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EXTRAÇÃO DE ADOÇANTES DE *Stevia rebaudiana* POR PERCOLAÇÃO EM MODO SEMICONTÍNUO A PARTIR DE FOLHAS NÃO TRATADAS E FOLHAS PRÉ-TRATADAS COM ETANOL

SIMONE ROCHA CIOTTA

Maringá
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Dissertação apresentada ao programa de Pós-Graduação em Ciência de Alimentos da Universidade Estadual de Maringá, como parte dos requisitos para obtenção do título de mestre em Ciência de Alimentos.

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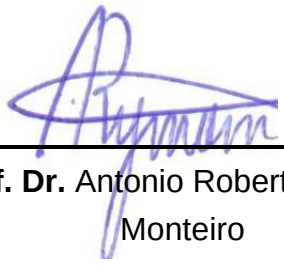

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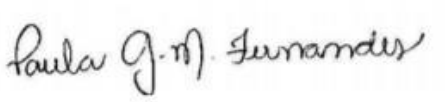

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BIOGRAFIA

Simone Rocha Ciotta nasceu na cidade de Marechal Cândido Rondon, no oeste paranaense. Em 2014 deu início a sua carreira acadêmica cursando Bacharelado em Bioquímica na Universidade Estadual de Maringá, campus Maringá. Durante a graduação teve o imenso prazer de ser membro do Programa de Educação Tutorial em Química da Universidade Estadual de Maringá (PET Química – UEM), onde pôde desenvolver atividades extracurriculares que tinham como objetivo integrar a comunidade acadêmica à comunidade externa, levando conhecimento científico para a comunidade de forma acessível, leve e didática através de, por exemplo, atividades lúdicas e feiras. Ainda durante a graduação conheceu laboratórios na área da microbiologia e imunologia. Ao final da graduação, realizou o estágio obrigatório para a conclusão do curso no NEPRON, Núcleo de Estudos em Produtos Naturais da Universidade Estadual de Maringá, o qual tem a planta *Stevia rebaudiana* como o principal objeto de estudo. Em 2019 ingressou no mestrado na área de Ciência de Alimentos e continuou no NEPRON, sob a orientação do professor Silvio Claudio da Costa, dando continuidade às pesquisas iniciadas durante o estágio. Durante o mestrado pôde trabalhar junto a um incrível grupo de pesquisa no processo de cultivo e colheita da Stevia, seguindo com os processos de extração e purificação dos glicosídeos de esteviol presentes na planta. Atuou na otimização de processos de extração tanto de adoçantes como de outros compostos de interesse presentes na planta e realizou análises de identificação e quantificação de adoçantes por cromatografia líquida de alta eficiência. Também trabalhou com a quantificação de compostos bioativos, padronização de técnicas e determinação da atividade antioxidante por espectrofotômetro.

Dedico

Aos meus pais, Tarcilio e Sueli.
Ao meu irmão, Tales.

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APRESENTAÇÃO

Esta dissertação de mestrado está apresentada na forma de um artigo científico. O artigo em questão foi submetido à revista Journal of Food Processing and Preservation, conceito B1 na área de Ciência de Alimentos.

1 Ciotta, S. R., Zorzenon, M. R. T., Hodas, F., Dacome, A. S., Fernandes, P. G. M., Costa, S. C. **Extraction of sweeteners from Stevia rebaudiana by semicontinuous percolation of untreated leaves and leaves pretreated with ethanol.** Journal of Food Processing and Preservation.

GENERAL ABSTRACT

INTRODUCTION

Steviol glycosides are compounds with sweetening power extracted from the *Stevia rebaudiana* plant, a shrub of the Asteraceae family native to South America. These sweeteners have a sweetening power that can be up to 450 times greater than the sweetening power of sucrose and among them are stevioside, steviolbioside, rebaudiosides A, B, C, D, E, F and dulcoside. Among these, rebaudioside A is the one with the best sensory profile. To overcome some problems in plant cultivation, NEPRON, Nucleus of Research in Natural Products at the State University of Maringá, selected an elite plant called Stevia UEM-13, which has rebaudioside A as the major glycoside. This variety is multiplied by seeds, has a better rooting level and a lower level of plant mortality. To obtain the sweeteners, extraction processes are necessary, which can be conventional or unconventional. In general, researchers seek the highest rate of glycoside recovery using the lowest possible solvent:leaf ratio. These extraction processes commonly generate crude extracts with high levels of soluble solids and suspended materials, making it impossible to directly use these products without a purification process. In an attempt to obtain crude extracts with better sensory profiles and facilitate clarification and purification, pretreatments performed with different solvents are used in order to selectively extract characteristic Stevia compounds that have a bitter aftertaste such as some antioxidant compounds, jhanol, austroinulin and others labdanic alcohols and at the same time keep the sweeteners in the leaves so that they can be later extracted.

AIMS

This work aimed to study the extraction of sweeteners from untreated leaves (UL) and leaves pretreated with ethanol (PL) from the Stevia UEM-13 variety, using the semicontinuous percolation method with water as solvent, in order to obtain a crude extract that can be used directly in the preparation of teas or coffees or that has the potential to achieve purity above 70 % by using a simple microfiltration step.

MATERIAL E METHODS

Stevia rebaudiana plants were harvested at their vegetative growth peak and dried in a forced air circulation oven at 60 ºC until the moisture content was lower than 10 %. Then, stems and leaves were separated and crushed in a knife mill. The leaves were stored in a polyethylene bag protected from the light to be later analyzed and extracted. For the ethanolic pretreatment, the leaves were packed with absolute ethanol in a column. This same solvent eluted through the column and 14 fractions of 300 ml were collected. These fractions were dried and stored for other purposes while the leaves were placed in a stainless steel tray for drying and then stored away from the light for further analysis. The quantification of steviol glycosides from leaves *in natura*, leaves pretreated with ethanol and from the extracts obtained from glycoside extraction was performed by HPLC. The extraction of steviol glycosides was performed with untreated leaves and leaves pretreated with ethanol by semicontinuous percolation, using water

as a solvent at a ratio of 1:40 (w/v). For this purpose, 200 ml of boiling deionized water were percolated through 5 g of leaves that were placed on an analytical paper filter. This process was repeated 4 more times totaling 1000 ml of solvent percolated and the filtrates were collected and stored separately. The extracts obtained in each cycle had their volume and total solids content determined. Color and turbidity analyses were performed by measuring the absorbance at 420 and 670 nm. The contents of soluble and insoluble impurities were estimated through calculations that correlate the absorbance with these contents.

