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Characterization of the Permeate Fraction from Nanofiltration Step in Purification Process of Stevia Sweeteners by Uhplc-Ms/Ms-Qtof

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In this study the composition of the stevia extracts obtained in different stages of the process in a pilot unit of extraction and purification was monitored by high performance chromatography (HPLC) analyzes. The permeate from the nanofiltration step that has been discarded as residue was sampled and subjected to analysis to determine its antioxidant potential and its composition by UHPLC-MS / MS-QTOF. The permeate showed 58% of phenolic compounds and 3.3% of steviol glycosides. In this fraction, ten phenolic compounds, with high potential to be uses as food or food additive were identified. Considering that expressive volumes of this compounds are generated during the purification of stevia sweeteners, its recovery could be important in order to make better use of the substances with functional effects present in stevia leaves.
Key words: Stevia rebaudiana, sweeteners, UHPLC-MS / MS-QTOF, nanofiltration.

1. Introduction

Stevia rebaudiana (Bert) Bertoni is an herbaceous plant, belonging to the family Asteraceae (Compositae) native to Paraguay. Stevioside, rebaudioside-A, rebaudioside C and dulcoside A are the major steviol glycosides present in stevia leaves. They are high intensity sweeteners, stable and has sweetness power of about 300 in relation to sucrose. The sensory profiles of commercial stevia extracts depend directly on the variety of stevia and the extraction and purification methods employed in its production. Processing of stevia leaves based on membrane separation processes has gained importance due to several advantages over conventional processes, among which, it is worth mentioning the non-use of organic solvents and the possibility of recovery and use of bioactive several stages of the process (Chhaya et al, 2012). The use of stevia extracts as a food additive or medicinal product requires safe and sustainable purification techniques. The use of solvents and chemicals that pose a health risk should be avoided as much as possible. Although sweeteners can be extracted and purified in aqueous media, many industrially adopted processes still make use of organic solvents that compromise the safety and natural character of the products obtained (Carakostas et al., 2008).
Between 1986 and 1999, a large number of patents were deposited, the majority being based on conventional methods. Several of them dealt with extraction of steviosides using different technologies. Kumar (1986) described a process involving several steps. The steps involved are: aqueous extraction, clarification by the use of coagulants, and fractional crystallization methods of steviol glycosides (using solvent). In 1999 the first attempts were made to use membrane technologies to clarify and purify the stevia extracts. In most cases, the

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processes involving the use of membranes are hybrids, that is, convection purification methods are associated (Chhaya et al, 2012). The development of membrane-based separation processes arouses great interest since they can be operated at room temperature and do not involve phase change. In addition, they are easy to scale up (Kutowy et al., 1999, Abou-Arab et al., 2010, Chhaya et al., 2012 and Rao et al., 2012).
Kutowy et al. (1999) was the first to describe a hybrid membrane based purification process. After the extraction column its process basically consists of three phases operated by membranes in the filtration and diafiltration mode: microfiltration, ultrafiltration and nanofiltration. The process has the advantages of not using organic solvents and also the fact that the waste generated in each unit operation can be recycled, aiming the recovery of other important bioactive.
Considering the importance of the nanofiltration step in the processing of stevia leaves in order to obtain stevia extracts of good quality and although the substances removed in this one (permeate and nanofiltration diafiltrates) have not yet been described in the literature, this work had as objective isolate this so-called fraction of permeated fraction (PF), determine its composition by means of UHPLC-MS / MS-QTOF and evaluate its potential as a source of bioactives to be used in food.

