



Clarification of *Stevia rebaudiana* Bertoni extract by Ca based silica microspheres

Gamze Gunduz Meric^a, Fatih Gegic^a, Sema Leblebici^b, Sifanur Gungor^b, Levent Degirmenci^{a,*}

^a Bilecik Seyh Edebali University, Department of Chemical Engineering, Bilecik, 11210, Turkey

^b Bilecik Seyh Edebali University, Department of Horticulture, Bilecik, 11210, Turkey

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ABSTRACT

Pre-treated *Stevia rebaudiana* Bertoni extract was clarified via using calcium (Ca) based silica (Si) microspheres. Both adsorption and chemical treatment were applied to extracts, and their effects on clarification were determined by comparison. The study aimed to maintain the highest level of clarity by eliminating chemicals from the treatment procedure. Although close in range with chemical treatment, the highest level of clarity was obtained in the presence of sole Ca/Si application. Clarification was achieved in an interval of 95–99 % with applied procedures. Sustainable utilization of Ca/Si microspheres was investigated by conducting three consecutive adsorption cycles with used microspheres. Results indicated minor changes in clarification levels at the end of three straight runs with used microspheres. Sustainable adsorption of impurities from the extract without the use of additional chemicals was the highlight of the present study.

1. Introduction

Stevia rebaudiana Bertoni is the only species giving the sweet essence among 230 species of the genus *Stevia*. *Stevia* and glycosides, namely stevioside and rebaudioside are extracted from its leaves, and these natural ingredients are currently being used as a sweetener alternative (Zhang et al., 2017; Yucesan et al., 2016). Although being cultivated in South America, this herbaceous plant is also found in North America, Asia, and Europe (Zhang et al., 2017; Yu et al., 2017; Yildiz-Ozturk et al., 2015). Extraction of stevioside and rebaudioside is conducted by infusion of powdered dry leaves in water or certain organic solvents such as methanol, ethanol, and butanol (Martins et al., 2016; Mahl et al., 2010; Siddique et al., 2016). Highest yields in the separation of glycosides were obtained via aqueous separation. However, pigments such as chlorophyll, carotene, and xanthophyll are also leached to solution as impurities (Martins et al., 2016; Mahl et al., 2010). In other words, the removal of these impurities rather than glycosides is the main problem in extraction.

In order to solve impurity problem in solution, either extraction process is modified to yield products with higher clarity using organic solvents (Siddique et al., 2016) or aqueous extract is purified with specific methods involving two phases aqueous extraction, consecutive

utilization of metallic ions and organic solvents, ultra-filtration, combined nano and ultra-filtration and membrane technology (Phong et al., 2018; Moraes and Machado, 2001; Chhaya et al., 2011; Chhaya et al., 2012; Reis et al., 2007; Das et al., 2015). Both approaches were proven to be effective in increasing clarity of the extract. However, utilization of organic solvent in the extraction required the separation of organic solvent from the extract which would cause an increase in manufacturing cost and more importantly these solvents are harmful to human health even if they remained in solution in trace amounts. Among these, chloroform used in extraction can be given as an example. This chemical had previously been classified as group 2B carcinogen (Verma et al., 2018). Membrane technology is highly effective in clarification. However, this is an emerging technology still coping with clogging problems and operational costs to actuate this process are very high. Health problems could be reduced by utilization of water as the solvent in the extraction and increase in clarity of the extract could be maintained using adsorbents, which are less priced compared to membranes. An example of these adsorbents is alginate beads, which were utilized at low pH levels in the work of Mahl et al. (2010) for successful clarification of pigments present in extract solution. High levels of clarification were achieved in the study at the expense of glycosides loss by adsorption. NaX and NaA zeolites were used in the work of Moraes and Machado (2001). These were

* Corresponding author.

E-mail address: levent.degirmenci@bilecik.edu.tr (L. Degirmenci).

ion-exchanged with calcium (Ca), and barium (Ba) ions and CaX, CaA, BaX, and BaA zeolites were used in the clarification of Stevia. Results indicated 70–80 % clarification in the presence of Ca-X contacted with aqueous extract. Clarification was further increased to 91.75 % in the work of [Silva et al. \(2007\)](#) with combined utilization of membrane filtration and Ca-X zeolite.

