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Programa de Pós-Graduação em Ciência de Alimentos

Métodos não convencionais de extração de Glicosídeos e Compostos Bioativos da Stevia (*Stevia rebaudiana* Bertoni)

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

Maringá

2022

Orientador

Prof. Dr. Silvio Cláudio da Costa

Co-orientadora

Profa. Dra. Camila da Silva

DJÉSSICA TATIANE RASPE

"MÉTODOS NÃO CONVENCIONAIS DE EXTRAÇÃO DE GLICOSÍDEOS E COMPOSTOS BIOATIVOS DA STEVIA (*STEVIA REBAUDIANA* BERTONI)."

Tese apresentada à Universidade Estadual de Maringá, como parte das exigências do Programa de Pós-graduação em Ciência de Alimentos, para obtenção do grau de Doutor em Ciência de Alimentos.

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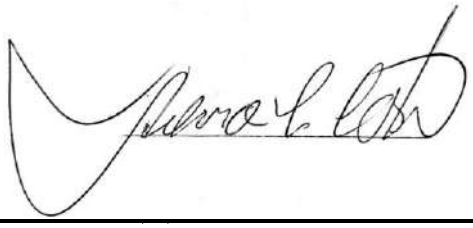

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BIOGRAFIA

Djéssica Tatiane Raspe, filha de Ivete Rosane Leonhardt Wiethölter e Eldir Raspe, nasceu em 05 de março de 1992 na cidade de Pato Bragado (Paraná).

Em 2009 ingressou no curso de Tecnologia em Alimentos pela Universidade Estadual de Maringá, graduando-se em 2012. No mesmo ano, iniciou o curso de Pós-Graduação em Bioenergia como bolsista CAPES com a dissertação intitulada "Hidrólise enzimática do óleo de Macaúba (*Acrocomia aculeata*)", concluída no ano de 2014. Em ambos os períodos, teve a oportunidade de trabalhar em projetos de pesquisa que visavam a modificação e análise de óleos e gorduras para obtenção de biocombustíveis e aplicação na indústria química e de alimentos, por meio de rotas e técnicas alternativas, com orientação e supervisão da Profa. Dra. Camila da Silva. Estes trabalhos, a possibilitaram participar de eventos de cunho científico à nível nacional e internacional, bem como, a publicar seus primeiros artigos científicos.

De 2016 à 2018 atuou no setor administrativo da Poersch Indústria Metalúrgica, empresa de fabricação de implementos agrícolas voltados à suinocultura, onde pode vivenciar experiências do ramo, contribuindo para o aperfeiçoamento de produtos e serviços. Além disso, realizou o cadastramento dos principais produtos fabricados pela empresa nas linhas de financiamento da Agência Especial de Financiamento Industrial (FINAME), do Banco Nacional de Desenvolvimento Econômico e Social (BNDES).

Em 2019, iniciou o Doutorado no Programa de Pós-Graduação em Ciência de Alimentos pela Universidade Estadual de Maringá como bolsista CAPES, sob orientação do Prof. Dr. Silvio Cláudio da Costa e co-orientação da Profa. Dra. Camila da Silva, com a proposta de atuar com rotas alternativas na obtenção de compostos de interesse de uma matriz vegetal com grande importância na indústria de alimentos e fitoterápica. Neste período, desenvolveu concomitantemente, projetos de pesquisa, ensino e extensão. Ministrou cursos, palestras, participou da organização de eventos e co-orientou projetos de iniciação científica, além da participação e apresentação dos resultados de seus trabalhos em eventos de cunho científico. Em 2021 ingressou no curso de graduação em Ciências Biológicas pela Universidade Estadual de Maringá, e nos meses iniciais de 2022, concluiu o Curso de Especialização em Docência para a Educação Profissional e Tecnológica, pelo Instituto Federal de Educação Ciência e Tecnologia de Rondônia (IFRO), ambos pela modalidade EaD.

Em paralelo à sua tese de doutorado, colaborou com outros projetos que geraram os seguintes artigos e capítulo de livro publicados em periódicos científicos:

FORMIGONI, M.; ZORZENON, M. R. T.; MILANI, P. G.; RASPE, D. T.; CIOTTA, S. R.; DACOME, A. S.; COSTA, S. C. Conventional Extraction Techniques. Steviol Glycosides: Production, Properties, and Applications. 1ed.: Elsevier, 2020, v. 1, p. 133-157. https://doi.org/10.1016/B978-0-12-820060-5.00006-6

RASMUSSEN, P.; STEVANATO, N.; RASPE, D. T.; GARCIA, V. A.; SILVA, C. Babassu kernel oil: Enhanced extraction and chemical characterization. Journal of Food Processing and Preservation, v. 1, e16559, 2022. https://doi.org/10.1111/jfpp.16559

GUERRA, A. P.; ROSA, A. C. S.; RASPE, D. T.; SILVA, C. Síntese de ácidos graxos livres do óleo de caroço de algodão. Revista Brasileira de Meio Ambiente, 2022. (aceito para publicação)

RASPE, D. T.; STEVANATO, N.; MASSA, T. B.; SILVA, C. Obtaining hydrolysate from macauba oil and its application in the production of methyl esters. Grasas y Aceites, 2022. (aceito para publicação)

SILVA, H. R. P.; FEITEN, M.; RASPE, D. T.; SILVA, C. Hydrolysis of macauba kernel oil: Ultrasound application in the substrates pre-emulsion step and effect of the process variables. Anais da Academia Brasileira de Ciências, 2022. (aceito para publicação)

TRENTINI, C. P.; MELLO, B. T. F.; POSTAUE, N.; OLIVEIRA, V. F. C.; SILVA, C. Sequential process to obtain fatty acid esters from crambe oil using a mixture of acyl acceptors under pressurized conditions. Journal of Supercritical Fluids, 2022. (aceito para publicação)

Dedico

Aos meus pais, Eldir Raspe e Ivete Wiethölter,
pelo inestimado apoio, compreensão e paciência nesta fase.
A minha avó Lorena Wiethölter (*in memorian*),
referência de força, coragem e amor.

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A Deus, acima de tudo, por me guiar, proteger e abençoar sob todas as circunstâncias.

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Aos colegas, a Profa. Dra. Paula Gimenez Milani e ao técnico responsável, Antônio Sérgio Dacome (NEPRON), por terem me ensinado com grande paciência as particularidades da matriz vegetal e as metodologias necessárias para a realização de algumas etapas deste trabalho. As queridas Natália Stevanato e Najla Postaue, pelo companheirismo, empatia e amizade durante este período. Aos demais colegas do PRO-IQA e Bloco B do DTC, que de alguma forma contribuíram para minha formação e tornaram meus dias mais leves e divertidos.

APRESENTAÇÃO

Em consonância com as regras do Programa de Pós-graduação em Ciência de Alimentos, esta tese está estruturada na forma de artigos científicos. Um total de quatro artigos, que contemplam o estado da arte e as investigações realizadas, configuram os resultados obtidos, conforme apresentado a seguir:

Artigo 1. RASPE, D. T.; SILVA, C.; COSTA, S. C. Compounds from *Stevia rebaudiana* Bertoni leaves: An overview of non-conventional extraction methods and challenge. Food Bioscience, v. 46, 101593, 2022. https://doi.org/10.1016/j.fbio.2022.101593

Artigo 2. RASPE, D. T.; CIOTTA, S. R.; ZORZENON, M. R. T.; DACOME, A. S.; SILVA, C.; MILANI, P. G.; COSTA, S. C. Ultrasound-assisted extraction of compounds from Stevia leaf pretreated with ethanol. Industrial Crops and Products, v. 172, 114035, 2021. https://doi.org/10.1016/j.indcrop.2021.114035

Artigo 3. RASPE, D. T.; CIOTTA, S. R.; MELLO, B. R. F.; MILANI, P. G.; SILVA, C.; COSTA, S. C. Pressurized liquid extraction of steviol glycosides from *Stevia rebaudiana* leaves. Chemical Engineering Transactions, v. 87, p. 301-306, 2021. https://doi.org/10.3303/CET2187051

Artigo 4. RASPE, D. T.; SILVA, C.; COSTA, S. C. Pressurized liquid extraction of compounds from Stevia leaf: Evaluation of process variables and extract characterization. (submetido)

Cabe ressaltar que, contemplando parte desta pesquisa, o trabalho intitulado “Efeito de diferentes métodos de extração no rendimento e obtenção de compostos bioativos de folhas de Stevia”, recebeu menção honrosa por mérito científico no V Congresso Internacional de Ciência, Tecnologia e Inovação da Universidade Paranaense, no ano de 2021.

GENERAL ABSTRACT

INTRODUCTION AND AIMS:

Stevia rebaudiana (Bertoni) is a perennial herbaceous shrub that has benefits due to its antioxidant, antimicrobial, antifungal, antitumor and antidiabetic properties. This plant has a sweetish flavor in its leaves, characteristic of compounds called steviol glycosides (SG), among which stevioside (Stv) and rebaudioside A (Reb A) stand out mainly, and rebaudioside C (Reb C) and dulcoside A (Dul A), as well as traces of rebaudiosides B, D, E and F. The presence of this matrix in the scenario of compounds with food and phytotherapic relevance and its possible attributions are widely discussed in the literature, mainly because their particularity is to provide up to 450 times more sweetening power than sucrose, not generating energy accumulation or any change to the glycemic index. However, there are still few reports involving obtaining its compounds through techniques that envision higher yields with the application of technology, linked to the precepts of green chemistry. With a view to contributing to the improvement of the matrix and techniques investigations, bringing unprecedented findings of the application of strategies still little explored in the literature, the main objectives of the four articles that make up this thesis were: **1)** To present and discuss recent experimental reports, bringing together strategies that have not yet been explored, focused on obtaining compounds from *Stevia rebaudiana* leaves through unconventional techniques on the rise, such as ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), and extractions under pressurized conditions, by pressurized liquid (PLE), subcritical water (SWE) and supercritical fluid (SFE). **2)** Investigate the UAE of compounds from *Stevia rebaudiana* leaves, evaluating the influence of ultrasound power intensity on leaves submitted and not submitted to ethanolic pretreatment, and through the application of an experimental design, examine the effect of experimental variables (temperature, solvent/leaf ratio and percentage of ethanol in the extracting solvent), under the mass yield (Y_M) and sweeteners (Y_S), SG content, as well as its active compounds, represented by the content of total phenolic compounds (TPC) and antioxidant activity (AA) of pre-treated leaves. **3)** Evaluate the efficiency of PLE by obtaining SG from leaves of *Stevia rebaudiana* submitted and not submitted to ethanolic pretreatment, investigating the effect of experimental variables (solvent/leaf ratio and percentage of ethanol in the extracting solvent) on Y_M it was. **4)** Investigate the extraction of compounds from pre-treated *Stevia rebaudiana* leaves under pressurized conditions, through the effect of experimental variables (percentage of ethanol in the extracting solvent, static time, pressure and temperature) on Y_M, TPC and AA, and through of the condition that resulted in the maximization of the response variables, to determine the content of active compounds (TPC, total flavonoid content - TFC and AA) and SG, aiming to compare the extracts obtained with those resulting from the extraction by Soxhlet and UAE.

METHODS:

1) For the development of this survey, articles and patents were consulted in journals from the main databases (Science Direct, Scielo, Web of Science, Scopus, Springer and Wiley, and SciFinder, respectively). For the compilation of data, only scientific articles and patents published in the last 12 years (2009–2021) were considered, limiting themselves to data referring to **i)** extraction of compounds from *Stevia rebaudiana* leaves, **ii)** data referring to compounds obtained in extraction and **iii)** data referring to the types of unconventional extraction. The selection and screening process to form the basis of the writing was carried out independently, summarized in the analysis of a total of 35 articles and 09 patents. **2)** In this study, the intensity of ultrasound power of 0, 50 and 100% (0, 83 and 165 W, respectively) was evaluated in the extraction of compounds from Stevia leaves with and without ethanolic pretreatment, in Y_M, Y_S, SG, TPC and AA, the latter represented by the concentration of

extract capable of reducing the DPPH radical by 50% (EC_{50}). A Box-Behnken experimental design with three levels, three variables and five repetitions of the central point was applied to examine the effect of temperature (35, 50 and 65 °C), solvent/leaf ratio (5, 10 and 15 mL g^{-1}) and percentage of ethanol in the extracting solvent (10, 40 and 70%, v/v), aiming to reach the conditions that maximize the Y_M and Y_S, within the tested experimental range. Subsequently, these extracts were characterized in terms of composition, and the correlation between the operational conditions and the evaluated responses was used in the analysis of principal components. **3)** To evaluate the PLE efficiency in obtaining the Y_M and Y_S, the variables temperature, pressure and time were kept fixed at 120 ºC, 100 bar and 60 min, respectively. An experimental apparatus operated in semi-continuous mode was used to conduct the evaluation of the effect of the solvent/leaf ratio (30 to 90 mL g^{-1}) and ethanol concentration in the extracting solvent (100 and 70%, v/v) in the extraction of leaf compounds with and without ethanolic pretreatment. **4)** In this study, the effect of the percentage of ethanol in the extracting solvent (40 and 70%, v/v), static time (10, 20 and 30 min) and pressure (50 and 100 bar) were investigated in the Y_M, TPC and AA of pre-treated Stevia leaf compounds. The conditions that maximized the response variables were then investigated in the extraction kinetics (100, 125 and 150 °C). Subsequently, a comparison was made between the extract obtained by PLE resulting from the maximization of the response variables, Soxhlet (50 mL g^{-1} for 8 hours) and UAE (15 mL g^{-1} for 3 cycles of 10 min), in relation to the Y_M, Y_S, SG, and active compounds (TPC, TFC and AA).

MAIN RESULTS AND DISCUSSION:

1) The reports leveraged through unconventional extraction methods, contemplate promising technological criteria in the world market demand for natural compounds, where *Stevia rebaudiana* presents itself as a matrix with efficiency and profitability in the recovery of its sweeteners and active compounds. They allow quick and easy operation, through the use of renewable solvents under operating conditions that do not affect the quality of the extract obtained. Among these processes, techniques involving ultrasound acoustic cavitations, non-ionizing microwave irradiation, compressed fluids in pressurized liquid and subcritical water extraction, as well as supercritical fluid extraction, are highlighted on the rise. In addition, there were no reports of the application of these techniques linked to strategies that could improve the process and the sensory characteristics of the extract obtained, as is the case of the application of ethanolic pre-treatment. **2)** The application of maximum power (165 W) of the ultrasound cavitations provided the attainment of higher Y_M and Y_S, and extracts with higher contents of TPC and AA. The increase in the solvent/leaf ratio and the percentage of ethanol in the extracting solvent provided the highest values of Y_M and Y_S, while the temperature promoted an increase only in the Y_M. Maximum values of Y_M (44.7 wt%) and Y_S (88.5 wt%) were obtained using solvent with 40% ethanol, 50 °C and 15 mL g^{-1} (solvent/sheet). The extract obtained had ~26.0 wt% SG, corresponding to 9.5, 4.1 and 12.0 wt% of Stv, Reb C and Reb A, respectively. The analysis of the principal components indicated a high correlation of the variables percentage of ethanol in the extracting solvent and solvent/leaf ratio in obtaining extracts with higher levels of active compounds (TPC and AA), in addition to demonstrating that there is no correlation in obtaining sweeteners and compounds active. **3)** Under pressurized conditions, the results showed greater extraction of SG when pre-treated leaves were used. Increasing the solvent/leaf ratio from 30 mL g^{-1} to 90 mL g^{-1} did not favor the extraction of sweeteners, demonstrating that an excess of solvent in the medium does not result in higher yields. These results made it possible to preliminarily evaluate the effects of this technique on the matrix, gathering data and information for further investigations. **4)** The increase in the percentage of ethanol in the extractor solvent favored the Y_M, TPC and AA, while the increase in static time and pressure (>10 min and >50 bar) did not affect the extraction of compounds under the conditions evaluated. The temperature provided an increase in the Y_M and the highest levels of active compounds were obtained after 30 min of the process. The Y_M was similar between the extraction techniques evaluated, with PLE (125°C) providing greater recovery of TPC and AA, and Soxhlet favoring the extraction of SG and TPC. The composition of the extract obtained by PLE was ~26.0 wt% of SG,

corresponding to 9.5, 3.9 and 12.68 wt% of Stv, Reb C and Reb A, respectively, representing 87.8% of the total obtained by Soxhlet. The active potential (TPC, TFC and AA) of the PLE extract was 3.6 and 11.0% higher than that of Soxhlet and UAE, respectively.

CONCLUSIONS:

1) The UAE, MAE, PLE, SWE and SFE techniques address environmental and food safety issues by allowing the use of renewable solvents, in addition to being aligned with the development of alternative processing routes, contemplating sustainability concepts. With a view to contributing to the improvement of these techniques and adding information to future research related to the matrix, by **2)** evaluating the UAE, efficiency in obtaining an extract with high yield, TPC and AA content, and great potential for use as a food additive, it was verified. In terms of the feasibility of the process, the use of an unconventional technique of rapid processing, together with a binary mixture between ethanol and water as an extracting solvent, stands out, which provides reduced production costs compared to using only ethanol and easier solvent separation when compared to using water. **3)**, **4)** When investigating PLE, higher Y_M and active compounds (TPC and AA) was observed, through the use of less energy and solvent, which combined with operational strategies such as the pre-treated matrix and the application of a binary mixture of ethanol and water as a solvent, allowed the conduction of a process in less time, with ease of separation of the extracting solvent and, at the same time, potential reduction of operational costs. Therefore, obtaining compounds from this matrix through the unconventional methods proposed here, in addition to being a viable and adequate alternative for improving the solubility and availability of analytes, has been shown to provide a reduction in operating conditions, enabling the profitable use of GRAS (generally recognized as safe) solvents in these processes. that fit with merit in the precepts of green chemistry, generating an extract with high phytotherapic and food use potential. Although a possible implementation of these processes on a pilot scale presents itself as a relevant alternative, as it allows for fewer inconveniences when compared to conventional processes, the reduction in consumption of inputs and the feasibility of reusing the solvent in the process still need to be improved in their investigation. Combined or sequential processes could be applied as alternatives to this problem, but their exploitation has not yet been reported, as well as the consequences of exposing this matrix to extreme conditions of some of the mentioned technologies, mainly in relation to the possible degradation of the target compounds. These gaps and the lack of information about these techniques are the main challenges in expanding the use of these technologies, emerging as perspectives for future work.

KEY-WORS: Active compounds; Antioxidant activity; Green solvents; Natural sweeteners; Phenolic compounds; Sweeteners; Technology application.

RESUMO GERAL

INTRODUÇÃO E OBJETIVOS:

Stevia rebaudiana (Bertoni) é um arbusto herbáceo perene que apresenta benefícios devido às suas propriedades antioxidantes, antimicrobianas, antifúngicas, antitumorais e antidiabéticas. Esta planta possui em suas folhas sabor adocicado, característico de compostos denominados glicosídeos de esteviol (GS), dentre os quais se destacam principalmente o esteviosídeo (Stv) e o rebaudiosídeo A (Reb A), e minoritáriamente o rebaudiosídeo C (Reb C) e dulcosídeo A (Dul A), além de traços de rebaudiosídeos B, D, E e F. A presença dessa matriz no cenário de compostos com relevância alimentar e fitoterápica e suas possíveis atribuições são amplamente abordadas na literatura, principalmente por terem como particularidade fornecer até 450 vezes mais poder adoçante que a sacarose, não gerando acúmulo energético ou qualquer alteração ao índice glicêmico. No entanto, ainda são escassos os relatos envolvendo a obtenção dos seus compostos por meio de técnicas que vislumbrem maiores rendimentos com a aplicação de tecnologia, atrelado aos preceitos da química verde. Vislumbrando contribuir para o aprimoramento das investigações da matriz e das técnicas, trazendo constatações inéditas da aplicação de estratégias ainda pouco exploradas na literatura, os principais objetivos dos quatro artigos que compõem esta tese foram: **1)** Apresentar e discutir relatos experimentais recentes, reunindo estratégias ainda pouco exploradas, concentradas na obtenção de compostos das folhas de *Stevia rebaudiana* por meio das técnicas não convencionais em ascensão, como a extração assistida por ultrassom (EAU), extração assistida por microondas (EAM), e as extrações em condições pressurizadas, por líquido pressurizado (ELP), água subcrítica (EAS) e fluido supercrítico (EFS). **2)** Investigar a EAU dos compostos das folhas de *Stevia rebaudiana*, avaliando a influência da intensidade da potência do ultrassom em folhas submetidas e não submetidas ao pré-tratamento etanólico, e por meio da aplicação de um delineamento experimental, examinar o efeito das variáveis experimentais (temperatura, relação solvente/folha e percentual de etanol no solvente extrator), sob o rendimento em massa (R_M) e de adoçantes (R_A), teor de GS, bem como seus compostos ativos, representados pelo teor de compostos fenólicos totais (CFT) e atividade antioxidante (AA) de folhas pré-tratadas. **3)** Avaliar a eficiência da ELP mediante a obtenção dos GS a partir de folhas de *Stevia rebaudiana* submetidas e não submetidas ao pré-tratamento etanólico, investigando o efeito das variáveis experimentais (relação solvente/folha e percentual de etanol no solvente extrator) no R_M e R_A. **4)** Investigar a extração de compostos das folhas de *Stevia rebaudiana* pré-tratadas sob condições pressurizadas, mediante o efeito das variáveis experimentais (percentual de etanol no solvente extrator, tempo estático, pressão e temperatura) no R_M, CFT e AA, e por meio da condição que resultou na maximização das variáveis resposta, determinar o teor de compostos ativos (CFT, teor de flavonóides totais – TFT e AA) e GS, vislumbrando comparar aos extratos obtidos ao resultantes da extração por Soxhlet e EAU.

METODOLOGIA:

1) Para o desenvolvimento deste levantamento, foram consultados artigos e patentes em periódicos das principais bases de dados (Science Direct, Scielo, Web of Science, Scopus, Springer e Wiley, e SciFinder, respectivamente). Para o compilado de dados, foram considerados apenas artigos científicos e patentes publicadas nos últimos 12 anos (2009–2021), limitando-se a dados referentes à **i)** extração de compostos de folhas de *Stevia rebaudiana*, **ii)** dados referentes aos compostos obtidos na extração e **iii)** dados referentes aos tipos de extração não convencional. O processo de seleção e triagem para formar a base da redação foi realizado de forma independente, resumido-se na análise do total de 35 artigos e 09 patentes. **2)** Neste estudo, a intensidade da potência do ultrassom de 0, 50 e 100% (0, 83 e 165 W, respectivamente) foi avaliada na extração de compostos de folhas de Stevia com e sem pré-tratamento etanólico, no R_M, R_A, GS, CFT e AA, este último representado pela concentração de extrato capaz de reduzir o radical DPPH em 50% (EC_{50}). Um delineamento experimental Box-Behnken com três níveis,

três variáveis e cinco repetições do ponto central foi aplicado para examinar o efeito da temperatura (35, 50 e 65 °C), relação solvente/folha (5, 10 e 15 mL g^{-1}) e percentual de etanol no solvente extrator (10, 40 e 70%, v/v), objetivando o alcande das condições que maximizam o R_M e R_A, dentro da faixa experimental testada. Posteriormente, esses extratos foram caracterizados quanto à composição, e a correlação entre as condições operacionais e as respostas avaliadas foi utilizada na análise de componentes principais. **3)** Para avaliar a eficiência da ELP na obtenção do R_M e R_A, as variáveis temperatura, pressão e tempo foram mantidas fixas em 120 ºC, 100 bar e 60 min, respectivamente. Um aparato experimental operado em modo semicontínuo foi utilizado para a condução da avaliação do efeito da relação solvente/folha (30 a 90 mL g^{-1}) e concentração de etanol no solvente extrator (100 e 70%, v/v) na extração dos compostos das folhas com e sem pré-tratamento etanólico. **4)** Neste estudo, o efeito do percentual de etanol no solvente extrator (40 e 70%, v/v), tempo estático (10, 20 e 30 min) e pressão (50 e 100 bar) foram investigados no R_M, teor de CFT e AA dos compostos de folhas de Stevia pré-tratadas. As condições que maximizaram as variáveis resposta foram então investigadas na cinética da extração (100, 125 e 150 °C). Posteriormente, um comparativo foi realizado entre o extrato obtido por ELP resultante da maximização das variáveis resposta, Soxhlet (50 mL g^{-1} por 8 horas) e EAU (15 mL g^{-1} por 3 ciclos de 10 min), em relação ao R_M, R_A, GS, e compostos ativos (CFT, TFT e AA).

PRINCIPAIS RESULTADOS E DISCUSSÃO:

1) Os relatos alavancados por meio de métodos de extração não convencionais, contemplam critérios tecnológicos promissores na demanda do mercado mundial por compostos naturais, onde a *Stevia rebaudiana* apresenta-se como matriz com eficiência e rentabilidade na recuperação de seus edulcorantes e compostos ativos. Possibilitam operação rápida e fácil, por meio do uso de solventes renováveis sob condições de operação que não afetam a qualidade do extrato obtido. Dentre esses processos, técnicas que envolvem as cavitações acústicas do ultrassom, irradiação não ionizante do microondas, fluidos comprimidos na extração com líquido pressurizado e água subcrítica, bem como a extração com fluido supercrítico, são destacados em ascensão. Além disso, não haviam relatos da aplicação destas técnicas atreladas à estratégias que pudesses melhorar o processo e as características sensoriais do extrato obtido, como é o caso da aplicação do pré-tratamento etanólico. **2)** A aplicação da potência máxima (165 W) das cavitações do ultrassom proporcionou a obtenção de maiores R_M e R_A, e extratos com maiores teores de CFT e AA. O aumento da relação solvente/folha e do percentual de etanol no solvente extrator proporcionou os maiores valores de R_M e R_A, enquanto a temperatura promoveu aumento apenas no R_M. Os valores máximos de R_M (44,7% em peso) e R_A (88,5% em peso) foram obtidos usando solvente com 40% de etanol, 50 °C e 15 mL g^{-1} (solvente/folha). O extrato obtido possuia ~26,0% em peso GS, correspondentes a 9,5, 4,1 e 12,0% em peso de Stv, Reb C e Reb A, respectivamente. A análise dos componentes principais indicou alta correlação das variáveis percentual de etanol no solvente extrator e relação solvente/folha na obtenção de extratos com maiores teores de compostos ativos (CFT e AA), além de demonstrar que não há correlação na obtenção de adoçantes e compostos ativos. **3)** Sob condições pressurizadas, os resultados mostraram maior extração dos GS quando foram utilizadas folhas pré-tratadas. Aumentar a relação solvente/folha de 30 mL g^{-1} para 90 mL g^{-1} não favoreceu a extração dos adoçantes, demonstrando que um excesso de solvente no meio não resulta em maiores rendimentos. Esses resultados possibilitaram preliminarmente avaliar os efeitos desta técnica junto à matriz, reunindo dados e informações para as investigações posteriores. **4)** O aumento do percentual de etanol no solvente extrator favoreceu o R_M, CFT e AA, enquanto o aumento do tempo estático e da pressão (>10 min e >50 bar) não afetou a extração dos compostos sob as condições avaliadas. A temperatura proporcionou um aumento no R_M e os maiores teores de compostos ativos foram obtidos após 30 min do processo. O R_M foi semelhante entre as técnicas de extração avaliadas, com ELP (125°C) proporcionando maior recuperação dos CFT e AA, e o Soxhlet favorecendo a extração dos GS e CFT. A composição do extrato obtido pela ELP foi de ~26,0% em peso de GS, correspondendo a 9,5, 3,9 e 12,68% em peso de Stv, Reb C e Reb A, respectivamente, representando 87,8% do total obtido por Soxhlet. O potencial ativo (CFT, TFT e AA) do extrato da

ELP foi 3,6 e 11,0% superior ao de Soxhlet e EAU, respectivamente.