RESULTS AND DISCUSSION

Three hundred grams of *Stevia rebaudiana* leaves of the Stevia UEM-13 variety were pretreated. For this, 5.1 L of absolute ethanol were used and after drying the leaves, a mass of 280.14 g was obtained, resulting in a yield of 93.38 %, values compatible with those in the literature. The content of total glycosides for UL was 13.6 % and 13.1 % for PL, which shows the efficiency of the ethanolic pretreatment in selectively extracting undesirable substances leaving practically unaltered the sweetener content in the pretreated leaves. Both untreated leaves and those pretreated with ethanol kept rebaudioside A as the major sweetener in their composition, with a ratio of 1.58 and 1.52, respectively. The extraction by percolation allowed the recovery of 91.64 % of the glycosides for UL and 93.80 % for PL, leaving the bagasse with a glycoside content of 0.18 % and 0.28 % respectively. In the first extraction cycle, the purity content of the extract obtained was 25.68 % for UL and 24.14 % for PL, with a glycoside recovery of, respectively, 49.73 % and 42.33 %. In the second extraction cycle, there was an increase in the purity content of the extracts obtained, with 35.21 % for UL and 38.03 % for PL, with a recovery of glycosides present in the leaves of, 34.29 % and 37.82 %, respectively. In the third extraction cycle, the purity content of the extract obtained was 44.54 % for UL and 45.96 % for PL, with a sweetener recovery of 6.16 % and 10.31 %, respectively. Absorption values at 420 nm (color) ranged from 1.4933 to 0.0543 from the first to the fifth cycle for UL and from 1.2858 to 0.0696 for PL, causing a color reduction of 96.36 % and 94.59 %, respectively. At 670 nm (turbidity), the values ranged from 0.2653 to 0.0096 for UL and from 0.1999 to 0.0105 for PL, which generates a reduction in turbidity of 96.38 % and 94.75 %, respectively. With the pretreated leaves, 903.33 ml of extract were obtained and 623.2 mg of sweeteners were recovered. With the untreated leaves, 877.33 ml of extract were obtained, recovering 614.4 mg of sweeteners. It is concluded that from 5 g of UL, it was obtained enough sweeteners to sweeten 934.8 ml of coffee, while from PL it was obtained enough sweeteners to sweeten 921.6 ml of coffee. Five grams of untreated leaves and leaves pretreated with ethanol were mixed with 80 g of coffee powder and preliminary sensory tests unequivocally demonstrated that the coffee prepared with PL did not have an unpleasant, herbaceous taste as presented in the coffee prepared with UL. It is estimated that the removal of insoluble solids by microfiltration present in the lower purity extract has the potential to raise the level of purity of the UL extract from 25.68 % to 81.12 % and of the PL extract from 24.14 % to 87.26 %. Regarding the effect of extraction cycles on the levels of stevioside, rebaudioside C, rebaudioside A and RebA/Stev ratio, it was observed that the increase in extractive cycles was accompanied by a reduction in the RebA/Stev ratio. For UL and PL, 124.63 and 122.88 mg of total glycosides per gram of leaves were recovered respectively. Regarding the recovery of

rebaudioside A, the major glycoside, the values obtained were 66.53 mg/g for UL and 65.03 mg/g for PL.

CONCLUSIONS

The method of semicontinuous percolation using boiling water as solvent allowed steviol glycosides recovery levels higher than 90 % from both untreated leaves and leaves pretreated with ethanol, resulting in bagasse with very low glycoside contents. Extracts with lower purity can be easily purified by using microfilters, resulting in extracts with purity above 70 %. It is estimated that the removal of insoluble solids by microfiltration from the lowest purity cycle can result in an increase in the purity level of the UL extract from 25.68 % to 81.12 % and of the PL extract from 24.14 % to 87.26 %. The extracts generated from 5 g of leaves had enough sweeteners to prepare approximately one liter of coffee, with both UL and PL. Preliminary sensory tests indicated that extracts obtained from PL did not have the same pronounced and unpleasant herbaceous taste as the extracts obtained from the untreated leaves, which should allow the PL extracts to be used without any additional clarification or purification steps in the formulation of juices and beverages, in total or partial replacement of sucrose or other synthetic sweeteners.

Keywords: clarification, extraction, rebaudioside, steviol glycosides.

RESUMO GERAL

INTRODUÇÃO

Glicosídeos de esteviol são compostos com poder edulcorante extraídos da planta *Stevia rebaudiana*, um arbusto da família Asteraceae nativo da América do Sul. Estes adoçantes possuem poder edulcorante que pode chegar a ser 450 vezes maior que o poder edulcorante da sacarose e dentre eles estão o esteviosídeo, esteviolbiosídeo, rebaudiosídeos A, B, C, D, E, F e dulcosídeo. Destes, o rebaudiosídeo A é o que apresenta melhor perfil sensorial. Para contornar alguns problemas no cultivo da planta, o NEPRON, Núcleo de Estudos em Produtos Naturais da Universidade Estadual de Maringá, selecionou uma planta de elite denominada Stevia UEM-13 que apresenta o rebaudiosídeo A como o glicosídeo majoritário. Esta variedade é multiplicada por sementes, possui um melhor nível de enraizamento e um menor nível de mortalidade das plantas. Para a obtenção dos adoçantes, são necessários processos de extração os quais podem ser convencionais ou não convencionais. De forma geral, pesquisadores buscam a maior taxa de recuperação de glicosídeos utilizando a menor proporção solvente:folha possível. Estes processos de extração comumente geram extratos brutos com altos níveis de sólidos solúveis e materiais em suspensão, impossibilitando a utilização direta destes produtos sem que haja um processo de purificação. Em tentativa de se obter extratos brutos com melhores perfis sensoriais e facilitar a clarificação e purificação, surgem pré-tratamentos realizados com diferentes solventes que buscam extrair seletivamente compostos característicos da Stevia que possuem sabor residual amargo como alguns compostos antioxidantes, jhanol, austroinulina e outros álcoois labdânicos e ao mesmo passo mantendo nas folhas, os adoçantes para que estes possam ser extraídos posteriormente.

OBJETIVOS

Este trabalho teve como objetivo estudar a extração de adoçantes a partir de folhas não tratadas (FNT) e folhas pré-tratadas com etanol (FPT) da variedade Stevia UEM-13, empregando o método de percolação semicontínua com a utilização de água como solvente, a fim de se obter um extrato bruto que possa ser utilizado diretamente no preparo de chás ou cafés ou ainda que tenha potencial de atingir pureza acima de 70 % pelo emprego de uma simples etapa de microfiltração.