Microspheres, originally developed as carriers in controlled drug release, were successfully used in a variety of applications ([Ciriminna et al., 2011](#); [Yang et al., 2013](#); [Majewski et al., 2013](#); [Han et al., 2008](#); [Zhu et al., 2013](#)). Silica (Si) microspheres were also proven to be useful in our recent studies ([Degirmenci and Orbey, 2013](#); [Gunduz-Meric and Degirmenci, 2016](#); [Gunduz-Meric et al., 2017](#)). Ca/Si microspheres were utilized as an adsorbent in the present study to maintain efficient clarification of *Stevia rebaudiana* Bertoni extracts. Consecutive extraction of Stevia leaves was performed in the presence of chloroform, ethanol, and butanol. Ca/Si microspheres were utilized as the final step of clarification. The primary aim was to develop an alternative and also an economically feasible method to increase clarification of Stevia extract via Ca/Si microsphere utilization.

2. Materials and methods

2.1. Microsphere preparation

Ca-based silica microspheres were prepared by sol-gel microencapsulation method applied with minor modifications ([Gunduz-Meric and Degirmenci, 2016](#); [Gunduz-Meric et al., 2017](#)). 0.5 g CTAB (hexadecyltrimethyl ammonium bromide, Merck) and varying amounts of CaCl_2 (Merck) were dispersed in 20 ml deionized water by ultra-sonication for 15 min. The resulting solution was homogenized in a mixture of 50 ml ethanol and 10 ml 25 wt. % ammonia solution. 5 ml of TEOS (tetraethyl ortho silicate, Merck) was added dropwise to this resulting solution. The amount of Ca inside the microspheres was determined based on the weight of Ca to the amount of silicon inside TEOS and was altered between 1–8 %. Selection of the microsphere to be used in clarification experiments was conducted based on the amount of lowest loss of Ca during microsphere formation. Ca containing solution was mixed for 360 minutes at room temperature to facilitate the formation of spherical structure. The obtained product was washed with ethanol and deionized water for 3 times and dried at room temperature for 24 h. The microspheres were initially heated up to 750 °C with a heating rate of 1 °C/min. Calcination was then conducted at this temperature for 6 h.

2.2. Characterization of microspheres

X-ray diffraction (XRD), Scanning electron microscopy (SEM) and Atomic absorption spectrometry (AAS) techniques were used in the characterization of synthesized microspheres. The XRD patterns were obtained by a Panalytical Empyrean instrument ($\lambda: 1.5418 \text{ \AA}$) at 200 kV and 50 mA in the range of 2θ between 5° and 80° with a speed of 10°/min. The surface morphology of microspheres was determined via SEM analysis in a Zeiss Supra 40 V device.

AAS was applied to solutions obtained via consecutive washing of microspheres with water and ethanol to determine the amount of Ca in the washing solution. Results were interpreted, and Ca loading was selected according to the lowest amount of Ca in the washing solution. The result obtained from AAS was accepted as the threshold of Ca loading in the microsphere.

2.3. Extraction process

Dried Stevia leaf powder (5 g) were extracted by shaking bath in three consecutive steps involving pretreatment with chloroform, extraction with ethanol and pretreatment with butanol. All the chemicals utilized in the process was taken as 50 ml. Although these pretreatment procedures are an extraction procedure, we insisted on using this term as their effects

