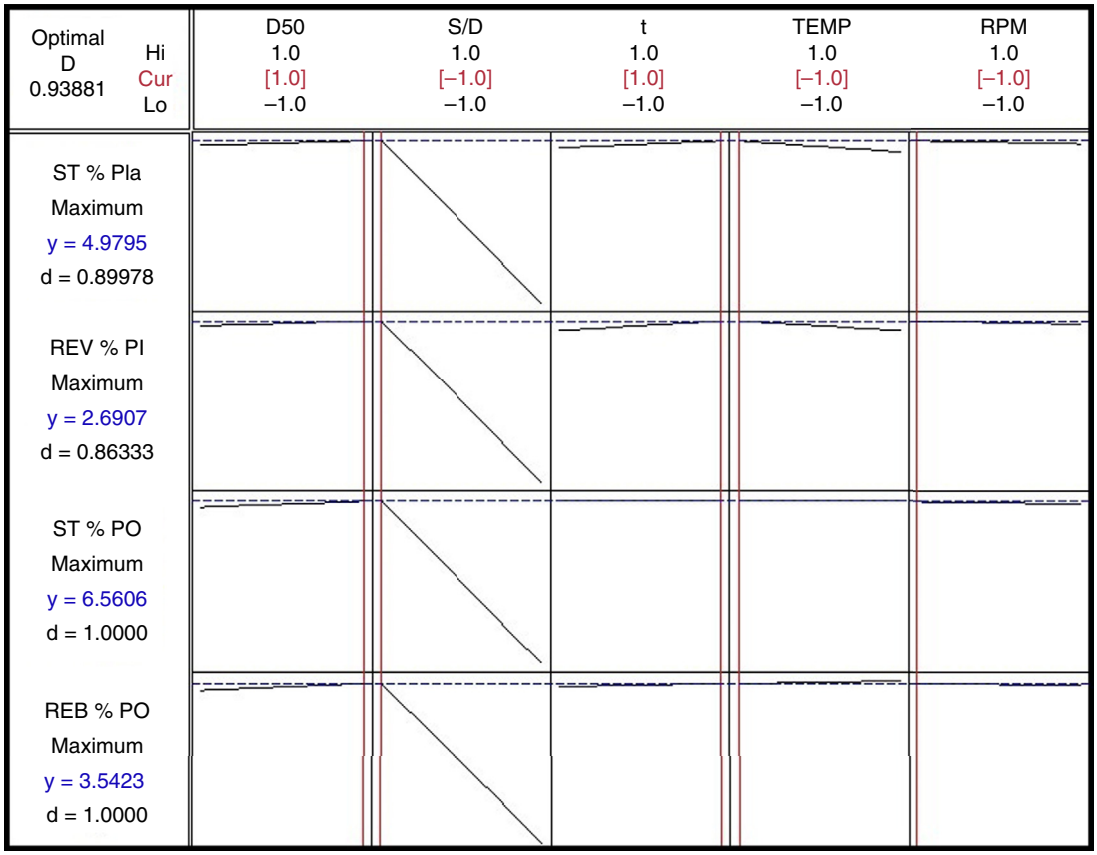

Fig. 6. Plot showing the desirability responses for stevioside and rebaudioside yields, YST and YREB, and their contents in dried extract, DST and DREB, for each of the following studied factors. Drug powder size: *D*50; temperature: *T*; time: *t*; turbolysis speed: SS, and solvent to drug ratio: *S*/*D*.

$$\text{YREB}\ \ (\%) = 1.281 - 1.2057\left(\frac{SD-20}{10}\right) + 0.071\left(\frac{t-10.5}{7.5}\right) - 0.071\left(\frac{T-51.5}{28.5}\right) \quad (9)$$

The values of stevioside and rebaudioside A content in dried extract, DST and DREB, respectively are given in Table 1. The stevioside content varied from 0.76 to 6.19% depending on turbo-extraction condition, but was affected significantly only by the solvent to drug ratio, *S*/*D*. On the other hand, DREB was affected significantly by *S*/*D* and also *D*50. The models were fitted to DST and DREB with adjusted *R* squared of 99.6 and 99.8%, respectively, and are given by Eqs. (10) and (11).

$$\text{DST}\ \ (\%) = 3.787 - 2.669\left(\frac{SD-20}{10}\right) \quad (10)$$

$$\text{DREB}\ \ (\%) = 2.049 + 0.059\left(\frac{D50-480.5}{299.5}\right) - 1.415\left(\frac{SD-20}{10}\right) \quad (11)$$

