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Stepwise membrane filtration for Rebaudioside A and Stevioside enrichment in aqueous and ethanolic stevia extracts

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ABSTRACT

Enriching steviol glycosides derived from the stevia plant is an important step in producing stevia natural sweeteners. This study investigated the enrichment of Rebaudioside A and Stevioside compounds of aqueous and ethanol stevia extracts using cascade membrane filtration techniques. Accordingly, extracts from stevia leaves were processed using membrane filtration techniques such as ultrafiltration and nanofiltration. In particular, the 30 kDa ultrafiltration membrane was highly effective in enriching steviol glycosides. The highest concentrations of Rebaudioside A (21.47 g L^{-1}) and Stevioside (19.97 g L^{-1}) compounds were reached at the 30 kDa ultrafiltration retentate fraction in both aqueous and ethanolic extracts. However, it was found that the permeate fluxes and the extracts obtained after the nanofiltration process had very low concentrations of these compounds. The findings highlight the importance of membrane selection in effectively enriching steviol glycosides.

1. Introduction

Steviol glycosides, which are the natural sweet components of stevia leaves, have recently been the subject of a substantial trend in their extraction. Several studies have advocated for the substitution of ethanol, an industrial process solvent, with water (Das et al. 2015; Díaz-Montes et al. 2020; Zoua Assoumou et al. 2024). This substitution is due to the environment-friendly attribute of water, as well as the non-toxic and non-flammable nature of it. Furthermore, the utilization of water as a solvent may lower the production expenses and enhance the sustainability of the extraction procedure (Díaz-Montes et al. 2021).

Recent developments have focused on the increase of the purity and yield of steviol glycosides by optimizing membrane filtration processes. Díaz-Montes et al. (2020) achieved a significant enrichment of Rebaudioside A by using a two-stage ultrafiltration process to fraction the liquid extracts of *Stevia rebaudiana*. Similarly, Karhan (2020) investigated fractional membrane filtration practices to purify stevia extracts and emphasized the importance of the membrane pore size in achieving desired purity levels. In addition, Zhang et al. (2000) suggested the use of resin or fluctuation agents to improve flow before membrane processing and prevent membrane clogging.

Many studies have been conducted to determine the effectiveness of multiple combinations of different membrane filtration techniques to fractionate target steviol glycosides as much as possible (Liu et al. 1991; Martínez-Alvarado et al. 2017; Díaz-Montes et al. 2020; Karhan 2020). Membrane filtration applications are frequently applied as important enrichment processes in the purification of steviol glycosides. These techniques can selectively separate molecules based on their size and molecular weight. However, testing different membrane filtration combinations is important to determine the change in the steviol glycoside profile and which application will be more successful when specific purification is desired. Therefore, this research was aimed at providing a comprehensive understanding of the factors influencing the enrichment of steviol glycosides, with a focus on the stepwise membrane filtration approach.

2. Materials and Methods

2.1. Material

The stevia plant (*Stevia rebaudiana* var. Levent 93), cultivated in the Department of Field Crops, Akdeniz University trial area, was employed as research material in the study. The freshly harvested leaves were dried in an oven at 70°C until the moisture content reached 5 g 100rte g^{-1} (dry basis). They were then stored in closed containers at room temperature until the time of analysis.

2.2. Production of raw aqueous and ethanolic stevia extracts

The dry stevia leaves were separated from extraneous substances such as stem, flower, waste, etc. then ground using a laboratory grinder (Waring Blender, USA). The dried stevia leaves were mixed with distilled water/ethanol (96% purity) in a ratio of 1:15 (dry ground leaf: water/ethanol) in a laboratory malaxer (Alfa Laval X, Sweden) for 30 min at 20°C. The mixture (Sample: E) was subjected to centrifugation with 2900xg force at 20ºC for 15 minutes. The collected supernatant was passed through a coarse filter paper to remove any solid particles (Sample: C). The same method was used to produce both

ethanolic stevia extracts as well as aqueous ones. The solvent (ethanol) was removed with a rotary evaporator (IKA RV10 Auto Pro V-C, Germany) at 40°C and 900 rpm only after the coarse filtration stage. Subsequently, distilled water was added to the remaining portion in an amount equivalent to the amount of liquid that had evaporated. Consequently, this mixture was employed as the raw ethanolic stevia extract throughout the analyses.