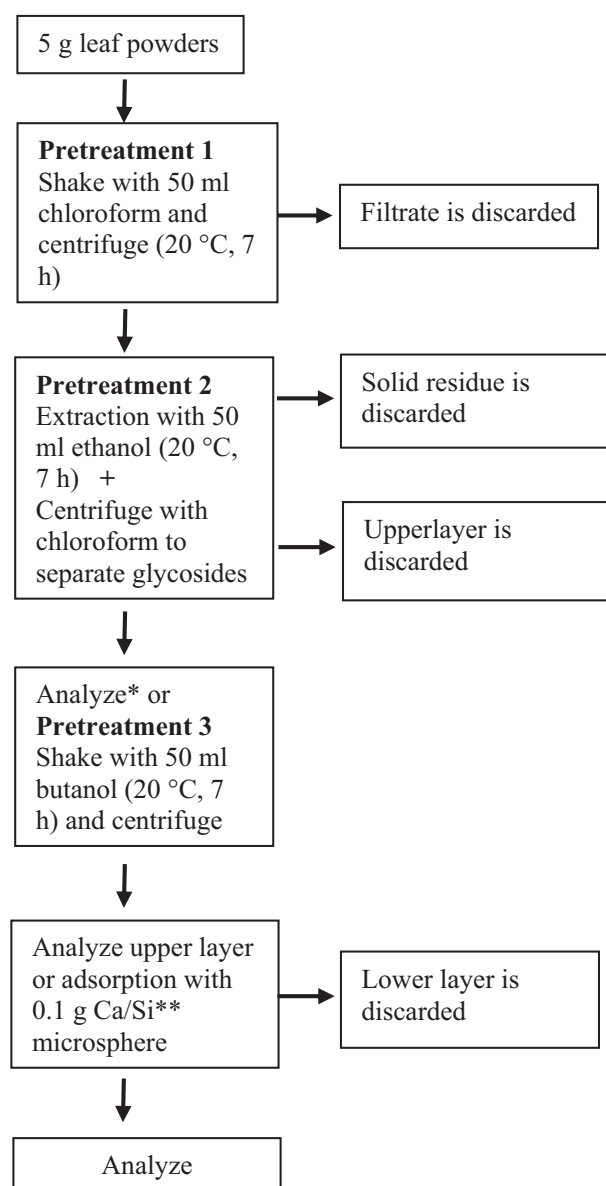


Fig. 1. Scheme of the procedures applied in the present study (* Stevia extract was crystallized in deepfreeze for 24 hours. The extracts were then analyzed by dissolving in ethanol to determine the change in total carbohydrates and clarity) (** The amount of microspheres (0.1 g) and their calcium content (4%) were identical for all procedures).

on clarification and total organic content were investigated by comparing their optional utilization during extraction and clarification. Adsorption experiments in the presence of Ca/Si microspheres were also conducted based on the choice to provide a comparison with treatment procedures. The procedures applied in this study were a modification of a procedure applied in the work of [Afandi et al., \(2013\)](#). The steps of a typical process were illustrated in [Fig. 1](#) and the applied procedures were summarized in [Table 1](#). Analyses applied for clarification, and total carbohydrates were elaborated in Section 2.4. Extraction and adsorption procedures applied in the course of study were illustrated in [Table 1](#). Reusability of Ca/Si microspheres was investigated with 3 consecutive adsorption cycles applied to Stevia extract obtained at the end of the 5th procedure ([Table 1.](#)).

2.4. Analysis of stevia extract

The clarification was determined as % clarification via measuring absorbances (A) at 420 and 670 nm. Analyses were conducted with

Table 1

Pretreatment, Extraction and Adsorption procedures applied for the clarification of Stevia extract.

Procedure No	Pretreatment with chloroform	Extraction with ethanol	Pretreatment with butanol	Adsorption with Ca/Si (4%) microspheres
1	++	+	+	–
2	–	+	+	–
3	+	+	–	+
4	+	+	–	+
5*	–	+	–	+
6	–	+	+	+

(* 5th procedure was applied thrice to determine reusability of Ca/Si microspheres).

(** “+” and “–” signs indicated whether treatment, extraction and adsorption was applied during procedure).