MATERIAL E METODOS

As plantas de *Stevia rebaudiana* foram colhidas no seu pico do crescimento vegetativo e secas em estufa de circulação de ar forçado a 60 ºC até que a umidade fosse menor que 10 %. Em seguida, caules e folhas foram separados e triturados em moinho de facas. As folhas foram armazenadas em saco de polietileno ao abrigo de luz para serem analisadas e extraídas posteriormente. Para o pré-tratamento etanólico, as folhas foram empacotadas com etanol absoluto em uma coluna. Este mesmo solvente eluiu pela coluna e foram coletadas 14 frações de 300 ml. Essas frações foram secas e armazenadas para outros fins enquanto as folhas foram colocadas em uma bandeja de inox para a secagem e em seguida foram armazenadas ao abrigo da luz para posteriores

análises. A quantificação de glicosídeos de esteviol das folhas *in natura*, folhas pré-tratadas com etanol e dos extratos obtidos da extração de glicosídeos foi realizada por CLAE. A extração de glicosídeos de esteviol foi feita com as folhas não tratadas e com as folhas pré-tratadas com etanol por percolação de forma semicontínua, utilizando água como solvente na proporção de 1:40 (p/v). Para tal, 200 ml de água deionizada fervente foram percolados por 5 g de folhas que estavam dispostas em um filtro de papel analítico. Este processo foi repetido por mais 4 vezes totalizando 1000 ml de solvente percolado e os filtrados foram colhidos e armazenados separadamente. Os extratos obtidos em cada ciclo tiveram o volume e o teor de sólidos totais determinados. Análises de cor e turbidez foram realizadas através da medida da absorbância a 420 e 670 nm. Os teores de impurezas solúveis e insolúveis foram estimados através de cálculos que correlacionam as absorbâncias a tais teores.

RESULTADOS E DISCUSSÃO

Foram pré-tratadas 300 g de folhas de *Stevia rebaudiana* da variedade Stevia UEM-13. Para isso foram utilizados 5,1 L de etanol absoluto e após secagem das folhas, obteve-se uma massa de 280,14 g, resultando em rendimento de 93,38 %, valores compatíveis com os da literatura. O teor de glicosídeos totais para FNT foi de 13,6 % para FPT de 13,1 %, o que mostra a eficiência do pré-tratamento etanólico em extrair seletivamente substâncias indesejáveis deixando praticamente inalterado o conteúdo de adoçantes nas folhas pré-tratadas. Ambas as folhas não tratada e pré-tratadas com etanol mantiveram o rebaudiosídeo A como o adoçante majoritário na sua composição, apresentado uma razão de 1,58 e 1,52, respectivamente. A extração por percolação permitiu a recuperação de 91,64 % dos glicosídeos para FNT e de 93,80 % para FPT, restando no bagaço um teor de glicosídeos de 0,18 % e 0,28 % respectivamente. No primeiro ciclo de extração, o teor de pureza do extrato obtido foi de 25,68 % para FNT e de 24,14 % para FPT, com recuperação de, respectivamente, 49,73 % e 42,33 % de glicosídeos. No segundo ciclo de extração, observou-se aumento no teor de pureza do extrato obtido, com 35,21 % para FNT e 38,03 % para FPT, com recuperação de, respectivamente, 34,29 % e 37,82 % dos adoçantes presentes nas folhas. No terceiro ciclo de extração, o teor de pureza do extrato obtido foi 44,54 % para FNT e de 45,96 % para FPT, com recuperação de, respectivamente, 6,16 % e 10,31 % dos adoçantes presentes nas folhas. Os valores de absorção a 420 nm (cor) variaram de 1,4933 a 0,0543 do primeiro para o quinto ciclo para FNT e de 1,2858 a 0,0696 para FPT, causando uma redução de cor de 96,36 % e 94,59 %, respectivamente. Já a 670 nm (turbidez), os valores variaram de 0,2653 a 0,0096 para FNT e de 0,1999 a 0,0105 para FTP, o que gera uma redução de turbidez de 96,38 % e 94,75 %, respectivamente. Com as folhas pré-tratadas, foram obtidos 903,33 ml de extrato e foram recuperados 623,2 mg de adoçantes. Já com as folhas não tratadas, 877,33 ml de extrato foram obtidos recuperando-se 614,4 mg de adoçantes. Conclui-se que a partir de 5 g de FNT foi obtido adoçante suficiente para adoçar 934,8 ml de café, enquanto que a partir de FPT foi obtido o equivalente para adoçar 921,6 ml de café. Cinco gramas de folhas não tratadas e folhas pré-tratadas com etanol foram misturadas com 80 g de pó de café e testes sensoriais preliminares demonstraram de forma inequívoca que o café preparado com FPT não apresentou gosto herbáceo e desagradável como apresentado pelo café preparado com FNT. Estima-se que a remoção de sólidos insolúveis por microfiltração presentes no extrato de menor pureza tem potencial de elevar o nível de pureza do

extrato de FNT de 25,68 % para 81,12 % e do extrato de FPT de 24,14 % para 87,26 %. Em relação ao efeito dos ciclos de extração sobre os teores de esteviosídeo, rebaudiosídeo C, rebaudiosídeo A e razão RebA/Stev observou-se que o aumento de ciclos extrativos foi acompanhado da redução da razão RebA/Stev. Para FNT e FPT, foram recuperadas, respectivamente, 124,63 e 122,88 mg de glicosídeos totais por grama de folhas. Em relação à recuperação de rebaudiosídeo A, glicosídeo majoritário, os valores obtidos foram de 66,53 mg/g para FNT e de 65,03 mg/g para FPT.

CONCLUSÕES

O método de extração por percolação semicontínua utilizando água fervente como solvente permitiu níveis de recuperação de glicosídeos de esteviol superiores a 90 % a partir de folhas não tratadas e pré-tratadas com etanol, resultando em bagaços de folhas com baixíssimos teores de glicosídeos. Os extratos de baixa pureza podem ser facilmente purificados pelo emprego de microfiltros, resultando em extratos com pureza acima de 70 %. Estima-se que a remoção de sólidos insolúveis por microfiltração do ciclo de menor nível de pureza, pode resultar na elevação do nível de pureza do extrato de FNT de 25,68 % para 81,12 % e do extrato da FPT de 24,14 % para 87,26 %. Os extratos gerados a partir de 5 g de folhas apresentaram adoçantes suficientes para o preparo de aproximadamente um litro de café, tanto com FNT como com FPT. Testes sensoriais preliminares indicaram que os extratos obtidos a partir de FPT não apresentam o pronunciado e desagradável gosto herbáceo dos extratos obtidos a partir de folhas não tratadas, o que deverá permitir que o extrato de folhas pré-tratadas seja empregado sem nenhuma etapa adicional de clarificação ou purificação na formulação de sucos e bebidas, em substituição total ou parcial da sacarose ou outros edulcorantes sintéticos.

Palavras chaves: clarificação, extração, rebaudiosídeo, glicosídeos de esteviol.

ARTICLE

Extraction of sweeteners from *Stevia rebaudiana* by semicontinuous percolation of untreated leaves and leaves pretreated with ethanol

Extraction of Stevia's sweeteners by percolation

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Abstract

Untreated leaves (UL) and leaves pretreated with ethanol (PL) of the Stevia UEM-13 variety were extracted by a percolation method using boiling water as solvent (1/40). Five extractive cycles were performed for each starting material. The total recovery of sweeteners was 91.64 % for UL and 93.80 % for PL. In the first extractive cycle the value of insoluble impurities was estimated at 55.44 % for UL and 63.12 % for PL, therefore the purity levels of the extracts from the first cycle could, respectively, be increased from 25.68 % to 81.12 % and from 24.14 % to 87.26 % by employing an additional microfiltration step. It was extracted enough sweeteners from five grams of both Stevia leaves to sweeten a liter of coffee, and preliminary sensory tests showed that, unlike the coffee prepared with UL, the coffee prepared with pretreated leaves did not have an unpleasant herbaceous taste.

