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Ultrasound-assisted Extraction Process of Glycosides from Stevia Rebaudiana Bertoni Leaves

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Stevia Rebaudiana Bertoni leaves contain glycosides that have a sweetening power of 100 to 300 times greater than sucrose. These glycosides are increasingly used as natural sweetener, mainly to replace aspartame, in order to reduce daily caloric intake. The two major glycosides are Stevioside and Rebaudioside A, which represent about 90 % [w/w] of total Steviol. The Rebaudioside A is the most interesting since it has the advantage of not having a bitter aftertaste, unlike stevioside. In recent years, the cultivation of organic stevia is growing in the Occitanie region in France. Thus, the valorization of this production chain is of economic interest for this region. It's known that conventional extractions to obtain these glycosides are time consuming and the extensive use of organic solvents may be harmful for human health and environment. Thus, the objective of this work was to develop a green extraction process suitable for industrial purposes, using water as solvent and ultrasound technique to obtain high yield of glycosides in the Stevia extract. The extractions are carried out in batch with recirculation and continuous assemblies at 45 °C. The effects of ultrasound power (at 20kHz), experience time/ residence time, and leaves concentration are studied. The soluble solids and stevia glycosides concentration are analyzed respectively by °Bx and HPLC. With the continuous assembly the experiments were carried out at 64 W and 144 W, which represents 50 % and 100 %, respectively, of the generator capacity. A low flowrate (or high residence time) is detrimental to the use of ultrasound, leaves sedimentation and their accumulation prevents probably the ultrasound diffusion in the medium, harming the steviol extraction. The extraction ultrasound-assisted allowed to increase in 9-10 % °Bx concentration for batch and continuous assembly, compared to extraction without ultrasound under the same conditions. Soluble species other than steviols are extracted with ultrasound. The HPLC results showed that the ultrasound favored lightly the extraction of stevioside.
Key-words: continuous extraction, green extraction, sweeteners.

1. Introduction

Stevia Rebaudiana is part of the Asteraceae family and is a perennial shrub of native to certain regions of South America (Gasmalla et al., 2017). It has been also cultivated in China or Southeast Asia for a few decades (Liu et al., 2010). Nowadays, there are many production fields of organic Stevia Rebaudiana in Occitanie Region in France. This herb became known for its natural non-caloric sweetness and it is considered a substitute for synthetic sweeteners.
Stevia Rebaudiana Bertoni plant contains several glycosides. Stevioside and Rebaudioside A, represent about 90 % [w/w] of total Steviol, whereas Rebaudioside C, D, E, F, Dulcoside A, steviolbioside and Rubusoside have been identified as minor components of the stevia. These glycosides are on average about 200-300 times sweeter than sucrose (Chranioti et al., 2016). To being sweeter than sucrose and because of its several advantages, extraction processes of steviol glycosides are increasing market interest.
It is known that conventional extraction processes to obtain these glycosides present many disadvantages such as the use of toxic organic solvents in high quantities, time consuming, difficult to remove residual solvent completely, possibility of thermal degradation due to high temperature and lengthy extraction period

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(Easmin et al., 2015). Therefore, to contribute to the reduction and/or elimination of them, in recent years, the food and pharmaceutical industry express growing interest in the development and application of new methods. Several studies aim to use non-conventional methods such as microwaves, ultrasound, high voltage electrical discharges, etc., which can reduce the extraction time, decrease the operating temperature and solvent consumption, in addition to obtaining greater efficiency of the extracted components and lower energy consumption compared to conventional methods (Kujundžic, et al., 2017).
The application of ultrasound has proven to be extremely effective in the extraction of various types of compounds, compared with classical methods like maceration and heat extraction for example. Because of the mechanical effects of ultrasound, which provide greater penetration of solvent into cellular materials and the mass transfer of compounds that dissolve in the solvent is substantially improved. The ultrasound cavitation energy alone will enable the destruction of the plant cell walls, and thus facilitate the release of cell contents into the solvent (Gasmalla et al., 2017). Thus, the objective of this work was to develop a green extraction process suitable for industrial purposes, using water as solvent and ultrasound technique.

