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High-pressure technologies as a green approach for recovering and valorizing bioactive compounds from acerola, soursop, and strawberry agri-industrial by-products

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High-pressure technologies as a green approach for recovering and valorizing bioactive compounds from acerola, soursop, and strawberry agri-industrial byproducts

O presente trabalho em nível de doutorado foi avaliado e aprovado por banca examinadora composta pelos seguintes membros:

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Dr. Edy Sousa de Brito - Membro Externo - Embrapa Alimentos e Territórios

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Prof. Dr. Marcelo Lanza

Orientador

Florianópolis, 2023.

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Em todas as coisas da natureza existe algo de maravilhoso.

Aristóteles

RESUMO

A graviola (*Annona muricata*), acerola (*Malpighia emarginata* DC) e morango (*Fragaria x Ananassa*), são processadas na região Nordeste do Brasil e utilizadas para a fabricação de sucos, polpas, sorvetes, doces, entre outros. A agroindústria de frutas produz 30-60% de resíduos (casca, caroço ou sementes e bagaço) que são pouco explorados embora ricos em fitoquimicos. Dessa forma, o objetivo deste estudo foi recuperar os compostos potencialmente bioativos, por técnicas não convencionais, como extração fluido supercrítico (SFE), água subcrítica (SWE) e assistida por micro-ondas (MAE) em diferentes combinações e condições operacionais avaliando comparativamente com técnica convencional de extração (Soxhlet-SOX). Os extratos foram avaliados em relação ao conteúdo fenólico total (TPC), a capacidade antioxidante (DPPH, ABTS e FRAP), caracterização química e atividade antiproliferativa (*in vitro*) contra linhagens de células cancerígenas. Os extratos da semente de graviola, tiveram a fração lipídica recuperada por SFE (30 MPa/40 ºC), composta por 70.0% de MUFA (ácidos oleico, 10,13-octadecadienoico e linoleico). O valor de TPC (77.3 mg GAE·g^{-1}) foi maior na extração sequencial (SFE +SWE) e capacidade antioxidante foi superior às extrações simples SFE, SWE e SOX. Foram identificados por LC-ESI-MS/MS, 41 compostos fenólicos, destes 29 foram detectados pela primeira vez, predominantemente ácidos fenólicos e flavonoides. Em relação ao extrato de resíduo de acerola, foram comparadas extrações SWE a 70, 90, 110 e 130°C utilizando residuo fresco e seco (8 h; 40 °C). O valor de TPC variou entre (348.3 - 362.0 mg GAE. g^{-1}), seguido de uma redução considerável a 90°C e tornando a subir a 110 e 130°C. Mesmo comportamento para atividade antioxidante (DPPH, FRAP e ABTS), devido a neoformação de compostos antioxidantes derivados de reações de Maillard e de caramelização associada à degradação do ácido ascórbico. O perfil fitoquímico foi acessado por UPLC-ESI-QTOF-MS^E, onde foram identificados, ácido málico, ácido ascórbico e compostos fenólicos (flavonoides), sendo quercetina *O*-ramnosídeo, quercetina pentosil-*O*-hexosídeo e kaempferol *O*-ramnosídeo mais abundantes e intensos nos extratos SWE com amostra fresca a 90°C do que na seca, destacando seu potencial de reaproveitamento. A fração fenólica do resíduo do morango foi recuperada por extrações simples (SWE, MAE e SOX) e combinadas com despressurização rápida SP+MAE. O melhor valor de TPC foi para SWE 130°C (269.10 mg GAE. g^{-1}) e atividade antioxidante variaram, para DPPH entre (EC_{50}. 2.53 e 0.47 mg mL^{-1}), ABTS de (438.08 e 1205.16 µmol TE g^{-1}), e FRAP de (360.35 - 1010.99 µmol TE g^{-1}). O perfil fitoquímico foi obtido por UPLC-ESI-QTOF-MS^E e identificados 16 metabólitos, incluindo ácidos orgânicos, elagitaninos, procianidinas e outros compostos fenólicos que variaram em quantidades e intensidade conforme técnica de extração. Os extratos foram utilizados em ensaios de atividade antiproliferativa contra B16F10, 4T1, SF295, SK-MEL-28, e PC-3 linhagens de células cancerígenas sendo que os extratos de semente de graviola SFE 30MPa 40°C e SWE 130°C foram capazes de reduzir o crescimento tumoral em 35% e 31% para B16F10 e 4T1, respectivamente. A utilização de técnicas de extração ecologicamente atraentes, agregam valor a estes resíduos agroindustriais possibilitando aplicações alimentícias, nutracêuticas, farmacêuticas, químicas e cosméticas além de auxiliar na promoção de uma economia circular ao reaproveitar biomassa que seria descartada.

Palavras-chave: Resíduo agroindustrial. Compostos bioativos. Extração por fluidos supercrítico (SFE). Extração por água subcrítica (SWE). Extração assistida por microondas (MAE). Atividade antiproliferativa.

ABSTRACT

Soursop (*Annona muricata*), acerola (*Malpighia emarginata* DC), and strawberry (*Fragaria x Ananassa*) are processed in the Northeast region of Brazil and used for the manufacture of juices, pulps, ice cream, and sweets, among others. The fruit agroindustry produces 30-60% of residues (peel, core or seeds, and bagasse), which are little explored although rich in phytochemicals. Thus, the objective of this study was to recover potentially bioactive compounds by unconventional techniques, such as supercritical fluid (SFE), subcritical water (SWE), and microwave-assisted extraction (MAE) in different combinations and operating conditions, evaluating comparatively with conventional extraction technique (Soxhlet-SOX). The extracts were evaluated for total phenolic content (TPC), antioxidant capacity (DPPH, ABTS, and FRAP), chemical characterization, and antiproliferative activity (in vitro) against cancer cell lines. Soursop seed extracts had the lipid fraction recovered by SFE (30 MPa/40 ºC), composed of 70.0% UFA (oleic, 10,13-octadecadienoic and linoleic acids). The value of TPC (77.3 mg GAE·g^{-1}) was higher in the sequential extraction (SFE +SWE), and antioxidant capacity was superior to the simple extractions SFE, SWE, and SOX. Forty-one phenolic compounds were identified by LC-ESI-MS/MS, of which 29 were detected for the first time, predominantly phenolic acids and flavonoids. Concerning acerola residue extract, SWE extractions at 70, 90, 110, and 130°C using fresh and dry residue (8 h; 40°C) were compared. The TPC value varied (348.3 - 362.0 mg GAE.g^{-1}), followed by a considerable decrease at 90°C and rising again at 110 and 130°C. The same behavior for antioxidant activity (DPPH, FRAP, and ABTS) is due to the neoformation of antioxidant compounds derived from Maillard reactions and caramelization associated with ascorbic acid degradation. The phytochemical profile was accessed by UPLC-ESI-QTOF-MSE, where malic acid, ascorbic acid and phenolic compounds (flavonoids) were identified, with quercetin *O*-rhamnoside, quercetin pentosyl-*O*-hexoside and kaempferol *O*-rhamnoside being the most abundant and intense in the SWE extracts with fresh sample at 90◦C than in the dry one, highlighting its reuse potential. The phenolic fraction of the strawberry residue was recovered by simple extractions (SWE, MAE, and SOX) and combined with SP+MAE rapid depressurization. The best TPC value was for SWE 130°C (269.10 mg GAE. g^{-1}) and antioxidant activity varied, DPPH (EC50. 2.53 to 0.47 mg mL^{-1}), ABTS (438.08 to 1205.16 μmol TE g^{-1}), and FRAP (360.35 to 1010.99 μmolTEg^{-1}). UPLC-ESI-QTOF-MSE accessed the phytochemical profile, and 16 metabolites were identified, including organic acids, ellagitannins, procyanidins, and other phenolic compounds that varied in amounts and intensity according to the extraction technique. The extracts were used in antiproliferative activity assays against B16F10, 4T1, SF295, SK-MEL-28, and PC-3 cancer cell lines, and soursop seed extracts SFE 30MPa 40°C and SWE 130°C were able to reduce tumor growth by 35% and 31% for B16F10 and 4T1, respectively. The use of ecologically attractive extraction techniques adds value to these agro-industrial residues, enabling food, nutraceutical, pharmaceutical, chemical and cosmetic applications, as well as helping to promote a circular economy by reusing biomass that would otherwise be discarded.