Keywords: clarification, extraction, rebaudioside, steviol glycosides.

1. Introduction

Stevia rebaudiana is a shrub of the Asteraceae family, native to South America. Sweeteners called steviol glycosides are extracted from the leaves of this plant, which have a sweetening power that can be up to 450 times greater than the sweetening power of sucrose (Das et al., 2015). Among the glycosides found in Stevia are stevioside, steviolbioside, rebaudiosides A, B, C, D, E, F and dulcoside (Ahmed & Mukta, 2017), being rebaudioside A, among the major glycosides, the one with the best sensory profile.

NEPRON, the Nucleus of Research in Natural Products at the State University of Maringá, obtained an elite plant named Stevia UEM-13 that has rebaudioside A as its main glycoside. This variety can be multiplied by seeds, which improves the level of rooting, reduces the cost of planting crops and reduces the level of plant mortality, significantly improving the production process of Stevia leaves with a high content of rebaudioside A (Milani et al., 2017).

The methods for obtaining Stevia sweeteners have been classified into two large groups: conventional and unconventional (Formigoni et al., 2021). The vast majority of methods, both conventional and unconventional, have the following steps in common: primary pretreatment (drying and grinding of the starting material), extraction, clarification and purification.

In the extraction step, the aim is to obtain the highest sweetener recovery rate, using the lowest possible solvent/stevia leaf ratio. This strategy often generates crude extracts with high levels of soluble solids and suspended matter, which can hardly be used directly as a sweetening agent, requiring exhaustive methods of clarification and purification and often employing substances that compromise the safety of the final product.

Pretreatment with nonpolar solvents has been used to obtain extracts of better sensory quality and facilitate the purification steps by employing membrane separation processes (Rao et al., 2012).

Kutowy et al. (1999) claim that the selectivity of the steviol glycosides extraction step in percolation mode with water as the solvent can be modulated by controlling the pH, solvent temperature in the range of 0 to 10 ºC and solvent:leaves ratio between 1/10 and 1/50.

Pasquel et al. (1999) used supercritical pretreatment to remove substances that compromise the sensory profile of Stevia extracts, having characterized the extract obtained in the pretreatment using gas chromatography. Yoda et al. (2003) reported that the pretreatment of Stevia leaves using supercritical CO_2 reduces the aftertaste by removing some compounds that would be responsible for the bitter taste, among which jhanol, austroinulin and other labdanic alcohols stand out, adding up to 40 % of the substances removed by the pretreatment.

Maioral (2015) in characterization studies of the Stevia UEM-13 variety, observed that the extraction of Stevia leaves with absolute ethanol removed, preferentially, classes of substances other than steviol glycosides, generating an ethanolic extract with an extremely bitter taste, preserving in the extracted leaves most of the glycosides present in the *in natura* leaves. This observation drew attention to the possibility of employing the ethanolic extraction as a pretreatment before the glycoside extraction step.

Formigoni et al. (2018) characterized the ethanolic extract by LC-MS, determining the presence of various classes of substances. Also, observed that the Stevia extracts with high purity content obtained from the pretreated leaves had a better sensory profile than the extracts obtained from the untreated leaves.

Silva et al. (2021) developed cereal bars with untreated Stevia leaves and also with leaves pretreated with ethanol, demonstrating that the bars formulated with pretreated leaves had a better sensory profile, with the important functional property of improving the condition of rats with hepatic steatosis.

This work aimed to study the extraction of sweeteners from pretreated (with absolute ethanol) and untreated leaves of the Stevia UEM-13 variety, using the method of percolation with boiling water as solvent, in order to obtain a crude extract that could be used directly in the preparation of teas or coffees or even that has the potential to achieve purity above 70 % by using a simple microfiltration step.

2. Materials and methods

2.1. Starting material

The starting material were *Stevia rebaudiana* leaves of the seminal Stevia UEM-13 variety, selected and cultivated at the Nucleus of Research in Natural Products, located at the State University of Maringá, Maringá. The plants were harvested at their vegetative growth peak (50-60 days after pruning), when the content of steviol glycosides in the leaves was at its maximum. The plants were dried in a forced air circulation oven at 60 ºC until the humidity was lower than 10 %. Stems and leaves were separated and ground in a knife mill and the leaves were stored in a polyethylene bag protected from the light to be later analyzed and extracted.

2.2. Pretreatment of Stevia leaves with absolute ethanol

The ethanolic pretreatment was performed according to Formigoni et al. (2018). Previously ground Stevia leaves (300 g) were packed with absolute ethanol into a column. This same solvent eluted through the column and 14 fractions of 300 ml were collected. These

fractions were dried and stored for other purposes while the leaves were placed in a stainless steel tray for drying and then stored away from the light for further analysis.

2.3. Quantification of steviol glycosides by high performance liquid chromatography (HPLC)

Steviol glycosides present in *in natura* leaves, leaves pretreated with ethanol and the obtained extracts were quantified by high performance liquid chromatography (HPLC) according to Dacome et al. (2005), using a chromatograph (Gilson, model 307) coupled to a refractive index detector device (IR) with a 5 μm NH2 column and dimensions of 125 x 4.6 mm. The mobile phase used was acetonitrile:water (80:20, v/v) at a flow of 0.5 ml/min.

To analyze the untreated and pretreated leaves, a sample (2 g) was placed in a beaker with 100 ml of deionized water and brought to a boil for 5 minutes. Then, the extract was vacuum filtered. This procedure was repeated 2 more times with 100 ml and 50 ml of deionized water, respectively. Subsequently, the volume was adjusted to 250 ml. For the analysis of the extracts, a sample of the extract was mixed with acetonitrile (20:80, v/v).

2.4. Extraction of steviol glycosides by percolation

For the extraction by semicontinuous percolation, five cycles were carried out at 1/40 ratio (w/v). For this, 200 ml of boiling deionized water percolated five grams of pretreated leaves that were placed on an analytical paper filter. This process was repeated 4 more times and the filtrates were collected and stored separately, characterizing the minimally processed Stevia extracts. At the end of the extraction, there was a total percolation of 1000 ml of solvent. The extracts obtained in each cycle had their volume and total solids content determined and were stored for further analysis. The same extraction procedure was repeated for the untreated Stevia leaves.

2.5. Color and turbidity analysis

Color and turbidity analysis was performed in order to analyze pigment removal and ensure that suspended particles were removed. For this, a mathematical formula that correlates absorbance measurements to clarification was used, according to Bunhak et al. (2002) (Equation 1).

$$\% D = \left[1 - \left(\frac{(Abs\lambda)\ after}{(Abs\lambda)\ before}\right)\right] x\ 100 \qquad \text{(Equation 1)}$$

The extracts obtained from untreated and pretreated leaves had their absorbances measured at wavelengths of 420 and 670 nm, characterizing the color and turbidity analysis, respectively. The reading of the absorbances called "before" occurred right after the extraction, while the readings of the absorbances called "after" were taken 24 hours after the extraction, to observe possible precipitation.