2. Material and methods

Material: shredded stevia leaves from Occitanie region in France, are kindly provided by Oviatis (Agen, France). The size distributions are measured by laser granulometry. The Sauter diameter, D [3;2], is given in the text. For the extractions assisted by ultrasound (US), an ultrasonic horn at 20 kHz controlled by a NEXTGEN LAB 750 generator from SINAPTEC (France) is used. The amplitude of sonication is adjustable from 0 to 100 %. The values indicated during the tests give an order of magnitude of the dissipated power: 50 % (64 W) and 100 % (144 W). Two devices are used for the extractions (continuous and batch with recirculation), as shown in Figure 1. The volumes of the cell equipped with ultrasound horn and of the reactor used in the device with recirculation are 123 mL and 1 L, respectively. For the batch experiments, the leaves are introduced into water at 45 °C. During the extraction, samples are taken and filtrated at the exit of the cell in continuous mode or in the vessel in the batch mode. Analysis of soluble solids in °Bx are performed using a Leica AR600. HPLC method are carried out in order to measure the concentrations of Stevioside and Rebaudioside A using a column (Zorbax carbohydrate, 150 mm x 4.6 mm, 5 µm) and UV detector set at 205.4 nm. The mobile phase is composed of 70 % acetonitrile and 30 % water (v/v) with a flowrate of 1 mL/min. The total steviol concentration is represented by the sum of Rebaudioside A and Stevioside concentrations.

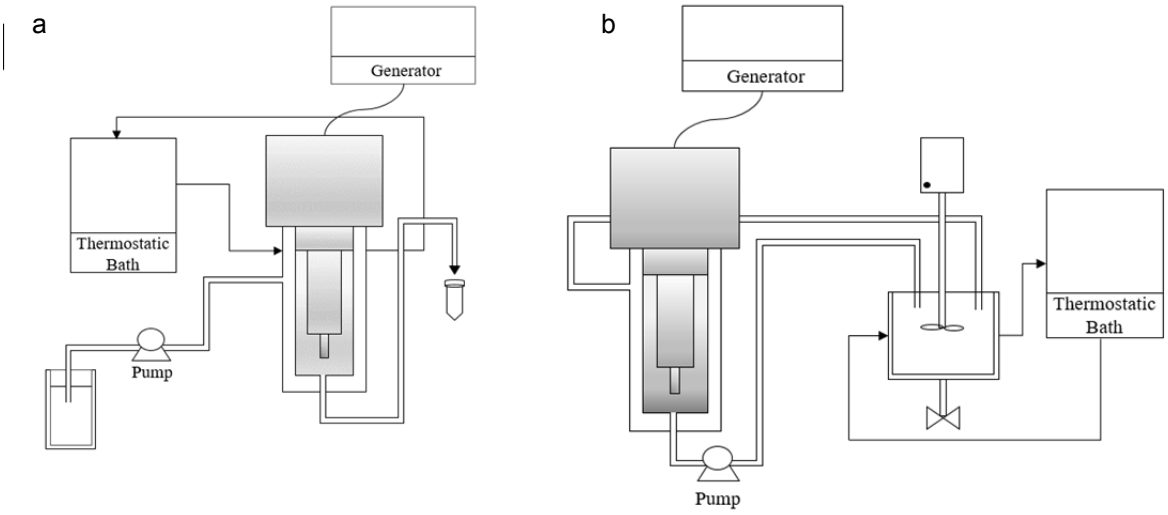

Figure 1: Representative scheme of continuous (a) and batch with recirculation (b) devices (schematic diagram, the size between equipment is not respected).

3. Results

Batch extraction

Batch extractions are performed in a stirred batch vessel of 1 L with the objective of knowing the maximum concentration of steviol extracted. The extractions are carried out at 45 °C for approximately 22 h with different leaves concentration (50 to 150 g of leaves/L of water). Distillated water is used as solvent. The results are summarized in Table 1.

Three sets of leaves are used. The first one is supplied grinded by Oviatis. The second one is obtained by grinding leaves and branches for 30 s using an IKA M20 grinder. The third corresponds to the size class below 250 µm obtained after sieving the set 2 (leaves and branches) before grinding.
The set 2 presents a slightly higher steviol content 0.400 instead of 0.300-0.337 g/100 g solution. As it is possible to see in Table 1 the variation in °Bx and steviol content, for the same set, is proportional to the leaves concentration.

