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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 extraction and purification process carried out in a pilot unit was monitored by high liquid performance chromatography (HPLC) analysis. The permeate from the nanofiltration step that has been discarded as residue was sampled and subjected to analysis to determine its antioxidant activity 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 used as food or food additives were identified. Considering that significant volumes of this waste stream 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 have 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 the 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 deal 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 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 has the objective isolating the so-called nanofiltration waste (NW) fraction, determine its composition by UHPLC-MS / MS-QTOF analysis and evaluate its potential as a source of bio actives to be used in food.

* 1. 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 according to 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, Brazil). Deionized water (18 MΩ·cm) by Milli-Q plus system was purchased from Induslab (Londrina, Paraná, Brazil). All the reference compounds were provided by Sigma-Aldrich (Brazil).

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).

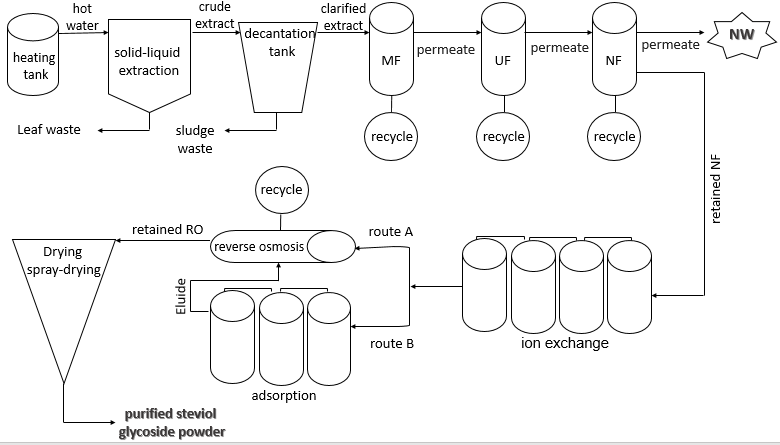


Figure 1: Design of the pilot unit for the extraction and purification of steviol glycosides

Stevia leaves were extracted with water at 60 °C. The aqueous crude extract was treatment with calcium oxide and decanted. The supernatant clarificated was microfiltrated. The permeated of microfiltration was ultrafiltrated. In the nanofiltration, the permeate extract (NW) was discarded as a residue and the retained was sent to ion exchange system. The ion exchange eluate can follow two paths. The first is the reverse osmosis process for volume reduction and subsequent treatment in the adsorption resins. The second is to follow directly from the ion exchange resins to the adsorption resins. We used in this case the first option (figure 1 – route A). At the end, the purified aqueous extract was dried in spray dryer to obtain powdered extract purified.

2.3 Characterization of the nanofiltration waste (NW)

2.3.1 HPLC Analysis

The glycosides total of steviol present in the NW 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 in isocratic mode.

2.3.2 UHPLC-MS/MS-QTOF Analysis

The dry residue of the nanofiltration (80 mg/mL of NW) 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 Data Analysis 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

Phenolic compounds in NW were determined according to the method described by Singleton et al. (1999). For the quantification of total phenolic compounds, 0.2 ml of sample (1 mg / ml) and 1 ml of Folin-Ciocalteu reagent were used. This mixture was homogenized for 2 minutes, and then 0.8 ml of 7.5% sodium carbonate was added. The final blend was incubated for 90 minutes at room temperature. The absorbance was measured at 760 nm. The concentration of total phenolics was expressed in mg gallic acid equivalent (mgEAG) / g extract, using its standard curve. Quantification of total flavonoids was determined according to Jia et al., 1999. For the determination of total flavonoids, 0.5 ml of sample (1 mg / ml), 0.3 ml of 5% sodium nitrite (NaNO2) were added. This mixture remained under constant stirring for 5 minutes, and was added 0.3 ml of 10% aluminum chloride (AlCl 3). After 6 minutes at rest the mixture received 2 ml of a solution of NaOH (1M). The sample was read at 510 nm absorbance. Data were expressed as quercetin equivalents (mgEQ) / g extract. The free radical scavenging activity of NW was measured by the ability to eliminate DPPH radicals (Blois, 1958). The 1.0 ml amount of the 0.3 mM DPPH solution was added to 2.5 ml of the sample (1 mg / ml). After 30 minutes at rest and protected from light, the absorbance was measured at 517 nm. Gallic acid was used as the reference compound. The results were expressed as percent inhibition of free radicals.

* 1. Results and discussion

3.1 Extraction and purification of steviol glycoside

Aqueous stevia extract obtained was semi purified by membrane separation processes following the methodology described by Zhang et al. (2000). The authors report that up to 45% 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 residue of nanofiltration.