2.6. Estimation of soluble and insoluble impurities contents of the extracts obtained from untreated leaves and from leaves pretreated with ethanol

The extracts from the first three cycles of both extractions had the total soluble solids estimated through the correlation of the absorbances of the extracts measured at 420 and 670 nm, as shown in equations 2 and 3, respectively:

$$\text{SS} = \frac{\text{Abs}}{4{,}8415} \qquad \text{(Equation 2)}$$

Where SS and Abs are, respectively, the total soluble solids and the absorbance measured at 420 nm.

$$\text{SS} = \frac{\text{Abs}}{0{,}6514} \qquad \text{(Equation 3)}$$

Where SS and Abs are, respectively, the total soluble solids and the absorbance measured at 670 nm.

The contents of insoluble solids were calculated by subtracting the value of total soluble solids from the total mass of the extract. Once the values of total soluble and insoluble solids were obtained (de Paula Moraes & Machado, 2001), the contents of soluble and insoluble impurities were calculated according to equations 4 and 5, respectively:

$$SI = \frac{(SS - SM) * 100}{EM} \quad \text{(Equation 4)}$$

Where SI: soluble impurity content; SS: total soluble solids; SM: mass of sweeteners in the extract; EM: total extract mass.

$$II = \frac{IS * 100}{EM} \quad \text{(Equation 5)}$$

Where II: insoluble impurity content; IS: total insoluble solids; EM: total extract mass.

3. Results and discussion

3.1. Ethanolic pretreatment of Stevia UEM-13 leaves

A 300 g sample of *Stevia rebaudiana* leaves of the Stevia UEM-13 variety was pretreated with 5.1 liters of absolute ethanol in percolation mode. Fourteen fractions were collected, pooled and the solvent removed in a rotaevaporator. The pretreated leaves (PL) had a mass of 280.14 g, resulting in a yield of 93.38 %, compatible with the recovery values obtained by Maioral (2015) and Formigoni et al. (2018).

3.2. Characterization of untreated Stevia leaves and leaves after ethanolic pretreatment

Dry samples of PL and untreated leaves (UL) were analyzed by high performance liquid chromatography (HPLC) according to the methodology described by Dacome et al. (2005). The total glycoside contents were 13.6 and 13.1 % for UL and PL respectively, demonstrating that

the ethanolic pretreatment was extremely selective, removing other classes of substances and leaving the sweeteners content in PL practically unchanged (Table 1) (Figure 1).

The rebaudioside A/stevioside ratio (RebA/Stev) for UL was 1.58 and for PL it was 1.52, which indicates that the relationship between rebaudioside A and stevioside presented in the elite variety was not compromised by the pretreatment with ethanol. Both samples had rebaudioside A (Reb A) as their major glycoside, followed by stevioside (Stev) and rebaudioside C (Reb C).

Table 1. Quantification of steviol glycosides in untreated Stevia leaves (UL) and in leaves pretreated with ethanol (PL).

Steviol glycosides	UL (%)	PL (%)
Stevioside	4.5	4.4
Rebaudioside A	7.1	6.7
Rebaudioside C	2.0	2.0
Total glycosides	13.6	13.1
RebA/Stev	1.58	1.52

UL: untreated leaves; PL: leaves pretreated with ethanol.

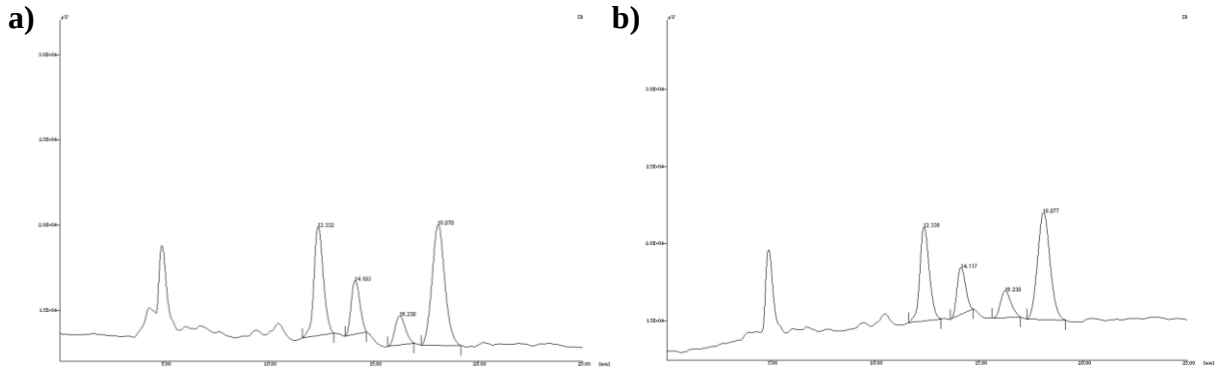

Figure 1. Chromatographic profile determined by HPLC for UL (a) and PL (b).

3.3. Extraction of steviol glycosides by percolation of untreated leaves and leaves pretreated with ethanol

Samples (five grams) of UL and PL were extracted by percolation using boiling water as solvent at a ratio of 1/40 (w/v). For each sample, five extraction cycles were performed (Tables

3 and 4). The recovery of glycosides for UL was 91.64 % and for PL was 93.80 %, considering the content of glycosides present in the starting materials. The glycosides content in the bagasse was 0.18 % for UL and 0.28 % for PL (Figure 2), which is in agreement with the high glycoside recovery values determined in this work.

Table 2. Yields of steviol glycosides with untreated (UL) leaves and leaves pretreated with ethanol (PL) compared to the literature.

References	Stevioside (mg/g)	Rebaudioside A (mg/g)	Rebaudioside C (mg/g)	Total glycosides (mg/g)
UL	43.81	66.53	14.29	124.63
PL	43.05	65.03	14.79	122.88
Formigoni et al. (2018)	-	-	-	74.88
Raspe et al. (2021)	33.89	45.78	17.25	96.92
Erkucuk et al. (2009)	41.10	18.80	-	59.90
Martins et al. (2017)	49.80	27.00	-	76.80
Yilmaz et al. (2021)	70.40	42.90	-	133.30
Ameer et al. (2017)	19.60	15.30	-	34.90
Yen et al. (2021)	-	-	-	88.94
Yildiz-Ozturk et al. (2015)	-	-	-	21.21
Németh et al. (2019)	125.00	34.70	-	159.70
Peng et al. (2020)	5.56	22.19	-	27.75
Arslan Kulcan et al. (2021)	-	-	-	120.40
Abou-Arab et al. (2010)	75.30	-	-	-

UL: untreated leaves; PL: leaves pretreated with ethanol.