2.3. Hot clarification

Before the implementation of membrane filtration techniques, a hot clarification procedure was carried out to prevent the membrane filters from becoming fouled. Pre-treatments, such as the usage of lime or flocculating agents, are suggested before membrane processing such as ultrafiltration for improved flux (Zhang et al. 2000).

During the hot clarification phase, clarification aids were added to the stevia extract in a water bath (JeioTech, BS-06/31, Seoul, Korea) kept at 50°C in the quantities determined by preliminary trials (bentonite: 1%, gelatine: 1%, kieselsol: 3%). The clarification was terminated after 3 hours, and the clear part on the upper side (Sample: HC) was filtered through the coarse filter without removing the sediment that had formed at the bottom.

2.4. Stepwise membrane filtration

Polyethersulfone (PESU) membrane with a pore size of 30 kDa (Sartocon Slice, Sartorius Stedim Biotech GmbH, Germany) was used for ultrafiltration, and Hydrosart membrane with a pore size of 5 kDa (Sartocon Cassette, Sartorius Stedim Biotech GmbH, Germany) was used for nanofiltration. Applications of stepwise membrane filtration were implemented at a membrane pressure of 2 bar and a permeate flux of 90%. Accordingly, the translucent fraction that passed through the coarse filter after the hot clarification process was initially filtered by ultrafiltration using a 30 kDa PESU membrane filter. Following that, the permeate was further filtered by nanofiltration using a 5 kDa Hydrosart membrane filter (Figure 1).

2.5. Methods

2.5.1. Total soluble solids and pH analyses

The total soluble solids and pH values of the stevia extracts were measured using a digital refractometer (Isolab, Germany) and a pH meter (FE20-Five, Mettler-Toledo, Ohio, USA) (Cemeroğlu 2007).

2.5.2. Color measurement

Color (L*, a*, b*) values of the samples were measured using an UltraScan-VIS spectrophotometer (Hunterlab, USA) equipped with a CIE-Lab color model. The CIE-Lab model characterizes color by employing three parameters: The L* metric lightness function represents the degree of lightness on a scale from 0 (black) to 100 (white). The a* and b* chromaticity coordinates reflect opposing scales of red–green (+a for reds, -a for greens) and blue-yellow (+b for yellows, -b for blues).

2.5.3. Quantification of Stevioside and Rebaudioside A

An external standard method with HPLC was used to determine the Stevioside and Rebaudioside A amounts in the stevia extracts (Wölwer-Rieck et al. 2010). For the analysis, a High-Performance Liquid Chromatography (HPLC) instrument (Shimadzu, LC 20 AD) was used. The instrument was equipped with a C18 column (Dimensions: 5 µm, 250 x 4.6 mm, ID) along with a photodiode array (PDA) detector system set at a wavelength of 210 nm. A mixture (68:32) of phosphate buffer (10 mmol L^{-1} sodium phosphate, pH 2.6) and acetonitrile (HPLC quality, Merck) was used as the mobile phase and the flow rate was set to 0.8 ml min^{-1} (Wölwer-Rieck et al. 2010).

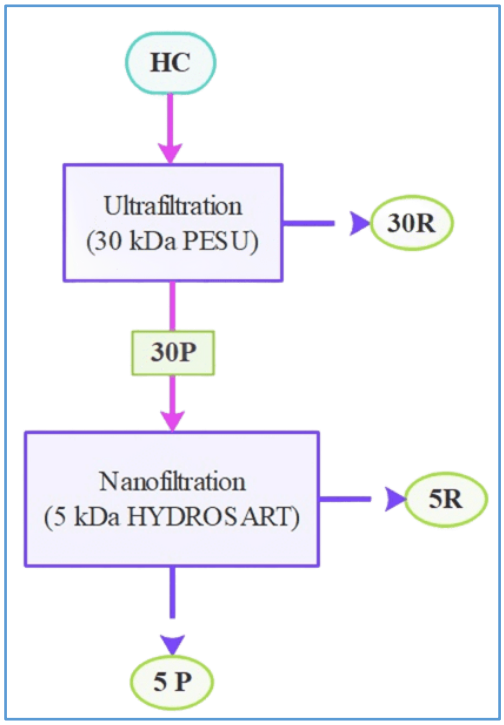

Figure 1. Stepwise membrane filtration procedures.