Agilent Cary 60 UV-Vis spectrophotometer. Total carbohydrates were also determined as % by measuring the absorbance of the reaction mixture at 490 nm. Calculations were conducted in comparison with standard solutions. Samples were considered as “clarified” only after pretreated with butanol and treated with Ca/Si microsphere. % clarification was calculated by Eq. (1), according to Moraes and Machado (2001):

$$\% \text{ Clarification} = \left[1 - \frac{(A_{420} \text{ or } A_{670}) \text{ before}}{(A_{420} \text{ or } A_{670}) \text{ after}} \right] \times 100 \quad (1)$$

The subscripts “before” and “after” relate to absorbance measured before and after clarification process.

The total carbohydrate (TCH) was calculated by Eq. (2), according to Moraes and Machado (2001):

$$TCH (g \%) = \left[\frac{L_A}{L_S} \right] \times 4,5045 \quad (2)$$

With L_A and L_B corresponding to sample and standard absorbance, respectively. Analyses solutions for total carbohydrates determination was prepared by mixing 10 μ l of the extract with 10 ml water. Sample, blank, and standard solutions are prepared according to Moraes and Machado (2001) as:

Sample solution: 500 μ l phenol +5 %, 500 μ l extract diluted with 2.5 ml concentrated sulfuric acid.

Blank solution: 500 μ l phenol +5 %, 500 μ l water diluted with 2.5 ml concentrated sulfuric acid.

Standard solution: 500 μ l phenol +5 %, 500 μ l glucose 4.5 % (w/w) diluted with 2.5 ml concentrated sulfuric acid.

The mixtures were kept for 15 minutes and then analyzed in a UV-Vis spectrophotometer at 490 nm, against the blank solution.

3. Results and discussion

Atomic absorption spectroscopy analyses conducted on washing solutions of microspheres were illustrated in Table 2. Results indicated an increase of Ca in washing solution with loading amount except for 4 % Ca loading. Hence 4 % was selected as the threshold of loading in microsphere synthesis.

Table 2

Atomic absorption spectrometry (AAS) analysis of Ca microspheres (Analyses were conducted in GBC933AA device with hollow cathode Ca lamp (Wavelength 422.7, GBC).

Ca % content in microsphere	Absorbance
1	0.059
2	0.105
4	0.025
8	1.531

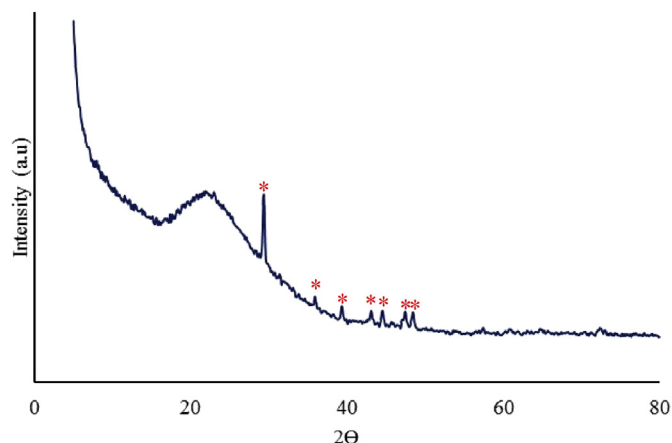


Fig. 2. X-ray diffraction patterns of Ca/Si (4%) microspheres (λ :1.5418 Å) at 200 kV and 50 mA in the range of 2θ between 5° and 80° with a speed of 10°/min; * indicates CaO crystal phases).

Next step for the characterization of microspheres was XRD analysis to validate the presence of Ca inside microsphere. XRD analysis revealed the presence of Ca with 2θ values obtained at 9.3°; 39.3°; 43.1°; 44.5°; 47.5°; 48.4° and 72.2° (Fig. 2). Peak values, determined from Inorganic Crystal Structure Database (ICSD), corresponded to CaO formation in microsphere and were expected due to calcination procedure applied to microspheres. SEM analysis validated microsphere formation via applied synthesis procedure (Fig. 3).