CONCLUSÕES:

1) As técnicas de EAU, EAM, ELP, EAS e EFS abordam questões ambientais e de segurança alimentar ao permitirem o uso de solventes renováveis, além de estarem alinhadas com o desenvolvimento de rotas alternativas de processamento, contemplando conceitos de sustentabilidade. Vislumbrando contribuir no aprimoramento dessas técnicas e agregar informações às futuras pesquisas relacionadas à matriz, ao **2)** avaliar a EAU, eficiência na obtenção de um extrato com alto rendimento, teor de CFT e AA, e grande potencial para uso como aditivo alimentar, foi verificado. Em termos de viabilidade do processo, destaca-se a utilização de uma técnica não convencional de processamento rápido, em conjunto com uma mistura binária entre etanol e água como solvente extrator, que proporciona custos de produção reduzidos em relação ao uso apenas de etanol e separação mais fácil do solvente, quando comparado ao uso da água. **3), 4)** Ao investigar a ELP, maior R_M e de compostos ativos (CFT e AA) foi observada, por meio do uso de menos energia e solvente, que aliado a estratégias operacionais como a matriz pré-tratada e a aplicação de uma mistura binária de etanol e água como solvente, possibilitaram a condução de um processo em menor tempo, com facilidade de separação do solvente extrator e paralelamente, potencial redução de custos operacionais. Assim sendo, a obtenção dos compostos dessa matriz por meio dos métodos não convencionais aqui propostos, além de serem alternativa viável e adequada na melhoria da solubilidade e disponibilidade dos analitos, demonstrou fornecer redução das condições operacionais, possibilitando a utilização rentável de solventes GRAS (geralmente reconhecido como seguro) nesses processos que se encaixam com méritos nos preceitos da química verde, gerando um extrato com alto potencial fitoterápico e de uso alimentício. Embora uma possível implementação desses processos em escala piloto se apresente como uma alternativa relevante, pois permite menos inconvenientes quando comparado aos processos convencionais, a redução no consumo de insumos e a viabilidade do reaproveitamento do solvente no processo ainda precisam ser ser aprimorados em sua investigação. Processos combinados ou sequenciais poderiam ser aplicados como alternativas a esse problema, mas sua exploração ainda não foi relatada, bem como as consequências de expor essa matriz a condições extremas de algumas das tecnologias mencionadas, principalmente em relação à possível degradação dos compostos alvo. Essas lacunas e a falta de informações sobre essas técnicas configuram os principais desafios na ampliação do uso dessas tecnologias, surgindo como perspectivas à trabalhos futuros.

PALAVRAS-CHAVE: Aplicação de tecnologia; Compostos ativos; Compostos fenólicos; Atividade antioxidante; Adoçantes naturais; Edulcorantes; Solventes verdes.

ARTIGO 1

Compounds from *Stevia rebaudiana* Bertoni leaves: An overview of non-conventional extraction methods and challenge

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Compounds from *Stevia rebaudiana* Bertoni leaves: An overview of non-conventional extraction methods and challenges

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ABSTRACT

To overcome the inconveniences related to its conventional obtainment of natural sweeteners and active compounds from *Stevia rebaudiana* Bertoni, methods involving application of technology, such as ultrasound assisted extraction, microwave assisted extraction, extraction under pressurized conditions by means of pressurized liquid extraction, subcritical water extraction and supercritical fluid extraction have been investigated. In this review, these emerging techniques were analyzed and discussed, the process variables and operational strategies, their impacts on the extraction and their comparison against the conventional techniques were demonstrated. Simplicity and the possibility of operational automation, the use of less energy and solvents, in addition to the reduced complexity of the subsequent purification steps, make up viable alternatives suitable for a possible industrial application. These processes leverage the concept of green chemistry, but still suffer limitations related to acquisition and maintenance costs, in addition to the effects of the action of some of these technologies remaining unexplored.

1. Introduction

The demand for functional products rich in fiber, natural antioxidants and low in calories has increased considerably in the last decade, due to their beneficial effects on health (Alizadeh, 2021; Gençdağ et al., 2021; Pereira et al., 2021a; Velotto et al., 2021). *Stevia rebaudiana* appears in this context, not only as a non-calorie sweetener, but also as a valuable nutritional supplement ingredient (Stamataki et al., 2020; Yu et al., 2017), since its compounds present a series of phytochemicals, antioxidants and antiglycation properties (Ali et al., 2021; Pirgozliev et al., 2021; Villaño et al., 2021), prominent attributes without any mutagenic, teratogenic or carcinogenic effect (Ahmad et al., 2020; Momtazi-Borojeni et al., 2017).

Stevia rebaudiana Bertoni belonging to the Asteraceae family, is native to Paraguay and cultivated in many countries, including Brazil (Halim et al., 2016) and it has an important role in the food industry because its leaves present diterpenic glycosides (SGs) with a sweet taste, among which stand out mainly, the stevioside (Stv) and the rebaudioside A (Reb A) (Wölwer-Rieck, 2012). Minority compounds such as rebaudioside C (Reb C) and duloside A (Dul A) (Yadav et al., 2011), and traces of rebaudiosides B, D, E and F (Aranda-González et al., 2015) are also reported. SGs have the particularity of providing up to 450 times more sweetening power than sucrose (Mondal and De, 2014), and because they are thermally stable, these chemical compounds have been used as a sweetening agent, flavor modifier and sugar substitute (Gençdağ et al., 2021), as they do not promote changes in the glycemic index, not generating energy accumulation (Kurek et al., 2021).

Active and functional compounds with antiviral properties and therapeutic effect in the treatments of neuralgia, lumbago, anaemia, eczema, rheumatism and dermatitis have been reported for Stevia leaf extract (Salehi et al., 2019). Antiamnesic activity (Noreen et al., 2020), antibacterial properties (Lemus-Mondaca et al., 2018; Atas et al., 2018) and antifungal (Ramírez et al., 2020) were also found, in addition to having antioxidant activities (Barba et al., 2015; Carbonell-Capella et al., 2017; Kovačević et al., 2018), that promote a reduction in the development of chronic-degenerative diseases through immunomodulatory actions (Boonkaewwan and Burodom, 2013) anti-hyperglycaemic (Ahmad; Ahmad, 2018), anti-hypertensive (Ferri et al., 2006), anti-inflammatory (Fengyang et al., 2012), anti-tumor (Chen et al., 2018), diuretic and anti-diarrheal (Chatsudthipong and Muanprasat, 2009). Furthermore, its compounds may provide support to the human immune system in fighting COVID-19 (Boyacı-Gündüz et al., 2021;

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Galanakis, 2020).

Stv traditionally constitutes the majority of glycosides (60%–70% of the total) (Carbonell-Capella et al., 2017; Görgüç et al., 2019; Yildiz-Ozturk et al., 2015; Yilmaz et al., 2021) and its therapeutic value lies in the ability of this compound to stimulate insulin secretion in the pancreas in the treatment of diabetes and to control other metabolic disorders (Jeppesen et al., 2000; Milani et al., 2017). Although sweetness is reported, this compound has a degree of pungency with a prolonged effect (Yadav et al., 2011) that reduces its acceptability. Reb A is reported as the glycoside that has no accumulation of bitter aftertaste (Gallo et al., 2017), characteristic attributed to the presence of an extra glucose atom in its structure (Chaturvedula et al., 2011) and although its share in glycosides is smaller (30%–40% of the total), its sweetness and stability are greater than that of the Stv (Nalesso-Leão et al., 2020) and its structure has been proven to improve oral bioavailability hydrophobic nano-drug delivery systems with great potential antitumor (Wang et al., 2021).

The compounds from Stevia leaves are mainly obtained by conventional extraction methods, such as pressing, infusion, maceration, percolation, decoction, continuous reflux with hot solvent (Soxhlet) and orbital agitation. These processes require more time and amount of solvent, demanding considerable energy consumption (Periche et al., 2015; Yang et al., 2019; Milani et al., 2020; Yılmaz et al., 2021). The low selectivity, associated with the low yields and quality of the extract obtained, intensified the development and improvement of techniques that exert minimal influence on the extracted content, aligning with the concepts of green chemistry and meeting the Sustainable Development Goals, referring to responsible consumption and production, as well as the guarantee of health and well-being (United Nations Development Programme, 2021).

Non-conventional extraction techniques such as ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), extraction under pressurized conditions by means of pressurized liquid extraction (PLE), subcritical water extraction (SWE) and supercritical fluid extraction (SFE), have been reported with prominence in the process of obtaining sweeteners and active compounds from Stevia, aiming to avoid low yields and damage to the product quality and, in parallel, to develop new methods that are able to favor the removal of target compounds, with less consumption of energy, reagents and time, as well as less waste generation. In addition, these emerging technologies have the characteristic of generating minimal deterioration to the nutritional and functional characteristics of the compounds obtained (Galanakis, 2021), taking into account the perspectives of innovations in the agri-food and nutraceutical sectors (Galanakis et al., 2021).

This work aimed to present and discuss the general approach on the current scenario of obtaining compounds from *Stevia rebaudiana* Bertoni leaves, associated with the application of non-conventional extraction methods, taking into account recent studies that have been evaluating the use of different solvents and experimental variables with these techniques. Furthermore, the investigated strategies to maximize the attainment of target compounds, aiming to overcome the main challenges imposed by each technique are mentioned, thus offering a discussion of the advantages, disadvantages and limitations of these approaches. Finally, trends and perspectives on these alternative processing technologies are presented. The information summarized in this review can help develop strategies to improve the reported extraction techniques without compromising existing technological aspects.

2. Methodology

For the development of this study, articles were consulted in journals with Qualis Capes specialized in the area of Food Science, in databases such as Science Direct, Scielo, Web of Science, Scopus, Springer and Wiley. Scientific articles published in the last 12 years (2009–2021) were selected due to the growing interest of the scientific community in this area observed during this period, written exclusively in English, which were added to the EndNote software for structuring in this study, whose division consisted of 3 steps: 1) data regarding the extraction of compounds from *Stevia rebaudiana* Bertoni leaves; 2) data referring to the compounds obtained in the extraction and 3) data referring to the types of non-conventional extraction. The keywords used as a search strategy for articles were "*Stevia rebaudiana*" and "Extraction", individually or together, with the terms: “Glycosides”, “Rebaudioside A”, “Stevioside”, “Bioactive compounds”, “Antioxidants”, “Phenolic compounds”, “Ultrasound-assisted extraction”, “Microwave assisted extraction”, “Pressurized liquid extraction”, “Subcritical water extraction” and “Supercritical fluid extraction”.

Reference lists were exported from electronic platforms and counted in the EndNote software, which was used to remove duplicate references and eliminate by exclusion criteria. Review articles and conference papers, as well as papers that did not coincide with the theme, such as case study, case-control and repeated articles available on different platforms, were excluded. Articles from previous years were kept in the article database if the reported findings were considered important for the discussion. Patents were selected by title and abstracts in the SciFinder database, a resource of the Chemical Abstracts Service (CAS), which has curated chemical and bibliographic information covering various scientific fields (Gabrielson, 2018). The search on this platform was limited to patents and performed according to the step search strategy, in the same time interval (2009–2021) considered for scientific articles. The selection and screening process of articles and patents to form the basis of the writing of this general review was carried out independently, as summarized in Fig. 1.

A total of 61 articles were searched and collected, being subsequently screened for duplicity, 11 of the 46 articles selected for the entire reading were excluded for meeting at least one of the exclusion criteria. A total of 35 studies were evaluated and categorically subdivided considering: I – Composites of *Stevia rebaudiana* leaves obtained with non-conventional extraction methods (UAE, MAE, PLE, SWE and SFE); and II – compounds from *Stevia rebaudiana* leaves obtained through non-conventional methods, focusing on sweeteners (glycosides, Reb A and Stv) and bioactive compounds (antioxidants and phenolic compounds). Of this total, 6 contained more than one extraction technique in the same article, justifying their repeated appearance in the text. For patents, after the categorization step, a total of 9 documents were found and correlated with non-conventional extraction methods and obtaining the compounds from the plant matrix in question.

3. Compounds from *Stevia rebaudiana* Bertoni leaves

Stevia rebaudiana Bertoni is a perennial herbaceous shrub, which presents in its leaves, flowers, stems, seeds and roots, levels of steviol glycosides (Bondarev et al., 2003). Most of the investigations focus on the leaves, a portion that corresponds to the economic value of the plant and that presents the highest contents of these compounds. The constituents of its extract are functional ingredients for use in the food and nutraceutical industry, with potentials reported in Fig. 2.

Stevia leaves contain a significant amount of nutrients such as proteins and essential amino acids, lipids, saccharides, vitamins and minerals, however, their composition varies according to the management techniques and climatic conditions of their cultivation (Clemente et al., 2021; Tavarini et al., 2015). Until now, 64 steviol glycosides were found in Stevia leaves (Myint et al., 2020), where, in addition to the main (Stv and Reb A) and minor (Reb B–F and Dul A) glycosides, corresponding to the sweetness characteristic, substances do not sweeteners such as the triterpenes amyrin acetate, lupeol, stigmasterol, sitosterol and campesterol (Agostino et al., 1984; Madan et al., 2010) have also been reported.

The contents of photosynthetic pigments (chlorophylls and carotenoids), plant pigments involved in photosynthesis, have also been reported with antioxidant properties (Kovačević et al., 2018). These compounds are associated with the prevention of chronic diseases such as cancer and cardiovascular diseases, in addition to enhancing the

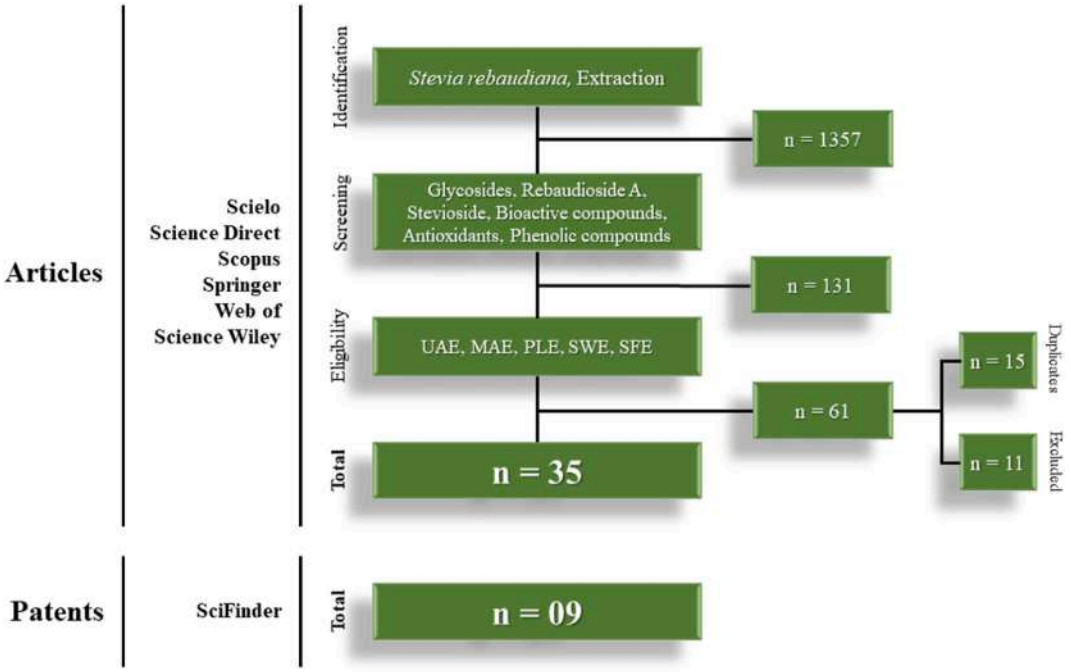

Fig. 1. Flowchart for the selection of articles and patents included in the review.

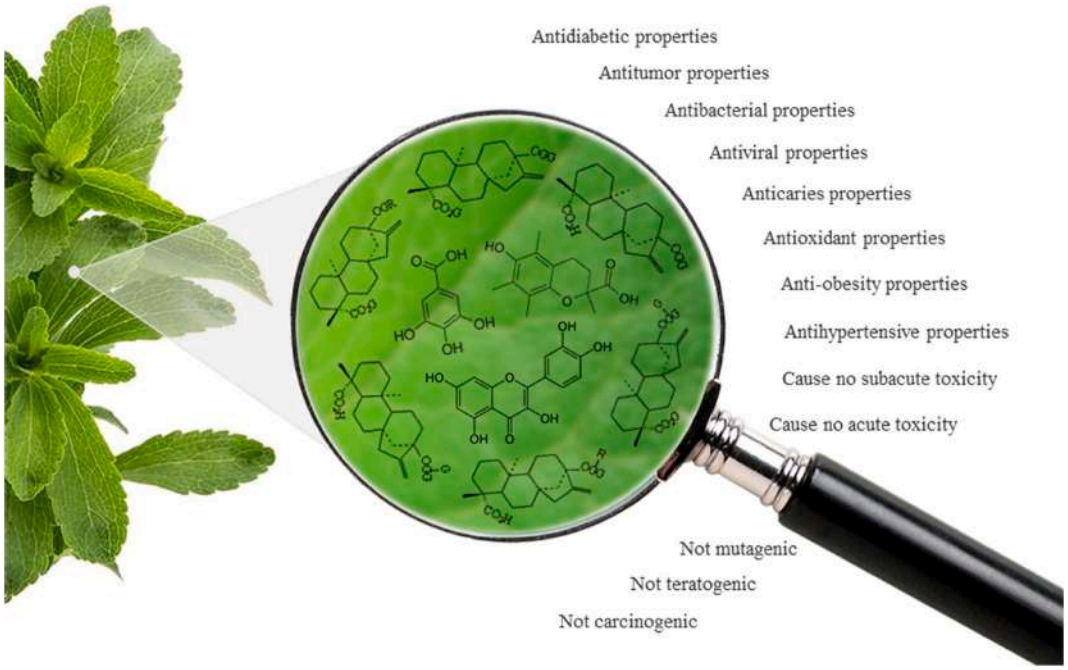

Fig. 2. Main health effects of Stevia rebaudiana leaf compounds reported in *in vitro* and/or *in vivo* studies.

immune response and promoting mental health (Bendokas et al., 2019; Braga et al., 2018; Ocean et al., 2019). Compounds such as chlorogenic acid, coumaric acid and sinapic acid (Periche et al., 2015) appear prominently in the composition of the extract, helping to control blood pressure, regulate blood glucose, and control cholesterol (Yan et al., 2020), in addition to the ability to mitigate toxicities (Chen, 2015).

Polyphenols, reported against lipid peroxidation (Galanakis, 2018) and physical and chemical UV filters (Galanakis et al., 2018), have been reported in Stevia leaves (Pacifico et al., 2019). Flavonoids such as centaureidin, epigallocatechin gallate, luteolin-glucoside (Laguta et al., 2019), described with beneficial effects on glucose homeostasis (Xiao et al., 2014), as well as the kaempferol glycosides, flavonols (quercetin) and flavones (luteolin and apigenin) (Karaköse et al., 2015; Pacifico et al., 2019) have synergistic effect against herpes simplex virus (Kumar and Pandey, 2013), in addition to antioxidant activity (Carrera-Lanestosa et al., 2019), anticancer effects (Kopustinskiene et al., 2020) and induction of apoptosis and autophagy (Kopustinskiene et al., 2020). Recently, the flavonoids diosmetin, casticin, dionflavone and kaempferol-7-o-glucoside were identified in Stevia leaf extract (Yılmaz et al., 2021), presenting the ability to induce apoptosis (Soares et al., 2019).

Antibacterial ability against *Staphylococcus aureus*, *Enterococcus*

faecalis, *Pseudomonas aeruginosa*, *Escherichia coli, Candida albicans* (Atas et al., 2018), *Salmonella typhimurium*, *Klebsiella pneumonia*, *Bacillus cereus* (Kabir et al., 2014) and *Listeria monocytogenes* (Sansano et al., 2017), and antifungal against *Fusarium oxysporum* (Ramírez et al., 2020) and *Candida albicans* (Herawati et al., 2021) was reports for the extract of Stevia leaves. Potential anti-inflammatory use has been demonstrated, *in vitro* and *in vivo* (Cho et al., 2013; El-Taib et al., 2020; Lemus-Mondaca et al., 2018), in which natural diterpenoids such as austroinulin and 6-Oacetyl austroinulin isolated have been shown to inhibit production of nitric oxide, inducible nitric oxide synthase, and pro-inflammatory cytokines (Cho et al., 2013). In addition, austroinullin and its derivatives are vasodilator cardiotonic and anesthetic (Siddique et al., 2014), and fermentable dietary fibers such as fructooligosaccharides, associated with prebiotic effects (Davani-Davari et al., 2019) have also been reported.

The lipids obtained from Stevia leaves are considered a good source of monounsaturated fatty acids, as oleic, and polyunsaturated fatty acids as linoleic or α-linolenic (Wölwer-Rieck, 2012), with the potential to reduce the level of cholesterol in the blood (Virsangbhai et al., 2020). Aromatic substances have been reported with the presence of important sesquiterpenes (δ-caryophyllene, trans-δ-farnesene, humulene) and monoterpenes (linalool, terpinen-4-ol and terpineol) (Hossain et al., 2010; Madan et al., 2010), with antiseptic, antibacterial, anti-inflammatory, calming, hypotensive properties with analgesic and immunomodulating effects (Price and Price, 2007).

Sensorially, aftertaste is reported as the biggest limiting factor in the use of sweeteners and compounds in Stevia leaves. This characteristic is described as bitterness, licorice, and metallic taste (Espinoza et al., 2014) and it is attributed to the magnitude and quality of flavor differing between its molecules in these compounds (Hellfritsch et al., 2012), by different glucose units in the steviol aglycone (Ohta et al., 2010), in addition to the presence of sesquiterpene lactones, essential oils, tannins, flavonoids, caryophyllene and spathulenol (Phillips, 1987; Soejarto et al., 1983; Tsanava et al., 1991; Zeng et al., 2013).

Oxalic acid and tannins have been reported and linked to Stevia leaves as anti-nutritional compounds (Savita et al., 2004). Oxalic acid can reduce the digestibility of calcium and other minerals, playing a key role in hyperoxaluria, with the formation of calcium oxalate stones in the kidneys (Higashijima et al., 2020). Tannins, although reported to have antibacterial and antioxidant activities, in large amounts can limit nutrient digestibility (Lemus-Mondaca et al., 2012). However, no adverse effects of genotoxicity and subchronic oral toxicity were reported (Zhang et al., 2017).

4. Extraction of compounds from Stevia leaves by non-conventional methods

Primary and secondary metabolites can be extracted from Stevia leaves by different techniques, which associated with operational variables, play an important role in the quality of the extracts obtained. This process basically involves separating the compound from the solid matrix through solubilization in a certain solvent, and its efficiency generally depends on the chemical nature of the compounds to be extracted, the particle size of the plant material, pH of the extracting medium, time and temperature of extraction, agitation speed, leaf to solvent ratio and solvent used.

The operational variables and extraction techniques applied in maximizing the extraction process will depend on the subsequent use to which the extract will be designated (sweetener or active compound) (Das et al., 2015; Kovačević et al., 2018; Periche et al., 2015; Raspe et al., 2021b). Conventional techniques are based on the use of liquid solvents and mostly hot, which have drawbacks related to low yield (Jaitak et al., 2009; Yılmaz et al., 2021), product quality, mainly due to the degradation of compounds that can influence their purity (Barba et al., 2015; Das et al., 2015), processing costs, due to the high amount of solvent and energy involved (Javad et al., 2014; Jentzer et al., 2015; Žlabur et al., 2015) and the need for long extraction times, which are required to obtain a substantial amount of the compounds (Ciulu et al., 2017; Yang et al., 2019). In addition, the presence of interfering substances that require separation and purification are also linked to these techniques (Díaz-Montes et al., 2020; Galanakis, 2015).

To facilitate and increase the yield of these extraction techniques and provide a reduction in the aftertaste of bitterness in the extract, the enzymatic transglycosylation of Stv and its congeners (Abelyan et al., 2004) and the use of chemical or enzymatic pre-treatments can be applied (Formigoni et al., 2018a, 2018b). However, the considerable volume of solvent, the uncertain fate of the waste generated and the cost of the enzyme make these processes questionable and dependent on further investigation.

Aiming to contribute to the fulfillment of requirements for the development of faster techniques, with high yields and that provide extracts with higher quality and yield, reduced energy consumption and that meet market and legal requirements through the use of solvents with GRAS certification (Generally Recognized As Safe), extraction techniques with process intensification concepts have been addressed. The use of cavitation in the UAE, non-ionizing radiation in the MAE, and the use of solvent under pressurized conditions in the PLE, SWE and SFE have particularities and factors that determine their environmental and economic viability, boosting their industrial competitiveness.

Although the industrial application of these technologies can be a challenge at this time because the costs of equipment are relatively high and data on scaling up processes is still scarce, laboratory research continues to be developed and allow for the expansion of the understanding of these processes, enabling the achievement of unpublished findings for these extraction techniques. Ciulu et al. (2017) reported for the first time isomers of ethyl chlorogenate, caffeic acid ethyl ester and dimethoxycynnamoyl-caffeoylquinic acid, as well as a fragment ion kaempferol-3-O-rutinoside/luteolin-7-O-rutinoside and a fragment ion kaempferol-O-glycoside/orientin/isorientin, in Stevia extracts obtained by PLE. Yılmaz et al. (2021) mentioned the unprecedented identification of the esculetin and 3-Hydroxycoumarin as coumarin derivatives, and the flavonoids diosmetin, casticin, dioonflavone and kaempferol-7-o-glucosidein, in extracts obtained by MAE and UAE. The presence of some amino acids (L-Tyrosine, D-Tryptophan, tryptophan derivative kynurenic acid and histidine derivative urocanic acid), fatty acids (stearidonic, stearic, stearamide and melissic), as well as the butaprost, glycerophosphocholine and trigonelline, have also been reported in an unprecedented way in Stevia leaf extracts.

4.1. Ultrasound-assisted extraction (UAE)

UAE allows the quick and less costly extraction of cellular material, presenting as its greatest attraction its high yields and reproducibility, lower consumption of solvent and energy, requiring little maintenance (Prado et al., 2017) and manipulation ability (Chemat et al., 2017) and installation (Patist and Bates, 2008). It stands out in terms of sustainability, being considered an innovative approach in increasing the efficiency of processes, and can be easily integrated into existing devices as part of the technological plant (Pereira et al., 2021b), or set up as a standalone process (Periche et al., 2015; Raspe et al., 2021b; Yildiz-Ozturk et al., 2015; Yılmaz et al., 2021; Žlabur et al., 2015).

This technique is based on the production of sound waves that create cavitation bubbles in the extraction system, generating a mechanical effect close to the plant matrix tissue (Koubaa et al., 2015) which, when imploded, result in an impact on the surface of the cell wall, causing its rupture. As a result, there is a reduction in particle size and an increase in the contact surface for solvent penetration, providing greater dissolution of the intracellular content and intensification of mass transfer (McDonnell and Tiwari, 2017; Prado et al., 2017), as represented by the schematic diagram of Fig. 3. In addition to increasing yield, this is a promising technique for allowing the application of a reduced volume of solvent, at atmospheric pressure and at mild temperatures, when

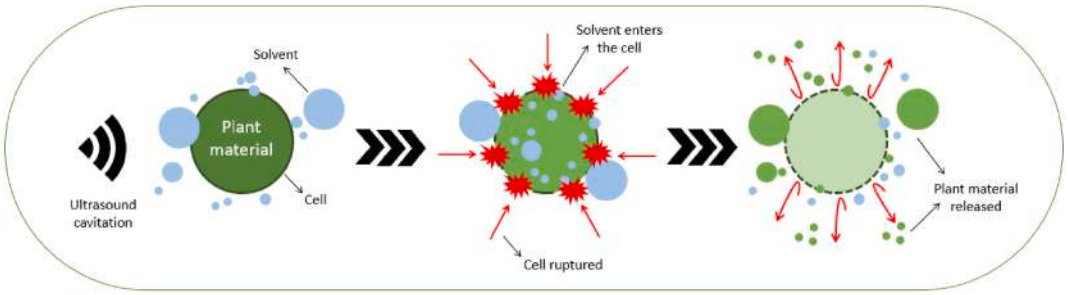

Fig. 3. Schematic diagram of ultrasound assisted extraction system.

compared to other extraction techniques (Periche et al., 2015; Raspe et al., 2021a; Rouhani, 2019; Yilmaz et al., 2021; Žlabur et al., 2015), enabling the extraction of thermolabile compounds.