2. Materials e methods

2.1 Sample and reagents

Stevia leaves of rebaudiana of the seminal variety, Stevia UEM-13, were obtained at the experimental site for the Nucleus of Research in Natural Products (NEPRON) study located at the State University of Maringá. The plants were collected in the flower bud formation stage (approximately 50–60 days after pruning) when the glycosides content of steviol was at a maximum. Afterward, they were dried in a forced circulation air oven until the humidity reached levels below 10%, crushed and stored. Concentrations of major glucosides in leave extract as determined by HPLC analysis by Dacome et al., 2005, were stevioside, 4.1; rebaudioside C, 2.0 and rebaudioside A, 4.4 g per 100g of dry leaves. All solvents and standards were LC grade or higher. Absolute ethanol (99.9%) by Merck (Londrina, Paraná, Brasil). Deionized water (18 MΩ·cm) by Milli-Q plus system was purchased from Induslab (Londrina, Paraná, Brasil). All the reference compounds were provided by Sigma-Aldrich (Brasil).

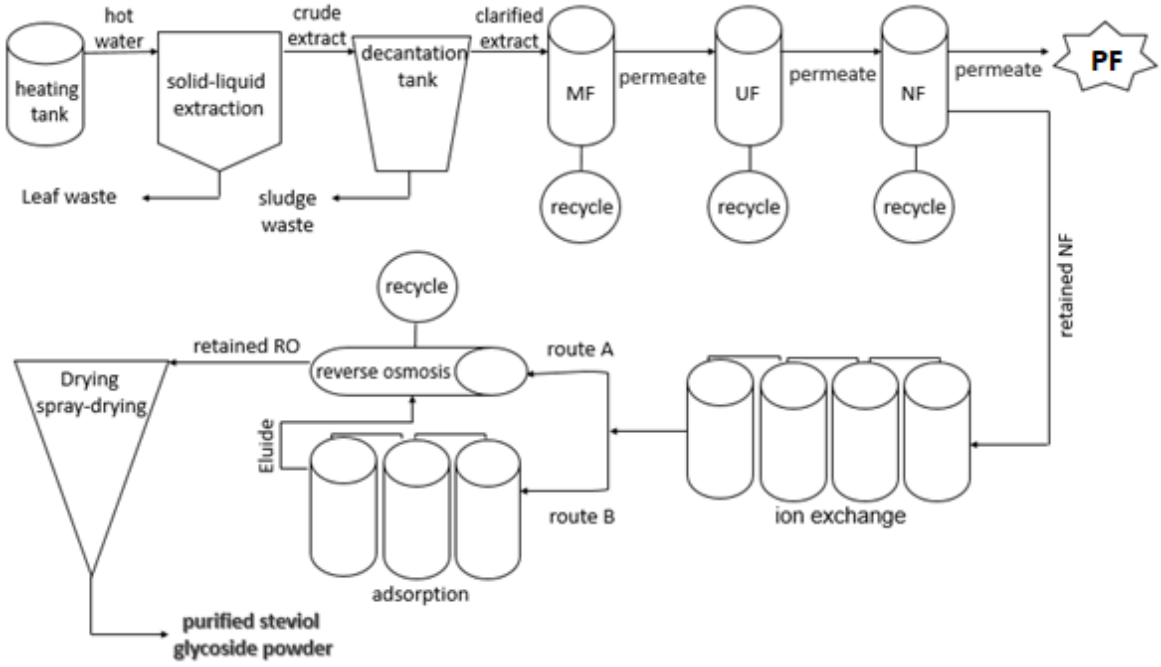

Figure 1: Design of the pilot unit for the extraction and purification of steviol glycosides

2.2 Extraction and purification of steviol glycoside

Stevia leaves of the Stevia UEM-13 variety were extracted and the aqueous extract was purified by membrane separation methods on a pilot scale according to methodology described by Zhang et al. (2000) (Figure 1).

Stevia leaves were extracted with water at 60 ° C. The crude extract was shipped to a settling tank to remove impurities. The permeated microfiltration material followed for ultrafiltration. In the nanofiltration, the permeated fraction (PF) was stored for further study and the retained was sent to ion exchange membranes, percolating through the cation and anion columns, respectively. The ion exchange permeate can then follow two paths. The first is the reverse osmosis process for volume reduction and subsequent percolation in the adsorption column. The second is to follow directly from the ion exchange columns to the adsorption column. At the end, the purified aqueous extract was dried in dryier spray to obtain purified powdered extract.