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

3.2 Characterization of the nanofiltration waste (NW)

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

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

|  |  |
| --- | --- |
| Analysis | NW |
| 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 de powder) | 82.17 |
| Antioxidant activity (EC50 mg EAG/ g) | 54.46 |
| Total glycosides (g/100 g of powder) | 3.30 |

The NW 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 bioactives such as phenolic compounds and the labdane diterpenes (sterebin B 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.

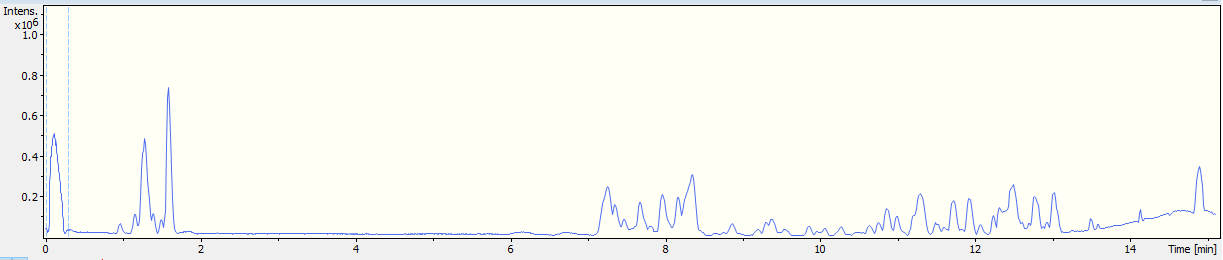


Figure 03. Chromatogram of the fraction NW obtained in UHPLC-MS/MS/QTOF.

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

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Family** | **Compound** | **Molecular Formula** | **Retention Time** | **Mass** | **m/z** | **Fragment** |
| Phenolic compounds |  |  |  |  |  |  |
| Flavonoids | Kaempferol-3-Glucoside | C21H20O11 | 7.19 | 449.1078 | 449.1071 | 287, 145 |
|  | Luteolin-3',7-di-O-glucoside | C27H30O16 | 7.46 | 611.1607 | 611.1593 | 611, 449, 288, 287 |
|  | Quercetin-3-neohesperidoside-7-rhamnoside | C33H40O20 | 7.62 | 757.2186 | 757.2159 | 757, 611, 449, 448, 147 |
|  | Rutin | C27H30O16 | 7.82 | 611.1607 | 611.1588 | 465, 449, 303, 287, 129 |
|  | Datiscetin-3-O-rutinoside | C27H30O15 | 7.92 | 595.1657 | 595.1636 | 449, 287, 85 |
|  | Kaempferol-3-Galactoside-6''-Rhamnoside-3'''-Rhamnoside | C33H40O19 | 8.79 | 741.2237 | 741.2221 | 595, 449, 287, 147 |
|  | Kaempferol-3-glucoside | C21H20O11 | 9.39 | 595.1657 | 595.1636 | 449, 287, 245, 195, 163, 145, 103 |
|  | Apigenin7-O-Glucoside | C21H20O10 | 9.83 | 433.1129 | 433.1119 | 433, 271 |
|  | 3,7,4'-Trihydroxyflavanone | C15H12O5 | 9.87 | 273.0757 | 273.0752 | 273, 255, 153, 107 |
|  | Luteolin 4'-glucoside | C21H20O11 | 9.95 | 449.1078 | 449.1065 | 449, 430, 287, 269 |
| Terpenoids |  |  |  |  |  |  |
| Sesquiterpenoids | Sterebin B | C20H32O5 | 10.93 | 353.2323 | 353.2312 | 353, 317, 123 |
| Diterpenoids | Austroinulin | C20H34O3 | 12.18 | 323.2581 | 323.2575 | 323, 305, 287, 237, 121, 85 |
|  | Rebaudioside D | C50H80O28 | 10.18 | 1129.4909 | 1129.4854 | 487, 325, 163 |
|  | Stevioside | C38H60O18 | 10.92 | 805.3852 | 805.3820 | 463, 317, 147 |
|  | Rebaudioside C | C44H70O22 | 11.58 | 951.4431 | 951.4396 | 627, 465, 309, 147 |
|  | Dulcoside | C38H60O17 | 11.62 | 789.3903 | 789.3878 | 627, 465, 309, 147 |
|  | Rebaudioside A | C44H70O23 | 11.67 | 967.4381 | 967.4343 | 625, 325, 163 |
|  | Steviol | C20H30O3 | 14.14 | 319.2268 | 319.2258 | 319, 301, 283, 273, 255, 165 |

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-Viedma et al., 2017; 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, 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.

* 1. 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 residue of the nanofiltration 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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