The UAE can be conducted in direct and indirect contact equipment, through a bath and an ultrasonic probe, respectively. The frequency (kHz) and power (W) of the equipment are parameters that arise in this process and provide for the action and intensity of the cavitation bubbles during extraction (Chemat et al., 2017). Although for Stevia leaves reports were found involving mild potency and frequency (≤1200 W and ≤25 kHz) in relation to other matrices, inadequate conditions of these variables can compromise the efficiency of the extraction of target compounds, due to the formation of free radicals resulting from the oxidative pyrolysis that occurs inside the cavitation bubbles (Sivasankar et al., 2007). Table 1 presents a compilation of the main published works concerning the UAE of the compounds from Stevia leaves, as well as the extracting solvent and the operational conditions indicated by the authors to obtain the maximum extraction of the compounds.

In a study conducted by Žlabur et al. (2015), the impact of the UAE (400 W) in obtaining the SG, total phenolic compounds (TPC), total flavonoids (TF) and antioxidant activity (AA) was evaluated. In this study, using ultrasound, it was possible to obtain an increase of 16.7%, 51.4%, 37.3%, 64.7% and 15.2% in the contents of Stv, Reb A, TPC, TF and AA, respectively, in a shorter extraction time (10 min), than when performed by conventional extraction using orbital shaking (24 h). The efficiency of the UAE (50 °C in 1 min) in obtaining of SG was investigated by Periche et al. (2015), where twice the levels obtained by extraction conducted in a thermostatic bath (50 °C in 5 min) were verified. Increases of 146.6% and 65.6% in SG and TPC in the extract,

Table 1
Experimental conditions and extractant solvent to maximize the ultrasound-assisted extraction (UAE) of Stevia leaf compounds.

Reported compounds	Solvent	Recommended experimental conditions: power, time, solvent to leaf ratio, temperature	Results[1]	Reference
SG	Water	100 W, 20 min, 10 mL g^{-1}, 25 °C	~35 mg^2	Alupului and Lavric (2009)
SG, Stv, Reb A	Methanol 80% (v/v)	nr, 30 min, 100 mL g^{-1}, 35 °C	7.2%, 5.0% and 2.2% in extract	Jaitak et al. (2009)
SG, Stv, Reb A	Water	60 W, 32 min, 10 mL g^{-1}, 68 °C	72.48%, 43.62%, 28.86% in extract	Liu et al. (2010)
CA	Water	400 W, 2 min, 16 g g^{-1}, nr (pre-treatment)	1.5 g^3	Barba et al. (2015)
TPC, AA			15.98 mg GAE[3] and 15.0 mM TEAC[3]	
Reb A	Isopropanol 60% (v/v)	360 W, 18 min, 10 mL g^{-1}, 30 °C	3.56 g^2	Gasmalla et al. (2015)
SG, Stv, Reb A, Reb C, Dul A	Water	nr, 1 min, 100 mL g^{-1}, 50 °C	61.43 mg, 39.06 mg, 14.12 mg, 6.25 mg and 2.0 mg^2	Periche et al. (2015)
TPC, TF, AA		nr, 20 min, 100 mL g^{-1}, 50 °C	80.0 mg GAE[3], 43.0 mg and 81.0 mg^3	
SG, Stv, Reb A	Ethanol	300 W, 45 min, 15 mL g^{-1}, 90 °C	14.90 mg, 10.24 mg and 4.66 mg^2	Yildiz-Ozturk et al. (2015)
TPC, TF, AA			86.57 mg GAE[2], 126.70 mg^2 and 92.4% DPPH radical scavenging[2]	
SG, Stv, Reb A	Water	400 W, 10 min, 400 mL g^{-1}, 81.2 °C	133.4 mg, 96.48 mg and 36.92 mg^2	Žlabur et al. (2015)
TPC, TF, AA			77.89 mg, 62.48 mg GAE[2] and 2.58 mM TEAC[2]	
SG, Stv, Reb A	Water	400 W, 1.2 min, 30 mL g^{-1} (pre-treatment), nr	~77.0 mg, 50.0 mg and ~22.0 mg^2	Carbonell-Capella et al. (2017)
AA, TPC, TF, CA			25.6 mg GAE[2], 20.2 μmol, 7.8 μmol, 66.9 μmol TEAC[2]	
Reb A	Isopropanol 60% (v/v)	480 W, 18 min, 10 mL g^{-1}, 30 °C	371.0 mg^3	Gasmalla et al. (2017)
SG, Stv, Reb A	Ethanol 50% (v/v)	104 W, 10 min, 10 mL g^{-1}, nr	192.15 mg, 93.18 mg and 98.97 mg^2	Covarrubias-Cárdenas et al. (2018)
TPC			163.0 mg GAE[2]	
Stv	Glycerol	200 W, 40 min, 30 mL g^{-1}, 70 °C	8.81 mg^3	Rouhani (2019)
SG, Stv, Reb A	Deep eutectic 90% (v/v)	130 W, 40 min, 10 mL g^{-1}, 59.4 °C	84.0 mg, 38.0 mg and ~46.0 mg^3	Milani et al. (2020)
Stv	Water	144 W, 40 min, 50 mL g^{-1}, 45 °C	~4.2 mg^3	Lima et al. (2021)
SG, Stv, Reb A, Reb C	Ethanol 40% (v/v)	165 W, 3 cycles of 10 min, 15 mL g^{-1}, 50 °C	~257.0 mg, 95.0 mg, 120.0 mg and 41.4 mg^2	Raspe et al. (2021b)
TPC, AA (EC_{50})			280.0 mg GAE[2] and 20.75 μg mL^{-1}	
SG, Stv, Reb A	Water	250 W, 15 min, 10 mL g^{-1}, 60 °C	249.6 mg, 147.1 mg and 102.5 mg^3	Stramarkou et al. (2021)
SG	Ethanol 70% (v/v)	550 W, 30 min, 100 mL g^{-1}, 75 °C	~90.0 mg^2	Yen and Quoc (2021)
SG, Stv, Reb A	Ethanol 50% (v/v)	540 W, 43 min, 118 mL g^{-1}, 50 °C	113.3 mg, 70.4 mg and ~43.0 mg^2	Yılmaz et al. (2021)
TPC, TF, AA			68.6 mg GAE[2], 47.7 mg CE[2] and 853 μmol TEAC[2]	

Steviol glycosides (SG); Stevioside (Stv); Rebaudioside A (Reb A); Rebaudioside C (Reb C); Dulcosíde A (Dul A); Total phenolic pompounds (TPC); Carotenoids (CA); Antioxidant activity (AA); Total flavonoids (TF); Gallic acid equivalent (GAE); Trolox equivalent (TEAC); Catechin equivalents (CE). Not reported (nr). [1]Values corresponding to the compounds reported in the first column; [2]per g of extract and [3]per g Stevia leaf.

with a reduction in processing time from 120 to 5 min, were also reported in the comparison between maceration and UAE investigated by Covarrubias-Cárdenas et al. (2018), respectively.

Considering that cavitation power intensity plays the most important role in UAE efficiency, Gasmalla et al. (2015) reported a 33.72% increase in Reb A content in the 62.5%–75% power range of ultrasound (480 W). According to the authors, from this intensity onwards, changes in the physical characteristics of the liquid extract started to be verified, such as polarity, viscosity and surface tension. Yildiz-Ozturk et al. (2015) verified that intensities close to 100% (400 W) caused adsorption of the compounds on the matrix surface, increasing the time required for extraction. On the other hand, 100% of the ultrasound power (200 W) was reported without adverse effects as responsible for the maximum removal of Stv (Rouhani, 2019). Raspe et al. (2021b) evaluated the effect of ultrasound in maximizing the extraction of compounds from pre-treated Stevia leaves and reported that the UAE in lower solvent to leaf ratio, temperature and extraction time provided 16.5% higher yield in Reb A than that obtained through extraction in orbital agitation (Formigoni et al., 2018b).

In the compilation shown in Table 1, the range of solvent to leaf ratio, temperature and time for works involving the UAE of Stevia leaf compounds were from 10 to 400 mL g^{-1}; 25–90 °C and 1–45 min, respectively. However, most studies worked with solvent to leaf ratios ≤10 mL g^{-1}, temperature ≤50 °C and time ≤40 min, contrary to the information on the reduction of operating conditions for this technique, previously highlighted. This effect may be related to the fact that Stevia leaves have a high wetting capacity (Mishra et al., 2010), which promotes rapid absorption of the solvent by the matrix, generating excessive swelling and, consequently, its swelling. Therefore, for the effect of cavitations to be propagated homogeneously to the system and the extraction to be promoted, operating conditions superior to those reported for this technique to other matrices are necessary. Furthermore, it is noteworthy that most studies were conducted with water as a solvent, due to its benefits as a green solvent. However, because the possibility of extraction with this solvent being less selective due to the increased solubility not only of the desired analyte, as well as the possibility of degradation of compounds (Castro-Puyana et al., 2017), the most recent reports have investigated binary mixtures between water and ethanol as an extracting solvent.

To assess the effect of these binary mixtures on UAE, Covarrubias-Cárdenas et al. (2018) investigated the phenolic content and antioxidant activity of extracts from Stevia leaves, comparing different concentrations of ethanol in water (0%, 25% and 50%, v/v). The authors found that the increase in the proportion of ethanol in water (0%–50%) provided an increase of 44.1% in TPC and 142.6% in AA, in 15 min of extraction. Raspe et al. (2021b) demonstrated that increasing the proportion of ethanol in water from 10% to 70% (v/v) at 30 °C and solvent to leaf ratio of 10 mL g^{-1} allowed an increase of 5.5% in SG yields, however, a reduction in the content in sweeteners of ~3.5% were verified.

It is evident that the mixture between these solvents affects the viscosity and polarity during the extraction, directly interfering with the process yields. Special attention has been given to binary mixtures of ethanol and water in intermediate percentages and polarities, as they provide the simultaneous attainment of higher levels of glycosides and active compounds. Yilmaz et al. (2021) when carrying out this investigation, by increasing the proportion of ethanol in the extractant solvent from 25% to 75%, verified an increase of 9.0% in the Stv content, with a parallel reduction in the contents of TPC (7.12%) and TF (10.2%), and when the proportion of ethanol investigated was from 0% to 50%, an increase of 10.1%, 13.7%, 35.3%, ~33.5% and 14.0% in the contents of Reb A, Stv, TPC, TF and AA were verified, respectively. Similarly, Raspe et al. (2021b) by increasing the percentage of ethanol from 10% to 40% in the extraction mixture, promoted simultaneous increase in Reb A and TPC, in the order of 1.7% and ~8.5%, respectively.

4.2. Microwave-assisted extraction

MAE uses non-ionizing microwave irradiation to promote interactions between molecules present in the extraction mixture only by heating them, without affecting the molecular structure of the matrix or generating damage to the compounds obtained (Chemat et al., 2020). The main works with the application of MAE to obtain compounds from Stevia leaves are presented in Table 2, as well as the extracting solvent and the operational conditions indicated by the authors to obtain the maximum extraction of the compounds.

MAE is efficient only for materials or solvents with permanent dipoles, limited to those that absorb electromagnetic waves from microwaves (Vinatoru et al., 2017). Water is the most common solvent used in SG extractions from Stevia leaves, however, as in UAE, its use in binary mixtures with ethanol has enabled the application of MAE to a wider variety of analytes, in addition to providing higher yields to the process. Mustafa and Turner (2011) highlight that extractions with binary mixtures explore two aspects, the ability of water to break the hydrogen bond between the matrix and the analytes, while ethanol increases the solubility of the extracted species. This was evidenced by Yılmaz et al. (2021) when performing the MAE of compounds from Stevia leaves using a binary mixture of ethanol and water, where the AA was significantly higher than the extract resulting from the process conducted only with water (Carbonell-Capella et al., 2017). At the same time, when analyzing the contents of Stv and Reb A from the application of the binary mixture of ethanol and water obtained by Yılmaz et al. (2021), and compare with the results obtained by Periche et al. (2015) in the aqueous extraction, increase of 51.5% and ~152.0% in these SGs were verified, respectively.

The power (≤700 W) linked to temperature (25–100 °C) plays a crucial role in this extraction technique, as the efficiency of microwave heating depends on the material's ability to absorb electromagnetic energy and dissipate heat, as the phenomena of heat and mass transfer occur from the inside of the plant cell to the outside (Li et al., 2013), as represented by the schematic diagram of Fig. 4. Therefore, temperatures close to the boiling point of the extracting solvent, in a potency sufficiently capable of removing the compounds from the matrix, without damage or loss being caused, are necessary, further promoting a reduction in processing time. When investigating the relationship between power (300–500 W) and temperature (40–90 °C), Yildiz-Ozturk et al. (2015) found that the increase in these variables provided an increase of ~300% and ~330% in the removal of Reb A and Stv, respectively. However, with an excessive increase in power (>400 W), a decrease in SG was verified, possibly due to the adsorption of these compounds in the matrix. Similarly, Jaitak et al. (2009) found an increase in Reb A in the power and temperature range of 20–80 W and 10–50 °C, respectively, with a reduction observed when the increase in these variables reached 160 W and 90 °C.

Although reports mention that a greater amount of solvent increases the solubility of the solute in plant matrices, low volumes (≤10 mL g^{-1}) could be identified in most works for Stevia leaves (Table 2). This relationship is also important as the MAE requires the material to be completely immersed in the extracting solvent to ensure a uniform system heating rate (Thanh-Thuy et al., 2020). Furthermore, because the cell absorbs more microwaves and reaches a higher temperature than the solvent during this heating period (Taqi et al., 2020), and considering that Stevia leaves have the characteristic of absorbing the applied solvent (Németh and Jánosi, 2019), large volumes of this component are dispensed with in conducting the extraction, since in this case, the heating process takes place simultaneously.

Long periods of exposure to microwaves are also not necessary, since the temperature that allows the extraction in this system is reached more quickly. With the exception of the work by Yildiz-Ozturk et al. (2015), relatively short time intervals (≤20 min) were verified, highlighting this technique compared to conventional. This can be proven by Yılmaz et al. (2021) when comparing the maceration and microwave techniques,

Table 2
Experimental conditions and extractant solvent to maximize the microwave-assisted extraction (MAE) of Stevia leaf compounds.

Reported compounds	Solvent	Recommended experimental conditions: power, solvent to leaf ratio, temperature and time	Results[1]	Reference
SG, Stv, Reb A	Methanol 80% (v/v)	80 W, 100 mL g^{-1}, 50 °C, 1 min	10.98%, 8.64% and 2.34% in extract	Jaitak et al. (2009)
Stv	Water	200 W, 10 mL g^{-1}, nr, 2 min	~0.77 mg[2]	Javad et al. (2014)
SG, Stv, Reb A, Reb C, Dul A	Water	3.30 W per g extract, 100 mL g^{-1}, nr, 2 min	72.14 mg, 46.48 mg, 17.03 mg, 6.6 mg and 2.03 mg[3]	Periche et al. (2015)
TPC, TF, AA		1.98 W per g extract, 100 mL g^{-1}, nr, 3 min	81.0 mg GAE[2], 45.0 mg CE[2] and 96.0 mg TEAC[2]	
SG, Stv, Reb A	Ethanol	400 W, 10 mL g^{-1}; 90 °C, 45 min	21.21 mg, 17.0 mg and 4.21 mg[2]	Yildiz-Ozturk et al. (2015)
TPC, TF, AA			80.13 mg GAE[2], 111.16 mg QE[2] and 91.39% DPPH radical scavenging[2]	
SG, Stv, Reb A	Ethanol 75% (v/v)	160 W, 10 mL g^{-1}, nr, 4 min	34.88 mg, 19.58 mg and 15.3 mg[3]	Ameer, Chun, and Kwon (2017)
SG, Stv, Reb A	Water	400 W, 30 mL g^{-1}, ~25 °C, 1.2 min (pre-treatment)	~71.0 mg, ~48.0 mg and ~23.0 mg[3]	Carbonell-Capella et al. (2017)
TPC, TF, AA, CA			25.7 mg AAE[3], 19.9 mg QE[3], 67.3 μmol TEAC[3] and 2.1 mg[3]	
TPC	Ethanol 75% (v/v)	nr, 10 mL g^{-1}, 100 °C, 20 min	45.0 mg GAE[2]	Ciulu et al. (2017)
SG, Stv, Reb A	Water	500 W, 10 mL g^{-1}, 53 °C, 16 min	88.1 mg, 62.5 mg and 25.6 mg[3]	Görgüç et al. (2019)
TPC			20.7 mg GAE[3]	
SG, Stv, Reb A	Ethanol 50% (v/v)	700 W, 118 mL g^{-1}, 51 °C, 16 min	113.3 mg, 70.4 mg and 42.9 mg[3]	Yılmaz et al. (2021)
TPC, TF, AA			68.6 mg GAE[3], 47.7 mg CE[3] and 853.7 μmol TEAC[3]	

Steviol glycosides (SG); Stevioside (Stv); Rebaudioside A (Reb A); Rebaudioside C (Reb C); Dulcosíde A (Dul A); Total Phenolic Compounds (TPC); Carotenoids (CA); Antioxidant activity (AA); Total flavonoids (TF). Ascorbic acid equivalent (AAE); Gallic acid equivalent (GAE); Trolox equivalent (TEAC); Catechin equivalents (CE); Quercetin equivalents (QE). Not reported (nr). [1]Values corresponding to the compounds reported in the first column; [2]in g Stevia leaf and [3]in g of extract.

where it was possible to reduce the extraction time from 112 to 16 min, respectively. In addition, the extract obtained by MAE showed an increase of 11.5% in the content of active compounds (AA, TF and TPC). Jaitak et al. (2009) in addition to reducing the process from 12 h to 1 min, obtained ~42.0% increase in SG in the extracts.

Considering that the UAE and MAE techniques work with similar variables, their correlation has been investigated and compared. Jaitak et al. (2009) demonstrated an increase of ~78.0% in the SG obtained by MAE when compared to UAE. For Yildiz-Ozturk et al. (2015) this increase in SG extraction was 42.3%. However, for the active compounds, the UAE was more prominent, resulting in the highest levels of TF, TPC and DPPH radical scavenging. Time and potency are parameters that directly affect the efficiency of the extraction, allowing that higher contents of compounds without damage to their quality can be obtained, in slightly longer times and lower potency, than in extreme conditions.

Although little explored, the use of microwaves as a pre-treatment step, and its use through a process combined with the action of enzymes, have also been reported as strategies to increase the removal of compounds from Stevia leaves. With pretreatment, Carbonell-Capella et al. (2017) aimed to promote cracks in the matrix cell wall without causing damage to the compounds, aiming at the later extraction of SG and active compounds under mild conditions (100 rpm, 20 °C for 1 h). UAE associated with enzymes (10.9 FBG unit g^{-1} Viscozyme L) was reported by Görgüc et al. (2019), with the aim of hydrolyzing and degrading the cell wall to contribute to the release of intracellular constituents. In both cases, low volumes of water ($\leq$30 mL g^{-1}) were used as solvent, and the extracts had higher contents of Stv and Reb A and lower contents of TPC, compared to those obtained by Periche et al. (2015) with this same extracting solvent and without pre-treatment. However, although it is ecologically correct, efficient and easy to conduct, extraction with enzymes adds costs to the process, limiting its use.

4.3. Extraction under pressurized conditions

Technologies that enable the use of compressed fluids for extraction, pressurized liquid extraction (PLE), subcritical water extraction (SWE) and supercritical fluid extraction (SFE), are techniques used to selectively obtain compounds contained in natural matrices, that minimize the co-extraction of unwanted materials, in addition to promoting high operational efficiency (Herrero and Ibañez, 2018). Through the simultaneous action of pressure and temperature, these techniques enable rapid extraction kinetics in static or dynamic operation (Bubalo et al., 2018; Gallego et al., 2019), allowing easy operation and solvent removal, and promoting these processes economic and environmental prominence. In addition to these variables, the other operating parameters must also be considered for maximizing the extraction, since in a balanced way, can recover analytes that have different functional groups and physicochemical properties (Andreu and Picó, 2019). The main published works of PLE, SWE and SFE on the compounds of Stevia leaves, as well as the extraction solvent used and the operating conditions indicated by the authors to obtain the maximum extraction of these processes, are presented in Table 3.

4.3.1. Pressurized liquid and subcritical water extraction

PLE and SWE compose extraction techniques that employ the solvent at temperatures above its boiling point, which are pressurized in order to be kept in a liquid state, as represented by the schematic diagram of Fig. 5. Elevated temperatures improve extraction efficiency due to increased diffusion rate and solubility of compounds in the solvent, resulting from disruption of analyte-matrix interactions caused by van der Waals forces, hydrogen bonding, and dipole attraction (Andreu and Picó, 2019), while the viscosity and surface tension values of the solvent will be lower than those at room temperature (Koubaa et al., 2015). On the other hand, elevated temperatures can simultaneously increase the rate of analyte degradation, especially when combined with longer

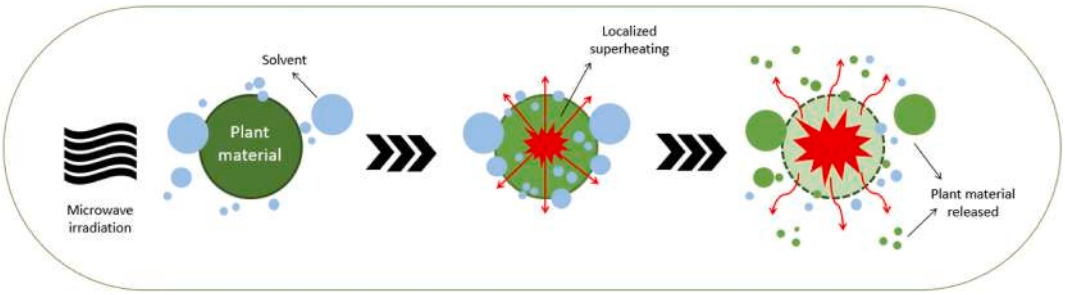

Fig. 4. Schematic diagram of microwave assisted extraction system.

Table 3
Experimental conditions for maximizing extraction under pressurized conditions of compounds from Stevia leaves.

Technique	Reported compounds	Solvent	Recommended experimental conditions: solvent to leaf ratio, time, temperature and pressure	Results[1]	Reference
PLE	TPC	Ethanol 50% (v/v)	2.75 mL g^{-1}, 20 min, 200 °C, 10.3 MPa	79.0 mg GAE[2]	Ciulu et al. (2017)[5]
	SG, Stv, Reb A, Reb C	Ethanol 70% (v/v)	30 mL g^{-1}, 60 min, 125 °C, 10.0 MPa	269.1 mg, 94.1 mg, 127.1 mg and 47.9 mg[2]	Raspe et al. (2021a)[6]
SWE	SG, Stv, Reb A TPC, TF, CA, AA	Water	36 mL g^{-1}, 45 min, 125 °C, 23.0 MPa	74.35 mg, 38.67 mg and 35.68 mg[3] 48.63 mg GAE, 29.81 mg QE[3], 5.71 mg[4] and 92.5% DPPH radical scavenging	Yildiz-Ozturk et al. (2014)[6]
	SG	Water	20 mL g^{-1}, 1 cycle 4 min, 100 °C, 10.3 MPa	91.8%[3]	Jentzer et al. (2015)[5]
	SG, Stv, Reb A TPC, CA	Water	30.6 mL g^{-1}, 3 cycles 10 min, 160 °C, 10.3 MPa	144.3 mg, 105.9 mg and 38.4 mg[2] 8.85 mg GAE and 3.79 mg[4]	Kovačević et al. (2018)[5]
	SG, Stv, Reb A	Water	5 mL g^{-1}, 20 min, 121 °C, 0.2 MPa	54.0%, 42.0% and 11.6%[3]	Németh and Jánosi (2019)[5]
	SG, Stv, Reb A, Reb B, Reb C, Stb	Water	100 mL g^{-1}, 1 min, 140 °C, 3.0 MPa	15.93%, 9.7%, 4.2%, 0.5%, 1.3% and 0.23%[2]	Yang et al. (2019)[5]
	TPC	Water	30.6 mL g^{-1}, 3 cycles 10 min, 160 °C, 10.34 MPa	18.6 mg GAE[2]	Sandra et al. (2020)[5]
SFE	SG, Stv, Reb A	CO_2 + ethanol 70% (v/v)	3.33 mL g^{-1}, 60 min, 80 °C, 21.1 MPa	17.4%, 36.66 mg and 17.79 mg[3]	Erkucuk et al. (2009)[6]
	SG, Stv, Reb A TPC	CO_2 + ethanol	nr, 70 min, 45 °C, 22.5 MPa	17.83%, 98.56 mg and 65.07 mg[3] 25.76 mg GAE[3]	Ameer et al. (2017a)[6]
	Stv	CO_2 + water	100 mL g^{-1}, 180 min, 35 °C, 30,0 MPa	7.0 mg[2]	Cui et al. (2019)[5]

Pressurized liquid extraction (PLE); Subcritical water extraction (SWE); Supercritical fluid extraction (SFE); Steviol glycosides (SG); Stevioside (Stv); Rebaudioside A (Reb A); Rebaudioside B (Reb B); Rebaudioside C (Reb C); Steviobioside (Stb); Total Phenolic Compounds (TPC); Carotenoids (CA); Antioxidant activity (AA); Total flavonoids (TF); Gallic acid equivalent (GAE); Quercetin equivalents (QE). Not reported (nr). [1]Values corresponding to the compounds reported in the first column; [2]in g Stevia leaf; [3]in g of extract; [4]in 100 g of extract; [5]Static extraction and [6]Dynamic extraction.

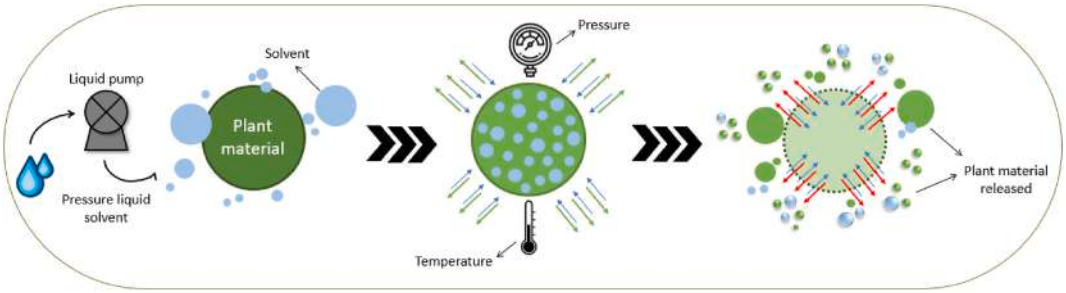

Fig. 5. Schematic diagram of subcritical water and pressurized liquid extraction system.

periods of extraction (Plaza and Turner, 2015; Teo et al., 2010). Furthermore, the solubility of matrix components also increases with this variable, resulting in darker extracts, susceptible to interfering constituents, which can affect the steps after extraction (Cheah et al., 2010).

For the extraction of compounds from Stevia leaves, PLE proves to be a recent and still little explored technique and used the binary mixture of water and ethanol in different proportions as an extracting solvent (Table 3). On the other hand, SWE, which has water as a solvent, presented a broader approach and investigation. These solvents are non-toxic, allow the preservation of analytes, and their mixture favors the interaction and extraction of different classes of compounds, when compared to the use of pure solvents, due to the thermodynamic properties that this binary mixture assumes (Herbst et al., 2021). Therefore, the choice of solvent for extractions under pressurized conditions is essential to obtain the compounds in a profitable way, and thus, their polarity plays an important role in this process (Mustafa and Turner, 2011).