Procedures mentioned in Table 1 were applied in a shaken batch system operated at 20 °C. Clarification and TCH values of the extract were illustrated in Fig. 4. Clarity % was low for procedures 3 and 4, whereas the highest clarity was obtained with procedures 2 and 5, which were conducted without chloroform treatment. On the other hand, Stevia leaves were pretreated with chloroform before extraction in procedures 3 and 4. This result indicated that chloroform had been ineffective in the clarification of Stevia extract. Procedure 5 included the utilization of only Ca/Si microspheres as a pretreatment/adsorption step before extraction. Procedure 2, included only butanol pretreatment before extraction. The clarification % for both procedures was in close range, and results implied utilization of either procedure be adequate for efficient clarification. However, removal of butanol from extract would be an economic challenge and considering health problems mentioned earlier, the application of the 5th procedure would be beneficial. Comparing TCH values of 2nd and 5th procedures indicated higher values when the 5th procedure was applied. This result implied lower loss of the material

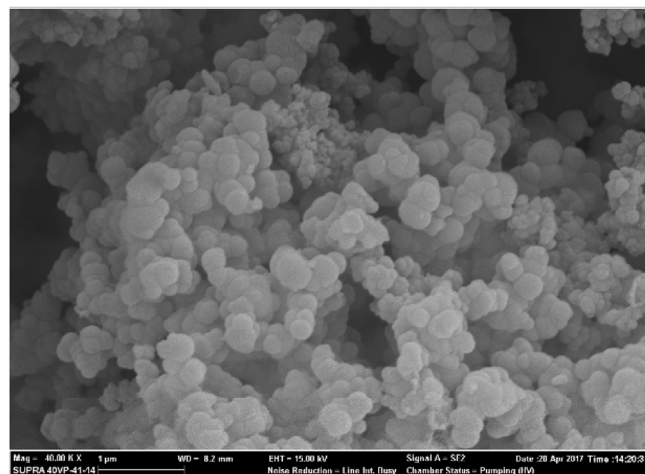


Fig. 3. SEM images of the Ca/Si (4%) microspheres.