Keywords: Agroindustrial waste. Bioactive compounds. Supercritical fluid extraction (SFE). Subcritical water extraction (SWE). Microwave-assisted extraction (MAE). Anti-proliferative activity (AlamarBlue assay).

RESUMO EXPANDIDO

Introdução

O consumo de frutas tropicais está aumentando nos mercados doméstico e internacional devido ao crescente reconhecimento de seu valor nutricional e terapêutico. Além dos nutrientes essenciais, a maioria das frutas apresenta quantidades consideráveis de micronutrientes, como minerais, fibras, vitaminas e compostos fenólicos secundários. Evidências crescentes mostram a importância desses micronutrientes para a saúde humana. A agroindústria de frutas produz 30-60% de resíduos resultantes do processamento de sucos e polpas. Estes resíduos são constituídos por casca, caroço ou sementes e bagaço, gerados em diferentes etapas do processo de produção e normalmente não utilizados, desperdiçados ou descartados, tornando-se fontes de contaminação ambiental. Levando em consideração o alto conteúdo do compostos bioativos presentes nos subprodutos do processamento de polpas de frutas, estes resíduos poderiam ser aproveitados para isolar fitoquímicos específicos como fonte potencial de ingredientes nutracêuticos, aditivos alimentares e funcionais, contribuindo como alternativas para a redução e recuperação dos resíduos de processos industriais e diminuindo o impacto ambiental. Os frutos incluídos neste estudo desempenham um papel econômico importante nos mercados nacional e regional. A graviola, acerola e morango são colhidas e processadas para posterior comercialização na região Nordeste do Brasil, e a grande maioria dos frutos é destinada ao processamento em agroindústrias, para a fabricação de sucos, sorvetes, doces, licores, frutas cristalizadas, entre outros. A extração e o processamento de compostos de interesse presentes nos resíduos de acerola, graviola e morango podem ser boas opções para agregar valor a esses subprodutos da agroindústria. Os resíduos resultantes do processamento das frutas contêm uma enorme quantidade de compostos fenólicos que podem ser recuperados por técnicas de extração e fracionamento, obtendo-se extratos vegetais com características potencialmente nutracêuticas e funcionais. A extração de compostos bioativos a partir de resíduos agroindustriais tem sido relatada utilizando métodos convencionais e não convencionais. A maioria dos métodos convencionais de extração utiliza solventes orgânicos e apresenta algumas limitações, como o uso de solventes tóxicos (riscos relacionados à saúde), altas temperaturas e elevados tempos de processo (degradação de compostos de interesse). Dessa forma, torna-se necessário o estudo de técnicas de extração com melhor seletividade e eficiência. Dentre essas tecnologias, processos intensificados baseados no uso de ultrassom, microondas (MAE, do inglês *Microwave Assisted Extraction*) e extração supercrítica por fluídos pressurizados (SFE) e extração com água subcrítica (SWE, do inglês *Subcritical Water Extraction*) estão ganhando atenção para a extração e recuperação de compostos bioativos a partir de diferentes matrizes naturais. Essas técnicas representam uma alternativa ambientalmente segura e economicamente viável de processo. Poucos estudos avaliaram a combinação de tecnologias a alta pressão e, até o presente momento, nenhuma pesquisa avaliou a extração simples e em combinação da recuperação de compostos fenólicos dos resíduos propostos e coletou dados experimentais visando a caracterização química e aplicação de atividade antiproliferativa .

Objetivos

O objetivo geral deste trabalho é estudar o aproveitamento do resíduo agroindustrial proveniente da produção de polpa de frutas de graviola (*Annona muricata* L.), acerola (*Malpighia emarginata* DC) e morango (*Fragaria x ananassa*) avaliando e comparando os compostos bioativos presentes para possível uso nas áreas alimentícia e/ou farmacêutica.

Metodologia

As sementes de graviola e bagaço de acerola e morango utilizados neste estudo foram cedidas pela empresa nome fantasia Tropicássia Polpa de Fruta, localizada na cidade de Fortaleza/Ceará. As sementes são subprodutos do processamento de polpa de frutas. As resíduos foram coletados na empresa, porcionados em sacos plásticos, congeladas a -18 ºC e transportadas para o Laboratório de Termodinâmica e Tecnologia Supercrítica (LATESC) da Universidade Federal de Santa Catarina (UFSC). O teor de umidade das sementes de graviola foi determinado por secagem direta em estufa de acordo com a metodologia nº 925.09 (AOAC, 2005a). Os extratos bioativos foram obtidos pela técnica convencional de Soxhlet (SOX) foi realizada de acordo com o método 920.39C (AOAC, 2005a). As extrações ESC foram realizadas em uma unidade de extração do Laboratório LATESC, que opera em pressões de até 40 MPa e vazões de solvente de até 1,9769 x 10^{-3} kg.h^{-1}nas condições de pressão e temperatura de 15 MPa/55ºC; 20 MPa/50ºC; 25 MPa/ 50 ºC e 30 MPa/40ºC, estabelecendo uma vazão de solvente a 0,7 kg CO_2 Kg h^{-1}. Os experimentos de extração com água subcrítica (SWE) foram realizados em uma unidade desenvolvida no LATESC/UFSC. As condições experimentais foram realizadas em modo dinâmico, com vazão e pressão fixa de 4 mL min^{-1} e 10 MPa, respectivamente, e temperaturas de 70, 90, 110 e 130 ºC para as amostras simples e em combinação. As extrações assistidas por microondas foram realizadas em reator de microondas (Monowave 300 da Anton Paar GmbH) equipado com magnetron único de 850 W. O tratamento combinado SFE, foi realizado a 30 MPa com CO_2 (99,95% puro, White Martins LTDA, Joinville/SC – Brasil) e mantido sob compressão estática por 30 min, em temperatura de 40 ºC. Após o término do tempo estático, o CO_2 foi liberado em um fluxo de 1,97 x 10^{-3} kg h^{-1}, o que permitiu que a pressão diminuísse até o restabelecimento da pressão atmosférica, rapidamente. Em seguida, a matéria-prima pré-tratada do vaso de extração foi coletada para posterior extração com MAE (processo denominado PS+MAE). O teor de compostos fenólicos totais (TPC) foi avaliado segundo a metodologia de Folin-Ciocalteau e a atividade antioxidante foi obtida através de ensaios DPPH, FRAP e ABTS . O perfil químico foi obtido através de cromatografia gasosa acoplada ao espectrometro de massas, LC-ESI-MS/MS e UPLC-QTOF-MS^E. A atividade antiproliferativa dos extratos foi avaliada através de ensaio de viabilidade célular (AlamarBlue) contra B16F10, 4T1, SF295, SK-MEL-28, e PC-3 linhagens de células cancerígenas.