Envisioning the maximum extraction of TPC from Stevia leaves, Ciulu et al. (2017) investigated different percentages of ethanol in the

extractant solvent (0%, 15%, 50%, 85% and 100%, v/v) in order to explore a wide range of dielectric constant values as a measure of polarity (19.00–59.09). The authors found that the use of binary mixtures resulted in greater removal of these compounds, allowing an increase of up to 49.0% when associated with an increase in temperature (40–200 °C). On the other hand, in terms of SG, the solvent composition was investigated by Raspe et al. (2021b), where reduction in the contents of Stv, Reb A and Reb C could be verified when comparing pure ethanol to a binary mixture of 70% ethanol (v/v), demonstrating the negative influence of the addition of a portion of water in the extracting solvent for obtaining these compounds.

Pressurized water and/or water under subcritical conditions present a significant variation in its polarity with temperature, which allows achieving the selective extraction of polar, moderately polar and non-polar organic compounds, by changing the extraction parameters (Liang and Fan, 2013). However, this particularity can compromise the purity of the extracts obtained, as a result of the parallel co-extraction of analytes, reflecting in lower yields of SWE when compared to PLE. This finding can be made by comparing the TPC content obtained by Ciulu et al. (2017), with the results of Kovačević et al. (2018) and Sandra et al. (2020), which provided significantly higher levels, requiring less time and solvent to leaf ratio. For Reb A, 25.7% higher contents were obtained with the application of PLE (Raspe et al., 2021b), compared to those reported using SWE (Kovačević et al., 2018).

In PLE, the increase in the ratio (solvent to leaf) from 30 to 90 mL g^{-1} showed a reduction in SG (Raspe et al., 2021b). On the other hand, Yildiz-Ozturk et al. (2014) found that the ratio of 36 mL g^{-1}, when compared to 18 mL g^{-1}, increased the SG content in the extract obtained by SWE. According to the authors, the extraction of highly concentrated samples is provided by larger volumes of solvent, as these interrupt the equilibrium of the matrix surface, resulting in greater mass transfer of the analytes. A similar effect could have been verified in the extraction of active compounds, since the addition of cycles increases the solvent to sample ratio in the extraction due to the renewal of the extracting solvent. This explains the results of Kovačević et al. (2018) in the TPC and CA contents, where an increase of ~70.0% and ~4.0%, respectively, was verified by increasing the proportion from 10.2 (1 cycle) to 30.6 mL g^{-1} (3 cycles) in the SWE. In the same solvent to sample ratios, Sandra et al. (2020) obtained an increase of ~23.0% and 57.2% in the contents of TPC and TF, respectively.

PLE and SWE occurred at intervals from 1 to 60 min (Table 3), a difference that can be explained by the type of extraction (static or dynamic), since dynamic processes demand longer operational periods. In static mode, the efficiency of the process strongly depends on the equilibrium partition constant and the solubility of the compounds at temperatures that are generally higher, due to the limited volume of solvent used (Teo et al., 2009). In dynamic extraction, time and flow rate are crucial to maximize the extraction, with time being strongly dependent on temperature (Teo et al., 2009). Using PLE, in addition to reducing the extraction time from 12 h to 20 min, Ciulu et al. (2017) observed an increase of ~132.0% in the TPC contents obtained when compared to maceration at room temperature. Jentzer et al. (2015) and Yang et al. (2019), when comparing the SWE with the orbital agitation (60 °C) verified an increase in the SG contents, with a reduction in the time from 2 h to 20 and 1 min, respectively.

The temperature and pressure range used in the applications reported in PLE and SWE ranged from 100 to 200 °C and 0.2–30.0 MPa, respectively, with ~125 °C and 10.3 MPa being the most used conditions. This temperature is above the boiling point of all reported solvents and low enough to prevent analyte degradation. Through the reported pressure, the solvent is kept in a liquid state and is forced to penetrate the matrix pores, due to its high adsorptive capacity (Segura-Campos et al., 2014), not exerting any other influence on the extraction efficiency. This is reiterated by reviewing the studies, since there are no reports involving pressure variation in the recovery of compounds from Stevia leaves to date.

The application of cycles in extractions under pressurized conditions (Jentzer et al., 2015; Kovačević et al., 2018; Sandra et al., 2020), proposes to induce these techniques to a pseudo-dynamic process, as a tactic to contribute to the selectivity of the extraction and, consequently, obtaining better results, with the possibility of reducing the operating temperature. Sandra et al. (2020) obtained in an equivalent and even superior way the compounds of Stevia leaves through the process without cycles, however, they defended its use. Jentzer et al. (2015) highlighted that, in addition to extraction, additional time is required for the other steps inherent to the method, with no feasibility in its implementation. In addition, the application of cycles requires the renewal of the extracting solvent, resulting in an increase in operating cost. Extractions under pressurized conditions are mainly regulated by temperature, while cycles and extraction time are parameters related to solvent saturation (Vergara-Salinas et al., 2012). Therefore, although the intention of the cycles was to minimize the exposure of compounds to high temperature, with no losses being reported in the extractions, their application is not justified for Stevia leaves.

4.3.2. *Supercritical fluid extraction*

SFE uses solvents which are presented in a single phase, non-condensable at a temperature and pressure above its critical point, as represented by the schematic diagram of Fig. 6. Under these conditions, the fluid does not present a distinction between the liquid and gas phases, which allows for some physicochemical properties typical of both to be assumed (Uwineza and Waśkiewicz, 2020). So far, this technique has been little explored to obtain compounds from Stevia leaves, with application only the use of CO_2 in the presence of co-solvents has been reported (Table 3).

The added co-solvent acts by allowing the modulation of CO_2 polarity, which is originally low and less effective in extracting more polar compounds (Lefebvre et al., 2021), substantially influencing the direction of the analyte to be removed. This scenario was investigated by Erkucuk et al. (2009), who verified a significant increase in SG contents when adding 20 wt% of the binary mixture of ethanol and water (70 wt %, v/v) in relation to the mass of CO_2 was applied to the process, at 60 °C and 35 MPa. Expressive increase in TPC contents were reported by Ameer, Chun, and Kwon (2017), by increasing the percentage of ethanol as a co-solvent (0 wt%-40 wt%) in the process at 45 °C and 22.5 MPa. At the same time, Cui et al. (2019) when evaluating the addition of water (0 wt%-1.38 wt%) as a co-solvent in obtaining SG through micro-emulsions of CO_2+water + surfactant (polyethylene glycol trimethylnonyl ether), verified greater removal of compounds from Stevia leaves in the presence of maximum of that mediator. These co-solvents with polar characteristics induce changes in the cellular structure of the matrix, breaking the bonds between analyte-matrix, and providing an increase in the attainment of target compounds (Pimentel-Moral et al., 2019).

Operating parameters such as temperature and pressure can control the SFE process. Ameer, Chun, and Kwon (2017) found a ~90.0% increase in TPC contents in the range of 35–55 °C (15 MPa), while an increase in pressure from 15 to 30 MPa provided an increase of 62.8% (35 °C). Furthermore, the correlation of these variables (45 °C and 75 MPa to 65 °C and 22.5 MPa) also contributed to the increase in TPC contents (10.65%). Considering that the increase in pressure influences the solubility of the solute (Khaw et al., 2017), its combination with temperature affects the physical properties of the solvent (density, viscosity and diffusivity), allowing to promote selectivity in the extraction of compounds active (Pimentel-Moral et al., 2019). On the other hand, taking into account these variables and their interaction in obtaining the GS, Erkucuk et al. (2009) only verified an increase due to the influence of temperature (40–80 °C), with an increase of ~163.0% and 80.7% in the contents of Stv and Reb A, respectively, and by combining this increase in temperature with the increase in pressure (25–35 MPa), a 5.5% reduction in these contents was verified, which may be related to the behavior of the system, which remains totally unknown in the SG +

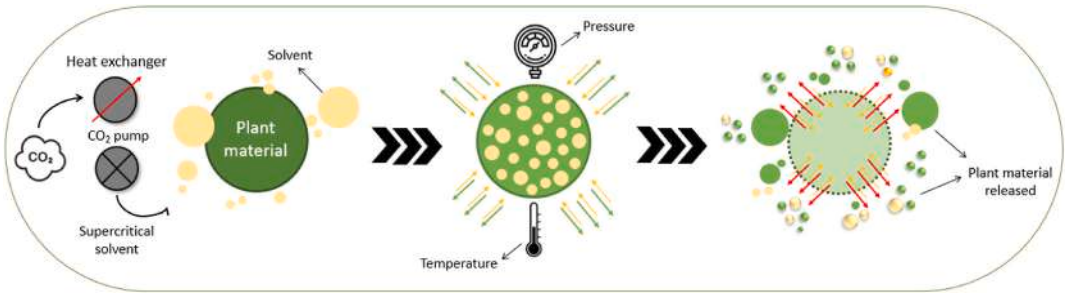

Fig. 6. Schematic diagram of supercritical fluid extraction system.

CO_2+co-solvent phase equilibrium (Yoda et al., 2003).

SFE was compared and, in general, it also stood out compared to conventional extraction techniques. Erkucuk et al. (2009) obtained contents ~12.3% higher than those resulting from Soxhlet extraction, faster and with a lower solvent to leaf ratio. Likewise, when comparing the efficiency of SFE with maceration, Ameer, Chun, and Kwon (2017) found that the highest contents of SG, Reb A and TPC were supplied by SFE. It is noteworthy that in addition to quick extraction, greater obtainment of compounds, reduced consumption and easy removal of solvent (Ameer et al., 2017b), this technique allows for lower energy consumption, as evidenced by Ameer, Chun, and Kwon (2017) when comparing the energy demand in the SFE with the maceration, whose reduction of ~67.0% was verified.

5. Patents

A compilation of the main patents registered in the last 12 years referring to the application of non-conventional methods of extracting Stevia leaves is presented in Table 4. It can be seen from the information presented in this table that, in relation to the application of the techniques, they are only reported in patents the UAE, SWE and SFE. The improvement of the characteristics of the compounds obtained as a result of the applied process, are described and linked only to two patents, by the UAE process to obtain the Stv (CN 106831906A), obtaining extracts with higher contents of this compound when compared to the conventional method, and for SWE (CN 102199178A), in which the process using subcritical water resulted in an extract that after purified showed 90% of Reb A. The other patents mention the use of extraction techniques in the process as an aid in obtaining compounds devoid of unpleasant smell and bitterness (CN 106632539A and IN 274074B); to obtain aqueous concentrate from roots and leaves (KR 2001111560A); obtaining a sweetener with a degree of sweetness 300 times higher than that of sucrose (CN 101461452A); simultaneous obtainment of Stv and total flavonoids (CN 101062077B and CN 101062078B) and obtainment of an extract rich in cannabidiol, with purity above 99.0% (CN 111099970A). Given the number of articles published in the area, patents are still scarce.

Table 4
Non-conventional compound extraction techniques from *Stevia rebaudiana* reported in patents.

Technique	Number	Title	Author
UAE	CN 106632539 (A)	Method for extracting stevioside	Jia (2017)
	CN 106831906 (A)	Ultrasonic-assisted method for extracting stevioside from *Stevia rebaudiana*	Yang et al. (2017)
SWE	KR 2001111560 (A)	A method for preparing concentrated solution of *Stevia rebaudiana* Bertoni	Kim (2001)
	CN 102199178 (A)	Process for extracting rebaudioside a from *Stevia rebaudiana*	Hu (2011)
SFE	IN 274074 (B)	An improved process for making natural sweetener from stevia leaves	Mukhopadhyay and Panja (2019)
	CN 101461452 (A)	Method for preparing sweetener for feed	Ye (2009)
	CN 101062078 (B)	Method for extracting total steviosides and flavones from Stevia	Shi et al. (2011a)
	CN 101062077 (B)	Method for simultaneously preparing total steviosides and total flavonoids from *Stevia rebaudiana*	Shi et al. (2011b)
	CN 111099970 (A)	Extraction method suitable for large-scale industrial prodn. of cannabidiol (CBD) from *Stevia rebaudiana* by supercritical carbon dioxide extraction, molecular distillation and supercritical fluid chromatographic separation	Chu et al. (2020)

Ultrasound assisted extraction (UAE), Subcritical water extraction (SWE) and Supercritical fluid extraction (SFE).

6. Final considerations

Contemplating promising technological criteria in the global market demand for natural compounds, *Stevia rebaudiana* Bertoni presents efficiency and profitability in the recovery of its sweeteners and active compounds through non-conventional methods of extraction, which enable quick and easy operation, through use of renewable solvents under operating conditions that do not affect the quality of the extract obtained. Among these processes, techniques involving ultrasound acoustic cavitations, non-ionizing microwave irradiation, compressed fluids in the extraction with pressurized liquid and subcritical water, as well as the extraction with supercritical fluid, are highlighted on the rise. These techniques address environmental and food safety issues by allowing the use of renewable solvents, in addition to being aligned with the development of alternative processing routes, contemplating sustainability concepts.

Although a possible implementation of these processes on a pilot scale is presented as a relevant alternative, as it allows for fewer inconveniences when compared to conventional processes, the reduction in the consumption of inputs and the feasibility of reusing the solvent in the process still need to be improved in their investigation. Combined or sequential processes could be applied as alternatives to this issue, but their exploration has not yet been reported, as well as the consequences of exposing this matrix to extreme conditions of some of the technologies mentioned, mainly in relation to the possible degradation of the target compounds. These gaps and the lack of information about these techniques, configure the main challenges in the expansion of the use of these technologies, which can serve as vectors to direct and drive new investigations, in order to contribute to the exploration of this promising segment.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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ARTIGO 2

Ultrasound-assisted extraction of compounds from Stevia leaf pretreated with ethanol

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Ultrasound-assisted extraction of compounds from Stevia leaf pretreated with ethanol

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ABSTRACT

In this study, ultrasound-assisted extraction (UAE) of *Stevia rebaudiana* leaf compounds previously treated with ethanol was investigated. Ultrasound power intensity was evaluated and an experimental design was applied to examine the effect of temperature, solvent to leaf ratio and ethanol percentage in the solvent on mass (Y_M) and sweeteners (Y_S) yield, total phenolic content (TPC) and antioxidant activity (AA). The application of the maximum power (165 W) provided the obtaining of greater Y_M and Y_S, and extracts with higher TPC contents and AA. The increase in the solvent/leaf ratio provided the highest Y_M and Y_S, as the increase of ethanol in the solvent, while the temperature promoted an increase only in the Y_M. Maximum values of Y_M (44.7 wt%) and Y_S (88.50 wt%) were obtained using solvent with 40 % ethanol, 50 °C and 15 mL g^{-1} (solvent/leaf). The extract obtained is constituted by ~26 wt% of steviol glycosides (GS), corresponding to 9.5, 4.1 and 12.0 wt% of Stevioside, Rebaudioside C and Rebaudioside A, respectively. The analysis of the principal components indicated a high correlation of the variables ethanol percentage in the solvent and solvent to leaf ratio in obtaining extracts with higher TPC content and AA.

1. Introdution

Stevia rebaudiana (Bertoni) exhibits therapeutic benefits due to its antioxidant, antimicrobial, antifungal, antitumor and antidiabetic properties (Kurek and Krejpcio, 2019). Its leaves are used as a natural sweetener, due to its composition in steviol glycosides (GS), among which stevioside (Stv) and rebaudioside A (Reb A), which give the plant sweetness, on average 250–300 times more sweetness than sucrose (Hajela et al., 2017). The extract obtained from Stevia leaves, rich in bioactive compounds, stands out with phytotherapic properties (Ganjiani et al., 2020), with benefits against hyperglycemia, hypertension, cystic fibrosis and obesity (Milani et al., 2017), and for presenting non-teratogenic, mutagenic or carcinogenic effects, in addition to the absence of acute and subacute toxicity (Momtazi-Borojeni et al., 2017).

The extraction of *Stevia rebaudiana* compounds traditionally takes place through the use of conventional techniques, involving the use of hot water (Formigoni et al., 2020), which requires long periods of extraction, high solvent consumption, low efficiency, as it promotes the simultaneous extraction of a variety of compounds (Vieites et al., 2018), in addition to the degradation of thermolabile compounds (Žlabur and Brnčić, 2014). Infusions and decoctions, often used to obtain herbal extracts (Li et al., 2014), have been reported with low efficiency due to the low recovery rate of the compounds of interest and their degradation when at high temperatures (Lemus-Mondaca et al., 2012). Maceration, a simple and suitable process for extracting thermolabile compounds, results in low process efficiency (Rouhani, 2019).

Ultrasound-assisted extraction (UAE) has been shown to be an efficient and promising method for obtaining plant compounds, due to the high extraction yields, using low temperatures and shorter process times (Periche et al., 2015; Yilmaz et al., 2020). This technique explores the cavitation process that is based on the production of sound waves that generate a mechanical effect, creating cavitation bubbles close to the tissue of the plant matrix, which cause a mechanical erosion that can break or break the cell walls, reducing the size particles and promoting

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increased penetration of the solvent into the cell (Wijngaard et al., 2012). These effects increase the interaction between solute and solvent, resulting in greater diffusion of intracellular content into the solvent (Barba et al., 2014; Roselló-Soto et al., 2015), with a consequent increase in the efficiency of the extraction process (Žlabur et al., 2015; Milani et al., 2020).

Water is the most selected solvent for the recovery of GS from stevia leaves as it is preferable for the development of drugs and food (Xu et al., 2019; Yilmaz et al., 2020). However, aiming at maximizing the extraction yield, use of alcohols as methanol (Javad et al., 2014), ethanol (Yildiz-Ozturk et al., 2015) and isopropanol (Gasmalla et al., 2017) have been evaluated. Ethanol, considered a green, biodegradable solvent and produced on a large scale in Brazil, has the main advantage of promoting the extraction of natural compounds with low toxicity under operational safety, factors that characterize it as a GRAS solvent (generally recognized as safe) (Chemat et al., 2012; Bubalo et al., 2015).

In binary mixtures, the combination of ethanol with water changes the polarity and extraction capacity of these solvents (Celaya et al., 2016), accelerates the mass transfer process between liquid and solid, increasing the permeability of plant tissues (Muniz-Marquez et al., 2013) and helping to interrupt the connection between solutes and the plant matrix (Carbonell-Capella et al., 2017), providing greater extraction of bioactive compounds, such as phenolics, flavonoids and antioxidants, as well as sweeteners, such as Stv and Reb A (Žlabur et al., 2015; Medrano et al., 2019; Yilmaz et al., 2020). Extractions in water result in higher yields of GS, especially Stv, which has maximum solubility in this solvent (Javad et al., 2014; Martono et al., 2015). However, due to the sensory characteristic of this glycoside, binary mixtures between ethanol and water have been investigated as a strategy to maximize the recovery of Reb A, a glycoside that is superior in sweetness and flavor (Žlabur et al., 2015; Carbonell-Capella et al., 2017; Gasmalla et al., 2017; Martins et al., 2017; Yilmaz et al., 2020).

The aftertaste in Stevia leaf extract is attributed to the presence of essential oils, tannins and flavonoids (Phillips, 1987), sesquiterpene lactones (Soejarto et al., 1983), and caryophyllene and spathulenol (Tsanava et al., 1991). In addition, among the sweeteners present in the extract, Stv has a sweet taste, followed by a residual bitterness, which has decreased as the Reb A content increases in the sweetener mixture (Goto and Clemente, 1998). Recently, the use of ethanolic treatment of the leaves was proposed (Formigoni et al., 2018), which enabled the extraction of higher levels of phenolic compounds from the matrix and obtaining an extract with higher antioxidant activity. The authors also show an increase in the yield of sweeteners with an increase in the Reb A content.

UAE of *Stevia rebaudiana* compounds has been previously reported (Bubalo et al., 2013; Martono et al., 2015; Periche et al., 2015; Yildiz-Ozturk et al., 2015; Žlabur et al., 2015; Carbonell-Capella et al., 2017; Gasmalla et al., 2017; Rouhani, 2019; Milani et al., 2020; Yilmaz et al., 2020), however, this extraction technique has not yet been applied to leaves after ethanolic treatment, in addition, it is not known to us, a study aimed at maximizing the recovery of sweeteners taking into account the mass terms of the process.

Therefore, the objective of this study was to investigate the UAE performance of compounds from *Stevia rebaudiana* leaves pretreated with ethanol, using a binary mixture of water and ethanol as the extractor solvent. For this purpose, the effect of the ultrasound power intensity was determined on the mass yield (Y_M), sweeteners yield (Y_S), composition of the samples in terms of GS (Stv, Reb A and Reb C), total phenolic content (TPC) and antioxidant activity (AA). Subsequently, the effect of process variables (temperature, solvent to leaf ratio and ethanol percentage in the extractor solvent) on Y_M and Y_S was determined, and the operational conditions that maximize these responses. The determination of TPC and AA was carried out in the extracts obtained under the conditions that provided the greatest Y_M and Y_S and the principal component analysis was carried out, aiming to evaluate the correlation between the evaluated responses and the adopted experimental conditions.

2. Materials and methods

2.1. Materials

Stevia rebaudiana Bertoni leaves were used (Milani et al., 2017), Stevia UEM-13 seminal variety, grown at the Natural Products Research Center of the State University of Maringá, Paraná, Brazil (23°24′ and 21°9′ S; 51°56′ and 22°0′ W). For the pre-treatment of the leaves and extraction, absolute ethanol (Merck, 99.8 % purity) was used. In the remaining steps, were used: deionized water (18 MΩ cm) (Milli-Q plus, Induslab, Brazil), acetonitrile (JT Baker, 99.9 % purity), chromatographic standards of stevioside, rebaudioside C and rebaudioside A (Sigma-Aldrich), sodium carbonate (Anhydrol, >99.5 % purity), gallic acid (Sigma-Aldrich), Folin-Ciocalteu (Dinâmica), methanol (Neon, >99.8 % purity) and 2.2-diphenyl-1-picrilhidrazil (DPPH) (Sigma-Aldrich, 95 % purity).

2.2. Sample preparation

The bushes, harvested at the maximum stage of vegetative growth (~50 to 60 days after pruning), were previously dried in an air circulation oven (60 °C for 8 h) until reduced moisture content (<10 %). Subsequently, the leaves were separated from the stems and branches and crushed in a stainless steel knife grinder with a 2 mm opening sieve (Marconi, TE 340). The milled leaves were classified according to the standard series of Tyler sieves (Bertel, ASTM) and the fractions retained in the sieves with an average diameter between 28–48 mesh were used in the experiments. The leaves were then submitted in the ethanolic pretreatment (Formigoni et al., 2018), which makes it possible to obtain an extract with higher quality (in terms of antioxidant activity, phenolic compounds and sweeteners content), lower amount of Stv corresponding to the aftertaste without harming the Reb A content. At this stage, the leaves were being soaked and kept in contact with absolute ethanol for 30 min, with subsequent continuous elution at a flow rate of ~30 mL min^{-1}. Subsequently, the leaves were dried in an oven with air circulation (60 °C for 8 h), showing final moisture content of 4.57 ± 0.01 wt %. The composition of the pretreated leaves in terms of sweeteners showed 4.08 ± 0.07, 1.75 ± 0.01 and 6.83 ± 0.02 g per 100 g of extract of Stv, Reb C and Reb A, respectively, corresponding to 12.76 ± 0.07 wt % of GS. The leaves without pretreatment showed a composition in terms of sweeteners of 4.34 ± 0.04, 1.92 ± 0.02 and 6.98 ± 0.04 g per 100 g of extract of Stv, Reb C and Reb A, respectively, corresponding to 13.18 ± 0.06 wt% of GS. Finally, the leaves were stored in polyethylene bags, in the dark and at room temperature, for later extraction step.

2.3. Ultrasound-assisted extraction

The extractions were carried out in an ultrasonic bath with heating control (Ultronique, Q 5.9/40 A/165 W, Eco-Sonics), in which the samples were submitted to ultrasonic power by indirect contact. In each experiment, a flask (250 mL) containing the crushed leaves (~3 g) and the extracting solvent was connected to an Allihn-type condenser (Vidrolabor) coupled to the cooling bath (Marconi, MA 184), and both were positioned in the center of the ultrasonic bath.

Preliminary tests indicated that the application of 3 cycles of 10 min each in the extraction process resulted in an increase of 32 % in the removal of sweeteners, when compared to the extraction performed for 30 min (without using cycles). After the period of each cycle (10 min), the leaves were filtered on filter paper (8 μm) and replaced with a new solvent in the flask, so that in each cycle 1/3 of the total volume of the solvent was added to the extraction. After the end of the three extraction cycles, the leaves were separated by filtration and the filtrate was concentrated until the solvent was completely eliminated (Marconi rotary vacuum evaporator, MA 120). The Y_M was calculated considering

the relationship between the extracted mass and the initial mass of the sample introduced in the extraction flask.

2.3.1. Effect of ultrasound power intensity (UPI)

To assess the UPI effect, extraction was carried out at 50 °C, using a ratio of 10 mL g^{-1} (solvent/leaf) and 70 % (v/v) of ethanol in the extractor solvent (Martins et al., 2016), using different power intensities of 165 W: 0, 50 and 100 % (0, 83, 165 W, respectively). The extractions and analyzes were performed in duplicate (4 answers) and the results were expressed as mean values ± standard deviation (SD). To check the influence of the ultrasound power on the results obtained, analysis of variance and the Tukey test were performed, with a 95 % confidence interval, using the Statistica® 8.0 software (StatSoft, Inc., Tulsa, OK, EUA).

2.3.2. Effect of process variables

For the evaluation of the effect of the process variables (temperature - X_1, solvent to leaf ratio - X_2 and ethanol percentage in the extractor solvent - X_3) and determination of the conditions that maximize the value of the response variables (Y_M and Y_S), a Box-Behnken experimental design with three levels, three variables and five repetitions of the central point, generated by the software Statistica® 8.0 (StatSoft, Inc., Tulsa, OK, EUA), were conducted. Where X_1 varied from 35, 50 and 65 °C, X_2 from 5, 10 and 15 mL g^{-1} and X_3 from 10, 40 and 70 % (v/v), under 100 % ultrasound intensity (165 W). The experimental conditions were selected according to previous studies, X_1 being based on reports by Martins et al. (2016) and Ameer et al. (2017), X_2 in the studies by Yildiz-Ozturk et al. (2015) and Muthusamy and Munaim (2019) and X_3 based on investigations of Carbonell-Capella et al. (2017) and Martins et al. (2017), considering the answers of the referred works for the GS extraction.

Analysis of variance (ANOVA) was performed to evaluate the effects of independent variables (with a 95 % confidence interval) on response variables and a second order polynomial model was used to adjust the experimental data. The resulting model is shown in Eq. 1.

$$Y = \beta_o + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j \quad (1)$$

where Y is the response variable (Y_M and Y_S); X_i and X*j* are the coded independent variables (temperature, solvent to leaf ratio and ethanol percentage in the extracting solvent); β_0, β_i, β_{ii} and β_{ij} are the regression coefficients of the model (β_0 = constant term; β_i = linear effect; β_{ii} = quadratic effect; β_{ij} = linear interaction term).

To determine the conditions that maximize Y_M and Y_S, within the experimental range tested, the Derringer desirability function was applied. The predictive capacity of the models was evaluated based on verification experiments in the conditions of maximum extraction, in quadruplicate.

The samples obtained in this condition were characterized in relation to the TPC content and AA, as well as the experimental runs of the experimental design that resulted in higher values of Y_M and Y_S under different experimental conditions. The response variables and the experimental conditions were correlated from the principal component analysis (PCA), using the Past software (Paleontological Statistics, version 4.03), in order to simplify the set of data obtained.

2.4. Characterization of extracts

The extracts obtained were characterized, in quadruplicate, in relation to the composition in sweeteners, the content of total phenolic compounds and antioxidant activity. The contents of stevioside (Stv), rebaudioside C (Reb C) and rebaudioside A (Reb A) were determined according to Dacome et al. (2005). For analysis, 10 mg of the sample was redissolved with 10 mL of the mobile phase deionized water and acetonitrile (20:80 v/v), and the obtained solution was sonified for 5 min (Ultronique, Q 3.0/40 A/110 W, Eco-Sonics), filtered (hydrophobic membrane, 0.5 µm, Millipore) and 20 µL were injected into the high performance liquid chromatography system (Gilson, model 307), consisting of a low pressure pump (Gilson, model 5.SC), refractive index detector (IR 133, Gilson), column oven (West, model 2300) and NH_2 analytical column (125 mm x 4.6 mm x5 µm, Scientific Term, HyperSil gold amino). The column temperature was maintained at 30 °C for 30 min and as a mobile phase, a solution of deionized water and acetonitrile (20:80 v/v) was used in the isocratic mode (0.5 mL min^{-1}). Steviol glycosides were analyzed comparing the integrals of their peaks with those of a standard analytical curve and retention time (Zorzenon et al., 2020). The sweeteners yield (Y_S) was calculated as shown in Eq. 2:

$$Y_S(wt\%) = \left(\frac{M_L \times Y_M \times GS}{A_i}\right) \times 100 \quad (2)$$

where M_L is the leaf mass (g), Y_M is the mass yield (g dry extract g^{-1} leaf), GS is the glycoside content (Reb A, Reb C and Stv) (g g^{-1} dry extract) and A_i is the initial mass of sweeteners present in the leaf (g).