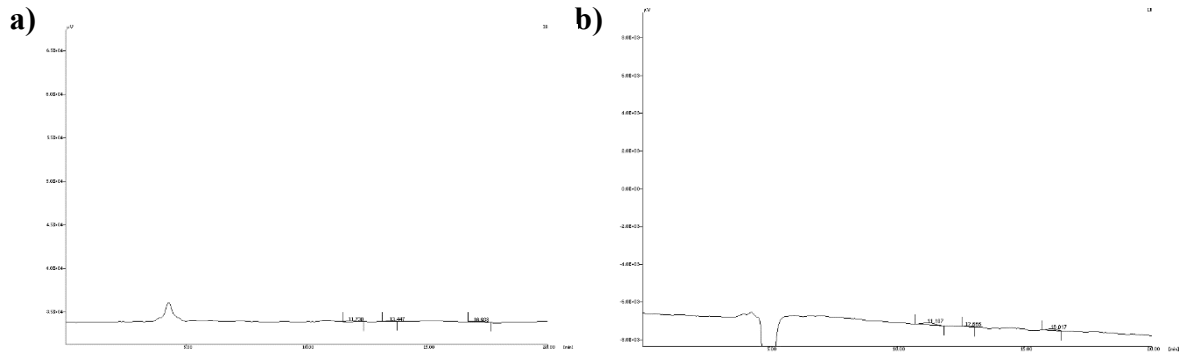

Figure 2. Chromatographic profile determined by HPLC of UL (a) and PL (b) bagasse.

In the first extraction cycle, the purity content of the extract obtained was 25.68 % for UL and 24.14 % for PL, with glycoside recovery of 49.73 % and 42.33 %, respectively. In the second extraction cycle, there was an increase in the purity content of the extracts obtained, with 35.21 % for UL and 38.03 % for PL, with a recovery of sweeteners of 34.29 % and 37.82 % respectively. In the third extraction cycle, the purity content was 44.54 % for UL and 45.96% for PL, with respectively glycoside recovery of 6.16 % and 10.31 %.

Table 3. Extraction parameters for UL.

Cycles	Extract volume (ml)*	Mass at a 5 ml aliquot (g)*	Extract mass (g)*	Mass yield (%)*	Sweeteners in the extract (g)*	Glycoside recovery (%)*	Purity (%)*
I	164.00 ± 1.15	0.0402 ± 0.0021	1.3170 ± 0.0669	26.34 ± 1.34	0.3382 ± 0.0206	49.73 ± 3.03	25.68 ± 1.08
II	182.00 ± 3.46	0.0182 ± 0.0015	0.6623 ± 0.0453	13.25 ± 0.91	0.2332 ± 0.0158	34.29 ± 2.32	35.21 ± 1.50
III	181.33 ± 3.71	0.0026 ± 0.0010	0.0940 ± 0.0349	1.88 ± 0.70	0.0419 ± 0.0090	6.16 ± 1.32	44.54 ± 9.41
IV	187.33 ± 1.33	Nd**	Nd	Nd	0.0099 ± 0.0003	1.46 ± 0.04	Nd
V	188.67 ± 1.33	Nd	Nd	Nd	Nd	Nd	Nd
Total	**903.33 ± 5.93**	**0.0610 ± 0.0011**	**2.0733 ± 0.0347**	**41.47 ± 0.69**	**0.6232 ± 0.0183**	**91.64 ± 2.69**	**30.06 ± 1.37**

* Mean values of triplicate ± SD; **Nd: not detected.

Table 4. Extraction parameters for PL.

Cycles	Extract volume (ml)*	Mass at a 5 ml aliquot (g)*	Extract mass (g)*	Mass yield (%)*	Sweeteners in the extract (g)*	Glycoside recovery (%)*	Purity (%)*
I	148.00 ± 7.57	0.0386 ± 0.0020	1.1487 ± 0.1103	22.97 ± 2.21	0.2773 ± 0.0213	42.33 ± 3.26	24.14 ± 0.70
II	180.67 ± 3.71	0.0180 ± 0.0020	0.6513 ± 0.0704	13.03 ± 1.41	0.2477 ± 0.0087	37.82 ± 1.33	38.03 ± 3.10
III	181.33 ± 2.40	0.0041 ± 0.0006	0.1469 ± 0.0186	2.94 ± 0.37	0.0675 ± 0.0081	10.31 ± 1.24	45.96 ± 1.43
IV	182.67 ± 2.91	Nd**	Nd	Nd	0.0208 ± 0.0048	3.18 ± 0.74	Nd
V	184.67 ± 2.40	Nd	Nd	Nd	0.0011 ± 0.0011	0.16 ± 0.16	Nd

Total	**877.33 ± 18.49**	**0.0607 ± 0.0004**	**1.9469 ± 0.0674**	**38.94 ± 1.35**	**0.6144 ± 0.0068**	**93.80 ± 1.04**	**31.56 ± 0.80**

* Mean values of triplicate ± SD. **Nd: not detected.

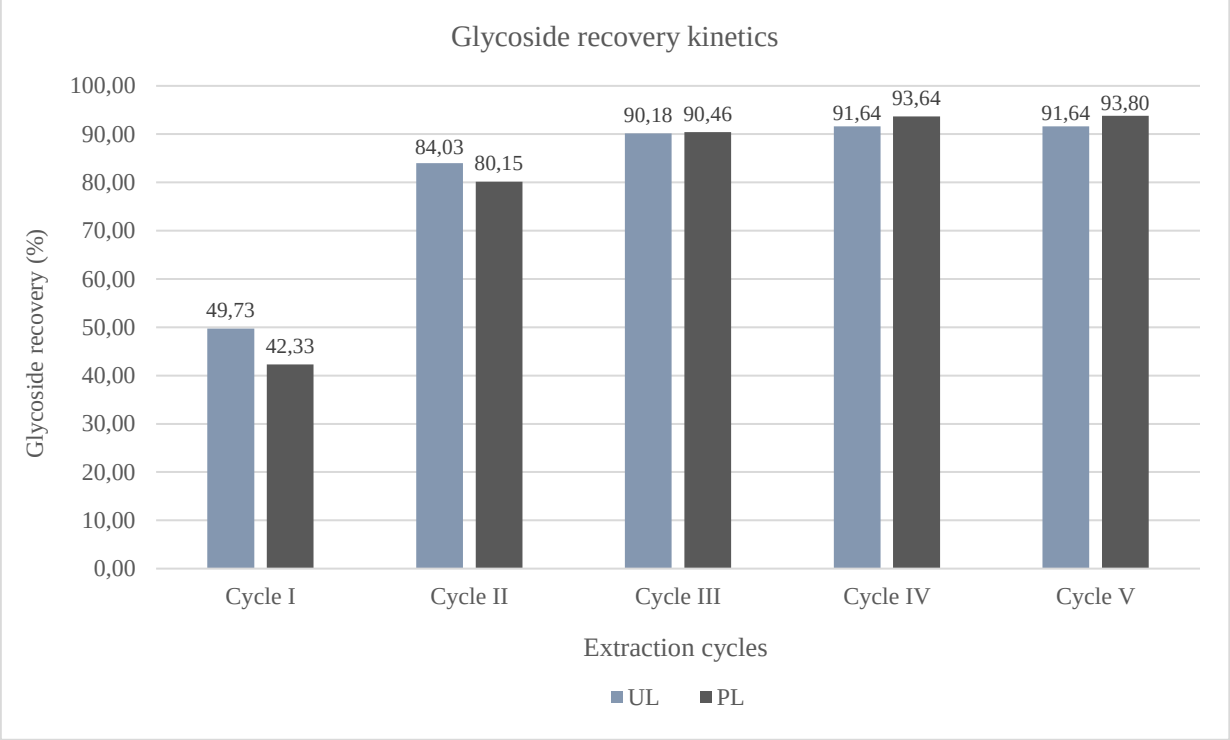

Figure 3. Glycoside recovery kinetics for UL and PL.