Resultados e Discussão

Os extratos da semente de graviola tiveram a fração lipídica recuperada por SFE (30 MPa/40 ºC), composta por 70.0% de UFA (ácidos oleico, 10,13-octadecadienoico e linoleico). O valor de TPC (77.3 mg GAE·g^{-1}) foi maior na extração sequencial (SFE +SWE) e capacidade antioxidante foi superior às extrações simples SFE, SWE e SOX. Foram identificados por LC-ESI-MS/MS, 41 compostos fenólicos, destes 29 foram detectados pela primeira vez, predominantemente ácidos fenólicos e flavonoides. Em relação ao extrato de resíduo de acerola, foram comparadas extrações SWE a 70, 90, 110 e 130°C utilizando resíduo fresco e seco (8 h; 40 °C). O valor de TPC variou (348.3 - 362.0 mg GAE. g^{-1}), seguido de uma redução considerável a 90°C e tornando a subir a 110 e 130°C. Mesmo comportamento foi verificado para atividade antioxidante (DPPH, FRAP e ABTS), devido a neoformação de compostos antioxidantes derivados de reações de Maillard e de caramelização associada à degradação do ácido ascórbico. O perfil fitoquimico foi acessado por UPLC-ESI-QTOF-MS^E, onde foram identificados, ácido málico, ácido ascórbico e compostos fenólicos (flavonoides), sendo quercetina *O*-ramnosídeo, quercetina pentosil-*O*-hexosídeo e kaempferol *O*-ramnosídeo mais

abundantes e intensos nos extratos SWE com amostra fresca a 90°C do que na seca, destacando seu potencial de reaproveitamento. A fração fenólica do resíduo do morango foi recuperada por extrações simples (SWE, MAE e SOX) e combinadas com despressurização rapida SP+MAE. O melhor valor de TPC foi para SWE 130°C (269.10 mg GAE. g^{-1}) e atividade antioxidante variaram, DPPH (EC_{50}. 2.53 - 0.47 mg mL^{-1}), ABTS (438.08 - 1205.16 μmol TE g^{-1}), e FRAP (360.35 - 1010.99 μmol TE g^{-1}). O perfil fitoquímico foi acessado por UPLC-ESI-QTOF-MS^E e identificados 16 metabólitos, incluindo ácidos orgânicos, elagitaninos, procianidinas e outros compostos fenólicos que variaram em quantidades e intensidade conforme técnica de extração. Os extratos foram utilizados em ensaios de atividade antiproliferativa contra B16F10, 4T1, SF295, SK-MEL-28, e PC-3 linhagens de células cancerígenas sendo que os extratos de semente de graviola SFE 30MPa 40°C e SWE 130°C foram capazes de reduzir o crescimento tumoral em 35% e 31% para B16F10 e 4T1, respectivamente.

Considerações Finais

A extração de compostos de interesse utilizando técnicas de extração a alta pressão de maneira simples ou em associação para sua intensificação, apresentou altos rendimentos e elevada qualidade dos extratos recuperados. A abordagem sugerida pode ser considerada uma alternativa sustentável para obter compostos com alto potencial de aplicação a partir de diferentes matrizes vegetais. A utilização de técnicas de extração ecologicamente atraentes, agregam valor ao resíduo agroindustrial e ensaios *in vitro, in vivo* e clínicos podem acompanhar futuros estudos para confirmar os benefícios à saúde das frações obtidas visando estabelecer suas aplicações alimentícias, nutracêuticas,farmacêuticas, químicas e cosméticas.

Palavras-chave: Resíduo agroindustrial. Compostos bioativos. Extração por fluidos supercrítico (SFE). Extração por água subcrítica (SWE). Extração assistida por microondas (MAE). Atividade antiproliferativa

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AA – Antioxidant activity

$ABTS^{\bullet+}$ - 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt

ANOVA – *Analysis* of variance test

Asc. Ac. - Ascorbic acid

BHT – Butylhydroxytoluene

BPI- Base peak intensity

B16F10 - Murine melanoma cell lines

CAGR- Compound annual growth rate

CER – Constant Extraction Rate

CO_2 – *Carbon dioxide*

DIF – Diffusion Controlled Rate

DPPH – 2,2-Diphenyl-1-picrylhydrazyl radical

EC_{50} – Half Maximal effective concentration

FAO - Food and Agriculture Organization of the United Nations.

FER – Falling extraction rate

FRAP - Ferric Reducing Antioxidant Power

4T1 - Animal model for stage IV human breast cancer cell line

GAE - Gallic acid equivalent

GC-MS – Gas chromatography-mass spectrometry

GEIMM - Grupo de Estudos de Interações entre Micro e Macromoléculas

HPLC- High-Performance Liquid Chromatography

IBGE - Instituto Brasileiro de Geografia e Estatística

MAE - Microwave-Assisted Extraction

PC-3 - Human prostate cancer cell line

PLE – Pressurized liquid extraction

SEM- Scanning Electron Microscopy

SFE – Supercritical fluid extraction

SF295 - Human Glioblastoma cell line

SOX – Soxhlet extraction

SK-MEL-28 - Human malignant melanoma cell line

SWE – Subcritical Water Extraction

TEAC - Trolox equivalent antioxidant capacity

TIC – Total ion Chromatogram

TPC – Total Phenolic Content

TROLOX- (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid

UFSC- Universidade Federal de Santa Catarina

UPLC-QTOF-MS- Ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry

X0 – Extraction yield

SUMÁRIO

CONCEPTUAL DIAGRAM

High-pressure technologies as a green approach for recovering and valorizing bioactive compounds from acerola, soursop, and strawberry agri-industrial by-products

Why?

The acerola, soursop and strawberry juices or pulps processing generate agro-industrial by-products that correspond to 30% to 70% of the fruit volume and are discarded without use, generating large volumes of organic residues;
The high content of antioxidant compounds with high biological value present in fruit residues indicates that they could be better used;
By-products from the fruit pulp processing could be used as a potential source of nutraceutical/pharmaceutical ingredients, reducing waste generation and adding value to a product that would otherwise be discarded;
The chosen solvents and techniques allow the waste generated by processing to be used for other purposes, as it does not use toxic solvents.
The use of unconventional extraction technologies makes it possible to obtain potentially bioactive products through cleaner technology and with less waste generation.

State of the art?

Conventional extraction methods to recover bioactive compounds from fruits and agroindustrial residues have been reported to provide good results;
The literature has few reports on high-pressure extraction methods from fruits like soursop, strawberry and acerola, or its agro-industrial residues, and "*in vitro*" studies of its biological activities are often not performed;
No references were found on using combined high-pressure technologies with green solvents to obtain the valuable compounds from the agro-industrial by-products of acerola, soursop, and strawberry.

Hypotheses

It is possible to use high-pressure extraction techniques with green solvents to obtain bioactive compounds from the agro-industrial by-products of soursop, acerola, and strawberry;
Acerola, soursop, and strawberry by-products have phenolics compounds, potential antioxidant activity, and antiproliferative activity compatible with those described in the literature;
The use of single and combined pressurized processes is efficient in recovering several compounds of interest with bioactive potential in these residues.

Which steps?

Study of SFE, SWE, and MAE process parameters to select the best recovery conditions for bioactive compounds compared with the SOX as a conventional bench method;
Evaluate the application of pressurized combined extraction methods according to solvent selectivity, obtaining extracts rich in bioactive compounds;
Evaluate the quality of the extracts through *in vitro* analyzes of total phenolic content, antioxidant activity and chemical profile by high-performance liquid and gas chromatography;
Perform a screening test to assess *in vitro* cytotoxicity against tumour cells.

Expected results

To elucidate the extraction parameters of SFE,SWE and MAE to recover bioactive compounds from acerola, soursop and strawberry by-products;
To produce bioactive extracts of acerola, soursop and strawberry agroindustrial by-products with antioxidant characteristics;
To combine pressurized extraction techniques to obtain potential bioactive extracts with different properties;
To propose the chemical profile of acerola, soursop and strawberry pomace extract by high-performance liquid and gas chromatography;
To Assess *in vitro* cytotoxicity against tumour cells;
Enable the obtaining of more products from fruits and potential reuse of waste in other processes, as it does not use toxic solvents.
These techniques + solvents can further expand the circularity of fruit processing
To promote a circular economy by reusing agro-industrial waste that would otherwise be discarded

THESIS METHODOLOGICAL SEQUENCE FLOWCHART

This document is divided into seven chapters:

Chapter 1 includes the introduction, thesis structure, research objectives, and conceptual diagram. The following chapters present bibliographic reviews and experimental results corresponding to articles written and published in scientific journals in Food Engineering.

Chapter 2 presents the literature review, contextualizing this work's state of the art.

Chapter 3 presents the study of extraction by unconventional methods of soursop seed, seeking to promote the use of this residue from industrial processing.

Chapter 4 presents the evaluation of the SWE technique under different operational conditions for the recovery of bioactive compounds from the agro-industrial residue from the processing of acerola pulp.