2.3 Characterization of the permeated fraction (PF)

2.3.1 HPLC Analysis

The glycosides total of steviol present in the PF were identified and quantified by High Performance Liquid Chromatography (HPLC) coupled to an index detector refraction with a 5µm NH2 column 125x4.6 mm in size using acetonitrile: water (80:20) v / v as the mobile phase with HPLC grade.

2.3.2 UHPLC-MS/MS-QTOF Analysis

The permeated fraction of the nanofiltration (80 mg/mL of PF) was analyzed in UHPLC-MS/MS-QTOF using a liquid chromatography system, Nexera X2, with LC-30AD pump and Shimadzu XR-ODSIII 150 x 2 mm column maintained at 38 °C with a linear gradient of elution using water (A) and acetonitrile (B) whit 0.1% formic acid. The mass spectrometer used was the Q-TOF type impact II (Bruker, Germany). All analysis was performed using collision-induced dissociation (DIC). The ion chromatogram and spectra (MS2) were visualized with DataAnalysis 4.3 software, in positive ionization mode (M+H), compared to the literature and identified using databases, such as Respect, MassBank and Human Metabolome Database. The error of precision assumed in the identification was a maximum of 4 ppm.

2.3.3 Total phenolic compounds, Flavonoids and Antioxidant activies

Total phenolic compounds in PF was performed using the method described by Singleton *et al.* (1999). The absorbance was measured at 760 nm. The total phenolic concentration was expressed as mg of gallic acid equivalent (EAQ)/mg extract. The quantification of total flavonoids was determined according to the method described by Jia et al. 1999. The PF was prepared at a concentration of 1 mg/ml of ethanol. The absorbance of the samples was measured at 510 nm. Data were expressed as quercetin equivalents. The free radical scavenging activity of extracts and aliquots was obtained by the ability to eliminate DPPH radicals (Blois, 1958). The absorbance was measured at 517 nm, and gallic acid was used as the reference compound. The results were expressed as percent inhibition of free radicals.

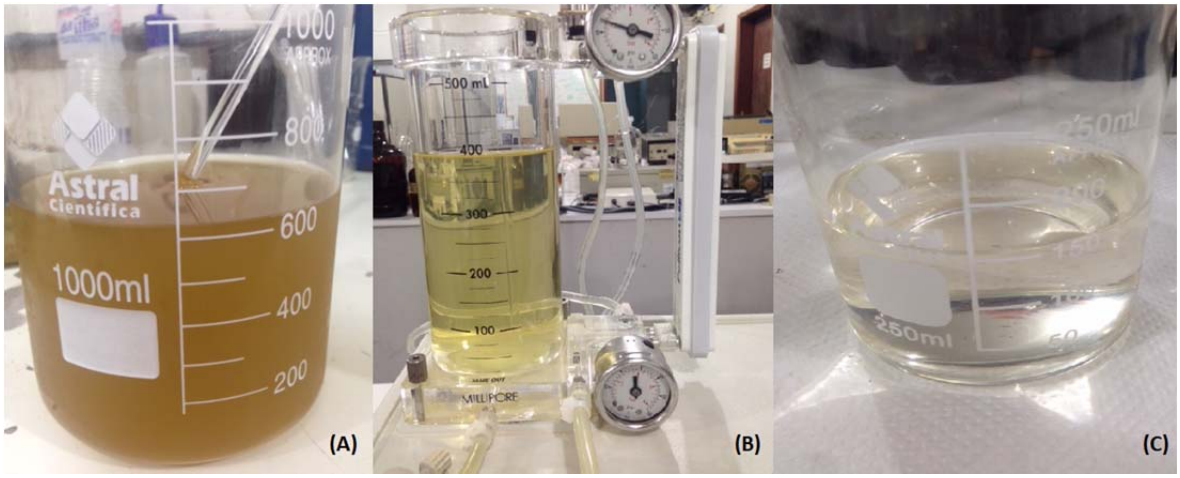

Figure 2. Decrease in coloration after purification processes. (A) Ultrafiltration output; (B) Nanofiltration output; (C) Ion exchange output.