3.4. Color and turbidity analysis

For UL, the absorption values at 420 nm (color) ranged from 1.4933 to 0.0543 from the first to the fifth cycle, while the absorption values at 670 nm (turbidity) ranged from 0.2653 to 0.0096 (Table 5). For PL, the absorption values at 420 nm ranged from 1.2858 to 0.0696 from the first to the fifth cycle, while the absorption values at 670 nm ranged from 0.1999 to 0.0105 (Table 6).

Table 5. Color and turbidity parameters for UL.

Color (420 nm)			**Turbidity (670 nm)**		
Cycles	**Absorption***	**Color reduction (%)**	**Cycles**	**Absorption***	**Turbidity reduction (%)**
I	1.4933 ± 0.1141	-	I	0.2653 ± 0.0193	-
II	1.0870 ± 0.0758	27.21	II	0.1938 ± 0.0147	26.94

III	0.2531 ± 0.0470	83.05	III	0.0393 ± 0.0061	85.17
IV	0.0919 ± 0.0054	93.85	IV	0.0146 ± 0.0008	94.51
V	0.0543 ± 0.0012	96.36	V	0.0096 ± 0.0001	96.38

* Mean values of triplicate ± SD.

Table 6. Color and turbidity parameters for PL.

Color (420 nm)			**Turbidity (670 nm)**		
Cycles	**Absorption***	**Color reduction (%)**	**Cycles**	**Absorption***	**Turbidity reduction (%)**
I	1.2858 ± 0.0812	-	I	0.1999 ± 0.0104	-
II	1.073 ± 0.0820	16.50	II	0.1710 ± 0.0078	14.44
III	0.3450 ± 0.0553	73.17	III	0.0466 ± 0.0040	76.67
IV	0.1348 ± 0.0237	89.52	IV	0.0194 ± 0.0018	90.31
V	0.0696 ± 0.0128	94.59	V	0.0105 ± 0.0015	94.75

* Mean values of triplicate ± SD.

a) **b)**

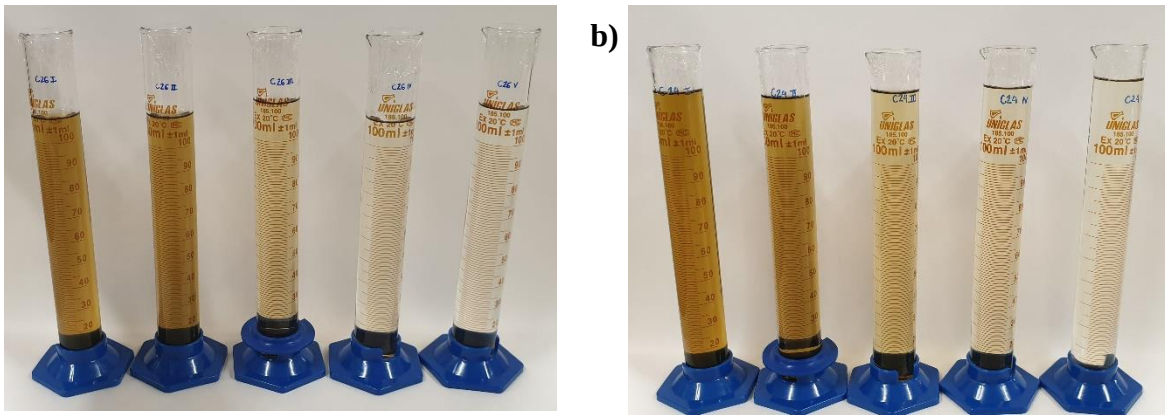

Figure 4. Extracts from the five cycles for UL (a) and PL (b).

3.5. Sweetness power of the extracts obtained

In 903.33 ml of extract obtained from UL, 623.2 mg of sweeteners were recovered, whilst for PL, in 877.33 ml of extract obtained, 614.4 mg of sweeteners were recovered. Considering that approximately 40 mg of steviol glycosides have a sweetening power equivalent to 5 g of sucrose (Cardoso et al., 2004) , it is concluded that from five grams of UL it was obtained enough sweetener to sweeten 934.80 ml of coffee, while from PL it was obtained the equivalent to sweeten 921.60 ml of coffee. Five grams of both untreated and pretreated leaves were mixed with 80 g of coffee powder and percolated with 1000 ml of boiling water. Preliminary sensory tests

have unequivocally demonstrated that the coffee prepared with PL does not have an herbaceous taste as presented in the coffee prepared with UL. Formigoni et al. (2018) showed that pretreatment of Stevia leaves with absolute ethanol selectively removes compounds such as phenolics and flavonoids that may contribute to the bitter aftertaste of the sweeteners extracted. Also, they performed a sensory analysis that showed that the sweeteners obtained from the leaves pretreated with ethanol presented a similar sensory profile to that of sucrose.

3.6. Estimates of soluble and insoluble impurities in extracts obtained from UL and PL in different extraction cycles

The soluble and insoluble impurities content of the extracts obtained from UL and PL for the first three cycles were estimated (Tables 7 and 8). From the first to the third cycle, there was an increase in the content of soluble impurities and a reduction in the content of insoluble impurities. Considering that the content of insoluble impurities would be easily removed through a simple microfiltration process, it would be possible to increase the purity of the extract from the first cycle obtained from the untreated leaves from 25.68 % to 81.12 %, removing the 55.44 % of insoluble impurities present in this extract. The same logic can be applied to the extract of the first cycle obtained from the pretreated leaves. Starting with a purity of 24.14 % and a content of insoluble impurities of 63.12 %, there is the potential to raise the purity of this extract from 24.14 % to 87.26 % through a simple microfiltration process. Such results give us the opportunity to process these first extracts in a different way, apart from the other extracts. Thus, these extracts, which previously had a very low purity, could easily become products of high interest, due to their high purity level.

Table 7. Soluble and insoluble impurities contents per cycle for UL.