Chapter 5 presents a comparative study of conventional and unconventional extraction techniques to obtain extracts with bioactive compounds from the agro-industrial residue from the processing of strawberry pulp juicing.

Chapter 6 presents a screening of potential antiproliferative activity from high-pressure extracts of agri-food by-products.

Finally, Chapter 7 presents general conclusions of the work and future study perspectives.

This study's general objective was to evaluate and compare bioactive compounds of commercial interest in acerola, soursop, and strawberry pulp agro-industry residue to generate new products in the food and/or pharmaceutical areas. In particular, data from the extraction of bioactive compounds by conventional techniques and new techniques were analyzed. This study aids in promoting a circular economy by reusing agro-industrial waste that would otherwise be discarded.

CHAPTER 1:

1.1INTRODUCTION

Brazil is one of the three largest producers of tropical, subtropical, and temperate fruits due to its continental dimensions and climates (BATISTA et al., 2018). About 47% of the national production is consumed fresh, and 53% is processed (BATAGLION et al., 2015; SILVA; OLIVEIRA; ABUD, 2016; MARQUES et al., 2018).

Consumption of tropical fruits is increasing in domestic and international markets due to increasing recognition of their nutritional and therapeutic value (PESCHEL *et al.*, 2006; RUFINO *et al.*, 2010). In addition to essential nutrients, most fruits contain numerous micronutrients, such as minerals, fiber, vitamins, and secondary phenolic compounds. Growing evidence shows the importance of these micronutrients for human health (AYALA-ZAVALA *et al.*, 2011; BATISTA *et al.*, 2018; RUFINO *et al.*, 2010).

The fruit agro-industry generates 30-70% of waste resulting from the processing of juices and pulps. These residues consist of bark, seeds or seeds, and bagasse, generated in different stages of the production process and usually not used, wasted, or discarded, becoming sources of environmental contamination(CHEOK *et al.*, 2018; SILVA; OLIVEIRA; ABUD, 2016; SOUSA; VIEIRA; LIMA, 2011).

Considering the high content of bioactive compounds present in the by-products of fruit pulp processing, these residues could be used to isolate specific phytochemicals as a potential source of nutraceutical ingredients, food, and functional or natural additives, contributing as alternatives to the reduction and recovery of residues of industrial processes and reducing the environmental impact (AYALA-ZAVALA *et al.*, 2011; DA SILVA; JORGE, 2017; GREGORIS *et al.*, 2013; KODAGODA; MARAPANA, 2017; PEREIRA *et al.*, 2015).

The fruits included in this study play an essential economic role in national and regional markets. More specifically, soursop, acerola, and strawberry are harvested and processed for later commercialization in the Northeast region of Brazil. The vast majority of the fruits are destined for processing in agro-industries to manufacture juices, ice creams, sweets, liqueurs, and candied fruits (JOSÉ *et al.*, 2014).

Soursop (*Annona muricata* L.) is a native fruit belonging to the *Annonaceae* family and has been widely used in recent decades (GAVAMUKULYA; WAMUNYOKOLI; EL-SHEMY, 2017; PATEL; PATEL, 2016; RADY *et al.*, 2018; SOLÍS-FUENTES;

HERNÁNDEZ-MEDEL; DURÁN-DE-BAZÚA, 2011a). The fruit has a bright white pulp, dark brown seeds, and green skin with a prickly, reticulated, leathery appearance (JOSÉ *et al.*, 2014; SANUSI; FADZELLY; BAKAR, 2018).

More than 212 bioactive compounds were found in *A. muricata*, the predominant ones classified as acetogenins, alkaloids, phenols, and others. The main plant parts studied were leaves and seeds, possibly due to their traditional use (CORIA-TÉLLEZ *et al.*, 2018). Moreover, different parts of soursop (leaf, bark, root, fruit, and seed) are rich in phytochemicals have a great potential for industrial application and can be valued in other sectors, in addition to reducing the environmental impact and generating income.

Acerola (*Malpighia emarginata*) is a fruit native to Central America whose culture has adapted well to Brazil (MALEGORI *et al.*, 2017). In the last decades, Brazil has expanded the areas of acerola cultivation, which is marketed mainly as fresh fruit, pulp, and juice (REZENDE; NOGUEIRA; NARAIN, 2017). The demand for this fruit has grown mainly due to its rich content of ascorbic acid, carotenoids, and phenolic compounds and its high antioxidant activity (JAESCHKE; MARCZAK; MERCALI, 2016; MEZADRI *et al.*, 2008).

Strawberry (*Fragaria spp*.) is a well-known non-climacteric fruit of significant natural and/or processed consumption (MONTERO *et al.*, 1996). In addition to its attractive color and flavor, strawberry is a good source of vitamin C and other antioxidant compounds such as flavonoids, phenolic acids, tannins, stilbenes, and anthocyanins (ROBARDS *et al.*, 1999).

The extraction and processing compounds of interest in acerola, soursop, and strawberry residues can add value to these agro-industry by-products. These residues, after processing, still contain a considerable amount of phenolic compounds that can be recovered by extraction and fractionation techniques, obtaining plant extracts with potential nutraceutical and functional characteristics (IGNAT; VOLF; POPA, 2011; GARCIA-CASTELLO et al., 2015). The extraction of bioactive compounds from agro-industrial residues has been reported using conventional and unconventional methods. Most conventional extraction methods use organic solvents and have some limitations, such as the use of toxic solvents (health-related risks), high temperatures, and long process times (degradation of compounds of interest) (AZMIR et al., 2013; GARCIA-CASTELLO et al., 2015).

Thus, studying extraction techniques with better selectivity and efficiency is necessary. Among these technologies, intensified processes based on the use of Microwave-Assisted Extraction (MAE), pressurized fluids as [Supercritical Fluid Extraction] (SFE), and Subcritical Water Extraction (SWE) are gaining attention for the extraction and recovery of bioactive

compounds from different natural matrices with green solvents (ANDRADE; TRIVELLIN; FERREIRA, 2017; ERŞAN *et al.*, 2018; MAZZUTTI *et al.*, 2017a). SFE has also demonstrated some advantages in the environmental field; for example, to reduces solvent waste, to get new useful compounds from industrial by-products, and to allow quantification and/or removal of toxic compounds from the environment (Herrero et al.,2010). These techniques represent an environmentally safe and economically viable process alternative.

In this context, it is worth highlighting that this thesis, belonging to the line of research in the area of sub/supercritical fluids of the Laboratory of Thermodynamics and Supercritical Technology (LATESC) of the Department of Chemical and Food Engineering at the Federal University of Santa Catarina, aimed to develop studies with a main focus on the extraction of bioactive compounds from biological matrices such as the use of waste generated by the fruit juice agroindustry in the Northeast of Brazil based on the principles of circular bioeconomy and environmentally friendly extraction techniques and by the use of green solvents.

1.2 OBJECTIVES

1.2.1Main objective

The general objective of this work is to study the use of agro-industrial residue from the production of pulp from soursop (*Annona muricata* L.), acerola (*Malpighia emarginata* DC), and strawberry (*Fragaria x ananassa*) fruits, evaluating and comparing the bioactive compounds present for possible use in the food and/or pharmaceutical areas.

1.2.2 Specific objectives

a) Obtain extracts from soursop seed, acerola, and strawberry residue using conventional (Soxhlet) and non-conventional (SFE, SWE, and MAE) extractions under different operational conditions.

b) Obtain extracts from soursop seed, acerola, and strawberry residue using combined extraction techniques under different operational conditions to increase the recovery of compounds of interest from the plant matrix.

c) Compare the global yields obtained by the different extraction methods.

d) Evaluate the quality of the extracts through *in vitro* analyses of total phenolic content, antioxidant activity, and chemical profile by high-performance liquid and gas chromatography;

e) Perform a screening test to assess *in vitro* antiproliferative assay against tumor cells.

CHAPTER 2 :

2 LITERATURE REVIEW

This chapter will present a brief literature review on the subjects relevant to understanding and contextualizing the work.