3. Results and discussion

3.1 Extraction and purification of steviol glycoside

Aqueous stevia extract obtained was purified by membrane separation processes following the methodology described by Zhang et al. (2000) (Figure 2). The authors report that up to 89% of the impurities present in the ultrafiltered extract are removed in the permeates and diafiltrates of the nanofiltration step, with the semi-purified steviol glycoside mixture remaining in the retentate. It was also observed that the fraction removed in

the permeate of the nanofilter had a residual bitter taste, being discarded as a permeated fractionof nanofiltration.

3.2 Characterization of the permeated fraction (PF)

The permeate and nanofiltration diafiltrates were pooled and named as permeated fraction (PF). Samples were concentrated to dryness in a rotavaporator and analyzed for the phenolic compounds content, antioxidant activity and total glycosides of steviol (Table 1). It is observed that the permeated fraction (PF) is rich in phenolic compounds (58.91%) and presents significant antioxidant activity. It was also determined PF the presence of a small amount of steviol glycosides (3.3%).

Table 1. Analysis of PF dry in rotaevaporator after the diafiltration process for obtaining stevia sweeteners

Analysis	PF
Phenolic compounds (g EAG/ 100 g of powder)	58,91
Flavonoids (g EQ/ 100 g of powder)	18,62
Antioxidant activity (% I / 0,5 mg of powder)	82,17
Antioxidant activity (EC50 mg EAG/ g)	54,46
Total glycosides (g/100 g of powder)	3,3

The PF had its composition analyzed by means of UHPL-MS / MS-QTOF according to a methodology described in the literature (Figure 03) (Molina-Calle et al., 2017; Ciulu et al., 2017). A total of 18 substances were identified (Table 02), among which a number of important bioaccents such as phenolic compounds and the labdane diterpenes (esterebina and austroinulin) are highlighted. The knowledge of the molar mass and the physicochemical characteristics of the substances being removed in the nanofiltration stage will allow the choice of nanofilters to be performed in a less empirical and more rational way.

Table 2. Identification of compounds in the FW sample in UHPLC-MS / MS-QTOF

Family	**Compound**	**Molecular Formula**	**Retention Time**	**Mass**	**m/z**	**Fragment**
Phenolic compounds						
Flavonoids	Kaempferol-3-Glucoside	$C_{21}H_{20}O_{11}$	7.19	449.1078	449.1071	287, 145
	Luteolin-3',7-di-O-glucoside	$C_{27}H_{30}O_{16}$	7.46	611.1607	611.1593	611, 449, 288, 287
	Quercetin-3-neohesperidoside-7-rhamnoside	$C_{33}H_{40}O_{20}$	7.62	757.2186	757.2159	757, 611, 449, 448, 147
	Rutin	$C_{27}H_{30}O_{16}$	7.82	611.1607	611.1588	465, 449, 303, 287, 129
	Datiscetin-3-O-rutinoside	$C_{27}H_{30}O_{15}$	7.92	595.1657	595.1636	449, 287, 85
	Kaempferol-3-Galactoside-6''-Rhamnoside-3'''-Rhamnoside	$C_{33}H_{40}O_{19}$	8.79	741.2237	741.2221	595, 449, 287, 147
	Kaempferol-3-glucoside	$C_{21}H_{20}O_{11}$	9.39	595.1657	595.1636	449, 287, 245, 195, 163, 145, 103
	Apigenin7-O-Glucoside	$C_{21}H_{20}O_{10}$	9.83	433.1129	433.1119	433, 271
	3,7,4'-Trihydroxyflavanone	$C_{15}H_{12}O_5$	9.87	273.0757	273.0752	273, 255, 153, 107
	Luteolin 4'-glucoside	$C_{21}H_{20}O_{11}$	9.95	449.1078	449.1065	449, 430, 287, 269
Terpenoids						
Sesquiterpenoids	Sterebin B	$C_{20}H_{32}O_5$	10.93	353.2323	353.2312	353, 317, 123
Diterpenoids	Austroinulin	$C_{20}H_{34}O_3$	12.18	323.2581	323.2575	323, 305, 287, 237, 121, 85
	Rebaudioside D	$C_{50}H_{80}O_{28}$	10.18	1129.4909	1129.4854	487, 325, 163
	Stevioside	$C_{38}H_{60}O_{18}$	10.92	805.3852	805.3820	463, 317, 147
	Rebaudioside C	$C_{44}H_{70}O_{22}$	11.58	951.4431	951.4396	627, 465, 309, 147
	Dulcoside	$C_{38}H_{60}O_{17}$	11.62	789.3903	789.3878	627, 465, 309, 147
	Rebaudioside A	$C_{44}H_{70}O_{23}$	11.67	967.4381	967.4343	625, 325, 163
	Steviol	$C_{20}H_{30}O_3$	14.14	319.2268	319.2258	319, 301, 283, 273, 255, 165