2.5.4. Statistical analysis

Statistical analysis of the data (per each sample, three repetitions, and two reading parallels) was performed at the level of 5% significance. The Shapiro-Wilk test was applied to determine the suitability of the data for normal distribution, then the Kruskal-Wallis test (one-way ANOVA), was used. When a significant difference was found between the groups as a result of the Kruskal-Wallis test, Dunn's multiple comparison test was applied (Origin 2019b OriginPro, USA).

3. Results and Discussion

3.1. Variations across extraction and membrane filtration stages

3.1.1. Total soluble solids and pH

The total soluble solids (TSS) contents of the stevia samples are given in Table 1. The findings showed that the large molecules were successfully concentrated by 30 kDa ultrafiltration in the retentate (30R) phase of the aqueous extract, leading to a significant rise in the TSS contents. Iwuozor et al. (2024) found that water extraction had a greater ability to extract soluble solids in comparison to methanol and methanol/water combinations, therefore confirming this observation. The effect of nanofiltration on soluble solids content after ultrafiltration was limited. In the process starting at 30P (0.63 °Bx), which is the permeate part of ultrafiltration, the permeate (5P: 1.39 °Bx) and retentate (5R: 1.80 °Bx) values obtained throughnanofiltration showed a limited concentration increase. This suggests that nanofiltration is not able to distinctly decompose the solution after ultrafiltration but does provide some concentration. In particular, the absence of large differences between the 5P and 5R values compared to 30P, which is the feed solution, reveals that nanofiltration does not provide complete separation.

Table 1. Soluble solids and pH values of aqueous and ethanolic stevia extracts

Parameter	Process	Aqueous	Ethanolic
Total Soluble Solids (°Bx)	E	3.10±0.00^{b}	4.03±0.07^{a}
	C	3.10±0.00^{b}	3.30±0.00^{b}
	HC	4.00±0.06^{b}	2.43±0.09^{c}
	30P	0.63±0.10^{c}	1.63±0.09^{d}
	30R	12.31±1.39^{a}	4.20±0.29^{a}
	5P	1.39±0.52^{c}	1.78±0.12^{d}
	5R	1.80±0.62^{c}	4.92±0.83^{a}
pH	E	5.81±0.00^{b}	6.09±0.02^{a}
	C	4.82±0.02^{d}	5.47±0.04^{c}
	HC	5.93±0.00^{b}	4.63±0.01^{d}
	30P	6.62±0.06^{a}	5.72±0.07^{b}
	30R	5.60±0.19^{c}	4.82±0.06^{d}
	5P	5.57±0.39^{c}	4.65±0.35^{d}
	5R	5.99±0.27^{b}	5.22±0.23^{c}

The values indicated by different letters show a significant difference between different processes under the same extraction method for the same color parameter (P<0.05). E: Extraction, C: Centrifugation, HC: Hot clarification, 30P: Ultrafiltration, 30 kDa-permeate flux, 30R: Ultrafiltration, 30 kDa-retentate flux, 5P: Nanofiltration, 5 kDa-permeate flux, 5R: Nanofiltration, 5 kDa-retentate flux.

When the ethanolic extract was subjected to membrane filtration, ultrafiltration, and nanofiltration techniques led to a more restricted enrichment, with limited increases in the concentration of soluble solids compared to the aqueous extract. This emphasizes the distinct impacts of ultrafiltration and nanofiltration procedures on the chemical characteristics of the extract, depending on the solvent employed (water or ethanol). For example, in the ethanolic extract, the total soluble solids concentration after ultrafiltration (30R) was 4.20±0.29 °Bx, whereas after nanofiltration (5R), it increased to 4.92±0.83 °Bx, showing a more restricted enrichment compared to the aqueous extract (Table 1).