2.1 OVERVIEW OF TROPICAL FRUIT PROCESSING AND AGROINDUSTRIAL BY-PRODUCTS VALORIZATION

Tropical fruits are essential in developing countries, both from a nutritional and commercial trade point of view. An estimated 99% of tropical fruit production originates from low-income countries, mainly cultivated at subsistence rather than at the commercial level by smallholder farmers who typically are endowed with, or have access to, less than 5 ha of land. In 2018, FAO (2018) estimated that world production of tropical fruits will reach about 100 million tons, an increase of 3.3% compared to the previous year (VILLACÍS-CHIRIBOGA *et al.*, 2021). As such, tropical fruits contribute directly and significantly to food security and nutrition in most producing zones (OECD-FAO Agricultural Outlook 2020-2029, 2020).

Consumption of tropical fruits is growing worldwide and is expected to increase even more in the coming years with an estimated annual growth of about 3.8%, in part probably due to the recognition of their nutritional and health-promoting benefits as well as their interesting organoleptic properties (SAYAGO-AYERDI *et al.*, 2021).

Brazil's fruit farming business managed to export fruits worth more than one billion dollars in 2021, achieving the mark highly desired by the sector that produces the third biggest volume of fruits globally, behind only China and India (VIDAL, 2022) (VIDAL, 2021; KIST; CARVALHO; BELING, 2021, 2022). The country shipped 1.217 million tons of fruits abroad in 2021, up 20% from the previous year (KIST; CARVALHO; BELING, 2022).

A study conducted in 2021 by the Brazilian Confederation of Agriculture and Livestock (CNA) points to a total production of 41.3 million metric tons of fruits in Brazil, based on information provided by such organs as the Brazilian Institute of Geography and Statistics (IBGE), 2017 Census of Agriculture and Municipal Agricultural Production (PAM, in the Portuguese acronym) of 2020. The production value surpasses R$ 49.8 billion from fruit

farming, and the cultivated area surpasses 2.6 million hectares (KIST; CARVALHO; BELING, 2022).

In the Brazilian Northeast region, despite the water and soil restrictions of the semi-arid region, fruit growing is also of high economic and social importance. In 2021 and 2022, the region accounted for 34,4% of the value of national fruit production (VIDAL, 2021, 2022).

The Brazilian Northeast region is the leading fruit exporter in the Country, accounting for 149.631 thousand tons and US$ 113.630 million (KIST; CARVALHO; BELING, 2021).

According to the Agropecuary cense from the Brazilian Institute of Geography and Statistics – IBGE, 60,996 tons of acerola, 7,569 thousand soursop fruits, and 139,508 tons of strawberries were produced in 2017 in Brazil (IBGE, 2017).

Regarding the regional production of these fruits, the Northeast region produced 47,608 tons of acerola, 6,861 thousand soursop fruits, and 2,857 tons of strawberries in 2017 (IBGE, 2020).

However, about 50% of the tropical and subtropical worldwide fruit production is intended for the fresh fruit market, and 50% is used in numerous processed forms (desserts, nectars, compotes, marmalades, sauces, syrups, snacks, jellies, flours, and wines), the most important being tropical fruit beverages (SAYAGO-AYERDI *et al.*, 2021). Brazil follows this trend, offering 53% as fresh fruits, and 47% are destined for agro-industrial processing in juices, pulps, and other formulations (COSTA; SOUZA, 2018; LOUSADA JUNIOR *et al.*, 2005)

Agribusinesses constantly invest in increasing the processing capacity of (tropical) fruits, generating large amounts of by-products, such as peels, seeds, pulps after juice extraction, and bagasse (NGUYEN *et al.*, 2019). All this waste is generally released into landfills or burned for energy production leading to the loss of the remaining economic and biological value(CAMPOS *et al.*, 2020). On the other hand, these by-products are an excellent source of bioactive compounds, such as phenolic compounds (phenolic acid, carotenoids, flavonoids), bioactive proteins (peptide isolate, amino acids), fatty acids, fibres, and so on (PATRA; ABDULLAH; PRADHAN, 2022).

World hunger increased in 2020 under the shadow of the COVID-19 pandemic. Despite the general concept of global food security, 928 million people across the globe suffer from hunger, representing 12 % of the global population. At the same time, 1.5 billion people are affected by one or more forms of malnutrition, i.g. micronutrient deficiency (FAO, 2021). To manage the nutritional problems of today's society, we require more composite nutritional sources.

The United Nations created the 17 sustainable development goals (SDGs) to approach the problems to protect the planet, end poverty, and ensure prosperity for all humanity. In order to effectively address the Zero Hunger (SDG 2) goal, global food production will have to increase by at least 60% by 2050 (CAMPOS *et al.*, 2020). Also, agriculture is dependent, connected, or essential to improving other SDGs, especially when practised sustainably. Hence, we can highlight the Responsible Consumption and Production (SDG 12) goal related to agriculture-friendly practices that contribute to the efficient management of natural resources (e.g., soil and water) and reduce food waste and waste production (VIANA *et al.*, 2022).

Therefore, the abovementioned fact, combined with the incessant demand for tropical fruits, implies vast amounts of agro-industrial food losses and waste, an environmental hazard that should be treated and managed appropriately (CÁDIZ-GURREA *et al.*, 2020).

Accordingly, these agri-food by-products can be valorized in an integrated way under the application of downstream processes by the industries, transforming the waste into secondary raw materials. Moreover, fruits and vegetables contain unreplaceable nutritional characteristics, containing several bioactive compounds, making them a potential ingredient in nutraceutical and pharmaceutical products with positive effects on human health (CAMPOS *et al.*, 2020; VILLACÍS-CHIRIBOGA *et al.*, 2021). This approach aligns with the circular economy concept that considers an integrated supply chain, where a by-product from one industry can become a resource for another (PAINI *et al.*, 2022).

2.2 ACEROLA, SOURSOP, AND STRAWBERRY-FRUIT PULP PROCESSING AND BY-PRODUCT VALORIZATION

2.2.1 Soursop (*Annona muricata* L.)

Annonaceae represents a generic name to designate the plants of the Annonaceae family, consisting of about 120 genera and around 2,300 species. In Brazil, 29 genera are registered, among which about 260 species, some of which are economically important. Among the most commercially important species of this family are soursop (*Annona muricata* L.), pine cone (*Annona squamosa* L.), cherimoya (*Annona cherimoya*, Mill.), and atemoya, a hybrid between *A. cherimoya* and *A. squamosa*, as shown in **figure 1.**

Figure 1- *Annonas* varieties with economic value

Annona muricata L | *Annona squamosa* L | *Annona cherimoia,* Mill | *Atemóia (A. cherimoia e A. squamosa.)*

Source: https://www.repletodevida.pt/wp-content/uploads/2019/03/graviola-png-2-1-300x156.png

Soursop is part of a group of fruit trees of economic importance in several countries, such as Venezuela, Colombia, Puerto Rico, Costa Rica, Mexico, Panama, Jamaica, Cuba, Spain, India, Honduras, Guyana, Suriname, Brazil, Peru, Senegal, and Singapore (SANUSI; FADZELLY; BAKAR, 2018). The primary soursop producer countries are Brazil, Venezuela, and Colombia (PINTO *et al.*, 2018).

Soursop is a green tropical fruit with a prickly rind and a sweet, white, juicy, aromatic, and slightly acidic pulp. The seeds are entirely covered by the pulp and, together with the husks, are often discarded (JOSÉ *et al.*, 2014; SILVA, 2016).

In Brazil, soursop was introduced by the Portuguese in the 16th century, with the states of Bahia and São Paulo being the largest producers. The soursop fruit reaches more than five kilos. It is usually sold in fresh fruit, pulp, or frozen and is also used to prepare juices, jellies, yoghurts, ice creams, and other food purposes(JOSÉ *et al.*, 2014; SAWANT; DONGRE, 2013). **Table 1** shows the proximate composition of the soursop seed.