Table 1: Summary of the results obtained by the batch tests performed at 500 rpm.

set	Concentration (g/L)	D [3;2] (µm)	°Bx (g/100g solution)	Steviol (g/100g solutions)
1	50	30 ± 1	2.05 ± 0.01	0.307 ± 0.002
1	100	30 ± 1	3.91 ± 0.01	0.627 ± 0.002
1	150	30 ± 1	6.73 ± 0.02	not measured
2	50	18 ± 1	1.60 ± 0.01	0.400 ± 0.002
3	50	17 ± 1	1.48 ± 0.01	0.337 ± 0.002

Continuous extraction

The Figure 2 shows the yield of extraction performed at 45 °C, with a residence time (RT) of 30 min and leaves concentration of 50 g/L (set 1). The yield is calculated as the ratio of °Bx measured at the cell output to that obtained in the batch test. For experiments conducted at 0 % and 50 % power, the steady state seems to be established after 30 min with a yield of 90 % and 85 % respectively. No stabilization of the yield is observed for the experiment at 100 % US. This can be explained by an accumulation of stevia leaves inside the cell over time, caused by the low flowrate and the size of "particles" (4 mL/min). At 60 min of extraction, the yield seems to be independent of the power. The result is very different in terms of total steviol concentration (Figure 2 (b)). The concentration seems stable whatever the operating conditions. The steviol concentration is respectively 0.191 ± 0.007 g steviol/100g solution without ultrasound and 0.212 ± 0.015 g steviol/100 g solution with 100 % US.

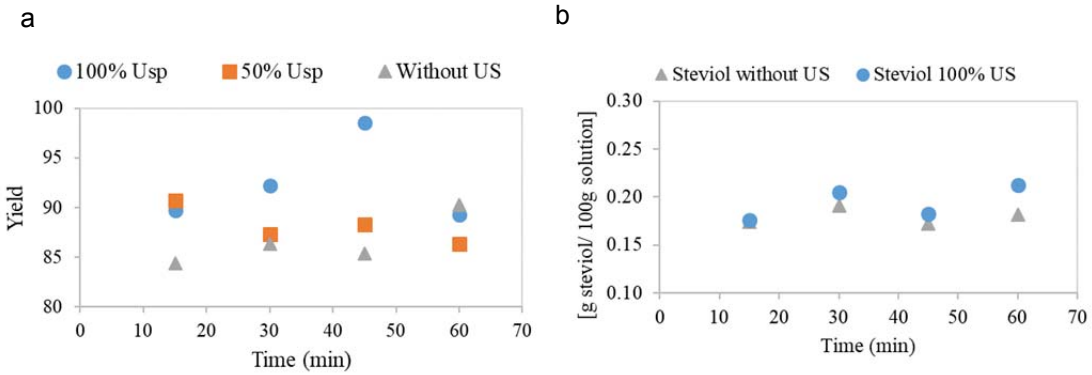

Figure 2: Experiments with a leaves concentration of 50 g/L (set 1), D [3,2] of leaves 30 µm and a residence time of 30 min (a) Yield calculated at the outlet cell and (b) total steviol concentration at the outlet cell measured by HPLC (not measured for experiment conducted with 50 % US).

Experiments are conducted with a leaves concentration of 100 g/L (set 1) (Figure 3).
For a residence time of 4 min, the yield is higher with ultrasound 90 % compared to 83-85 % without ultrasound. Moreover, in term of steviol concentrations, the stationary regime seems to be reached more quickly in the presence of ultrasound: lower than 15 min with ultrasound against 30 min without ultrasound. Although the yield is different between the experiments conducted without ultrasound and 100 % US, the amount of steviol is comparable and equal to 0.562 ± 0.002 g/100 g solution.
For a residence time of 30 min, the yield decreases when the ultrasonic power increases. This can be explained by the high concentration of stevia leaves which pass through the cell. This high concentration and an accumulation (due to the sedimentation of particles) prevent probably the ultrasound diffusion in the medium and a correct extraction with a low flowrate like for a leaves concentration of 50 g/L. The amount of steviol is comparable with and without US.