In determining the content of TPC, the Folin-Ciocalteau method was adopted (Singleton et al., 1999), with modifications. For this, 0.5 mL of aqueous extracts (500 µg mL^{-1}) was added to tubes containing 2.0 mL of Folin-Ciocalteau solution (10 %) and 2.5 mL of sodium carbonate solution (7.5 %). The mixture was homogenized and incubated at 50 °C for 5 min (Quimis®, Q334 M) in the absence of light and subsequently, the sample absorbance was determined at 760 nm (Shimadzu, UV-1900). To quantify the TPC content, a standard curve prepared with a solution of the gallic acid standard was used and the result was expressed in mg gallic acid equivalente (GAE) per g of dry extract.

The DPPH free radical assay was performed to evaluate the antioxidant capacity of the extracts (Brand-Williams et al., 1995). The dry extracts were diluted in methanol (2000 µg mL^{-1}) and volumes of 10, 15, 25, 50, 63 and 75 µL of this solution were then transferred to test tubes together with 2 mL of the DPPH solution (47 µg mL^{-1}). After 30 min of incubation in the dark at room temperature (~25 °C), the absorbance of the samples was determined at 517 nm (Shimadzu, UV-1900) against a methanol blank. The determination of antioxidant activity in relation to DPPH was calculated by Eq. 3:

$$AA_{DPPH}(\%) = \left(\frac{AA_{DPPH} - (A - A_B)}{A_{DPPH}}\right) \times 100 \quad (3)$$

where A_{DPPH} is the absorbance of the DPPH solution and A and A_B are the absorbance values for the samples and the blank, respectively.

The results were reported as EC_{50} values, which indicate the concentration of the extract capable of reducing the DPPH radical by 50 % and antiradical power (ARP), calculated from $1/EC_{50}$. For this purpose, a graph of the percentage of antioxidant activity versus concentration in µg dry extract mL^{-1} was constructed.

3. Results and discussion

3.1. UPI effect

Table 1 presents the results obtained from the extraction at 50 °C, with a proportion of 10 mL g^{-1} (solvent/leaf), using 70 % (v/v) of ethanol in the extractor solvent, varying the UPI from 0 to 100 % (0–165 W). From the data in this table, it appears that the composition of the extracts in terms of sweeteners (Reb A, Reb C and Stv) was an influence when the ultrasound was applied in the extraction, being possible to obtain a subtle increase of 4 and ~3% in the content of total glycosides by applying 50 and 100 % of the UPI, respectively, in addition to obtaining a higher Y_S, resulting from the higher Y_M. Additionally, the extracts obtained by application of ultrasound had higher TPC content, as well as higher antioxidant activity, as evidenced by higher ARP values (and lower EC_{50} values).

The application of ultrasound in the process (intensity of 100 %) led

Table 1
Effect of the intensity of ultrasonic power on the extraction of compounds from Stevia leaves.

Response variable		Power intensity (%)[1]			
		with pretreatment			without pretreatment
		0	50	100	100
Glycosides (g per 100 g of dry extract)	Stevioside	10.35 ± 0.02[a]	10.87 ± 0.03[b]	10.57 ± 0.00[c]	11.76 ± 0.14[d]
	Rebaudioside C	4.23 ± 0.08[a]	4.62 ± 0.01[b]	4.55 ± 0.06[b]	4.40 ± 0.16[b]
	Rebaudioside A	11.17 ± 0.11[a]	11.28 ± 0.20[a]	11.38 ± 0.07[a]	11.69 ± 0.33[a]
	Total	25.75 ± 0.01[a]	26.78 ± 0.22[b]	26.50 ± 0.01[b]	27.85 ± 0.32[c]
Y_M (wt%)		36.40 ± 0.57[a]	39.76 ± 0.42[b]	41.88 ± 0.03[c]	37.82 ± 0.38[a]
Y_S (wt%)		71.30 ± 0.04[a]	81.30 ± 0.66[b]	85.38 ± 0.04[c]	80.48 ± 0.93[b]
TPC (mg GAE per g dry extract)		240.41 ± 0.54[a]	258.57 ± 0.47[b]	269.41 ± 0.26[c]	262.98 ± 0.27[d]
EC_{50} (μg dry extract per mL)		26.45 ± 0.07[a]	24.40 ± 0.12[b]	23.57 ± 0.31[c]	26.78 ± 0.15[a]
ARP (μg dry extract per mL)		0.037 ± 0.00[a]	0.040 ± 0.00[b]	0.042 ± 0.00[c]	0.037 ± 0.00[a]

[1] Iin relation to the maximum power of the equipment of 165 W. Y_M: mass yield; Y_S: sweetener yield; TPC: total phenolic compounds; EC_{50}: efficiency concentration of the extract capable of reducing the DPPH radical by 50 % and ARP: antiradical power calculated from $1/EC_{50}$. Means followed by different letters on the same line indicate a significant difference ($p < 0.05$).

to an increase of ~15 % and ~20 % for Y_M and Y_S, respectively, when compared to the values obtained from extraction without ultrasound. Rouhani (2019), when evaluating extraction of stevioside from *Stevia rebaudiana* under various ultrasound powers, reported obtaining extracts with a concentration of 3 and 8 mg of Stv g^{-1} of dry leaf using a power of 40 and 160 W, respectively.

In the present study, an increase in ultrasonic power applied from 0 to 165 W led to an increase of ~12 % in TPC and a reduction of ~12.5 % in the value of EC_{50}. More and Arya (2021), when evaluating the effect of ultrasound power from 70 to 140 W, found an increase of ~26.5 % in TPC and 12.5 % in the antioxidant capacity of the extracts from the pomegranate peel. Luo et al. (2018) obtained ~4.5 % increase in UAE of TPC from red sorghum bran, in the power range of 0–150 W.

The selection of ultrasonic power is the initial step to avoid the undesirable degradation of the extracted compounds, which is caused due to the thermal and mechanical effects resulting from this condition having an influence on the extraction process (Tiwari, 2015). Increased extraction efficiency, proportional to the increase in ultrasound power, are the result of the mass transfer promoted by the greater number of cavitation bubbles and energy in the system (Rouhani, 2019).

For comparative purposes, the extraction of leaves without pretreatment with the application of 100 % of the UPI was investigated (Table 1). In this case, it is possible to verify that the application of UAE enables equivalent removal of sweeteners from Stevia leaves, with the exception of Stv, a compound that gives a bitter aftertaste and is partially removed through pre-treatment. It is noteworthy that the other extracted compounds were superior in quantity (Y_M and Y_S) and quality (TPC and AA) to leaves without pretreatment, which justifies their use.

3.2. Maximization of the UAE

3.2.1. Establishment of the mathematical model

Table 2 shows the experimental design adopted and the results obtained in terms of Y_M, composition of sweeteners in the extract and Y_S, resulting from the levels adopted for the factors in each experiment.

The response variables (Y_M and Y_S) were expressed in terms of coded variables and their interactions, adjusting to Eqs. 4 and 5:

$$Y_M(wt\%) = 43.81 + 0.29X_1 + 1.65X_2 - 0.80X_3 - 1.58{X_1}^2 - 1.09{X_2}^2 - 2.12{X_3}^2 - 0.51X_1X_2 + 0.50X_1X_3 + 0.22X_2X_3 \quad (4)$$

$$Y_S\ (wt\%) = 87.57 + 0.20X_1 + 3.23X_2 - 1.80X_3 - 2.11{X_1}^2 - 2.86{X_2}^2 - 3.10{X_3}^2 + 0.18X_1X_2 - 0.26X_1X_3 - 1.53X_2X_3 \quad (5)$$

The ANOVA results presented in Table 3 were used to validate the second order polynomial models (Eq. 4 and 5) adjusted to the experimental data, as well as to evaluate the influence of each factor in the responses. According to the statistical significance considered (Table 3), it can be seen in this table that Eq. 4 presented significance ($p < 0.05$) for the linear and quadratic effects, as well as its interactions, for the variables solvent to leaf ratio (X_2) and ethanol percentage in the extractor solvent (X_3). Eq. 5 was significant ($p < 0.05$) for linear, quadratic and interaction effects, with the exception of temperature (X_1), which presented $p > 0.05$ for the linear term, as well as for interaction with variables X_2 and X_3. According to the ANOVA data, F_{calc} for Y_M (52.19) and Y_S (139.26) were higher than F_{tab} of 3.37 and 4.10, respectively, and thus the equations were valid in relation to the experimental data.

Table 2
Experimental conditions of the Box-Behnken experimental design and results of the ultrasound-assisted extraction of compounds from pretreated Stevia leaves.

Run	Independent variables[1]			Glycosides (g per 100 g of dry extract)				Y_M (wt%)	Y_S (wt%)
	X_1	X_2	X_3	Stevioside	Rebaudioside C	Rebaudioside A	Total		
1	−1 (35)	−1 (5)	0 (40)	9.70 ± 0.24	4.94 ± 0.02	11.40 ± 0.10	26.04 ± 0.12	38.40	78.98 ± 0.18
2	1 (65)	−1 (5)	0 (40)	9.77 ± 0.15	4.63 ± 0.23	10.50 ± 0.06	24.89 ± 0.02	39.92	79.23 ± 0.03
3	−1 (35)	1 (15)	0 (40)	9.19 ± 0.20	4.76 ± 0.09	10.06 ± 0.23	24.02 ± 0.34	43.36	85.60 ± 0.61
4	1 (65)	1 (15)	0 (40)	9.17 ± 0.12	4.72 ± 0.53	10.84 ± 0.12	24.72 ± 0.29	42.85	86.60 ± 0.51
5	−1 (35)	0 (10)	−1 (10)	9.15 ± 0.29	4.41 ± 0.10	10.75 ± 0.02	24.31 ± 0.41	40.95	83.58 ± 0.76
6	1 (65)	0 (10)	−1 (10)	10.06 ± 0.14	4.37 ± 0.30	10.97 ± 0.13	25.40 ± 0.57	40.60	84.30 ± 0.94
7	−1 (35)	0 (10)	1 (70)	10.07 ± 0.04	4.56 ± 0.15	11.03 ± 0.15	25.65 ± 0.04	38.60	80.93 ± 0.06
8	1 (65)	0 (10)	1 (70)	9.44 ± 0.18	4.72 ± 0.18	10.38 ± 0.05	24.54 ± 0.05	40.26	80.61 ± 0.08
9	0 (50)	−1 (5)	−1 (10)	9.53 ± 0.21	4.74 ± 0.00	11.00 ± 0.04	25.27 ± 0.25	40.42	79.14 ± 0.41
10	0 (50)	1 (15)	−1 (10)	9.54 ± 0.15	4.22 ± 0.26	11.87 ± 0.54	25.63 ± 0.66	42.65	88.14 ± 0.43
11	0 (50)	−1 (5)	1 (70)	10.34 ± 0.55	4.98 ± 0.32	11.34 ± 0.02	26.66 ± 0.89	38.10	78.14 ± 1.33
12	0 (50)	1 (15)	1 (70)	10.10 ± 0.17	4.02 ± 0.32	11.82 ± 0.21	25.95 ± 0.28	41.23	81.02 ± 0.46
CP (13−17)[2]	0 (50)	0 (10)	0 (40)	9.51 ± 0.20	4.47 ± 0.02	11.00 ± 0.07	24.97 ± 0.64	43.81 ± 0.13	87.57 ± 0.38

[1] X_1 – temperature (°C); X_2 – solvent to leaf ratio (mL g^{-1}) and X_3 – ethanol percentage in the extractor solvent (v/v).
[2] Central point - average values of 5 experiments.

Table 3
Analysis of variance (ANOVA) of the quadratic model for the mass (Y_M) and sweeteners (Y_S) yield obtained from the ultrasound-assisted extraction.

	Y_M					Y_S				
	Sum of squares	Degrees of freedom	Mean square	F	p^a	Sum of squares	Degrees of freedom	Mean square	F	p^a
X_1 (L)	0.67	1	0.67	41.67	0.02	0.34	1	0.33	2.31	0.22
X_1 (Q)	10.62	1	10.62	655.04	<0.0001	17.88	1	17.88	121.65	<0.002
X_2 (L)	21.94	1	21.94	1353.44	<0.0001	83.68	1	83.68	569.30	<0.0002
X_2 (Q)	5.00	1	5.00	308.71	<0.0001	32.72	1	32.72	222.62	<0.001
X_3 (L)	5.16	1	5.16	318.24	<0.0001	26.10	1	26.10	177.56	<0.001
X_3 (Q)	18.95	1	18.95	1168.84	<0.0001	38.57	1	38.57	262.46	<0.001
X_1*X_2	1.04	1	1.04	64.27	<0.01	0.14	1	0.14	0.96	0.39
X_1*X_3	1.01	1	1.01	62.70	<0.01	0.26	1	0.26	1.83	0.26
X_2*X_3	0.20	1	0.20	12.72	0.02	9.36	1	9.36	63.68	<0.01
Lack of fit	0.95	3	0.31	19.61	<0.01	1.05	3	0.35	2.39	0.24
Pure Error	0.06	4	0.01			0.44	3	0.14		
Total	69.33	16				210.57	15			
R^2 = 0.985						**R^2** = 0.989				
$R^2_{adjusted}$ = 0.966						**$R^2_{adjusted}$** = 0.982				

[a] Statistical significance ($p < 0.05$); L - linear effect and Q - quadratic effect.

From the generated diagnostic graphs (data not shown) it was possible to verify that the predictive values of the models were consistent with the real values obtained by the experiment ($R^2 = 0.98$). In the evaluation of the normality residues of the models, the points of the residues were randomly distributed, indicating that the variations in the actual values observed were consistent with all responses. All residual values were less than 1 (±0.5) and there were no points outside the curve or unexpected errors, the reliability of the models was confirmed. Thus, it can be deduced that the established regression models can be used to predict the condition of maximum extraction.

3.2.2. *Temperature effect*

The favoring of the extractive process, linked to the increase in the process temperature of 35–65 °C, is due to the reduction of viscosity and increase in the diffusivity of the solvent, which provides for the wetting of the matrix and solubilization of the analytes (Jentzer et al., 2015; Rouhani, 2019; Dzah et al., 2020). The diffusion of analytes is facilitated, considering that there is more energy in the system, making it possible to break the analyte-matrix bonds more efficiently (Teo et al., 2010), in this way, the exit of the analyte from the matrix of the plant cell occurs easily.

In this process, the intermediate temperature (50 °C) investigated proved to be favorable to obtain higher process yields. At this temperature, the vapor pressure in the solvent is low, allowing cavitation bubbles to collapse violently, inducing matrix bonds to break, disrupting cell tissues (Tiwari, 2015) and causing higher compound extraction (Periche et al., 2015). On the other hand, higher temperatures can have a negative effect on the cavitation phenomenon, which can cause a reduction in the extraction yield (Milani et al., 2020) or even, degradation of the compounds obtained. In this case, the disruption of the solute-matrix interaction promotes the filling of voids with solvent vapors, resulting from the reduction in the intensity of cavitation (Tiwari, 2015), resulting in a decrease in the extraction rate. Furthermore, at higher temperatures, energy demand is greater (Rouhani, 2019), which can influence costs related to the feasibility of the process.

The increase in the extraction temperature from 35 to 65 °C had no influence on obtaining sweeteners, as reported in the literature. Periche et al. (2015) showed that the concentration of Stv and Reb A in Stevia leaf extracts obtained from UAE was not influenced by the temperature increase from 50 to 70 °C. Martins et al. (2017) report that the increase in temperature in the range of 23–80 °C did not contribute to the increase in the content of Stv and Reb A contents in fluid extract from *Stevia rebaudiana*. In the work of Xu et al. (2019), the extraction of Reb A was not affected by the increase in temperature, 40–50 °C.

3.2.3. *Effect of solvent to leaf ratio*

The greater availability of solvent in the extraction medium had the most pronounced effect on the process, providing greater values of Y_M and Y_S. Evidenced, for example, by experiments in which the proportion was increased from 5 to 15 mL g^{-1}, resulting in an increase in Y_M and Y_S of ~13 and ~8.5 % (runs 1 and 3), 7.3 and ~9.5 (runs 2 and 4), respectively. This effect is attributed to the better dissolution of the soluble components of the sample, since the diffusion rate of the compounds from the inside out of the plant matrix particle is favored (Vinatoru et al., 2017). This effect occurs due to the concentration gradient, which is the driving force, increases as the higher solvent ratio is used, leading to a greater diffusion of the analytes (Şahin and Şamlı, 2013). Moreover, the addition of extraction cycles allows the renewal of the solvent to disturb the equilibrium of the process (Jentzer et al., 2015), enabling the extraction solvent to increase its capacity for absorbing and transmitting the energy of the ultrasound (Shirsath et al., 2012).

Works related to obtaining compounds from Stevia leaf report that an excess of solvent in the extraction, in the order of 10 to 50 mL g^{-1}, is necessary to obtain an extract with high concentrations of GS (Javad et al., 2014; Carbonell-Capella et al., 2017). For extracting compounds from leaves treated with ethanol, Formigoni et al. (2018) obtained an increase of ~12 % in the extraction of GS (mg per g of dry leaf) with the increase in the solvent to leaf ratio from 10 to 30 mL g^{-1}. Yildiz-Ozturk et al. (2015) reported that the increase in the solvent to leaf ratio from 5 to 15 mL g^{-1} resulted in the extraction of 5.19 and 14.9 mg of GS per g of Stevia leaf, respectively.

3.2.4. *Effect of the ethanol percentage in the extractor solvent*

The increase in the percentage of ethanol in the extractor solvent from 10 to 70 % (v/v), causes a decrease in Y_M and Y_S, with a reduction of ~6 and ~3.5 % in the value of these variables, respectively, when comparing the results obtained in runs 5 and 7.

The extraction of phenolic compounds with the —OH group is promoted by the use of polar solvents (Yildiz-Ozturk et al., 2015; Osorio-Tobón, 2020), however, obtaining sweeteners (Stv and Reb A), is reported to be more efficient when performed using with intermediate polarity solvents, resulting from binary mixtures of water and ethanol (Žlabur et al., 2015; Gasmalla et al., 2017; Yilmaz et al., 2020). Vinatoru et al. (2017) point out that the use of water/ethanol mixture in the UAE causes the reduction of highly oxidizing species generated through the decomposition of water, since ethanol has greater stability than water in terms of homolytic cleavage and, thus, a mixture between the solvents prevents degradation of the extract and makes the extraction process more efficient.

Ameer et al. (2017) obtained an increase of ~9.7 % in the extraction of Reb A by increasing the percentage of ethanol in the extractor solvent from 25 to 75 %, at 45 °C and 45 min of extraction. In the study by Martins et al. (2017), the percentage of 70 % ethanol in the extracting solvent proved to be more efficient than 90 % in maximizing the extraction of Stv and Reb A. Yilmaz et al. (2020) showed an increase of ~9% in the extraction of Stv, in a process conducted at 48 °C, solvent ratio 38 mL g^{-1} after 93 min of extraction due to the increase in the percentage of ethanol in the extracting solvent from 25 to 75 %.

3.2.5. *Interaction effects*

The Fig. 1 shows the level curves constructed according to Eqs. 4 and 5, which illustrates the correlation of independent variables with the response variables. Each contour plot is a function of two variables, keeping the third at this central point.

An upward trend in the Y_M was verified with the progressive increase in the solvent to leaf ratio, while for temperature and percentage of ethanol an increase in the Y_M can be seen to some extent, followed by a slight decrease. Significant and positive interaction between temperature and percentage of ethanol resulted in a pronounced concavity of the curve (Fig. 1b), promoting higher Y_M in the central region of the curve. Fig. 1c shows that the level curves showed an increasing tendency of the dependent variable, in favor of an increase in the solvent leaf ratio and in intermediate levels of ethanol percentage.

For Y_S, however, the level curves for the interaction between the proportion of solvent sheet (Fig. 1d) and ethanol percentage (Fig. 1e)

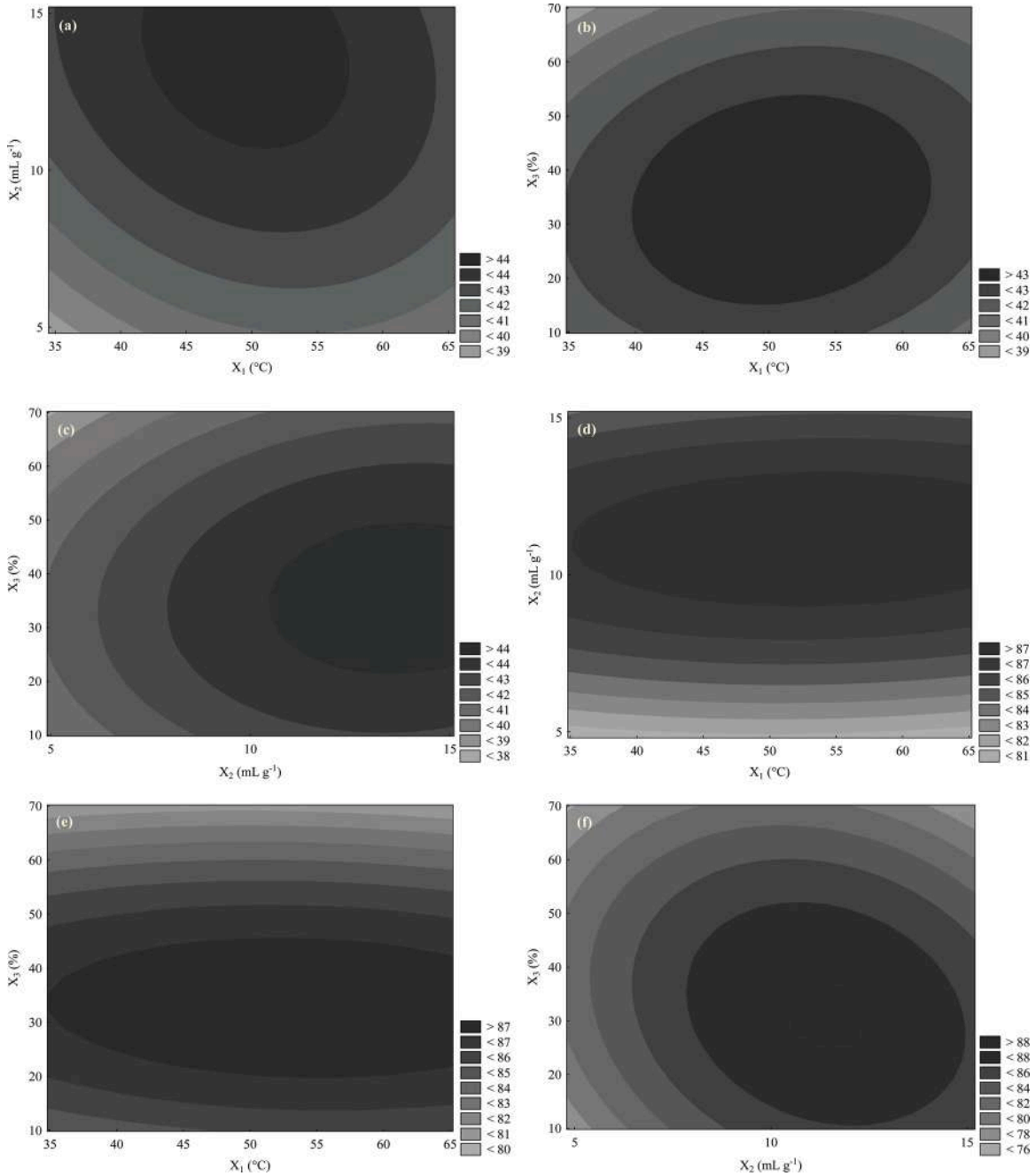

Fig. 1. Level curves showing of the effects of binary interactions between independent variables in mass yield (Y_M) and sweeteners yield (Y_S) as a function of: (a, d) temperature and solvent to leaf ratio; (b, f) temperature and ethanol in the extractor solvent; and (c, e) solvent to leaf ratio and ethanol percentage in the extractor solvent, respectively.

with temperature, did not show a significant effect. This is probably due to reaching the equilibrium of the extraction process in the investigated interval through the interaction with both independent variables (X_2 and X_3). The contour curves of the solvent to leaf ratio and ethanol percentage variables shown in Fig. 1f, makes it possible to verify significant influence and mutual sensitivity to intermediate levels in the interaction of these variables, resulting in a pronounced concavity of the curve in this region. However, the ascending behavior for the solvent to leaf ratio demonstrates a certain tendency to obtain higher Y_S contents in ratios slightly above the intermediate ones, similar to the conditions that provide the Y_M increases.

3.2.6. Glycoside profile

According to the results presented in Table 2, in general, the GS profile was not influenced by the experimental conditions adopted and was similarity between the extracts obtained with composition in Stv, Reb C and Reb A of ~37.0, ~16.0 and ~46.5 % respectively. The composition of Stevia leaf extracts varies according to cultivar, harvest time and the different methods and solvents used in the extraction (Yildiz-Ozturk et al., 2015; Milani et al., 2017). The GS content obtained from Stevia leaves is mainly represented by the components Stv and Reb A, responsible for at least 70 % of these compounds (JECFA, 2008). Periche et al. (2015) obtained extracts with ~86 % of Stv and Reb A, of which the largest portion was represented by Stv (61.5 %). From the GS obtained by Formigoni et al. (2018), 80 % corresponded to Stv (39 %) and Reb A (41 %).

3.2.7. Verification experiments

From the prediction Eqs. (4 and 5), the experimental conditions that maximize the response variables in relation to the evaluated conditions were determined at: 50 °C, solvent to leaf ratio of 15 mL g^{-1} and 40 % (v/v) of ethanol in the solvent extractor, resulting in a theoretical values of 44.38 wt% and 87.95 wt% for Y_M and Y_S, respectively. Verification experiments conducted under these conditions resulted in Y_M of 44.70 ± 0.21 wt% and Y_S of 88.50 ± 0.67 wt%. The prediction efficiency of the equations was verified through the Student test, with no significant difference ($p > 0.05$) between the experimental and predicted values, noting that the equations are predictive and robust. The maximum value of Y_S corresponds to obtaining an extract with 25.74 ± 0.20, 9.53 ± 0.00, 4.14 ± 0.13 and 12.07 ± 0.32 g 100 g^{-1} of dry extract of GS, Stv, Reb C and Reb A, respectively, and the removal of 11.5 g of GS 100 g^{-1} dry leaf. Milani et al. (2017) report obtaining extract from leaves of the same variety of Stevia (UEM-13) with 26 g 100 g^{-1} of dry extract of GS.

Values for GS and Reb A contents in the extracts obtained are higher than those reported by other authors, since the seminal variety of the cultivar of Stevia leaves used in this work, has the particularity of having constituents in higher proportions than wild varieties (Milani et al., 2017). Žlabur et al. (2015) obtained ~13.1 g and ~3.7 g 100 g^{-1} extract of GS and Reb A, respectively. Periche et al. (2015) reported obtaining ~6 g 100 g^{-1} extract in GS, of which ~1.3 g 100 g^{-1} extract corresponded to Reb A. Yilmaz et al. (2020) achieved extraction of 11.33 g per 100 g^{-1}extract from GS, where 4.29 mg 100 g^{-1} extract were Reb A.