Soursop is also reported to have good antioxidant activity, but its seeds have not been studied much for food purposes or oil extraction (MENEZES *et al.*, 2019; MOGHADAMTOUSI *et al.*, 2015; ONIMAWO, 2002; PINTO *et al.*, 2018). Moreover, little attention has been paid to the composition and physical and chemical properties that make it potentially attractive in the food sector. (SOLÍS-FUENTES *et al.*, 2010; SOLÍS-FUENTES; HERNÁNDEZ-MEDEL; DURÁN-DE-BAZÚA, 2011b). **Table 1** shows the proximate composition of the soursop seed.

The fatty acid composition in soursop seeds is predominantly composed of oleic acids (43%), linoleic acid (29.7%), palmitic acid (19.4%), and in a smaller proportion, we can find

stearic acids, palmitoleic, linolenic and dodecanoic (MENEZES *et al.*, 2019; PINTO *et al.*, 2018; SILVA; JORGE, 2014). Soursop fruit (pulp), leaves, and seeds have been recognized as sources of valuable bioactive compounds such as polyphenols, alkaloids, flavonoids, carbohydrates, cardio glycosides, saponins, tannins, phytosterol, terpenoids, proteins, acetogenins (AGUILAR-HERNÁNDEZ *et al.*, 2019; CORIA-TÉLLEZ *et al.*, 2018; GAVAMUKULYA *et al.*, 2014; JIMÉNEZ *et al.*, 2014; ORAK; BAHRISEFIT; SABUDAK, 2019; RAYBAUDI-MASSILIA *et al.*, 2014).

Table 1-Proximate composition of soursop seeds (*A.muricata* L).

	Valores (%)	Referências
Moisture	8.5 - 34.6	Solís-fuentes; Durán-de-Bazúa (2010)
Protein	2.4 - 27.3	Fasakin et al. (2008); Solís-fuentes et al. (2010); Agu; okolie (2017)
Carbohydrate	11-15	Pinto et al. (2018)
Fibers	8.0 - 43.4	Onimawo (2002); Fasakin et al. (2008); Pinto et al. (2018)
Ashes	2.3 - 13.6	Fasakin et al., (2008); Solís-fuentes et al.(2010)
Lipides	20.5 - 37.7	Solís-fuentes et al.(2010)

Source: Adapted from SOLÍS-FUENTES; HERNÁNDEZ-MEDEL; DURÁN-DE-BAZÚA (2011)

Soursop (*Annona muricata*) is widely used in traditional medicine in South America and the Caribbean and has become a nutritional supplement with potential medicinal properties. Different parts of the plant (leaf, bark, root, fruit, and seed) are used against various diseases, including hypertension, inflammation, diabetes, gastrointestinal disorders, respiratory diseases (asthma and bronchitis), intestinal parasites, lice, insects (BENITES *et al.*, 2015; CORIA-TÉLLEZ *et al.*, 2018; RADY *et al.*, 2018) and numerous studies on the anticarcinogenic properties of soursop have been found (RAYBAUDI-MASSILIA et al., 2014; ENDRINI; SUHRMAN; WIDOWATI, 2015; MOGHADAMTOUSI et al., 2015; PATEL; PATEL, 2016; CORIA-TÉLLEZ et al., 2018).

Soursop is widely used in the fruit pulp agro-industry, and the residue results in many seeds; a fruit can contain more than 200 seeds (3–8.5%), peels (7–20%) and loss corresponding to 30% of the pulp which is discarded and not used commercially (PINTO *et al.*, 2018; SANTOS *et al.*, 2023; SAWANT; DONGRE, 2013; SILVA, 2007) Soursop seeds could be a

source of biological compounds and pharmaceutical ingredients, so creating alternatives to reduce these residues' quantity is highly relevant.

2.2.2 Acerola (*Malpighia emarginata DC*)

Acerola, also known as Antilles cherry or Barbados cherry, is originally from the Antilles and belongs to the *Malpighiaceae* family and *Malpighia* genus; it is described in the literature as originating from two species, *Malpighia punicifolia,* and *Malpighia glabra*, but the scientific nomenclature currently accepted by taxonomists is *Malpighia emarginata* DC (ALVAREZ-SUAREZ *et al.*, 2017; GARCIA, 2016; PRAKASH; BASKARAN, 2018).

The planting of acerola trees was encouraged due to the high content of vitamin C, and under this incentive, the commercial planting of acerola trees began in Puerto Rico, later expanding to Cuba, Florida, and Hawaii (MENDES *et al.*, 2012).

In Brazil, large plantations began in 1949, coming from Puerto Rico(MENDES *et al.*, 2012). It is a rustic and resistant plant, spreading quickly in several regions with different climatic conditions (GONÇALVES *et al.*, 2019; MEZADRI *et al.*, 2008; PRAKASH; BASKARAN, 2018).

Acerola has a succulent pulp and protective rind, which, when ripe, changes color from green to reddish-yellow and finally to red or purple when fully ripened, depending on the variety (LEMOS *et al.*, 2019; MALEGORI *et al.*, 2017).

Acerola is considered a super fruit due to its high content of vitamin C (700 to 1400 mg/100 g^{-1}), high amount of nutrients such as β-carotene, and B vitamins such as thiamine (B1), riboflavin (B2), and niacin (B3), and minerals such as Calcium (Ca), Iron (Fe) and Phosphorus (P) (MOTOHASHI *et al.*, 2004a; RITZINGER; RITZINGER, 2011), a wide range of bioactive phytochemicals, such as phenolic acids, flavonoids, phytoestrogens, and carotenoids, among others, in addition to antioxidant activities(CHANG; ALASALVAR; SHAHIDI, 2019; MALEGORI *et al.*, 2017). Figure 2 shows the acerola tree, fruit, and seed.

Figure 2- Acerola tree, fruit, and seed



Source:https://tirolplantas.com/wp-content/uploads/2020/01/an463001089-1.jpg; https://www.healthbenefitstimes.com/9/gallery/acerola-cherry/Seeds-of-Acerola-cherry.jpg

Due to the aforementioned high concentration of bioactive compounds, acerola is a good option for developing functional foods due to its promising effects on human health (ALVAREZ-SUAREZ *et al.*, 2017; BELWAL *et al.*, 2018a, 2018b).

Table 2 shows the physicochemical properties of the residue (peel+seed) and edible portion of acerola.

Table 2- Physicochemical properties of the residue (peel + seed) and edible portion of acerola *M.emarginata* DC

	Residue (peels+seeds)	Edible portion
Total Soluble solids (◦Brix)	3.00 ± 0.04	9.20 ± 0.18
Acidity (g/100 g)	3.60 ± 0.40	34.40 ± 0.70
Protein (g/100 g)	0.00	0.40 ± 0.01
Reducing sugars (g/L)	15.29 ± 0.10	102.5
Moisture content (g/100 g)	4.90 ± 0.35	80,84±1,31
Ashes (g/100 g)	2.07 ± 0.07	0.20 ± 0.01
Lipidis (g/100 g)	2.92 ± 0.03	0.30 ± 0.01

Source: adapted de SANCHO et al., 2015 e U.S. DEPARTMENT OF AGRICULTURE, 2019.

Acerola is usually industrialized to produce juices, jellies, and pulps (MEZADRI et al., 2008), also reaching markets such as the dietary supplement and pharmaceutical industries, with its global market expected to reach U$ 17.5 billion by 2026 (BELWAL *et al.*, 2018a).

About 40% of the processed volume is residue from the pulping step, commonly consisting of residues, peels, seeds, and bagasse rejected (BORTOLOTTI *et al.*, 2013; NOGUEIRA *et al.*, 2019; SILVA *et al.*, 2020a). These residues are generally used in animal feed and organic fertilizers or discarded, causing environmental impact and energy loss (MALUCELLI *et al.*, 2017; SANCHO *et al.*, 2015).

According to Freitas et al. (2006), the Brazilian industry uses 34.4 thousand tons of acerola (7.16% of the whole fruit) and produces 18.0 thousand tons of juice and pulp (52.3%) and 16.4 thousand tons of waste (47.7%), with this production concentrated in the northeast region.