There were also changes in the pH values of stevia samples extracted in water and alcohol, depending on both extraction and membrane filtration type (Table 1). Samples extracted with ethanol initially have a more alkaline pH value, indicating that ethanol is better able to extract alkaline compounds. Mahl et al. (2010) found that ethanol extraction typically yields a greater quantity of alkaline chemicals, which is consistent with this finding. However, in the process of ultrafiltration, it was observed that alkaline chemicals were concentrated in the permeate phase of the aqueous extract, while acidic compounds were transferred to the permeate phase in the ethanolic extract. The nanofiltration procedure reverses this scenario for both types of extracts, resulting in an augmentation of acidic chemicals in the permeate and a concentration of alkaline compounds in the retentate. Arakawa et al. (2012) demonstrated that pH values significantly influence the adsorption and separation processes during filtration, which helps explain these results. These pH changes can be attributed to the differential permeability of the membrane to alkaline and acidic compounds, as well as the interactions between the membrane material and the solutes in the stevia extracts. During ultrafiltration, larger alkaline molecules may be retained more effectively, leading to their concentration in the retentate, while smaller acidic molecules permeate through the membrane. This selective separation can cause a shift in pH values, as noted by Zhang et al. (2000), who observed similar trends in other plant extract filtration processes.

3.1.2. Color

The color changes that occurred in the stevia extracts with the applied processes areshown in Table 2 and Figure 2. For the aqueous extract, it was observed that the decolorization continued to some extent with nanofiltration (5P) after UF (30P), showing a statistically significant increase in the L* value, and the green tone became slightly more dominant in terms of the a* value. Nevertheless, there were no significant alterations identified in relation to the b* value. Karhan (2020) observed that reducing the size of membrane pores during multi-stage membrane filtration operations led to an elevation in the L* value (representing lightness) of stevia extracts. The author attributed this variation to the removal of pigmented phenolic compounds from the permeate flux. In line with this, Kootstra et al. (2016) observed that the final stevia extract maintained a greenish-brown color even after nanofiltration. This implies that while ultrafiltration and nanofiltration improved transparency, they were not entirely successful in eliminating the color.

For the ethanolic extract, the (5P) a decrease in the L* value was observed after NF and there was a reduction in lightness. Very small changes were observed in the a* and b* values, i.e. no great progress was made in terms of achieving colorlessness. As a result, it can be said that discoloration with NF persists after UF in aqueous extracts, but this increase in lightness is limited.

Table 2. *L*, a*, b** values of aqueous and ethanolic stevia extracts

Color Parameter	Process	Aqueous	Ethanolic
*L**	E	30.51±0.03^{a}	31.46±0.66^{a}
	C	26.45±0.02^{b}	23.51±0.17^{c}
	HC	27.47±0.13^{b}	26.99±0.04^{b}
	30P	24.63±1.06^{c}	26.62±0.48^{b}
	30R	24.02±1.81^{c}	24.20±1.81^{c}
	5P	25.83±0.83^{b}	25.54±1.46^{b}
	5R	25.42±0.74^{b}	26.49±0.33^{b}
*a**	E	-0.66±0.06^{b}	-1.89±0.7^{a}
	C	-0.53±0.03^{b}	-0.48±0.07^{c}
	HC	-0.53±0.01^{b}	-0.25±0.51^{d}
	30P	-0.71±0.6^{a}	-0.73±0.32^{b}
	30R	0.67±0.09^{b}	0.74±0.05^{b}
	5P	-0.78±0.25^{b}	-0.73±0.06^{b}
	5R	-0.56±0.74^{b}	-0.75±0.07^{b}
*b**	E	4.77±0.59^{a}	10.28±0.34^{a}
	C	0.21±0.02^{c}	0.19±0.05^{d}
	HC	0.53±0.02^{b}	0.45±0.01^{b}
	30P	-0.54±0.06^{b}	0.36±0.62^{c}
	30R	0.45±0.18^{b}	0.51±0.59^{b}
	5P	-0.53±0.22^{b}	0.31±0.37^{c}
	5R	-0.49±0.12^{b}	0.09±0.09^{e}

The values indicated by different letters show a significant difference between different processes under the same extraction method for the same color parameter (P<0.05). E: Extraction, C: Centrifugation, HC: Hot clarification, 30P: Ultrafiltration, 30 kDa-permeate flux, 30R: Ultrafiltration, 30 kDa-retentate flux, 5P: Nanofiltration, 5 kDa-permeate flux, 5R: Nanofiltration, 5 kDa-retentate flux.