Recently, the application of multivariate analysis for pharmaceutical development has been recommended by the ICH-Q8-R2 (ICH, 2009) because this sort of approach turns possible the optimization of the process. Some methods of optimization, such as the desirability functions, allow the determination of the most suitable condition within the range of factors studied (Hu et al., 2008). Considering this, the models fitted in Eqs. (6)–(11) were used to carry out a desirability functions analysis (Hu et al., 2008) using Minitab 14 (Minitab, State College, USA). The settings to solve the models to find the conditions that maximize YST, YREB, DST and DREB simultaneously as well as the solution for best conditions are shown in Table 4. The desirabilities for each variable were 0.8998, 0.8633, 1.0000 and 1.0000 for YST, CST, YREB and CREB, respectively, which combined resulted in a global desirability of 0.9388. This is a very adequate global desirability and the best conditions for extraction correspond to *D*50 = 780 μm, *S*/*D* = 10 g/g; *t* = 18 min; *T* = 23 °C; SS = 5.000 rpm. The model could predict yields YST and YREB of 4.980% and 2.691%, while the glycosides contents were DST and DREB of 6.488% and 3.513%, respectively. The best extraction condition was carried out in triplicate and resulted in less than 5% deviation for the predicted values. These values are very close to the ones found for 18 h exhaustive extraction of *S rebaudiana* leafs, as shown in Table 2, demonstrating that turbolysis using ethanol 70% is very effective for stevioside and rebaudioside A extraction within a short time, moderate temperature and low solvent consumption. The literature on stevia reports solvent to drug ratio from 35 to 50 (Liu et al., 2010; Gasmalla et al., 2014; Koubaa et al., 2015) for better glycosides yields with water, but our data suggest best extraction with *S*/*D* of 10, which is not unusual ratio since many papers on herbal extracts support the results herein (Moura et al., 2005; Moura-Costa et al., 2012; Araújo et al., 2014; Jamal et al., 2014). Recently, the advantages of green extraction of *S. rebaudiana* by dynamic maceration (Martins et al., 2016) were reported, in a process that yielded 2.6 g of extract powder containing 12.89% of glycosides from 5 g of stevia leaves. The optimized condition of turbo-extraction in this work also resulted in 2.8 g of dried extract containing 10.0% of glycosides. However, the turbolysis process proposed herein can be advantageous since the optimal extraction time is only 18 min and is comparable to 1 h of dynamic maceration.

Table 4
Summary of desirability functions optimization.

Settings for optimization						Desirability	Maximized value
Responses	GOAL	LO	TGT	WT	IMP		
YST (%)	Maximize	3.00	5.20	1	1	0.8998	4.980
YREB (%)	Maximize	2.00	2.80	1	1	0.8633	2.691
DST (%)	Maximize	5.00	6.50	1	1	1.000	6.488
DREB (%)	Maximize	3.00	3.50	1	1	1.0000	3.513

Global desirability: 0.9388.
LO, lower value; TGT, target value; WT, weight of response; IMP, importance of response.
Optimum set of conditions for turbo extraction of STEVIA: $D50 = 780\ \mu m$, $S/D = 10\ (g/g)$; $t = 18$ min; $T = 23\ ^{\circ}C$; SS = 5000 rpm.
Stevioside and rebaudioside yields YST (%) and YREB (%). Stevioside and rebaudioside contents in dried extract, DST (%) and DREB (%).

Conclusions

The GRAS and green solvent ethanol 70% showed to be the best solvent for glycosides extraction from *S. rebaudiana* leaves, according to the turbolysis at room temperature. High yields of glycosides extraction can be attained by low solvent to drug ratios in turbo-extraction, coarse drug powder, low temperatures, low shear rates and shorter times. The stevioside and rebaudioside A yields obtained in this work are superior to the ones found previously using other solvents and other techniques. The results for the two glycosides turbolysis extraction are comparable to the ones recently published for dynamic maceration, but with the advantage of shorter extraction times. The results herein stimulate new research on *S. rebaudiana* glycosides extraction by turbolysis and the evaluation of those extracts in further purification steps.

Authors' contributions

PMM contributed in running the pharmacognostic characterization, extractions and manuscript preparation. ADL contributed to the chromatographic analysis and quantification of the glycosides. BNT contributed to manuscript preparation and critical reading. LAPF contributed to experimental design, data analysis and manuscript preparation. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

Financial support from CNPq for grant 401044/2013-0 under the "Cooperation Agreement MCTI-CNPq (Brazll) & DST (India)" and the scholarship (CNPq 202564/2014-1) to Prof P.M. Martins (Universidade de Brasília) are gratefully acknowledged.

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