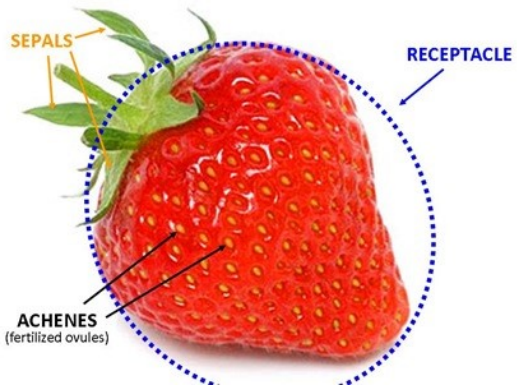
The recovery of fruit residues for use in food, cosmetics, and the pharmaceutical industry can be an essential alternative for sustainable development, as these residues can be sources of vitamins, carotenoids, polyphenols; and in addition, finding sustainable processes brings economic benefits (DUZZIONI *et al.*, 2013; NOGUEIRA *et al.*, 2019).

2.2.3 Strawberry (*Fragaria spp*)

Strawberry belongs to the *Rosaceae* family and Fragaria genus. The European wild strawberry originates from *Fragaria vesca* L. The cultivated varieties (*F. ananassa*) are F. chilosensis and F. Virginiana hybrids. The *F. ananassa* variety is cultivated worldwide; it is well adapted to temperate climates and plays an essential economic role (MÉNAGER; JOST; AUBERT, 2004).

The fruits grow on stems grouped in threes and are picked by hand at full maturity. They are called achenes by botanists, the edible part being a structure consisting of a fleshy red receptacle (OLIVA; OLIVA, 2018; SINHA, 2006). Figure 3 shows the anatomy of the strawberry (*Fragaria spp)*.

Figure 3- Strawberry (*Fragraria spp*) anatomy



Source: https://theses.hal.science/tel-01066753v1/document

Strawberry (*Fragaria spp*) is one of the most popular and appreciated fruits globally; it is considered a functional food mainly due to its biological activities and potential health

benefits (GIAMPIERI; ALVAREZ-SUAREZ; BATTINO, 2014; SKROVANKOVA *et al.*, 2015). This property is directly related to its phytochemical composition, consisting of bioactive and antioxidants, such as phenolic compounds, flavonoids, anthocyanins, vitamin C, K, and E, carotenoids, folic acid, potassium, manganese, iodine, omega 3 fatty acids, magnesium, and copper (CAO *et al.*, 2012; DIAS *et al.*, 2017; FELIX *et al.*, 2018; YANG *et al.*, 2016). Its consumption has been associated with preventing cardiovascular diseases, anti-inflammatory action, cancer prevention, reduction of oxidative stress, obesity, and diabetes (CHAVES; CALVETE; REGINATTO, 2017; GIAMPIERI; ALVAREZ-SUAREZ; BATTINO, 2014; THOMAS-VALDÉS *et al.*, 2018).

Ripe strawberries are consumed as fresh fruit but are also often found in processed products such as alcoholic beverages, liqueurs, syrup, jam, juice, pulp, ice cream, confectionery, concentrated flavor preparations, and agro-industrial processing (MÉNAGER; JOST; AUBERT, 2004). **Table 3** shows the strawberry's physicochemical properties and chemical composition (g/100g wet basis) (*Fragaria spp*).

Table 3 - Physicochemical properties and chemical composition (g/100g wet basis) of strawberry (*Fragaria spp*).

	Fresh strawberry	Strawberry residue
Total soluble solids (◦Brix)	10.50 ± 0.50	13.6 ± 5.1
Acidity(g ác.cítrico/100g)	0.86 ± 0.10	0.96 ± 0.08
Dietetic fiber (g/100g)	1.31 ± 0.18	60.0 ± 1.6
Proteins (g/100 g)	0.50 ± 0.02	17.3 ± 0.6
Carbohydrates (g/100g)	6.30 ± 0.13	13.6 ± 5.1
Moisture content (g/100 g)	92.68 ± 0.17	88,00
Ashes (g/100 g)	0.27 ± 0.0	5.7 ± 0.8
Lipidis (g/100 g)	0.25 ± 0.02	9.8 ± 0.5

Fonte: Adaptado de SÓJKA et al., 2013; DE SOUZA et al., 2014; VARGAS, 2015; AMARIZ et al., 2018

Processing of strawberries, in some cases, generates by-products; for instance, the residues (pomace) in juice production constitute approximately 4–11% of fruit weight (PUKALSKIENĖ *et al.*, 2021).

Strawberry pomace, consisting of achenes (seeds), stalks, and pulp, contains valuable nutrients such as phenolic compounds (anthocyanins, proanthocyanidins, ellagic and other

phenolic acids, ellagitannins), minerals, dietary fiber, and others (SAPONJAC *et al.*, 2015) and can be reused as a source of high-value substances and be added to products or transformed into products such as biofuels, functional food ingredients, pigments, flavors, animal feed or bio-adsorbents for wastewater treatment in good agreement on circular economy.

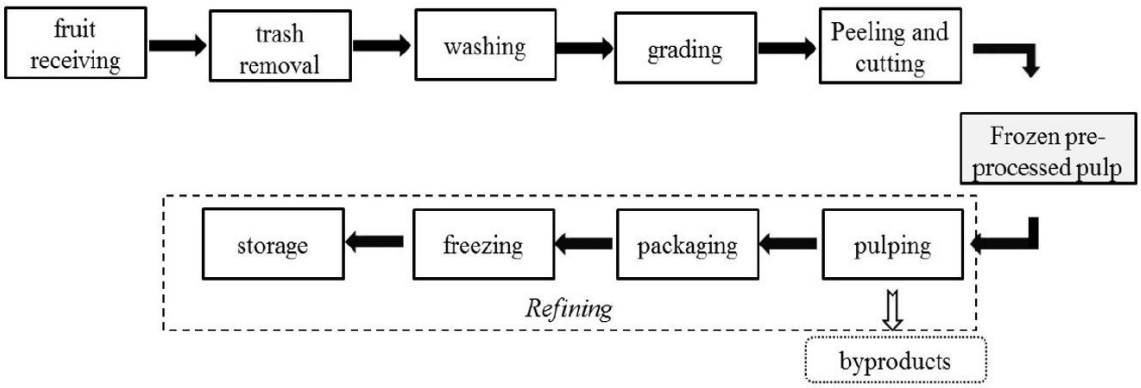
2.2.4 Processing technology and by-product valorization

Tropical and subtropical fruit production is used in numerous processed forms (desserts, nectars, compotes, marmalades, sauces, syrups, snacks, jellies, flours, and wines), the most important being tropical fruit beverages (MAIA *et al.*, 2019).

Nutrient-rich tropical fruit juices are gaining acceptance owing to consumers' desire for healthy products while experiencing new appealing sensorial features besides their undisputable nutritional value related to health-promoting activities (SAYAGO-AYERDI *et al.*, 2021).

The production of frozen fruit pulps has been highlighted as an essential alternative for using the fruits during the harvest, allowing the storage of the pulps for the time of production of the fruits *in nature* (SANTOS; COELHO; CARREIRO, 2008). **Figure 4** shows the frozen pulp processing flowchart.

Figure 4-General flowchart of frozen pulp processing



Source: (AMARIZ; LIMA; ALVES, 2018)

Fruit processing renders high amounts of byproducts, which lead to losses of biomass, nutrients and energy, and as a secondary side effect, they enhance environmental problems due to the great volume of residues inadequately stored, leading to soil and water pollution from the

leaching organic compounds, resulting also in public health problems (AMARIZ; LIMA; ALVES, 2018).

Depending on the species, tropical fruit's by-products can encompass a substantial amount of peel (10%–66%) and seed (1%–22%), and pulp leftovers, which are usually disposed of as wastes (SOUSA et al., 2011; SOUZA, 2015; ALVAREZ-RIVERA et al., 2020). Likewise, acerola, soursop, and strawberry agro-industrial processing into juices/pulps correspond to 20% to 70% of the waste volume (LOUSADA JUNIOR *et al.*, 2005; MARQUES; PRADO; FREIRE, 2009).