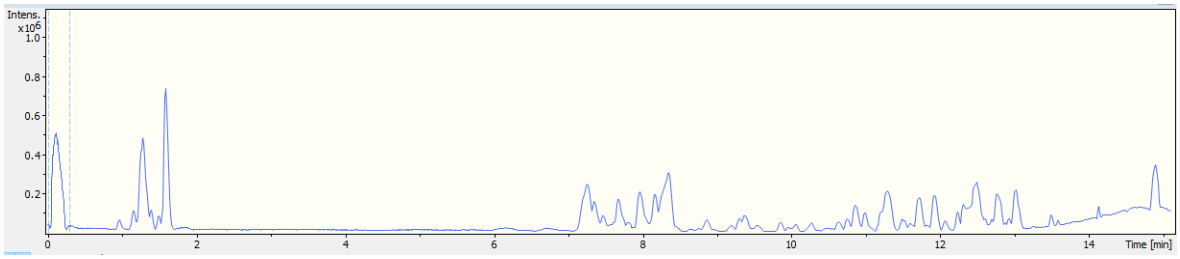

Figure 3. Chromatogram of the fraction PF obtained in UHPLC-MS/MS/QTOF.

Current studies are using stevia extracts as feed additives and confirming their potential for preventing lipid oxidation, reducing the growth of mesophilic microorganisms, or even bringing sweetness and functionality to foods (Ortiz-Virdma et al., 2018; Salazar et al., 2018).

In addition, Mathur et al. (2017) reviewed the pharmacological actions of stevia extracts and reported actions on energy and carbohydrate metabolism, effects on blood pressure and renal function, chromosomal and mutagenic effects, glucoregulation and hypotensive activity, medicinal potential as antihyperglycemic, insulinotropic, glucagonoss, hypotensive, anti-cancer, antiviral, antimicrobial, antioxidant, anti-inflammatory, immunostimulatory and chemopreventive agents, as well as for use as a digestive and dentistry tonic, and skin care and antioxidant, antifungal and antimicrobial for food and beverage applications. However, due to the high purity content required by legislation regulated by the European Union in 2016 (≥95% in steviol glycosides), a large proportion of these compounds are undesirable to commercial extracts and are therefore discarded in the purification steps applied.

4. Conclusions

In this study, it was possible to identify by UHPLC-MS / MS-QTOF analysis the substances that are removed in the nanofiltration step. The knowledge of the molar mass and its physicochemical properties is fundamental for the rational choice of nanofilters, in the sense of improving the separation process. In addition, the analysis showed that the permeated fraction that is being discarded is an important source of bioactive agents, among which phenolic compounds and labdanic diterpenes, with potential to be used in medicines and foods.

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