Cycle I			
Impurities (I)	**420 nm (%)***	**670 nm (%)***	**Average (%)***

Soluble impurity (SI)	12.73	25.04	18.89
Insoluble impurity (II)	61.59	49.28	55.44
Cycle II			
Impurities (I)	**420 nm (%)***	**670 nm (%)***	**Average (%)***
Soluble impurity (SI)	26.49	46.56	36.53
Insoluble impurity (II)	38.30	18.22	28.26
Cycle III			
Impurities (I)	**420 nm (%)***	**670 nm (%)***	**Average (%)***
Soluble impurity (SI)	56.29	71.94	64.12
Insoluble impurity (II)	Nd**	Nd	Nd

* Mean values of triplicate. **Nd: not detected.

Table 8. Soluble and insoluble impurities contents per cycle for PL.

Cycle I			
Impurities (I)	**420 nm (%)***	**670 nm (%)***	**Average (%)***
Soluble impurity (SI)	10.08	15.40	12.74
Insoluble impurity (II)	65.78	60.46	63.12
Cycle II			
Impurities (I)	**420 nm (%)***	**670 nm (%)***	**Average (%)***
Soluble impurity (SI)	23.48	34.80	29.14
Insoluble impurity (II)	38.48	27.16	32.82
Cycle III			
Impurities (I)	**420 nm (%)***	**670 nm (%)***	**Average (%)***
Soluble impurity (SI)	41.97	42.38	42.18
Insoluble impurity (II)	12.07	11.66	11.87

* Mean values of triplicate.

3.7 Effect of the extraction cycles on stevioside, rebaudioside C, rebaudioside A contents and RebA/Stev ratio

The results shown in Tables 9 and 10 present the glycoside composition of the extracts obtained from UL and PL. It was observed that, with the increase in extractive cycles, there was a reduction in the RebA/Stev ratio. Tables 11 and 12 show, respectively for UL and PL, the recovery of stevioside, rebaudioside C, rebaudioside A and total glycosides in relation to the dry mass of Stevia leaves. For UL and PL, 124.63 and 122.88 mg of total glycosides per gram of leaves were recovered, respectively. Regarding the rebaudioside A recovery, the values obtained

were 66.53 mg/g for UL and 65.03 mg/g for PL, which are higher than all values shown in Table 2.

Table 9. Glycosides extracted per cycle with UL.

Cycles	Stev (g)*	Reb C (g)*	Reb A (g)*	Total glycosides (g)*	RebA/Stev*
I	0.1174 ± 0.0079	0.0325 ± 0.0023	0.1883 ± 0.0123	0.3382 ± 0.0206	1.6044 ± 0.0038
II	0.0820 ± 0.0052	0.0313 ± 0.0025	0.1199 ± 0.0081	0.2332 ± 0.0158	1.4628 ± 0.0122
III	0.0159 ± 0.0035	0.0061 ± 0.0015	0.0198 ± 0.0039	0.0419 ± 0.0090	1.2448 ± 0.0277
IV	0.0038 ± 0.0001	0.0015 ± 0.0001	0.0046 ± 0.0003	0.0099 ± 0.0003	1.2105 ± 0.0816
V	0	0	0	0	-
Total	**0.2191 ± 0.0058**	**0.0715 ± 0.0052**	**0.3326 ± 0.0092**	**0.6232 ± 0.0183**	**1.5184 ± 0.0055**

* Mean values of triplicate ± SD. Stev: stevioside; Reb C: rebaudioside C; Reb A: rebaudioside A;

Table 10. Glycosides extracted per cycle with PL.

Cycles	Stev (g)*	Reb C (g)*	Reb A (g)*	Total glycosides (g)*	RebA/Stev*
I	0.0953 ± 0.0069	0.0291 ± 0.0019	0.1529 ± 0.0127	0.2773 ± 0.0213	1.6035 ± 0.0313
II	0.0871 ± 0.0031	0.0320 ± 0.0007	0.1286 ± 0.0055	0.2477 ± 0.0087	1.4774 ± 0.0110
III	0.0247 ± 0.0037	0.0095 ± 0.0006	0.0334 ± 0.0038	0.0675 ± 0.0081	1.3509 ± 0.0589
IV	0.0077 ± 0.0020	0.0033 ± 0.0005	0.0098 ± 0.0024	0.0208 ± 0.0048	1.2771 ± 0.0566
V	0.0005 ± 0.0005	0.0001 ± 0.0001	0.0005 ± 0.0005	0.0011 ± 0.0011	1.0000 ± 0.3333
Total	**0.2153 ± 0.0020**	**0.0740 ± 0.0014**	**0.3252 ± 0.0051**	**0.6144 ± 0.0068**	**1.5105 ± 0.0274**

* Mean values of triplicate ± SD. Stev: stevioside; Reb C: rebaudioside C; Reb A: rebaudioside A.

Table 11. Concentration of glycosides with UL.

Glycosides	g/5 g of dry leaves*	g/100 g of dry leaves*	mg/g of dry leaves*
Stev	0.2191 ± 0.0058	4.3813 ± 0.1164	43.81 ± 1.1645
Reb C	0.0715 ± 0.0052	1.4293 ± 0.1048	14.29 ± 1.0483
Reb A	0.3326 ± 0.0092	6.6527 ± 0.1842	66.53 ± 1.8423
Total	**0.6232 ± 0.0183**	**12.4633 ± 0.3659**	**124.63 ± 3.6586**

* Mean values of triplicate ± SD. Stev: stevioside; Reb C: rebaudioside C; Reb A: rebaudioside A.

Table 12. Concentration of glycosides with PL.

Glycosides	g/5 g of dry leaves*	g/100 g of dry leaves*	mg/g of dry leaves*
Stev	0.2153 ± 0.0020	4.3053 ± 0.0399	43.05 ± 0.3994
Reb C	0.0740 ± 0.0014	1.4793 ± 0.0277	14.79 ± 0.2767
Reb A	0.3252 ± 0.0051	6.5033 ± 0.1080	65.03 ± 1.0183
Total	**0.6144 ± 0.0068**	**12.2880 ± 0.1365**	**122.88 ± 1.3647**

* Mean values of triplicate ± SD. Stev: stevioside; Reb C: rebaudioside C; Reb A: rebaudioside A.

4. Conclusion

The semicontinuous percolation extraction method using boiling water as solvent allowed levels of steviol glycosides recovery higher than 90 % from both untreated leaves and leaves pretreated with ethanol, resulting in bagasse with very low glycoside contents. The extracts generated from five grams of leaves had enough sweeteners to prepare approximately one liter of coffee. Preliminary sensory tests indicated that extracts obtained from PL do not have the same pronounced and unpleasant herbaceous taste as the extracts obtained from untreated leaves do, which should allow the aqueous extracts from PL to be used without any additional purification or clarification step in the formulation of juices and beverages, in total or partial replacement of sucrose or other synthetic sweeteners. It is estimated that the removal of insoluble solids by microfiltration from the lowest purity cycle can result in an increase in the purity level of the UL extract from 25.68 % to 81.12 % and of the PL extract from 24.14 % to 87.26 %.

Acknowledgments

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