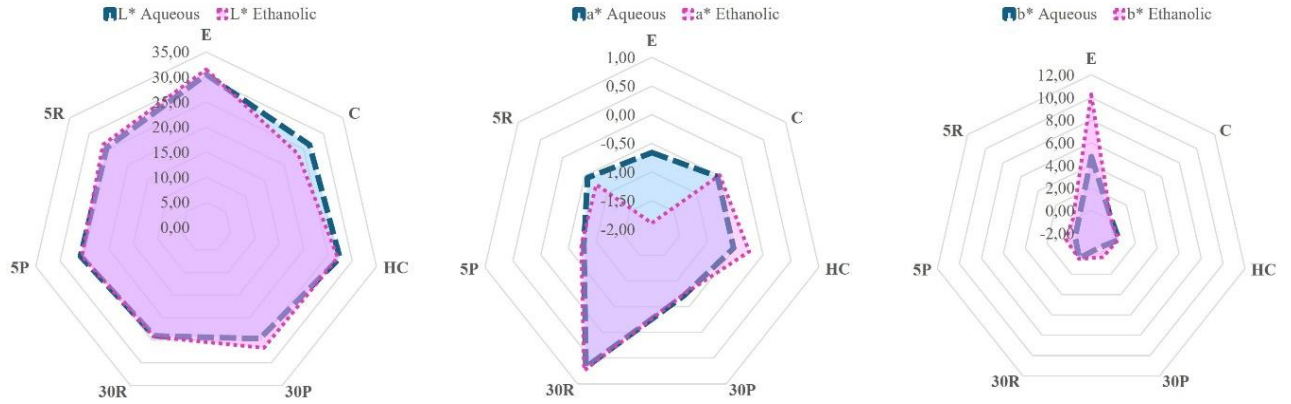

Figure 2. Color changes in aqueous and ethanolic stevia extracts throughout processing stages (E: Extraction, C: Centrifugation, HC: Hot clarification, 30P: Ultrafiltration, 30 kDa-permeate flux, 30R: Ultrafiltration, 30 kDa-retentate flux, 5P: Nanofiltration, 5 kDa-permeate flux, 5R: Nanofiltration, 5 kDa-retentate flux).

In ethanolic extracts, a slight darkening tendency was observed instead of an increase in lightness after NF. This suggests that NF is more effective on aqueous extracts but has a limited effect on ethanolic extracts if colorlessness is the main aim.

While aqueous and ethanolic extracts initially had the lightest color (L*), greenest (a*), and most yellow tones (b*), the ultrafiltration process caused a marked change in color tones, especially in the retentate (30R) stage, with red tones (a*) becoming dominant. After nanofiltration, a tendency to lighten was observed in aqueous extracts, while yellow tones (b*) were somewhat preserved in ethanolic extracts, but there was a shift to blue tones in general (Table 2). Except for the extraction (E) step, aqueous and ethanolic extracts exhibited a fairly similar trend of change in L*, a*, b* values during the processing stages (ultrafiltration and nanofiltration). Both types of extracts wentthrough parallel processes such as color darkening, tonal changes, and partial lightening (Figure 2). However, in the extraction phase (E), the ethanolic extract initially started in lighter, greener, and more yellow tones than the aqueous extract, indicating that the type of solvent had different effects on the color during the extraction phase. That is, although there is similarity in the processing stages, solvent-dependent differences were evident in the initial (E) stage.

3.1.3. Filtration effects on Rebaudioside A and Stevioside enrichment

The variation of steviol glycosides through different membrane filtration steps reveals that ultrafiltration in general (especially the 30 kDa membrane) is highly effective in enriching

both Rebaudioside A and Stevioside. In both aqueous and ethanolic extracts, it has been observed that the highest concentrations of these compounds are reached at the 30 kDa ultrafiltration retentate fraction. This result is consistent with Das et al.' (2015) findings, who reported that the 30 kDa membrane was the most effective in enriching Rebaudioside A, with minimal fouling behavior. In contrast, the concentrations of these compounds remained markedly low in the permeate fractions and nanofiltration steps. Similarly, Chhaya and Mondal (2012) discovered that nanofiltration had a minimal impact on the enrichment of Rebaudioside A and Stevioside, as a substantial portion of these components was retained by the membrane. This suggests that certain steps of membrane filtration play a critical role in the process of separation and enrichment of steviol glycoside (Figure 3).