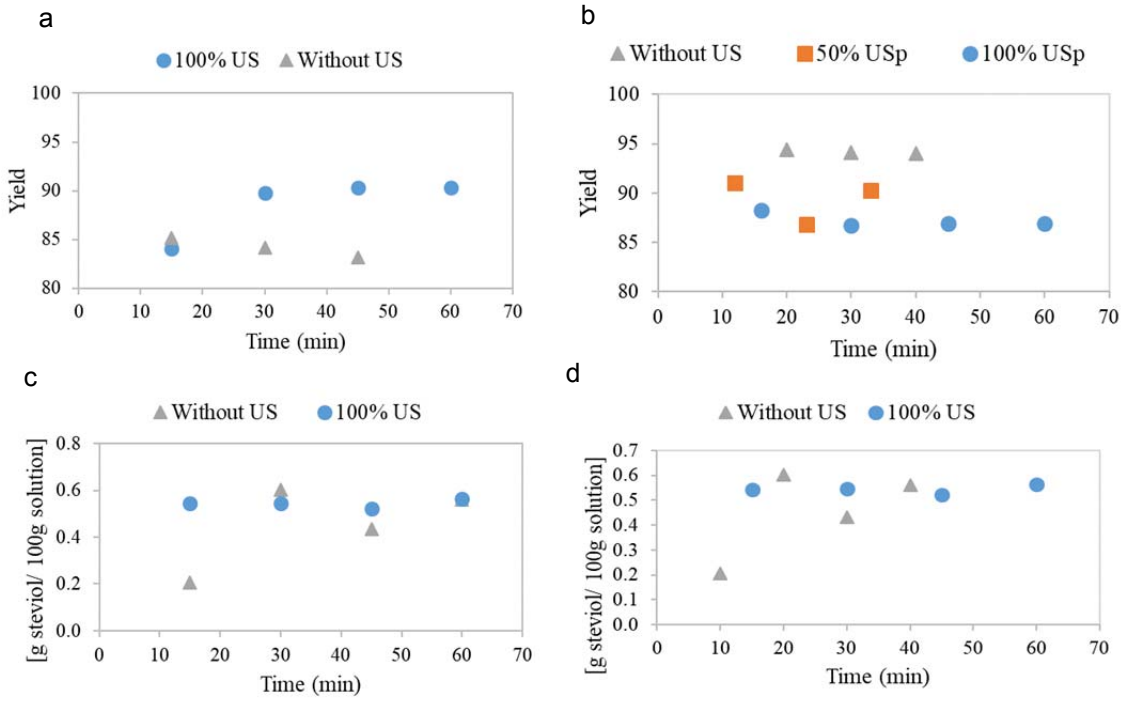

Figure 3: Experiments with a leaves concentration of 100 g/L (a) Yield of °Bx degrees calculated at the outlet cell for residence time (RT) of 4 min, (b) yield of °Bx degrees calculated at the outlet cell for residence time (RT) of 30 min, (c) steviol concentration measured by HPLC (RT of 4 min) and (d) steviol concentration measured by HPLC (RT of 30 min) (not measured for experiment conducted with 50 % US).

In order to verify the influence of ultrasound on the size distribution of the stevia leaves (D [3;2] 30 µm), two sampling are made, the first one in the inlet of the cell and the second one in the outlet. As it is possible to see in Table 2, ultrasound have no influence on the size distribution of stevia leaves during the extraction process on the following conditions: 100 % US, concentration 100 g/L, outlet temperature 45 °C.

Table 2: Characteristic diameters obtained from the granulometry analysis of Stevia leaves before and after US.

Diameter	Inlet (before US) (µm)	Outlet (After 100% US) (µm)
Dx (10)	14	14
Dx (50)	97	102
Dx (90)	299	292
Dx (97)	394	388
D [4;3]	129	129
D [3;2]	30	30

Batch extraction with recirculation

For these tests, the grinded leaves and branches (set 2) and leaves of size less than 250 µm obtained after sieving (set 3) with a concentration of 50 g/L are used.
Figure 4 (a) shows the results for the tests performed with set 3. It is possible to notice that with US the °Bx stabilizes since its first 15 min while without US reaches its stability after 30 min. This means that with ultrasound the extraction time is shorter, being at least 50 % faster than without US. In addition, the yield obtained with US is always higher than the one without US. Figure 5 (c) shows the total concentration of steviol measured by the HPLC. Analyzing the steviol content, the ultrasound favors the extraction: 0.172 ± 0.0018 g steviol/ 100g solution without US and 0.209 ± 0.0018 g steviol/100 g solution with 100 % US. Both of them are selective for Stevioside over Rebaudioside A extraction, presenting a ratio Stevioside/Rebaudioside A of 1.353 and 1.147, respectively.

These results are in accordance to Liu et al., 2010, which obtained the same trend using ultrasound-assisted batch extraction applied to Stevia Rebaudiana Bertoni. This can be explained by the mechanical action of the ultrasound on the stevia leaves cell walls, resulting in an increased solvent accessibility and consequently the extraction of the steviol glycosides.

Figure 4 (b, d) shows the results obtained by tests performed with the set 2. A constant behavior of yield and steviol concentration for the test performed without US (89 ± 0.73 % and 0.41 ± 0.0023 g steviol/100 g solution) is observed. With an amplitude of 50 % US, the yield and steviol concentration increase throughout the extraction. With 100 % US, steviol concentrations and yield are maximum at 40 min. It is possible to see that at 100 % ultrasound at 40 min of extraction, the yield exceeds the maximum concentration extracted per batch. This can be explained by the fact that the composition of the reconstituted leaves is more heterogeneous (e.g. leaves and branches). In addition, after 40 min the yield decrease, it is possible that long exposure to ultrasound might degrade steviol compounds. This assumption must be verified.