3.3. Characterization of dry extracts

The extracts obtained in the conditions that presented the highest Y_M and Y_S values in Table 2, as well as in the condition of maximum Y_M and Y_S were characterized in relation to the TPC content and antioxidant capacity. To verify the correlation between the operational conditions and the evaluated responses, principal component analysis was used and for this, a 5 × 3 matrix (rows x columns) was composed to express the data set, where the lines corresponded to the tests and the columns the responses obtained (TPC, EC_{50} and GS). The result was represented on a score graph, as shown in Fig. 2.

From Fig. 2, it can be seen that the main components 1 (CP1) and 2 (CP2) explain 97.73 % of the total variability obtained, making it possible to simplify the resulting data set. High correlation could be verified between EC_{50} and TPC ($R^2 = 0.82$, $p < 0.10$) through the Pearson correlation, which is evidenced by the proximity of the vectors of these variables in the PCA distribution.

The contribution of the ethanol percentage in the extractor solvent is evident in obtaining the TPC (Table 4), when verifying that the points of runs 4 and CP were closer to this vector. The tests conducted under the same solvent to leaf ratio (3 and 10) showed lower EC_{50} (Table 4), which characterizes greater antioxidant activity, justifying the opposite position of these tests to the respective vector. The experimental run MP, which resulted in a higher GS content, presented an TPC value intermediate at the points of tests 4 and PC, corresponding to the tests with higher values of this variable.

It can be seen that the variables that tend to generate the highest TPC and AA did not show correlation to those that promote an increase in the GS content. The CP test provided higher TPC, however, a reduction in GS content was verified. Periche et al. (2015) reported a negative

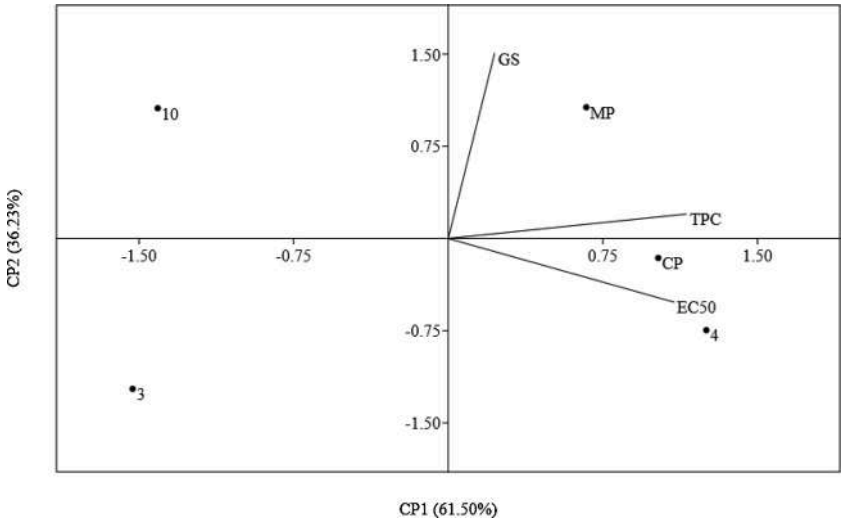

Fig. 2. Principal component analysis of the effect of different extraction conditions in the ultrasound-assisted extraction of compounds from pretreated Stevia leaves. The variables GS, TPC and EC_{50} are represented by total glycosides, total phenolic content and antioxidant activity, respectively. Points 3, 4, 10, CP and MP represent the experimental runs from the Table 4.

Table 4
Total glycosides (GS), total phenolic content (TPC), antioxidant activity (EC_{50}) and antiradical power (ARP) in extracts of pretreated Stevia leaves obtained from ultrasound-assisted extraction.

Run[1]	Independent variables[2]			GS (g per 100 g dry extract)	TPC (mg GAE per g dry extract)	EC_{50} (μg dry extract per mL)	ARP (μg dry extract per mL)
	X_1	X_2	X_3				
3	35	15	40	24.02 ± 0.34	256.91 ± 0.27	19.96 ± 0.10	0.050 ± 0.00
4	65	15	40	24.72 ± 0.29	277.74 ± 0.25	22.21 ± 0.07	0.045 ± 0.00
10	50	15	10	25.63 ± 0.66	258.11 ± 0.26	19.53 ± 0.01	0.051 ± 0.00
Central point (CP)	50	10	40	24.97 ± 0.64	280.89 ± 0.82	21.46 ± 0.05	0.050 ± 0.00
Maximum point (MP)	50	15	40	25.74 ± 0.20	279.92 ± 0.27	20.75 ± 0.02	0.047 ± 0.00

[1] As Table 2.
[2] X_1 – temperature (°C); X_2 – solvent to leaf ratio (mL g^{-1}) and X_3 – ethanol percentage in the extractor solvent (v/v).

correlation between the GS content and antioxidants obtained in Stevia leaf extracts obtained from different extraction methods. Kovačević et al. (2018) indicated no correlation between bioactive compounds and GS content in Stevia leaf extracts obtained by extraction with pressurized hot water.

TPC values in the extracts obtained in the present study (~257 to ~281 mg g^{-1} extract) were higher than those reported by Yildiz-Ozturk et al. (2015); Žlabur et al. (2015); Görgüç et al. (2019) and Yilmaz et al. (2020). Yildiz-Ozturk et al. (2015), who obtained ~87 mg g^{-1} extract in UAE at 90 °C, ethanol to leaf ratio of 15 mL g^{-1} after 45 min. Žlabur et al. (2015) using excess solvent (400 mL g^{-1}), in 10 min at ~82 °C, obtained ~78 mg g^{-1} extract. The UAE of compounds from Stevia leaves performed by Yilmaz et al. (2020), after 43 min at 50 °C, resulted in an extractor with a content of ~69 mg g^{-1} extract.

The extracts obtained from the pre-treated leaves showed a significant sequestering effect, with EC_{50} values of 19.53–22.21 μg dry extract per mL, as evidenced by the ARP values. Kim et al. (2011) reported that 200 μg mL^{-1} of extract resulting from the reflux process with distilled water at 100 °C and three cycles of 3 h are needed for ~30 % radical scavenging. Sukla et al. (2012) and Ruiz-Ruiz et al. (2015) reported 40 % reduction of DPPH radical using ~20 μg mL^{-1} and 250 μg mL^{-1} of aqueous extract from *Stevia rebaudiana*, respectively.

4. Conclusion

The use of maximum ultrasound power (165 W) caused an increase in the response of the variables Y_M (15 %), Y_S (20 %) TPC (9%) and the reduction in EC_{50} (57 %), when compared to extraction without ultrasound. Pre-treatment provided extracts with higher quality. From the experimental design, the maximum Y_M and Y_S values were determined, which resulted from the use of the solvent to leaf ratio (15 mL g^{-1}), at intermediate levels of the percentage of ethanol in the extracting solvent (40 %) and temperature (50 °C). Thus, an extract with ~26 wt% of GS was obtained, composed of Stv, Reb C and Reb A in percentages of 37, 16 and 46.5 %, respectively. From the PCA it was possible to verify that the use of solvent with 40 % of ethanol favored the obtainment of TPC, and the solvent to leaf ratio favored the increase of AA. The extraction method evaluated was efficient to obtain an extract with a high content of phenolic compounds, antioxidants and sweeteners, with great potential for use as a food additive. In terms of process feasibility, the use of an unconventional technique of rapid processing in conjunction with a binary mixture of ethanol/water as solvent stands out, which provides reduced production costs compared to using only ethanol and easier separation of the solvent, when compared to water use.

CRediT authorship contribution statement

Djéssica Tatiane Raspe: Conceptualization, Methodology, Writing - original draft. **Simone Rocha Ciotta:** Conceptualization, Methodology. **Maria Rosa Trentin Zorzenon:** Conceptualization, Methodology. **Antônio Sergio Dacome:** Conceptualization, Methodology. **Camila da Silva:** Writing - review & editing. **Paula Gimenez Milani:** Writing - review & editing. **Silvio Cláudio da Costa:** Supervision.

Declaration of Competing Interest

The authors report no declarations of interest.

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ARTIGO 3

Pressurized liquid extraction of steviol glycosides from *Stevia rebaudiana* leaves

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Pressurized Liquid Extraction of Steviol Glycosides from *Stevia rebaudiana* Leaves

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The present study aimed to evaluate the efficiency of pressurized liquid extraction (PLE) in obtaining steviol glycosides, natural sweeteners, from *Stevia rebaudiana* leaves. The extractions were conducted in an experimental apparatus operated in semicontinuous mode with fixed temperature, pressure and time at 120 ºC, 100 bar and 60 min, respectively, evaluating the effects of solvent to sample ratio (30 to 90 mL/g), concentration of ethanol in the extractor solvent (100 and 70 % v/v) for leaves with and without pretreatment. The results obtained were compared with the ultrasound-assisted extraction (UAE) conducted at 50 ºC, 60 min and solvent to sample ratio of 10 mL/g. Under pressurized conditions, the results showed greater extraction of sweeteners when pretreated leaves were used, reaching a yield of 70.94 ± 0.83%, which was higher than that obtained with the leaf without pretreatment (59.85 ± 1.14%). Increasing the solvent to sample ratio from 30 mL/g to 90 mL/g did not promote greater extraction in sweeteners from pretreated leaves (75.05 ± 1.89%), demonstrating that an excess of solvent in the medium does not result in higher yields. In parallel, little solvent (10 mL/g) did not contribute to obtaining sweeteners via UAE, promoting a yield of 46.78 ± 0.31%. The contents of sweeteners and mass obtained by PLE were about ~ 65% and ~ 55 % higher when compared to those obtained from UAE, respectively. The best results for sweeteners extraction (76.97 ± 2.70%) were obtained using pretreated leaves, at 30 mL/g and with 70% ethanol.

1. Introduction

The growing consumer awareness of the risks of diseases such as obesity and diabetes, has driven the industry in the search for low-calorie sweeteners, which aim to replace sugar. The most commercialized sweeteners today are synthetic compounds, such as saccharin and aspartame, which demonstrate long-term health impacts (Alkafafy et al., 2015). Therefore, aiming at the production of healthy foods, the search for natural sweeteners has been proposed and, in this sense, *Stevia rebaudiana* stands out. Cultivated for centuries in South America due to its sweetening properties (Puri et al., 2011), stevia has more than 20 steviol glycosides identified (Molina-Calle et al., 2017; Formigoni et al., 2018), among which are stevioside (Stv) and rebaudioside A (Reb A), the more abundant constituents and ~300 times sweeter than sucrose. Reb A has a better sensory profile than Stv, which is responsible for the characteristic bitter taste of the plant. In addition to their sweetening properties, these sweeteners do not cause caloric accumulation (Singh et al., 2019) and studies show that steviol glycosides have therapeutic properties, including antihyperglycemic, antihypertensive, anti-inflammatory, antitumor, anti-diarrheal, diuretic and immunomodulator effect (Ritu and Nandini, 2016; Ruiz-Ruiz et al., 2017).

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The main obstacle to the use of natural sweeteners in the food industry is the cost associated with their extraction and subsequent purification. The methodologies that have been proposed for the extraction of steviol glycosides are generally based on the use of superheated aqueous or alcoholic solvents, followed by several purification steps (Gasmalla et al., 2017; Ameer et al., 2017). Pressurized liquid extraction (PLE) is a technique that emerges as an alternative to conventional ones and it has as advantage the use of less solvents and energy, the greater selectivity and the better repeatability compared to other methods (Zhang et al., 2018), obtaining products with high purity and possibility of reducing the later stages. This process occurs with solvents at temperatures above their boiling point and below their critical point, associated with pressures sufficiently capable of maintaining them in the liquid state, allowing for increased solubility and promoting improvement in the mass transfer properties of the compounds of interest (Plaza and Turner, 2015). Due to these operating conditions, the physical-chemical properties of the solvents are modified, decreasing their surface tension and viscosity, causing an increase in the matrix's analyte diffusivity and desorption, due to the reduction of intermolecular interactions between analyte and matrix (Alvarez-Rivera et al., 2020). The pressure forces the solvent to penetrate areas of difficult access in atmospheric conditions, facilitating the extraction of analytes that are trapped in the pores of the matrix (Mustafa and Turner, 2011). Associated with the appropriate solvent, this type of extraction can promote appreciable yields in shorter periods of time (Pawliszyn, 2019), without causing losses in the composition of the obtained extract. Additionally, the pretreatment of the vegetable matrix can help to reduce the residual bitter taste of Stv (Formigoni et al., 2018), increasing the efficiency in obtaining sweeteners.

In this context, the aim of this work was to investigate the extraction by pressurized liquid (PLE) of steviol glycosides from *Stevia rebaudiana* leaves. The influence of the pretreatment, the effect of the ethanol concentration in the extracting solvent and the proportion of solvent and sample were evaluated, and the conditions that maximize the glycoside yield, mass yield and sweetener recovery were determined. The composition of the obtained extract was determined in relation to the total glycosides and the condition that led to the maximum yield in the PLE was compared to the result obtained by the ultrasound-assisted extraction (UAE).

2. Material and Methods

2.1 Sample and Reagents

Stevia rebaudiana Bertoni (Stevia UEM-13) plants were grown at the Nucleus of Research in Natural Products (NEPRON) located at the State University of Maringá (UEM, Paraná, Brazil). Harvested in the flower bud formation stage (~ 50 to 60 days after pruning), the leaves were separated from the stems, dried in an oven with air circulation at 60 □C for 8 hours, milled in a knife mill and had a final moisture content of <10% and average diameter of 0.30 - 0.60 mm. Part of the leaves were subjected to the ethanolic pretreatment (Formigoni et al., 2018), and later they were dried with the rest of the leaves and stored for the extraction. The composition in terms of sweeteners of the leaves used in this work with and without pretreatment are shown in Table 1. Ethanol (Honeywell, 99.9 % pure), ethanol (Anhydrol, 95.0 % pure) and distilled water were used as solvents in PLE and UAE. The deionized water (18 MΩ·cm) used in the chromatographic analysis was obtained by the Milli-Q plus system (Induslab, Brazil). All the reference standards were provided by Sigma-Aldrich (Brazil).

Table 1: Sweetener content in Stevia rebaudiana leaves before and after ethanolic pretreatment.

Analysis	Without pretreatment (g/100 g)	With pretreatment (g/100 g)
Stevioside	4.34 ± 0.04	4.08 ± 0.07
Rebaudioside C	1.92 ± 0.02	1.75 ± 0.01
Rebaudioside A	6.98 ± 0.04	6.83 ± 0.02
Total Glycoside	13.18 ± 0.06	12.76 ± 0.07

2.2 Pressurized liquid extraction

The experiments were carried out in a semicontinuous mode, as shown in Figure 1, and described by Rodrigues et al. (2017). The experimental apparatus consisted of a reservoir (SR) containing the solvent, which was continuously pumped by a high-pressure liquid pump (P). To enter the extraction bed (E) at the test temperature, the solvent passed through the preheating zone (P), which was monitored by a thermocouple (T).

At each end of the E there were synthesized steel filters. The oven (O) was heated to the desired temperature and, after reaching it, the E was allocated inside it with 2 g of stevia leaves, interspersed every 1 g with glass pearls.

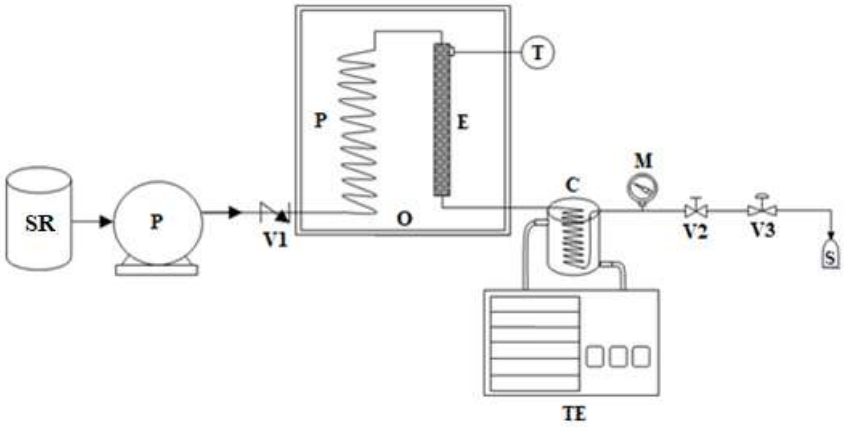

Figure 1: Semicontinuous experimental apparatus: (SR) solvent reservoir, (P) HPLC pump, (V1) check-valve, (O) oven, (P) preheater, (E) extractor, (T) thermocouple, (C) cooling unit, (TE) thermostatic bath, (M) manometer, (V2) pressure control valve, (V3) pressure reduction valve, and (S) sampling.

The extraction procedure is based on the heating of the system (O - Sanchis, BTT1050-00, Porto Alegre, Brazil) and the filling with solvent until the test pressure. Once the system was pressurized and the desired temperature was reached, the extractor was placed in the oven for 30 minutes in static time, after which the dynamic extraction was started. The samples (S) were collected after passing through a refrigeration system (C), connected to a thermostatic bath (TE). The system's pressure was monitored via a pressure indicator and controlled with a needle valve and a pressure reducing valve.
In the experiments, the extraction of steviol glycosides in leaves with and without ethanolic pretreatment was investigated. The proportion of ethanol in the extracting solvent of 100 and 70 % (v/v) and the proportion of solvent in the sample (R-L:S) of 30 and 90 mL/g were evaluated. The temperature and pressure used for the extractions were 125 □C and 100 bar, respectively, during the fixed time of 60 min. After the extraction period, the solvent was removed in a rotary vacuum evaporator (Marconi, MA 120) and dry extract was stored in a desiccator. The mass yield was calculated according to Eq (1), where q_o is the extract mass obtained (g) and q_s is the leaf mass (g) used in the experiment.

$$yield\ (\%) = \left(\frac{q_o}{q_s}\right) \times 100 \quad (1)$$

2.3 Ultrasound-assisted extraction

An ultrasonic bath with indirect contact and heating control (Ultronique, Q 3.0/40 A, Eco-Sonics), with a frequency of 40 kHz and power of 110 W, was used for the ultrasonic extraction. A flask (250 mL) containing the milled leaves and the solvent was connected to a condenser coupled to a cooling bath (Marconi, MA 184), which were positioned in the center of the ultrasonic bath. To evaluate the influence of the ultrasound on the extraction of steviol glycosides, 10 mL/g of 70% ethanol solvent were sonicated for 60 min, at 50 °C, with pretreated leaves (13.03 ± 0.02 g/100g of glycosides). After the extraction period, the leaves were separated by filtration, the solvent removed and the mass yield calculated according to Eq (1).

2.4 Quantification of steviol glycosides by HPLC

The total glycosides present in the leaves after the extraction steps were identified and quantified using a High Performance Liquid Chromatograph (HPLC). After extraction, the samples were concentrated to dryness in a vacuum rotary evaporator (Marconi, MA 120), being subsequently redissolved with 10 mL of the mobile phase (acetonitrile:deionized water, 80:20, v/v), as described by Dacome et al. (2005). For the analyses, a liquid chromatograph model CG 480-C (Brazil) equipped with a 5 µm (125 × 4.6 mm) NH_2 column was used, operated isocratically with a flow of 0.75 ml/min at room temperature and coupled to a Waters 410 DRI detector (coupled to an index refraction detector S:32).

2.5 Statistical analysis

All the analyses were performed in triplicate and the results were expressed as mean values ± SD. To verify the influence of the parameters evaluated in each step on the results obtained, analysis of variance (ANOVA; Excel® 2010 software) and the Tukey test, with a 95 % confidence interval, were carried out.

3. Results and Discussion

Table 2 shows the results of the extraction of steviol glycosides in terms of stevioside (Stv), rebaudioside C (Reb C) and rebaudioside A (Reb A), as well as the mass and sweetener yields obtained by PLE at temperature, pressure and time fixed of 125 □C, 100 bar and 60 min, respectively. Through the analysis of the data presented in Table 2, it is possible to verify that the ethanolic pretreatment had an influence on the mass yield and the content of sweeteners obtained by PLE, without causing significant losses in the composition of the extract obtained (experiments 1 and 2). The higher mass yield obtained (43.31%) resulted from an increase in the L:S ratio from 30 to 90 mL/g, however, significantly reduced the levels of Stv and Reb A in the extract (experiments 3 and 4), indicating that both are similarly affected by this factor, which is expected by the chemical similarity between these two glycosides. Increases in glycoside yields, proportional to the increase in the L:S ratio, were previously reported, indicating the influence of this variable in the extraction process (Martins et al., 2017; López-Carbón et al., 2019). The proportion of ethanol (EtOH) in the extractor solvent affected the glycosides yields, mass and sweeteners (experiments 2 and 3). Although the decrease in the proportion of ethanol from 100 % to 70 % has promoted a significant reduction in the contents of Stv, Reb C and Reb A, an increase in mass and sweetener yields has been verified. This is due to the change in polarity and extraction capacity that the water content in the ethanol promotes to the solvent (Celaya et al., 2016), making the dissolution of the constituents more effective (Carbonell-Capella et al., 2016), resulting in higher yields under lower proportions of EtOH (Martins et al., 2016; Medina-Medrano et al., 2019).

Table 2: Effect of variables on the yield of total glycosides, mass and sweeteners in the PLE of leaves with and without ethanolic pretreatment.

Run	Leaves	EtOH (%)	R-L:S (mL/g)*	Total Glycosides (g/100 g)**			Yield (%)	Sweeteners (%)**
				Stv	Reb C	Reb A		
1	Without pretreatment	100	30	12.51 ± 0.04^{a}	5.77 ± 0.38^{a}	13.48 ± 0.67^{a}	24.67	59.85 ± 1.14^{a}
2	With	100	30	11.28 ± 0.12^{bA}	5.88 ± 0.42^{aA}	13.42 ± 0.04^{aA}	29.21	70.94 ± 0.83^{bA}
3	pretreatment	70	30	9.41 ± 0.73^{B}	4.79 ± 0.21^{B}	12.71 ± 0.26^{B}	36.02	76.97 ± 2.70^{B}
4		70	90	7.86 ± 0.12^{C}	3.88 ± 0.54^{B}	10.07 ± 0.18^{C}	43.31	75.05 ± 1.89^{B}

*R-L:S, Ratio leaf:solvent; **Different lowercase letters in the same column indicate significantly different values between tests with leaves with and without pretreatment ($p < 0.05$); Different capital letters in the same column indicate significantly different values between tests with pretreated leaves ($p < 0.05$).

Studies report that pretreatments in vegetable matrices aims to improve the transfer of mass and heat, leading to shorter extraction times, less solvent consumption, energy savings, better yields, better quality and greater purity of compounds extracted (Amiri-Rigi et al., 2016; Llavata et al., 2020). Recently proposed, the ethanolic pretreatment in stevia leaves aims to improve the sensory profile of the obtained extract, reducing the sensory characteristic of bitterness caused by Stv (Formigoni et al., 2018). Regarding extraction, the PLE process has stood out in comparison to other emerging processes, such as microwave assisted extraction (MAE) (Ciulu et al., 2017), showing viability in the proposed investigation. For this purpose, in this work, the association of the pretreated vegetable matrix with PLE promoted appreciable mass (29.21%) and sweeteners yields (71.51 ± 0.84%), higher than those obtained through the leaf without pretreatment (24.67% and 63.22 ± 1.21%, respectively).

Table 3 presents the results of the best condition proposed in the PLE in comparison to the results obtained by the UAE, in relation to the extract composition, mass and sweeteners yields. Through Table 3, we can see that the PLE had yields higher than the UAE, corroborating with results found by Plaza et al. (2012), where PLE promoted yields of 36.43% while the UAE generated a yield of 4.79%, under the extraction of *Chlorella vulgaris*. Although no significant differences were obtained in relation to total glycosides when comparing the two extraction techniques, in which PLE promoted 26.91% and UAE resulted in 26.15%, PLE provided yields in sweeteners ~65% higher than those obtained by the UAE. This is mainly due to the fact that the process acts through the interaction between temperature and pressure, which, keeping the solvent in a liquid state

and above the boiling point, promote better analyte solubility, faster diffusion, lower solvent viscosity while weakens the interactions between the sample solution and the matrix (Kovačević et al., 2018).

Table 3: Yield in total glycosides, mass and sweeteners in PLE and UAE.

Extraction method		PLE*	UAE*
Total Glycosides (g/100 g)	Stevioside	9.41±0.73[a]	10.47±0.10[a]
	Rebaudioside C	4.79±0.21[a]	5.71±0.01[b]
	Rebaudioside A	12.71±0.26[a]	9.97±0.06[b]
Yield (%)		36.02	23.24
Sweeteners (%)		76.97±2.70[a]	46.78±0.31[b]

PLE of pretreated leaves, R-L:S 30 mL/g, 70 % EtOH, 125 □C, 100 bar and 60 min; UAE of pretreated leaves, R-L:S 10 mL/g, 70 % etOH, 50 □C and 60 min; *Different letters on the same line indicate significantly different values ($p < 0.05$).

4. Conclusions

The pressurized liquid extraction of steviol glycosides from *Stevia rebaudiana* leaves was investigated. It can be concluded that the ethanolic pretreatment of the leaves improves the mass and sweetener yield, without causing loss of the extracted compounds. Reduction of the ethanol content in the extracting solvent from 100% to 70% promoted an increase in mass yields (36.02%) and in sweeteners (76.97%) in the obtained extract. The application of a higher solvent flow (90 mL/g) improves the extraction efficiency, providing greater mass yields (43.31%), however, significant loss of Stv and Reb A are observed. PLE promoted yields of ~56% and ~65% of mass and sweetener recovery higher than that obtained by the UAE, respectively.

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ARTIGO 4

Pressurized liquid extraction of compounds from Stevia leaf: Evaluation of process variables and extract characterization

Pressurized liquid extraction of compounds from Stevia leaf: Evaluation of process variables and extract characterization

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ABSTRACT: The extraction of compounds from *Stevia rebaudiana* leaves under pressurized conditions was investigated. For this, the effect of the percentage of ethanol in the extracting solvent (40 and 70%, v/v), static time (10, 20 and 30 min), pressure (50 and 100 bar) and temperature (100, 125 and 150 ° C) on mass yield (Y_M), total phenolic compounds (TPC) and antioxidant activity (AA) was evaluated. The extract from PLE was characterized and compared to that obtained from Soxhlet extraction and ultrasound-assisted extraction (UAE). From the results, it appears that the increase in the percentage of ethanol in the extractor solvent favored Y_M, TPC and AA, while the increase in static time and pressure (>10 min and >50 bar) did not affect the extraction of compounds under the conditions evaluated. The temperature provided an increase in Y_M and the highest levels of active compounds were obtained after 30 min of the process, Y_M was similar between the extraction techniques evaluated, with PLE (125 °C) providing greater recovery of TPC and AA, and Soxhlet favoring the extraction of steviol glycosides (SG) and TFC. The composition of the PLE

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extract was ~26.0 wt% SG, corresponding to 9.5, 3.9 and 12.68 wt% of Stevioside, Rebaudioside C and Rebaudioside A, respectively, representing 87.8% of the total obtained by Soxhlet. The active potential (TPC, TFC and AA) of the PLE extract was 3.6 and 11.0% higher than that of the Soxhlet and UAE, respectively.

Keywords: *Stevia rebaudiana*, active compounds, steviol glycosides, ethanol, water.

1. Introduction

Stevia rebaudiana (Bertoni) leaves contain steviol glycosides (SG), including stevioside (Stv), rebaudioside (Reb) A to F, responsible for the sweet taste of the plant and corresponding to the commercial value that it presents in the world as a substitute of sugar in foods, beverages and medicines (Momtazi-Borojeni et al., 2017). Among these glycosides, Stv and Reb A and C are the main metabolites, which have, on average, 250 to 300 times more sweetness than sucrose (Gardana et al., 2010; Hajela et al., 2017), therapeutic effects against various diseases such as cancer, hyperglycemia, diabetes mellitus, hypertension, inflammation, cystic fibrosis, obesity and dental caries (Verma et al., 2019), and its components are metabolized without causing caloric and energy accumulation to the human body (Singh et al., 2019). In addition, studies indicate that these compounds are not teratogenic, mutagenic or carcinogenic and do not cause acute and subacute toxicity (Yadav and Guleria, 2012; Momtazi-Borojeni et al., 2017).