When the effect of membrane filtration applications on the enrichment of steviol glycosides was examined in more detail, it was determined that the aqueous extract reached the highest concentrations of 30 kDa ultrafiltration retentate, Rebaudioside A with 21.47 g L^{-1} and Stevioside with 19.97 g L^{-1} (Figure 4).

These values clearly show that steviol glycosides were effectively enriched in this step. On the other hand, in the permeate fractions (30PAq and 5PAq), rebaudioside A and Stevioside concentrations remained at very low levels of 0.62 g L^{-1} and 0.86 g L^{-1}, respectively. The nanofiltration process yielded similarly low concentrations, suggesting that a large proportion of these components were retained in the membrane and did not pass into the permeate fraction. These specific numerical details reveal the effect of each filtration step on the concentration of steviol glycosides.

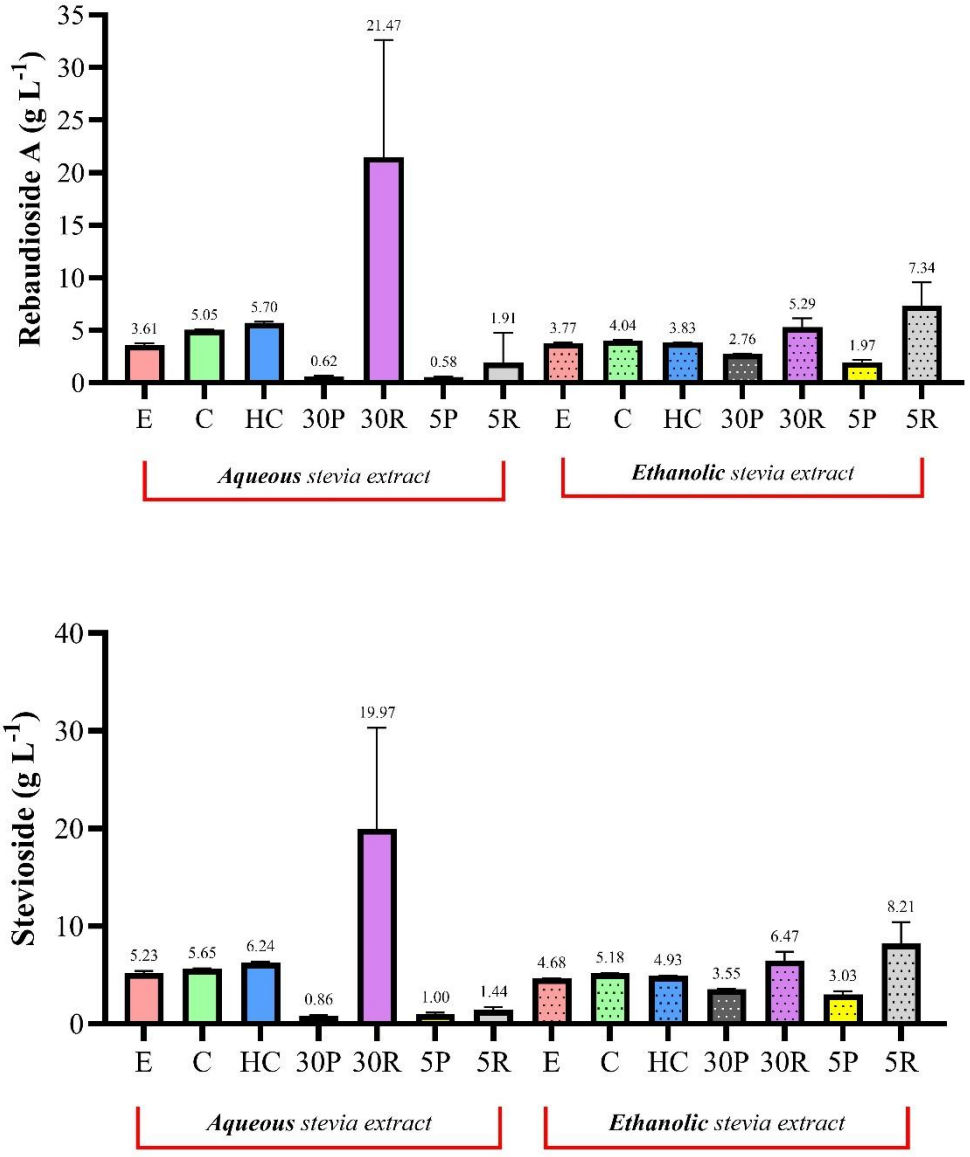

Figure 3. Rebaudioside A and Stevioside concentrations in aqueous and ethanolic stevia extracts across different processing stages (E: Extraction, C: Centrifugation, HC: Hot clarification, 30P: Ultrafiltration, 30 kDa-permeate flux, 30R: Ultrafiltration, 30 kDa-retentate flux, 5P: Nanofiltration, 5 kDa-permeate flux, 5R: Nanofiltration, 5 kDa-retentate flux).

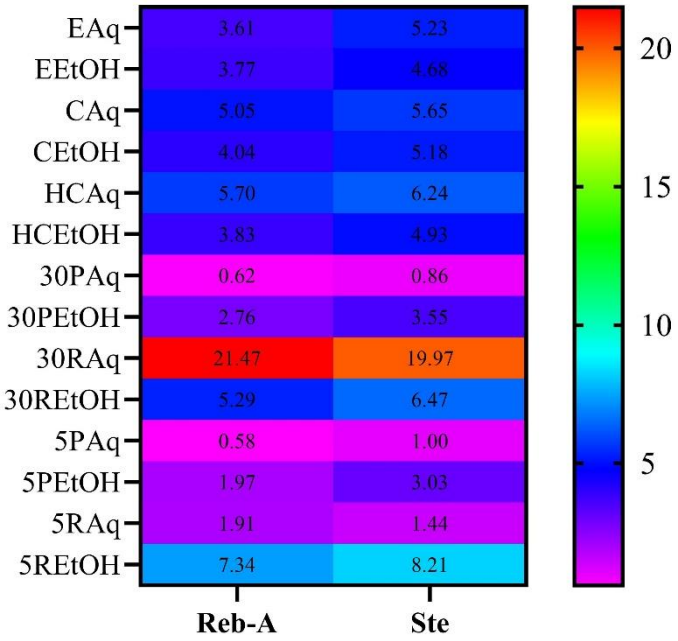