Comparing the °Bx and steviol content (Figures 4 b and d), it is possible to see that °Bx analysis present a higher yield than steviol content for ultrasound-assisted extraction like in continuous device. This might happen because the ultrasound permits to extract other molecules (soluble solids) present in leaves. Furthermore, the Stevioside extraction was favored over Rebaudioside A.

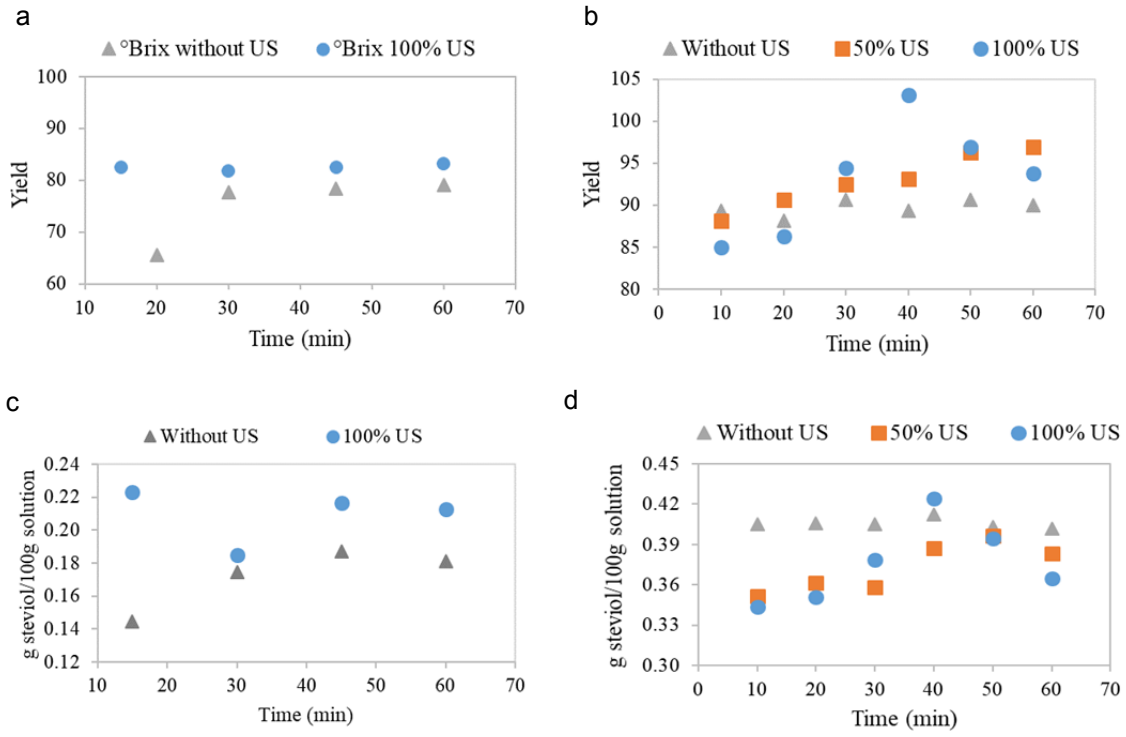

Figure 4: Yield calculated at the outlet cell with a leaves concentration of 50 g/L (a) °Bx concentration with D[3,2] of 17 µm (set 3) (b) °Bx concentration for D[3,2] of 18 µm (leaves and branches, set 2) (c) steviol concentration measured by HPLC (set 3), (d) steviol concentration measured by HPLC (set 2).

4. Conclusions

In this work, steviol extraction experiments were carried out at 45°C using distillated water and two procedures: a continuous and a batch extraction with recirculation with three sets of Stevia Rebaudiana Bertoni leaves.

In the case of the continuous experiments, ultrasound has an interesting extraction efficiency at low residence time. Ultrasound had no influence on the size distributions of stevia leaves. On the other hand, the extraction of soluble species seems to be favored.

Batch experiments have shown that the use of ultrasound is interesting for batches with a lower concentration of steviol to be extracted (set 3 versus set 2).

This study proved the pertinence in applying ultrasound to obtain a more efficient green extraction of steviol glycosides. However, the results are very dependent on the composition of stevia leaves.

Acknowledgments

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