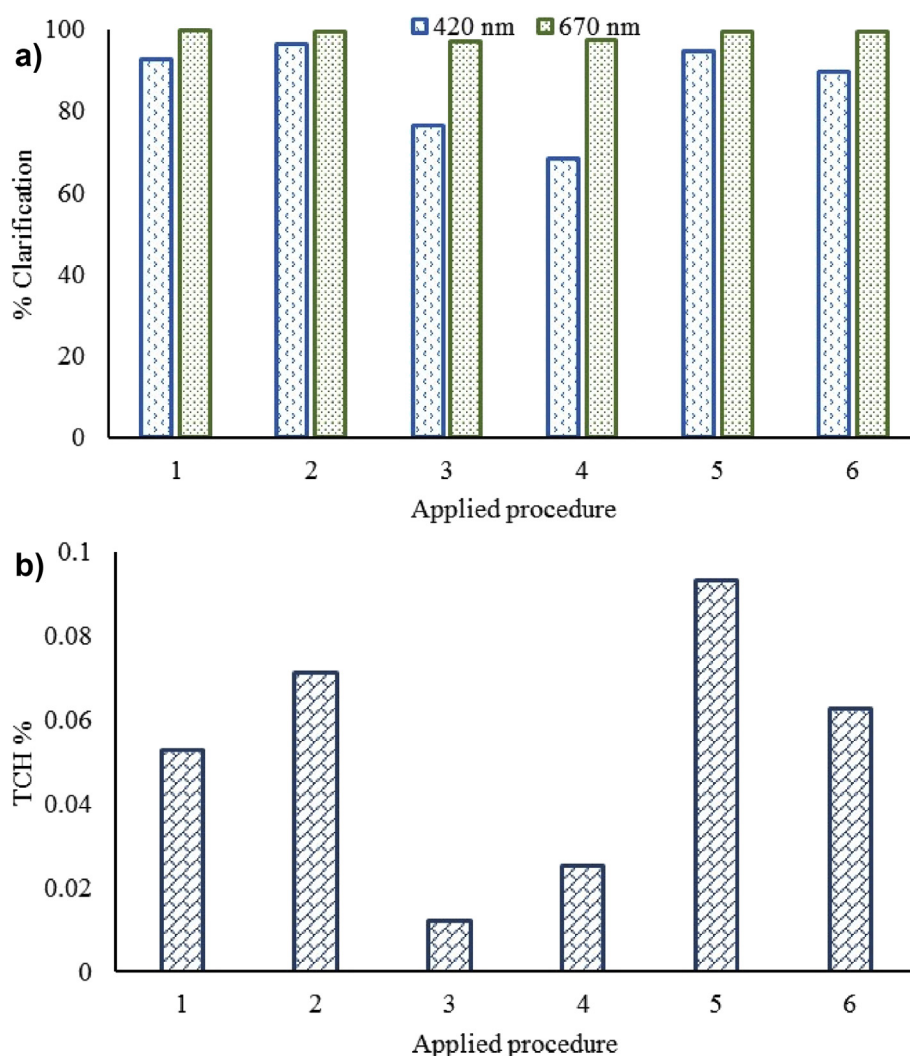


Fig. 4. Effect of applied procedure on a) clarity and b) TCH of % of Stevia extract.

during the procedure and more importantly, negligible or none glycoside adsorption during the procedure. In our opinion, the application of fewer chemical treatment steps would also be useful in decreasing losses of

active material emanated from the procedure. Hence 5th procedure should be preferred to minimize losses and maintain an economically feasible production. A similar conclusion could be reached with TCH

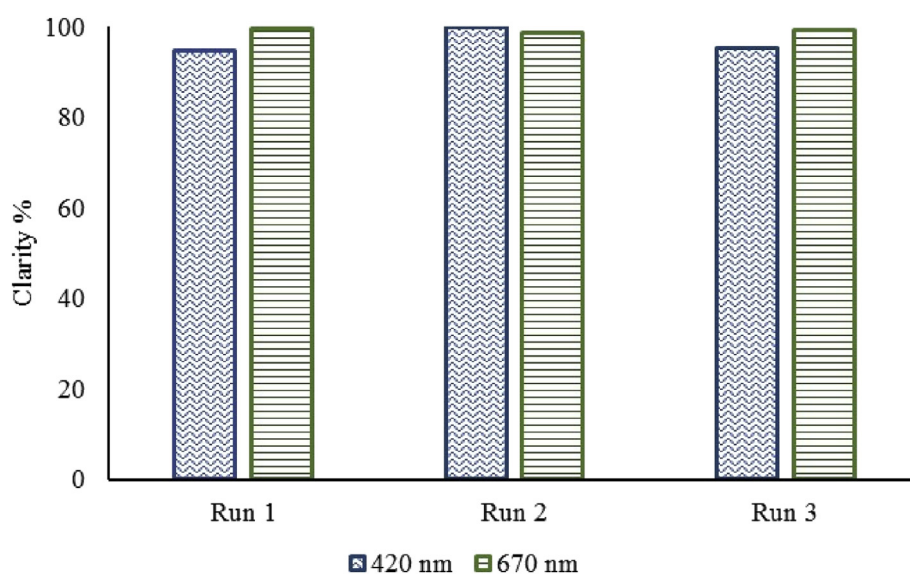


Fig. 5. Clarity values obtained with 5th procedure in repeated runs.

values of 2nd and 3rd procedures, which were the lowest among all procedures. We believed that loss of extract during the application of the procedures had been inevitable. Having said that, it could be minimized by decreasing the number of steps applied in extraction, the 5th procedure involved the lowest number of steps along with the 2nd procedure and also included a lower amount of chemical utilization, which was considered as an advantage over the 2nd procedure.

Adsorption when applied as treatment, was shown to be superior over chemicals utilized for clarification. Another advantage of these microspheres would be their repeated use without any loss in clarification values. Ca/Si microspheres utilized in adsorption were reused three times. Microspheres were removed from the system, and adsorption was conducted in the presence of microspheres obtained from the system without further treatment. Change of clarity values in repeated use was given in Fig. 5. Results indicated a negligible change of clarification, and the values remained higher than 94 %. This was one of the highlights of the study, along with the elimination of chemicals from treatment procedures.