Figure 4. Distribution of rebaudioside A and Stevioside levels in aqueous and ethanolic stevia extracts visualized by heatmap across processing stages (Aq: Aqueous, EtOH: Ethanolic, E: Extraction, C: Centrifugation, HC: Hot clarification, 30P: Ultrafiltration, 30 kDa-permeate flux, 30R: Ultrafiltration, 30 kDa-retentate flux, 5P: Nanofiltration, 5 kDa-permeate flux, 5R: Nanofiltration, 5 kDa-retentate flux).

4. Conclusion

The efficiency of using stepwise membrane filtration procedures during processing of aqueous and ethanolic stevia extracts was investigated in this study. The results indicated that the ultrafiltration (UF) process was highly effective in concentrating the analyzed steviol glycosides. Both Rebaudioside A and Stevioside glycosides were concentrated in the retentate flux of 30 kDa ultrafiltration membrane. However, if nanofiltration process (5 kDa) is to be combined with ultrafiltration, it is thought that using the retentate phase as a feed may be more effective in increasing the purity of the target compounds. The findings of this study indicate that the choice of membrane filtration type is crucial in the separation and concentration of steviol glycosides.

Acknowledgements

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Abbreviations

E: Extraction
C: Centrifugation
HC: Hot clarification
30P: Ultrafiltration, 30 kDa-permeate flux
30R: Ultrafiltration, 30 kDa-retentate flux
5P: Nanofiltration, 5 kDa-permeate flux
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