Although the nutraceutical attributions of this matrix are widely disseminated, sensorially the aftertaste is reported as the biggest limiting factor in the use of sweeteners and compounds from Stevia leaves. Attribute assigned to flavor quality, characteristic that is commonly described as bitterness, licorice, and metallic taste (Espinoza et al., 2014), and is due to different monosaccharides units in the steviol aglycone (Ohta et al., 2010), in addition

to the presence of sesquiterpene lactones, essential oils, tannins, flavonoids, caryophyllene and spathulenol in its extract (Soejarto et al., 1983; Phillips, 1987; Tsanava et al., 1991; Zeng et al., 2013). Strategies to reduce this inconvenience involving microencapsulation (Chranioti et al., 2016), modifiers and enhancers of flavor with artificial sweeteners (Gerwig et al., 2016), transglycosylation processes (Poele et al., 2018), biotransformation of Stv into Reb A (Adari et al., 2016) and development of new plant varieties (Kim et al., 2019) have been investigated, however, it is the performance of a pre-treatment in the matrix that has been demonstrating appreciable results sensorially (Formigoni et al., 2018), viability and extractive potential, especially when linked to unconventional techniques (Raspe et al., 2021a; Raspe et al., 2021b).

In this scenario, a recent and possible alternative proposal for extracting and obtaining the compounds from the leaves of this matrix, is the extraction by pressurized liquids (PLE) and subcritical water (SWE). These processes employ the use of solvents at high temperatures, above the boiling point and below the critical point, under enough pressure to keep them in a liquid state, allowing them to act interrelating the increase in the solubility of the compounds of interest in the matrix, improving the diffusion rate in short periods of time and smaller volumes of solvent (Pawliszyn, 2019). In these methods, the temperature modifies the physicochemical properties of the solvents (Plaza and Turner, 2015), improving matrix wetting and mass transfer, which takes place faster due to decreased surface tension and solvent viscosity. Furthermore, there is an increase in the diffusivity and desorption of the analyte to the solvent, due to the reduction of intermolecular interactions between the analyte and the matrix (Alvarez-Rivera et al., 2020). The use of pressure keeps the solvent below its boiling point and maintains a high fluid density, contributing to elution resistance (Ju and Howard, 2003; Freitas et al., 2013), forcing the solvent to penetrate areas that would not normally be reached under atmospheric conditions and thus facilitating the extraction of

analytes trapped in the matrix pores (Camel, 2001; Mustafa and Turner, 2011).

Aqueous extraction is the most used methodology to obtain compounds of interest to Stevia (Jentzer et al., 2015; Periche et al., 2015; Kovačević et al., 2018). However, it has been reported that the combination of water with ethanol provides an improvement in extraction yield (Martins et al., 2016; Ciulu et al., 2017; Raspe et al., 2021a; Raspe et al., 2021b). Binary mixtures between water and ethanol exploit the ability of water to break the hydrogen bond between the matrix and the analytes, while ethanol increases the solubility of the extracted species (Mustafa and Turner, 2011). Ethanol has the main advantage of promoting the green extraction of natural compounds, presenting operational safety, low toxicity (Carvalho, 2001), high purity and biodegradability, which characterizes it as a GRAS solvent (generally recognized as safe) (Bubalo et al., 2015), enabling its application in obtaining compounds for food purposes (Muthusamy and Munaim, 2019).

Although SWE, which has water as a solvent, has a broader approach and investigation for the recovery of compounds from Stevia leaves (Yildiz-Ozturk et al., 2014; Jentzer et al., 2015; Kovačević et al., 2018; Németh and Jánosi, 2019; Yang et al., 2019; Sandra et al., 2020), the use of PLE is a recent technique and still little explored in the maximization of processes through the use of a binary mixture of water and ethanol as extracting solvent (Ciulu et al., 2017), mainly involving a pre-treated matrix (Raspe et al., 2021a).

Based on the above, the objective of this study was to determine the effect of experimental variables (ethanol percentage in the extractor solvent, static time, pressure and temperature) on the PLE of compounds (mass, total phenolic and antioxidant activity) from Stevia leaf pretreated, and the operating conditions provide the maximum extraction of these compounds from the aforementioned method. The extract obtained by PLE was characterized in relation to the content and composition of glycosides, contents of phenolic compounds and flavonoids and antioxidant potential, and the values obtained compared to extracts from

Soxhlet and ultrasound-assisted extraction.

2. Materials and Methods

2.1. Materials

The *Stevia rebaudiana*, Stevia UEM-13 seminal variety, grown at the Natural Products Research Center of the State University of Maringá, Paraná, Brazil (23°24’ e 21°9’ S; 51°56’ e 22°0’ W). For extractions, ethanol (95.0% purity, Anidrol Produtos para Laboratório Ltd., Diadema, SP, BR) and deionized water (18 MΩ·cm) (Milli-Q plus, Induslab, Londrina, PR, BR) were used as solvents. For analysis of the content of total phenolic compounds, content of total flavonoids and antioxidant activity were used distilled water (TE-4007-20, Tecnal, Uninorte Distrito Industrial, Piracicaba, SP, BR), ethanol (99.5% purity, Anidrol), methanol (99.9% purity, Panreac, Castellar del Vallès, BCN, ES), sodium carbonate (≥99.5% purity, Anidrol), aluminum chloride (≥99.0% purity, Synth, Labsynth Produtos para Laboratório Ltd., Diadema, SP, BR), sodium acetate (≥99.0% purity, Dinâmica® Química Contemporânea Ltd., São Paulo, SP, BR), Folin-Ciocalteu reagent (Sigma Aldrich Chemical Co., St. Louis, MO, USA), 2,2-Diphenyl-1-picrylhydrazyl (DPPH) (Sigma Aldrich), 3,4,5-Trihydroxybenzoic acid (Gallic acid) (≥97.0% purity, Sigma Aldrich), 3,3’,4’,5,6-Pentahydroxyflavone (Quercetin) (≥95.0% purity, Sigma Aldrich), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (97.0% purity, Sigma Aldrich). To quantify the glycosides, acetonitrile (99.9% purity, J.T. Baker, Avantor™ Performance Materials, MEX, MX) and deionized water (18 MΩ•cm) were used as solvents, and stevioside, rebaudioside A and rebaudioside C (Sigma Aldrich) were used as chromatographic standards.

2.2. Preparation of sample

The bushes were harvested, ~60 days after pruning, at the flower bud formation stage and were immediately dried in an oven with air circulation at 60 ºC for 8 hours. Leaves and stems were separated and ground in a knife mill (TE 340, Marconi Equipamentos para Laboratório Ltd., Piracicaba, SP, BR), and the fractions retained in the Tyler sieves (ASTM E11, Bertel, Caieiras, SP, BR) with an average diameter between 28–48 mesh were used in the experiments. Subsequently, the leaves were submitted to ethanolic pre-treatment (Formigoni et al., 2018), and sequentially dried (room temperature, 24 hours) and stored for the extraction stage. The steviol glycoside composition of the leaves after pretreatment was 12.76 ± 0.07%, corresponding to 4.08 ± 0.07, 1.75 ± 0.01 and 6.83 ± 0.02 g per 100 g of extract of Stv, Reb C and Reb A, respectively, with a final moisture content of 4.57 ± 0.01 wt%.

2.3. Pressurized liquid extraction

The experiments were carried out in an experimental apparatus operated in semi-continuous mode, as outlined in Figure 1, which was previously described by Iwassa et al. (2019). The experimental apparatus consisted of a reservoir (SR), containing the binary mixture (ethanol and water) as a solvent, which was continuously pumped by means of a high pressure liquid pump (P – Waters, 515 HPLC Pump, Waters® Co., Milford, MA, USA). To enter the extractor bed (E) at the pre-set temperature, the solvent passed through the preheating zone (PH), with the temperature monitored by an indicator (T). At each end of the E, were coupled synthesized steel filters (Frits, 2 μm × 1/4" OD, Supelco®, Merck, Kenilworth, NJ, EUA), and placed inside, 2 g of Stevia leaf pretreated interspersed with glass spheres. The oven (O - BTT 1050-00, Sanchis Fornos Industriais, Irmãos Sanchis & Cia Ltd., Porto Alegre, RS, BR) was heated to the desired temperature and, in parallel, the E was filled with solvent until the pre-established pressure. After reaching the temperature and pressure of

149 the test, the E was conditioned inside the O and the pre-established static time counting is
150 started and later, the dynamic extraction is carried out, maintaining a fixed solvent flow rate
151 of 1 mL min^{-1}.
152
153 **Figure 1**
154
155 The samples (S) were collected after passing through a refrigeration system (C),
156 connected to a thermostated bath (TB - Q214M2, Quimis®, Quimis Aparelhos Científicos,
157 Diadema, SP, BR). The system pressure was monitored by manometer (M – Wika 600Psi,
158 Thermopress Comércio de Equipamentos Industriais, Curitiba, PR, BR) and controlled with a
159 needle valve (V2 - 10V2071, Needle Valves, Parker, Huntsville, AL, USA) and pressure
160 reducing valve (V3 - KPB120A415P20000, Stainless Steel BP Regulator, Swagelok® Co.,
161 Carapicuiba, SP, BR).
162 In the experiments, the effects of the percentage of ethanol in the extracting solvent,
163 static time and pressure were evaluated. The choice of solvents and their flow rate were
164 defined after preliminary tests, which indicated that binary mixtures of water and ethanol and
165 a lower proportion of leaf to solvent would make it possible to obtain an extract with higher
166 yields in mass and sweeteners (Raspe et al., 2021a). The extraction kinetics (destructive) was
167 obtained, fixing the extracting solvent, static time and pressure determined as responsible for
168 the maximization of of the response variables, at temperatures of 100, 125 and 150 °C in the
169 times of 5, 10, 20, 30, 45 and 60 min.
170 After the end of each extraction, the collected extract was concentrated until the
171 complete elimination of the solvent in a rotary vacuum evaporator (MA 120, Marconi). The
172 mass yield (Y_M) was calculated according to Equation 1, and the dry extract obtained was

stored in amber flasks and protected from light to determine the content of total phenolic compounds and antioxidant activity.

$$Y_M\ (wt\%) = \frac{w_o}{w_a} \times 100 \quad (1)$$

where w_o (g) is the mass of extract obtained and w_a (g) is the initial mass of leaves into the extractor.

The content of total phenolic compounds (TPC) was determined according to the method described by Singleton et al. (1999), in which 500 µL of aqueous extracts (500 µg mL^{-1}) were mixed with 2000 µL of aqueous solution of Folin-Ciocalteau reagent (10%) and 2500 µL of sodium carbonate (7.5%). The mixture was homogenized and incubated at 50 °C for 5 min (Q334M, Quimis®) in the dark, and then the absorbance of the mixture was determined at 760 nm in a UV-VIS spectrophotometer (UV-1900i UV-Vis Spectrophotometer, Shimadzu Scientific, TO, JAP). The total concentration of phenols of each extract was quantified using a standard curve prepared with gallic acid (R^2 0.99), and the results were expressed in mg of gallic acid equivalent (GAE) per g of leaf (considering the mass yield).

Free radical scavenging of extracts was measured by the ability to scavenge DPPH radicals as described by Brand-Williams et al. (1995), in which 100 µL of the ethanolic extracts (2000 µg mL^{-1}) were mixed with 3900 µL of the DPPH ethanolic solution (6.6×10^{-8} M). The mixture was homogenized and incubated in the dark at room temperature for 30 min, and then the absorbance of the mixture was determined at 517 nm in a UV-VIS spectrophotometer (UV-1900i, Shimadzu). The antioxidant activity (AA) of each extract was quantified using a standard curve prepared with trolox (R^2 0.99), the results being expressed in mmol of trolox equivalent (TEAC) per g of leaf (considering the mass yield).

198

199 **2.4. Soxhlet and ultrasound-assisted extraction**

200 For comparison with the PLE results, the extraction of compounds from pre-treated
201 Stevia leaves was carried out, in duplicate, by conventional extraction in Soxhlet and
202 ultrasound-assisted extraction (UAE). For both extractions, 3 g of dried and ground leaves
203 and a binary mixture of ethanol and water (70% ethanol, v/v) were used. Soxhlet extraction
204 was carried out in a solvent reflux extraction system coupled to a cooling bath (MA 184,
205 Marconi), using a solvent to leaf ratio of 50 mL g^{-1} for 8 hours. The UAE was performed in
206 an ultrasonic bath with indirect contact (Q 5.9/40 A, Ultronique, Eco-Sonics, Recreio
207 Campestre Jóia Indaiatuba, SP, BR) and heating control, as described by Raspe et al. (2021b)
208 using solvent to leaf ratio 15 mL g^{-1} for 30 min. After the extractions, the leaves were
209 separated by filtration and the removal of the extracting solvent was carried out, as reported in
210 section 2.3.

211

212 **2.5. Extract characterization**

213 The extracts obtained were characterized in relation to the content of glycosides,
214 contents of total phenolic compounds and total flavonoids and antioxidant activity.

215 The concentrations of total glycosides (stevioside, rebaudioside A and rebaudioside C)
216 present in the leaf extract were determined using a High Performance Liquid Chromatograph
217 (HPLC). In this step, the dry extract was redissolved with the mobile phase
218 (acetonitrile:water, 80:20, v/v) at a concentration of 1000 µg mL^{-1} (Dacome et al., 2005).
219 Subsequently, the extract was sonicated for 5 min (Q 3.0/40 A/110 W, Ultronique), from
220 which 20 µL were filtered through a hydrophobic membrane (0.5 µm, Millipore) to be
221 injected into an HPLC system (307, HPLC Piston Pump, Gilson, Champaign, IL, EUA).
222 Equipped with refractive index detector (IR 133, Gilson) and 5 µm NH_2 analytical column

(125 mm x 4.6 mm, Hypersil GOLD™ Amino HPLC Columns, Thermo Fischer Scientific, Waltham, MA, EUA), the system was operated isocratically with a flow of 0.5 mL min^{-1} by a low pressure pump (5.SC, Gilson), at 25 °C for 30 min (L-2300, Column Oven, West, Hitachi Elite LaChrom, Hitachi High Technologies America Inc., San Jose, CA, EUA). For the quantification of steviol glycosides, a standard analytical curve (R^2 0.99) was used to compare the peak integrations, with detection and quantification limits of 5 µg mL^{-1} and 10 µg mL^{-1}, respectively, with the results expressed in g per 100 g of leaf. The sweetener yield (Y_S) was calculated considering the ratio between the mass of the leaf used in the extraction, the Y_M value and the content of glycosides (Reb A, Reb C and Stv) in the dry extract resulting from the process, as a function of the mass of sweeteners present in the leaf.

The total flavonoid content (TFC) was determined using the aluminum chloride method described by Zaidan et al. (2019). An aliquot (500 µL) of the aqueous extracts (1300 µg mL^{-1}) was mixed with 1500 µL of methanol, 100 µL of aluminum chloride (10%), 100 µL of sodium acetate (1.0 M) and 2800 µL of water distilled. The mixture was homogenized and incubated in the dark at room temperature for 30 min, and then the absorbance of the mixture was determined at 450 nm in a UV-VIS spectrophotometer (UV-1900i, Shimadzu).The total concentration of flavonoids of each extract was quantified using a standard curve prepared with quercetin (R^2 0.99), and the results were expressed in mg of quercetin equivalent (QE) per g of extract.

Total phenolic compounds and antioxidant activity were analyzed as described in section 2.3, and the results were expressed per g of extract.

2.7. Analysis of data

The extractions and analysis of extracts were performed in triplicate, and the results were expressed as mean values ± standard deviation. To verify the influence of the parameters

evaluated at each stage on the results obtained, the comparison of the means obtained was evaluated by analysis of variance (ANOVA) using the Statistica® 8.0 software (StatSoft, Inc., Tulsa, OK, USA), followed by the Tukey test (with 95% confidence interval).

3. Results and discussion

3.1. Pressurized liquid extraction

3.1.1. Effect of ethanol percentage in the extractor solvent

Figure 2 shows the values for Y_M, TPC and AA of the dry extracts obtained from Stevia leaves from the investigation of different percentages of ethanol in the extracting solvent (40 and 70%, v/v) at extraction times of 30, 60 and 90 min. As can be seen in this figure, the dissolution of the constituents is more effective with increasing proportion of ethanol in the mixture. In this way, all investigations carried out using extracting solvent with 70% (v/v) of ethanol had an increase in their values, with the Y_M of this process on average ~8.0% higher than the result of the binary mixture with 40% (v/v) of ethanol, for the 3 extraction times investigated. Increasing capacity to extract TPC was verified using the solvent with 70% (v/v) of ethanol up to 60 min of process, while for the solvent composed of 40% (v/v) of ethanol, a reduction in these contents with the course of the process extraction time was observed. On the other hand, in the interval from 30 to 60 min of extraction, an increase of 18.8% in AA for both binary mixtures was verified, with no increase in their contents when this time was exceeded at 90 min.

Figure 2

Considering that the unconventional processes envision, among other factors, increasing the mass yield of extractions for a possible large-scale application (Raspe et al.,

2022), it appears that the percentage of ethanol in the extracting solvent directly inflicted on the PLE response in relation to Y_M (Figure 2). Correlating to this variable is the composition of the extract, which, by providing its quantitative increase, may favor the increase of its constituents. SG, such as Stv and Reb A, are the compounds that provide sweetening power to the extract of this matrix, and are reported to have poor solubility in ethanol and water (Celaya et al., 2016), however, through its mixture and proportions similar to those investigated in this work, expressive results can be obtained. Raspe et al. (2021a) when using the percentage of ethanol in the extracting solvent of 70% (v/v), reported obtaining an extract composed of SG ~13.5% higher than those obtained with 100% ethanol. At the same time, Martins et al. (2016) when using solvent with 70% (v/v) of ethanol could achieve ~90.0% of the SG in the first stage of exhaustive maceration, while the contents involving solvent with 90%(v/v) of ethanol were ~25.7% lower.

The choice of solvent is fundamental in the extraction processes, as it directly impacts the selectivity and, consequently, affects the chemical composition and functional properties of the final extract (Jacotet-Navarro et al., 2018). In general, proper solvent selection depends on the solubility of the target compound (Bubalo et al., 2015) and thus, the lower removal of TPC from Stevia leaves, obtained through processes using solvents with a higher proportion of water, can be explained by the low solubility of these compounds in this solvent (Yildiz-Ozturk et al., 2014). Binary mixtures between ethanol and water, however, provide in their different proportions a wide range of polarity in relation to the compounds to be extracted, because their solubilization implies electrostatic repulsions and attractions between the solvent and the solute (Jacotet-Navarro et al., 2018), more effectively promoting their solubilization and resulting in a higher extraction rate than when used pure (Celaya et al., 2016). In this work, an increase of ~32.5% was obtained in the TPC extraction by increasing the percentage of ethanol in the extracting solvent from 40 to 70% (v/v). Alara et al. (2018)

298 when investigating the influence of the percentage of ethanol in the extracting solvent from 40
299 to 60% (v/v), reported an increase of 13.63% in the TPC extracted from the leaves of
300 *Vernonia amygdalina*.

301 In PLE, the physicochemical properties of the solvent, such as the dielectric constant,
302 viscosity, surface tension and the diffusion coefficient change dramatically (Cheng et al.,
303 2021). Therefore, in addition to the binary mixture between ethanol and water promoting
304 susceptibility to changes in the solvent properties, the process allows its polarity to be
305 changed, making it possible to extract many substances simultaneously by adjusting the
306 temperature and pressure conditions of the solvent. extraction. This scenario, linked to the
307 results already highlighted, made it possible to obtain a dry extract with AA of 32.3% higher
308 using the binary mixture with 70% (v/v) of ethanol, evidencing its choice for conducting the
309 other investigations.

310

311 **3.1.2. Effect of static time**

312 The Y_M, TPC and AA of the dry extract of Stevia leaves obtained using solvent
313 extraction with 70% (v/v) ethanol at different static times (10, 20 and 30 min), followed by
314 dynamic extraction (30 and 60 min), as shown in Figure 3.

315

316 **Figure 3**

317

318 As shown in Figure 3, Y_M was not influenced by the different static times investigated
319 and, therefore, 10 min was considered sufficient to obtain ~45.0 wt% yields in 60 min of
320 extraction. For TPC, ~106.6 mg GAE g^{-1} leaf in static time of 10 min were obtained in 30 min
321 of extraction, value ~3.5 and 5.4% higher than those reported for static time of 20 and 30 min
322 for the same period of dynamic extraction, respectively. For AA, in the initial 30 min, the

increase in static time from 10 to 20 min resulted in similar values of ~3.70 mmol TEAC g^{-1} leaf, while 8.8% lower content was verified in the static time of 30 min.

In general, the static time makes it possible to obtain a greater recovery of the compounds of interest, where a longer contact time between solvent and matrix promotes a higher extraction yield (Setyaningsih et al., 2016). Sandra et al. (2020) reported that increasing the static time from 5 to 10 min promoted an increase of 10.5% in the TPC content extracted from Stevia leaves at 130 °C. However, in the present study, in the initial 30 min of dynamic extraction, an opposite effect was verified in the active compounds in the static times of 10, 20 and 30 min, which may be related to the fact that the phenolic acids that compose these compounds present chemical structure that differs from each other, therefore having variable suitability in different extraction conditions (Zoric et al., 2014; Plaza and Turner, 2015; Gil and Wianowska, 2017).

On the other hand, for the dynamic extraction time of 60 min, the TPC contents increased by ~21.0, 29.7 and 25.2% for 10, 20 and 30 min, respectively. For AA, after the same dynamic extraction time, an increase >13.0% was verified in the contents from the static time of 30 min. Similar behavior was reported by Kovačević et al. (2018), who verified an increase of 25.0% in the recovery of TPC obtained at 130 °C with increasing dynamic extraction time (10 to 30 min), represented by the application of 1 to 3 cycles of 10 min each.

During the period in which static time is applied in PLE, solubilization of compounds is provided from the matrix to the solvent, while the fluid flow is interrupted (Zia et al., 2020). However, this prolonged exposure can compromise the extraction efficiency, due to the saturation of the plant cell pores, arising from the achievement of equilibrium between the analytes still bound to the matrix and those already solubilized in the solvent (Herrero et al., 2013). Furthermore, it is reported the possibility of degradation of thermolabile compounds with increasing static time (Plaza and Turner, 2015), mainly as a result of exposure to the

348 temperatures at which the process is conducted. Once the dynamic extraction starts with the
349 continuous pumping of solvent to the PLE medium, the equilibrium is shifted by the
350 dissolution of the compounds, allowing an increase in the efficiency of the process, since the
351 flow reduces the contact of the analytes at high temperatures (Herrero et al., 2013). Thus, the
352 reported results demonstrated that a static extraction period of 10 min is sufficient for the
353 removal of compounds from Stevia leaves and, therefore, this condition was selected to
354 determine the effect of pressure and temperature.
355
356 **3.1.3. Effect of pressure**
357 Figure 4 shows the resulting values for YM, TPC and AA of the dry extracts obtained
358 from Stevia leaves from the investigation of different operating pressures (50 and 100 bar) at
359 125 °C, using extracting solvent with 70% (v/v) ethanol, in 10 min static time followed by
360 dynamic extraction conducted for 60 min. From the data presented in this figure, it is possible
361 to verify that in the first 30 min, the pressure of 50 bar promoted the highest Y_M and active
362 compounds contents. At the lowest pressure (50 bar), increase in extraction time at 60 min
363 resulted in ~19.0% increase in Y_M and 6.1% in TPC content, while for AA, ~2.0% reduction
364 was observed. When increasing the pressure to 100 bar, an increase of ~17.5% in Y_M was
365 verified from 30 to 60 min, and in the same time interval, an increase of 25.2% in TPC was
366 verified, with an increase of 11.7% in AA.
367
368 **Figure 4**
369
370 Pressure is the operational variable that allows greater diffusion of the solvent in direct
371 contact with the plant matrix (Fernández-Ponce et al., 2016), however, its increase can
372 interfere with the recovery of target compounds, due to bed compaction, which triggers the

reduction of solvent-solute contact, reducing porosity and formation of preferential channels in the matrix, which result in lower interstitial velocity (Osório-Tobón et al., 2014; Náthia-Neves et al., 2017). Mustafa and Turner (2011) mention that the pressure in the PLE allows the extraction process to be accelerated because the solvent remains in the liquid state and above its boiling point, being at the same time sufficiently capable of helping it to penetrate the plant matrix. Because it is restricted to this, in the PLE of active herbal compounds, pressures of 50 to 100 bar are generally used (Sánchez-Camargo et al., 2020). This finding is in agreement with the results obtained in this investigation, in which we can verify that high pressure is not necessary to obtain high yields and an extract rich in active compounds for the matrix in question.

Different authors mention that pressure does not influence the process of extracting active compounds. Santos et al. (2012) verified that the increase in pressure from 50 to 100 bar did not contribute to the recovery of TPC from jabuticaba skins during the PLE process with ethanol as extracting solvent, carried out at 80 °C. Huerta and Saldaña (2018) also verified this effect on the PLE of canola straw with a solvent composed of 60% (v/v) ethanol, in the range of 50 to 100 bar. It is worth noting that another factor that can infer this behavior may be related to the fact that the matrix in question is pre-treated, which facilitates solvent access and migration of analytes from the plant cell, promoting higher extraction yields, production of extracts with higher content of certain constituents (Vidović et al., 2014).

3.1.4. Extraction kinetics

Figure 5 shows the extraction kinetics when the effect of temperature (100, 125 and 150 °C) on obtaining the dry extract of Stevia leaves was analyzed. PLE was evaluated for the extraction of compounds from Stevia leaves in terms of YM, TPC and AA, using a solvent with 70% (v/v) ethanol and a static time of 10 min at 50 bar.

Figure 5

Temperature is reported as the main parameter that influences the physicochemical properties of the solvent and compounds to be extracted, and has a great influence on the extraction rate and PLE efficiency (Kamali et al., 2018). Based on the data presented in Figure 5, high temperatures promote extraction, with an increase of ~5.7 and ~30.5% in Y_M when raising the temperature from 100 to 125 and 150 °C, respectively, within 30 min of extraction (Figure 5a). For 60 min of extraction, in the same temperature range, this increase was even greater, being observed (~18.0 and 38.5%, respectively). Increase in extraction efficiency may be related to vapor pressure and thermal desorption of matrix compounds (Smith, 2002), which, when increased, promote a higher rate of diffusion and solubility of the compounds in the solvent. However, elevated temperatures can lead to a simultaneous increase in the rate of analyte degradation, especially when combined with longer extraction periods (Teo et al., 2010; Plaza and Turner, 2015), or lead to excess of co-extractants (Andreu and Picó, 2019), resulting in darker extracts, susceptible to interference by constituents. This was evidenced in the levels of AA of dry extract, which showed an increase of 9.6% in the range from 100 to 125 °C, with a subsequent reduction of ~5.0% when the temperature applied was 150 °C (Figure 5c). For AA, this effect was also verified at each of the temperatures investigated, where an increase in the contents was observed up to 30 min, with a subsequent reduction up to 60 min.

On the other hand, the TPC content increased as the operating temperature increased, resulting in increases of 9.4, 9.8 and 20.2% in the range of 100-125, 125-150 and 100-150 °C, respectively (Figure 5b). For PLE, Saravana et al. (2016) when investigating the range of 110-140 °C using a binary mixture with 25% (v/v) of ethanol as solvent, indicated an increase of

~46.0% in the TPC contents from *Saccharina japonica*, while for Allcca-Alca et al. (2021) the increase of these compounds from *Vitis vinifera* L. was ~68.0%, when evaluating the temperature increase in the range of 100 to 160 °C, using extracting solvent with 60% (v/v) of ethanol. In parallel, in the application of SWE, Yildiz-Ozturk et al. (2014) and Sandra et al. (2020) reported an increase of ~66.0 and 35.5% in the TPC contents of extract and Stevia leaves, in the temperature range of 125-150 °C and 100-160 °C, respectively. For TPC extraction, Iwassa et al. (2019) and Giombelli et al. (2020) report higher increases, from 86.6% to ~116.5%, when investigating temperatures of 100-130 °C and 100-160 °C in SWE of asparagus by-product and peach palm by-product, respectively.