In the present study, Ca/Si microspheres were introduced as an alternative for clarification of the extract. The extraction process was conducted in the presence of ethanol with chloroform and butanol used in pretreatment steps. Results indicated that the utilization of chemicals had not been necessary for increasing clarification of the extracts as similar values could have been obtained when Ca/Si microspheres were solely utilized in the procedure (5th procedure). TCH values were lower than expected, which could be increased by increasing the amount of active material used in the extraction and modifying the procedure applied for extraction. Some loss of extract was observed due to the high number of steps in treatment, increasing the possibility of experimental error. Ca/Si microsphere was introduced as a highly efficient, viable, adsorbent alternative with the present study.

4. Conclusion

Efficient and sustainable clarification of *Stevia rebaudiana* Bertoni extract was achieved by Ca/Si microsphere. Results indicated high values of clarification comparable to organic solvent utilization. Microsphere utilization in clarification would eliminate potential health risks associated with the use of organic solvents and an economical alternative with a feasible synthesis procedure was introduced to preexisting methods including consecutive utilization of metallic ions with organic solvents, aqueous two-phase organic solvent extraction, ultrafiltration, and membrane separation.

Declarations

Author contribution statement

Gamze Gunduz Meric, Fatih Gegic, Sema Leblebici: Performed the experiments.

Sifanur Gungor: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Levent Degirmenci: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