3.2. Effect of extraction technique

Table 1 presents the results of total glycosides, sweetener composition (Stv, Reb A and Reb C), Y_M, Y_S and content of active compounds (TPC, TFC and AA) of the extract of pre-treated Stevia leaves, obtained for different extraction techniques evaluated. According to the results presented in this table, the glycosidic profile was not influenced by the experimental conditions adopted in PLE when temperatures of 100 and 125 °C were applied, with similarity in relation to the composition in Stv, Reb C and Reb A. However, when corroborating these results with those obtained by the other techniques, differences can be verified. Soxhlet showed the highest yields in SG and in the quality of sweeteners, while the UAE and PLE techniques (100 and 125 °C) showed similarity in the removal of active compounds. Although the temperature of 150 °C in PLE provided the highest values of Y_M, a reduction in SG, Y_S and active compounds was observed.

Table 1

In PLE, the temperature acts causing a weakening of the chemical bonds present between the matrix compounds, increasing the solubility of these compounds in the solvent and the mass transfer (Mustafa and Turner, 2011). Together with the pressure, this variable allows a process with higher yields, quickly and that requires smaller volumes of solvents, especially when compared to conventional techniques, to be achieved (Sánchez-Camargo et al., 2017). This is evidenced in Table 1 in the tests carried out in pressurized media, where YM equivalent to that obtained by the exhaustive extraction promoted by Soxhlet, was verified. However, as already mentioned, despite the PLE operating conditions promoting better solubility and obtaining a higher crude quantity, high temperatures simultaneously promote the degradation rate of some analytes, especially when combined with long extraction periods, an effect observed at 150 °C. Furthermore, extracts with interfering constituents may have been obtained as a result of the co-extraction of compounds (Cheah et al., 2010), which may also have contributed to the reduction in the verified values of SG.

The inversely proportional behavior for sweeteners and active compounds is commonly verified in works involving the obtaining of extracts from Stevia leaves (Periche et al., 2015; Kovačević et al., 2018; Raspe et al., 2021b). Periche et al. (2015) verified this type of behavior in extracts obtained from UAE, MAE and decoction, and Kovačević et al. (2018) in extracts from SWE. Raspe et al. (2021b) reported that the UAE operational variables that tend to generate the highest TPC and AA are not correlated with those that promote an increase in SG content. Similarly, for PLE (100 and 125 °C), the obtainment of active compounds was superior to SG (Table 1), and inverse to that obtained by Soxhlet, which resulted in higher yields in SG and lower contents of active compounds.

Previous studies mentioned that the use of non-conventional techniques through the application of pressurized liquid technology, are promising in obtaining higher yields and recovery of target compounds (Ciulu et al., 2017; Santos et al., 2021; Chada et al., 2020).

Comparing the conventional technique (Soxhlet) with non-conventional techniques (UAE and PLE), it can be seen in Table 1 that the maximization of active compounds was observed in the extracts obtained through PLE (125 °C), while the resulting of SG from this condition corresponded to 87.8% of the total obtained by Soxhlet. This effect may be related to Soxhlet acting be led through exhaustive extraction, which takes place in a continuous process of reflux of the solvent at its boiling temperature, during hours of exposure. SG and TFC, despite being reported as thermolabile (Abou-Arab et al., 2010; Yildiz-Ozturk et al., 2014; Rosa et al., 2019; Li et al., 2020), had their greatest recovery through the use of Soxhlet. For UAE, although the Y_M obtained is equivalent to Soxhlet, its process conditions were possibly not sufficient to achieve the greatest removal of compounds from the plant matrix. But for both mentioned techniques (Soxhlet and UAE), the conditions applied were insufficient to maximize TPC and AA, which were obtained from PLE (125 °C). However, although these results are promising for obtaining an extract with active potential, marked degradation is reported for the extract obtained in PLE at 150 °C.

Finally, it is worth noting that the results obtained by the non-conventional techniques, in most parameters, were equivalent to those obtained by the conventional technique. The PLE (125 °C) and UAE techniques allowed the extraction of compounds from Stevia leaves, improving the efficiency of heat and mass transfer during the process, allowing a simple and fast operation through the use of renewable solvents, and having as a consequence, the favoring of Y_M, showing a higher content of active compounds to PLE. This result proves the involvement of fewer inconveniences in these techniques, when compared to conventional processes, such as the reduction in consumption of inputs and the feasibility of reusing the solvent in the process. Especially in the application of PLE, unpublished data could be obtained through the use of pre-treated Stevia leaves, since the reach of higher quality extracts, with great potential for use as a food additive, was evidenced.

4. Conclusions

This study reported on pressurized liquid extraction of compounds from pretreated Stevia leaves, using a binary mixture of ethanol and water as the extracting solvent. The highest yields of mass and active compounds were observed with the increase of the percentage of ethanol in the extracting solvent (70% v/v). Static time and pressure above 10 min and 50 bar, respectively, had no effect on the evaluated responses and maximum extraction resulted in Y_M, TPC and AA of ~46.0%, 125.0 mg GAE g^{-1} leaf and 4.3 mmol g^{-1} leaf, respectively. In the extraction kinetics, an increase in Y_M was obtained with increasing temperature, while the maximization of TPC and AA of the extract was achieved at 125 °C, with only 30 min of extraction. Above this temperature, the degradation of the compounds was verified. Thus, an extract with ~26.0% by weight of SG was obtained, composed of Stv, Reb C and Reb A in percentages of 36.4, ~15.0 and 48.5%, respectively. In the comparison with the extraction techniques (Soxhlet and UAE), it was possible to verify that the proposed technique was similar to Y_M, but favored obtaining the active compounds (TPC, TFC and AA), resulting in a extract with potential for use as a herbal medicine and food additive. In addition, the use of a non-conventional processing technique, which allows the use of less energy and solvent, linked to operational strategies such as the pre-treated matrix together with the application of a binary mixture of ethanol and water as solvent, allows a process in less time, with ease of separation of the extracting solvent, providing potential reduction of operational costs, and concomitantly, leveraging the concept of green chemistry.

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Contributions of Authors

Djéssica Tatiane Raspe: Data curation, Conceptualization, Investigation, Methodology, Writing-Original draft. **Camila da Silva:** Writing-Reviewing and Editing. **Silvio Claudio da Costa:** Reviewing and Supervision.

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Table 1. Yield in mass (Y_M) and in sweeteners (Y_S), total phenolic compounds (TPC), total flavonoid content (TFC) and antioxidant activity (AA) of extracts obtained by different extraction techniques from pretreated Stevia leaves.

Property		Technique				
		Soxhlet[1]	Ultrasound[2]	Pressurized liquid extraction[3]		
				100 °C	125 °C	150 °C
Steviol Glycosides (g per 100 g extract)	Stevioside	10.74 ± 0.11^{a}	10.10 ± 0.17^{ab}	9.75 ± 0.07^{abc}	9.51 ± 0.31^{abc}	7.51 ± 0.10^{d}
	Rebaudioside C	4.34 ± 0.01^{a}	4.02 ± 0.32^{ab}	3.97 ± 0.08^{abc}	3.91 ± 0.17^{bc}	2.90 ± 0.00^{d}
	Rebaudioside A	14.22 ± 0.42^{a}	11.82 ± 0.21^{ab}	13.11 ± 0.02^{abc}	12.68 ± 0.00^{abc}	9.60 ± 0.15^{d}
	Total	29.30 ± 0.07^{a}	25.95 ± 0.28^{b}	26.84 ± 0.03^{b}	26.12 ± 0.47^{b}	20.02 ± 0.32^{c}
Y_M (wt%)		40.83 ± 0.61^{a}	40.92 ± 0.44^{ab}	36.57 ± 0.67^{c}	38.65 ± 0.78^{abc}	47.70 ± 0.85^{d}
Y_S (wt%)		92.38 ± 0.76^{a}	81.02 ± 0.46^{b}	75.47 ± 0.10^{c}	75.74 ± 0.13^{c}	58.96 ± 0.95^{d}
TPC (mg GAE g^{-1} extract)		281.07 ± 0.68^{a}	278.75 ± 0.63^{b}	299.72 ± 0.25^{c}	310.41 ± 0.28^{d}	277.51 ± 0.26^{b}
TFC (mg QE g^{-1} extract)		104.05 ± 0.14^{a}	83.25 ± 0.15^{b}	91.28 ± 0.29^{c}	90.67 ± 0.27^{d}	78.85 ± 0.32^{e}
AA (mg TEAC g^{-1} extract)		11.46 ± 0.28^{a}	9.03 ± 0.02^{b}	11.09 ± 0.64^{ac}	11.09 ± 0.70^{ac}	8.07 ± 0.27^{b}

[1] solvent to leaf ratio of 50 mL g^{-1}, solvent with 70% (v/v) of ethanol, solvent boiling temperature (~80 °C) and 480 min;
[2] solvent to leaf ratio of 15 mL g^{-1}, solvent with 70% (v/v) of ethanol, 50 °C, 165 W and 30 min;
[3] solvent to leaf ratio of 15 mL g^{-1}, solvent with 70% (v/v) of ethanol, 50 bar and 30 min.
Means followed by different letters on the same line indicate a significant difference ($p<0.05$).

Figure Captions

Figure 1. Experimental apparatus used in pressurized liquid extraction: (SR) solvent reservoir, (P) high pressure liquid pump, (V1) check-valve, (O) oven, (PH) pre-heating, (E) extractor, (T) temperature indicator, (C) cooling system, (TE) thermostatic bath, (M) manometer, (V2) needle valve, (V3) pressure reduction valve and (S) sampling.

Figure 2. Effect of ethanol percentage (v/v) in extracting solvent (▨ and -△- 40%; ▭ and -●- 70%) on the mass yield (Y_M), total phenolic compounds (TPC) and antioxidant activity (AA) obtained at 125 ºC, static time of 30 min and 100 bar. Means followed by the same lowercase letters (time effect for each solvent) and upper-case letters (solvent effect for each time) did not differ statistically ($p>0.05$).

Figure 3. Effect of static time (▨ and -△- 10 min; ▭ and -●- 20 min; ▬ and -■- 30 min) on the mass yield (Y_M), total phenolic compounds (TPC) and antioxidant activity (AA) obtained at 125 ºC, percentage of ethanol in the extracting solvent of 70% (v/v) and 100 bar. Means followed by the same lowercase letters (time effect for each static time) and upper-case letters (static time effect for each time) did not differ statistically ($p>0.05$).

Figure 4. Effect of pressure (▨ and -●- 50 bar; ▭ and -△- 100 bar) on the mass yield (Y_M), total phenolic compounds (TPC) and antioxidant activity (AA) obtained at 125 ºC, percentage of ethanol in the extracting solvent of 70% (v/v) and static time of 10 min. Means followed by the same lowercase letters (time effect for each press) and upper-case letters (pressure effect for each time) did not differ statistically ($p>0.05$).

809 **Figure 5.** Extraction kinetic (-■- 100 °C, -●- 125 °C and -△- 150 °C) obtained by the
810 percentage of ethanol in the extracting solvent of 70% (v/v), static time of 10 min and
811 pressure of 50 bar on the: (a) mass yield (Y_M), (b) total phenolic compounds (TPC) and (c)
812 antioxidant activity (AA). Means followed by the same lowercase letters (time effect for each
813 temperature) and upper-case letters (temperature effect for each time) did not differ
814 statistically ($p>0.05$).

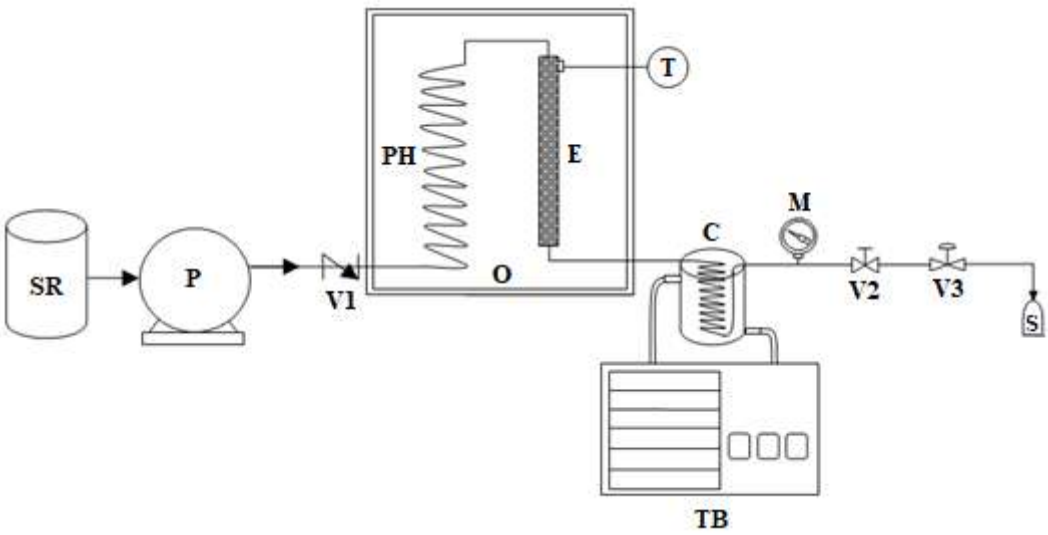


Figure 1

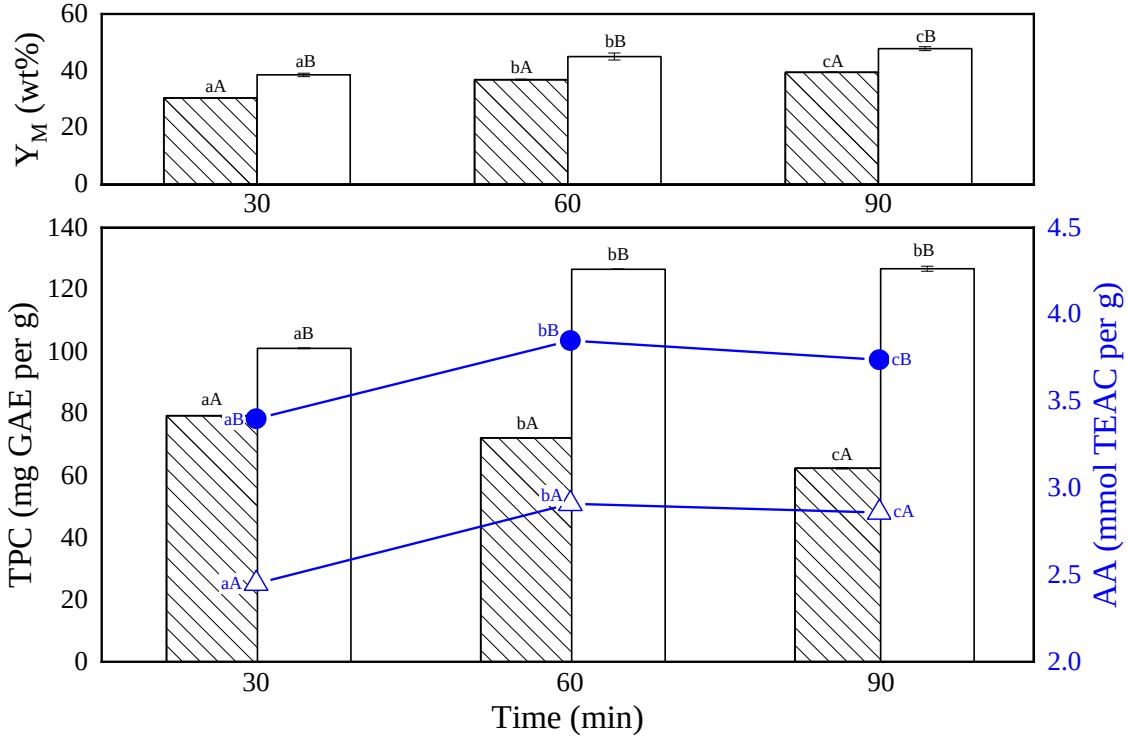
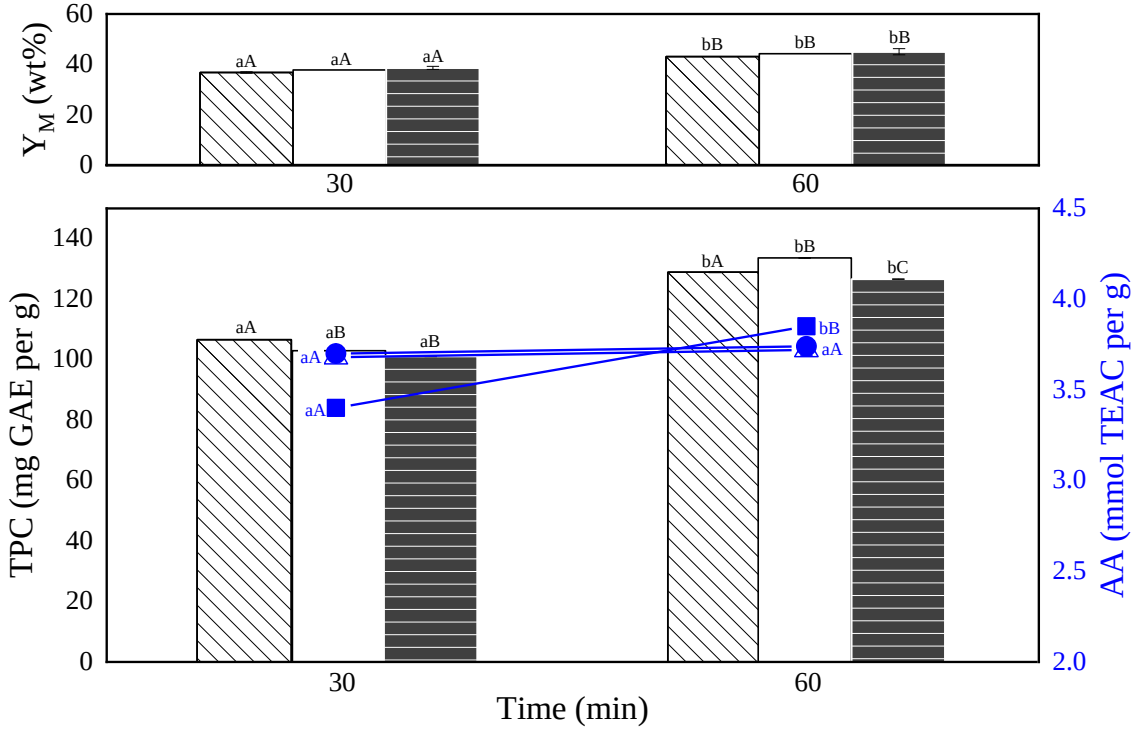

60
40
20
0
Y_M (wt%)
aA
aB
bA
bB
cA
cB
30
60
90
140
120
100
80
60
40
20
0
TPC (mg GAE per g)
4.5
4.0
3.5
3.0
2.5
2.0
AA (mmol TEAC per g)
Time (min)

Figure 2



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820

Figure 3

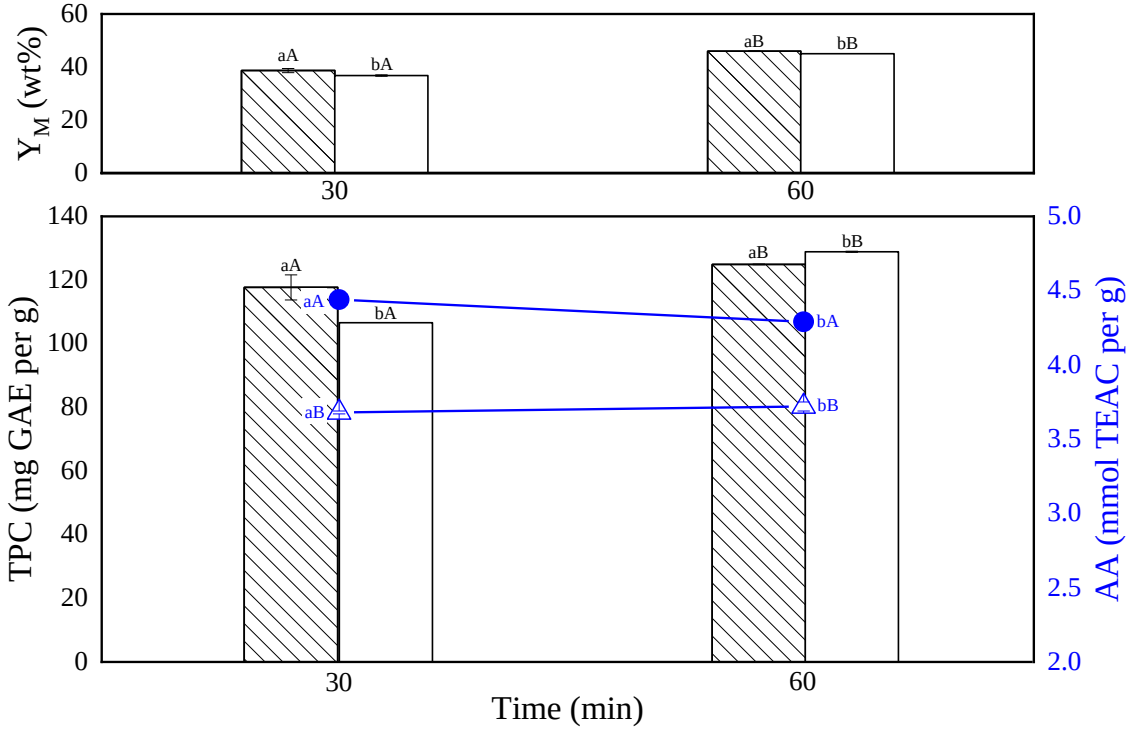

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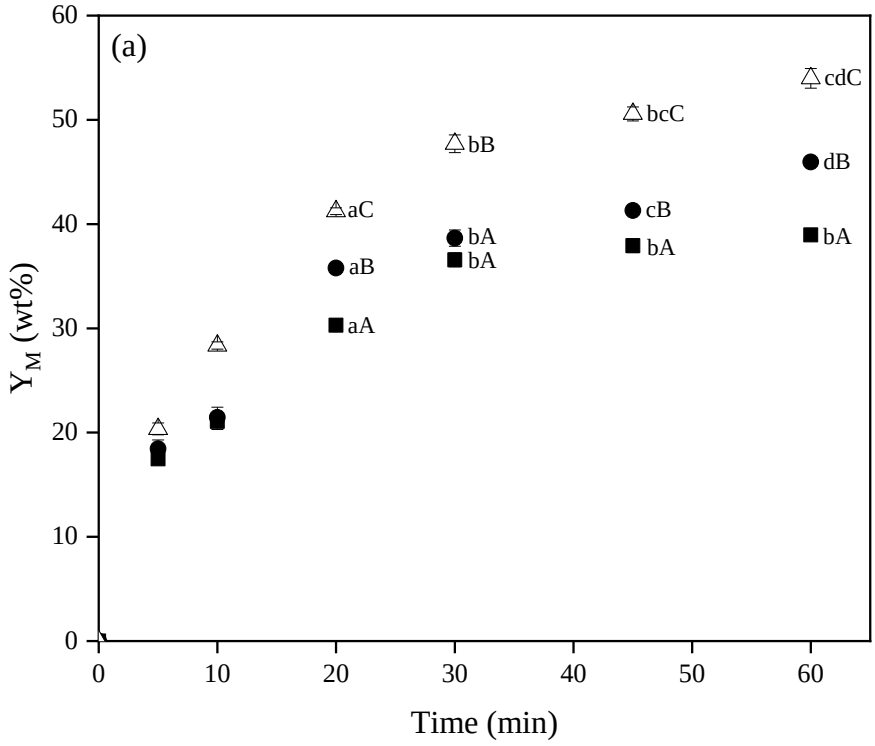
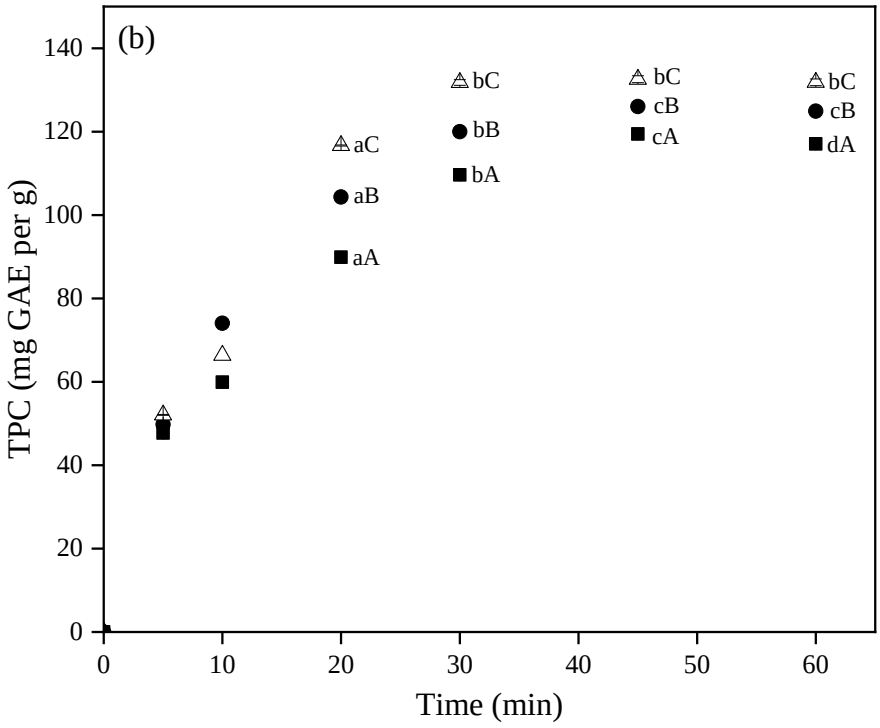
Figure 4


(a)
Y_M (wt%)
Time (min)
aA
aB
aC
bA
bA
bB
cB
bA
bcC
dB
bA
cdC

823


(b)
TPC (mg GAE per g)
Time (min)
aA
aB
aC
bA
bB
bC
cA
cB
bC
dA
cB
bC

824

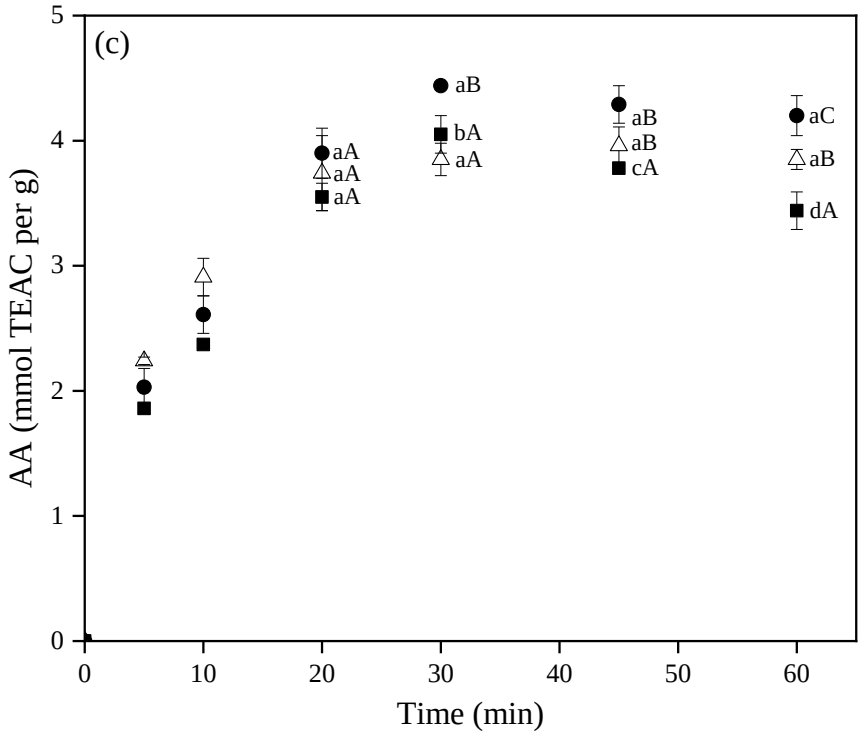


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Figure 5