References

- Afandi, A., Sarijan, S., Shaha, R.K., 2013. Optimization of rebaudioside A extraction from *stevia rebaudiana* (Bertoni) and quantification by high-performance liquid chromatography analysis. *J. Trop. Resour. Sustain. Sci.* 1 (1), 62–70. ISSN: 2289-3946.
- Chhaya, Sharma, C., Mondal, S., Majumdar, G.C., De, S., 2011. Clarification of Stevia extract by ultrafiltration: selection criteria of the membrane and effects of operating conditions. *Food Bioprod. Process* 90 (3), 525–532.
- Chhaya, Mondal, S., Majumdar, G.C., De, S., 2012. Clarification of stevia extract using cross flow ultrafiltration and concentration by nanofiltration. *Separ. Purif. Technol.* 89, 125–134.
- Ciriminna, R., Scirtino, M., Alonzo, G., de Schrijver, A., Pagliaro, M., 2011. From molecules to systems: sol-gel microencapsulation in silica based materials. *Chem. Rev.* 111, 765–789.
- Das, A., Paul, D., Golder, A.K., Das, C., 2015. Separation of Rebaudioside-A from stevia extract: membrane selection, assessment of permeate quality and fouling behavior in laminar flow regime. *Separ. Purif. Technol.* 144, 8–15.
- Degirmenci, L., Orbey, N., 2013. Microencapsulation of silicotungstic acid to retain catalytic activity. *Ind. Eng. Chem. Res.* 52, 16714–16718.
- Gunduz-Meric, G., Degirmenci, L., 2016. Silika ile mikroenkapsüle edilmiş Fe₂O₃ içeren kürelerin üretim prosesinin iyileştirilmesi ve katalitik aktivitelerinin belirlenmesi. *J. Faculty Eng. Archi. Gazi Univ.* 31, 277–283.
- Gunduz-Meric, G., Arbag, H., Degirmenci, L., 2017. Coke minimization via SiC formation in dry reforming of methane conducted in the presence of Ni-based core shell microsphere catalysts. *Int. J. Hydrogen Energy* 42, 16579–16588.
- Han, L., Shan, Z., Delong, C., Yu, X., Yang, P., Tu, B., Zha, D., 2008. Mesoporous Fe₂O₃ microspheres: rapid and effective enrichment of phosphopeptides for MALDI-TOF MS analysis. *J. Colloid Interface Sci.* 318, 315–321.
- Mahl, C.R.A., de Oliveira, S.P.D., da Silva, B.B., da Igreja, G., Simões, M.R., da Silva, C.F., 2010. Effect of pH on the clarification of *Stevia rebaudiana* Bertoni extract using alginate beads. *Adsorpt. Sci. Technol.* 28, 189–194.
- Majewski, A., Wood, J.J., Bujalski, W., 2013. Nickel-silica core@shell catalyst for methane reforming. *Int. J. Hydrogen Energy* 38 (34), 14531–14541.
- Martins, P.M., Thorat, B.N., Lanchote, A.D., Freitas, L.A.P., 2016. Green extraction of glycosides from *Stevia rebaudiana* (Bert.) with low solvent consumption: a desirability approach. *Resour. Efficient Technol.* 2, 247–253.
- Moraes, É.P., Machado, N.R.C.F., 2001. Clarification of *Stevia rebaudiana* (Bert.) Bertoni extract by adsorption in modified zeolites. *Acta Sci., Maringá-PR* 23, 1375–1380.
- Phong, W.N., Show, P.L., Chow, Y.H., Ling, T.C., 2018. Recovery of biotechnological products using aqueous two phase systems. *J. Biosci. Bioeng.* 126 (3), 273–281.
- Reis, M.H.M., Da Silva, F.V., Andrade, C.M.G., Rezende, S.L., Wolf Maciel, M.R., Bergamasco, R., 2007. Clarification and Purification of Aqueous Stevia Extract Using Membrane Separation Process.
- Siddique, A.B., Rahman, S.M.M., Hossain, M.A., 2016. Chemical composition of essential oil by different extraction methods and fatty acid analysis of the leaves of *Stevia Rebaudiana* Bertoni. *Arabian J. Chem.* 9, 1185–1189.
- Silva, F.V., Bergamasco, R., Andrade, C.M.G., Pinheiro, N., Machado, N.R.C.F., Reis, M., Araujo, A.A., Rezende, S.L., 2007. Purification process of Stevioside using zeolites and membranes. *Int. J. Chem. React. Eng.* 5 (40), 1–6.
- Verma, P., Tordik, P., Nosrat, A., 2018. Hazards of improper dispensary: literature review and report of an accidental chloroform injection. *J. Endod.* 44 (6), 1042–1047.
- Yang, D., Liu, Q., Guo, X., Rong, H., Cheng, M.L., Wang, G., 2013. Folic acid functionalized magnetic ZnFe₂O₄ hollow microsphere core/mesoporous silica shell composite particles: synthesis and application in drug release. *Mater. Sci. Eng. C* 33, 2879–2884.
- Yildiz-Ozturk, E., Nalbantsoy, A., Tag, O., Yesil-Celiktas, O., 2015. A comparative study on extraction processes of *Stevia rebaudiana* leaves with emphasis on antioxidant, cytotoxic and nitric oxide inhibition activities. *Ind. Crops Prod.* 77, 961–971.
- Yu, H., Yang, G., Sato, M., Yamaguchi, T., Nakano, T., Xi, Y., 2017. Antioxidant activities of aqueous extract from *Stevia rebaudiana* stem waste to inhibit fish oil oxidation and Identification of its phenolic compounds. *Food Chem.* 232, 379–386.
- Yucesan, B., Mohammed, A., Büyükgöçmen, R., Altuğ, C., Kavas, Ö., Gürel, S., Gürel, E., 2016. In vitro and ex vitro propagation of *Stevia rebaudiana* Bertoni with high Rebaudioside-A content- A commercial scale application. *Sci. Hortic.* 203, 20–28.
- Zhang, Q., Yang, H., Li, Y., Liu, H., Jia, X., 2017. Toxicological evaluation of ethanolic extract from *Stevia rebaudiana* Bertoni leaves: genotoxicity and subchronic oral toxicity. *Regul. Toxicol. Pharmacol.* 86, 253–259.
- Zhu, D., Zhang, J., Song, J., Wang, H., Yu, Z., Shen, Y., Xie, A., 2013. Efficient one-pot synthesis of hierarchical flower-like α-Fe₂O₃ hollow spheres with excellent adsorption performance for water treatment. *Appl. Surf. Sci.* 284, 855–861.