On the other hand, agro-industrial residue from pulp and fruit juice processing can be a source of valuable compounds that are often underestimated and underutilized (KUMAR *et al.*, 2017; SAGAR *et al.*, 2018). However, the latest studies on the chemical composition of tropical fruits by-products underlined the significant quantities of bioactive compounds present in non-edible fractions, even in a greater extender than edible ones, which are usually discarded (CÁDIZ-GURREA et al., 2020).

Therefore, agro-industrial by-products from tropical fruits are regarded as promising sources of valuable chemical and nutritional compounds, mainly pectin, proteins, fatty acids, polysaccharides, carbohydrates, fiber, vitamins, minerals, pigments, essential oils, enzymes, organic acids, antioxidants, and phenolic compounds, among other phytochemicals with acknowledged bioactive properties (SAYAGO-AYERDI *et al.*, 2021; VILLACÍS-CHIRIBOGA *et al.*, 2021). Fruit processing by-products can be sources of nutrients and bioactive compounds with potential health-promoting effects, reducing the risk of cardiovascular disease and cancer through its antioxidant and anti-inflammatory activity, which reduces oxidative stress (ABUD; NARAIN, 2010; SILVA et al., 2014; STRUCK et al., 2016; PEREIRA-NETTO, 2018).

Therefore, due to their proven health-promoting activities, extracting phytochemicals from tropical fruit by-products with claimed bioactive properties is an excellent practice to be exploited in food supplements, functional food, nutraceutical, cosmeceutical, pharmaceutical, and biofuel industries (CAMPOS et al., 2020).

Industrial strategies have aimed at revalorizing by-products to recover value-added substances that could be exploited for further use. This practice agrees with one of the leading sustainable development goals of the United Nations, which encourages responsible consumption and production (CÁDIZ-GURREA *et al.*, 2020).

The extraction process is the most crucial step for recovering these compounds. This process can be treated from different approaches: conventional extraction techniques, such as maceration (solid-liquid extraction) and Soxhlet extraction, and non-conventional techniques, such as supercritical fluid extraction (SFE), Microwave-assisted extraction (MAE), and subcritical water extraction (SWE)(GARCÍA-VILLEGAS *et al.*, 2022).

Green technologies have become a critical system for isolating valuable compounds from food by-products, focusing on a high extraction efficiency while minimizing the coextraction of unwanted materials (VILLACÍS-CHIRIBOGA *et al.*, 2021).

The recovery of bioactive compounds with promoting-health properties from agro-food by-products is an emerging trend in food science, which has proved to be a powerful alternative for the valorization of agri-food industrial processing generated wastes (ALVAREZ-RIVERA *et al.*, 2020).

In recent years, significant efforts have been focused on the phytochemical profiling of promising exotic fruit by-products or components, although some information can be found in the literature. Comprehensive food composition analysis demands analytical tools capable of characterizing volatile and non-volatile compounds in complex food matrices. Chromatographic analysis coupled to ultra-violet spectroscopy (UV), mass spectrometry (MS), or high-resolution mass spectrometry (HRMS) detectors, either via liquid chromatography (LC) or gas chromatography (GC), has been mainly used (CONG-CONG *et al.*, 2017; KHODDAMI; WILKES; ROBERTS, 2013).

Reusing by-products and putting them back into the supply chain as new up-grade products endorse optimizing natural resources, promoting a closed-loop processing and system moving from a linear economy to a circular economy, enabling the entire advance of by-products utilization to generate novel and safe value-added products.

Consequently, revalorizing these underutilized matrices has been cutting-edge research in the last years, and it is one of the main challenges addressed by the food industry (CÁDIZ-GURREA *et al.*, 2020).

2.3 EXTRACTION METHODS

The extraction of bioactive compounds from plant tissues is a crucial step (BELWAL *et al.*, 2018a). These compounds must be separated and recovered without affecting their

physicochemical properties, as they are valuable for nutraceutical and pharmaceutical fields (YAHYA; ATTAN; WAHAB, 2018).

Traditionally, phytonutrient extraction is done by conventional methods using prolonged heating, such as Soxhlet extraction. To improve the efficiency of extracting antioxidant components from plant materials, several unconventional green methods have been developed to reduce operating time and use of organic solvents, such as supercritical fluid extraction (SFE), pressurized liquid extraction (SWE), and microwave-assisted extraction (MAE) (XU et al., 2017; BELWAL et al., 2020).

2.3.1 Soxhlet extraction (SOX)

The Soxhlet extractor developed by chemist Franz Ritter Von Soxhlet (1879), designed primarily for lipid extraction, has been used to extract valuable bioactive compounds from natural sources. Sort and Soxhlet is a standard technique used as a reference compared to newly developed methodologies (AZMIR *et al.*, 2013; LÓPEZ-BASCÓN; CASTRO, 2020).

The Soxhlet extraction method is a classic technique. It has the advantage of being easy to operate, requiring no professional expertise (1) and a smaller amount of solvent than maceration (2). The sample is repeatedly brought into contact with fresh portions of the solvent, shifting the mass transfer equilibrium (3). There is no need for filtration to separate the raw material from the solvent (4), and the sample yield can be increased by simultaneous parallel extraction because the basic equipment is cheap (5) also conventional SOX extraction using ethanol and hexane as polar and nonpolar solvents, respectively, was performed to afford results for TPC (CASTRO; PRIEGO-CAPOTE, 2010; LÓPEZ-BASCÓN; CASTRO, 2020; SUWAL; MARCINIAK, 2018).

Despite being widely used, the technique has disadvantages compared to other conventional techniques for preparing solid samples: (1) the long time required for extraction, exposure to liquid organic solvents hazardous and flammable, with potential toxic emissions during extraction; (two) samples are generally extracted at the boiling point of the extractor, and the possibility of thermal decomposition of thermolabile compounds cannot be ignored; (3) the conventional Soxhlet extractor is unable to provide agitation, which would speed up the step; (4) due to a large amount of solvent used, and evaporation/concentration step after extraction is mandatory; 5) the technique is restricted to solvent selectivity and is not easily automated. In addition, (AZWANIDA, 2015; CASTRO; PRIEGO-CAPOTE, 2010; CHEMAT

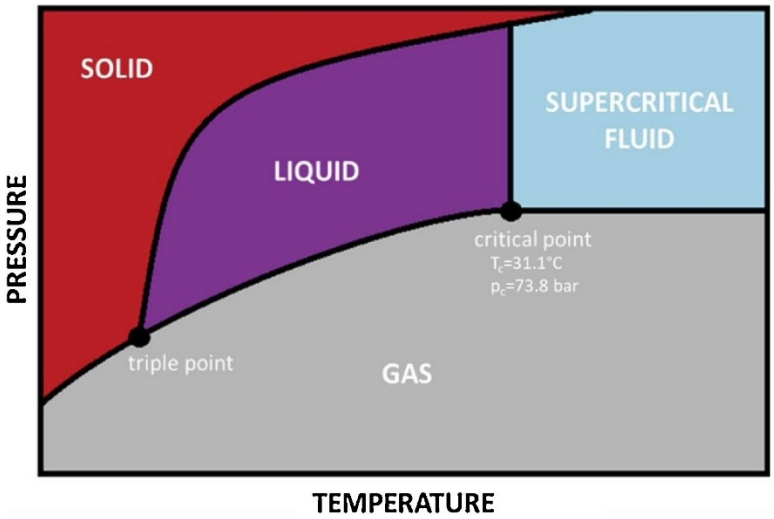
et al., 2020). In addition, soxhlet extraction can be used to extract compounds of various polarities, subject to the choice of solvent. For this reason, this technique was chosen to be a standard for comparison with the others, simply by changing the extraction solvent (CASTRO; PRIEGO-CAPOTE, 2010; RODRIGUES *et al.*, 2019).

2.3.2 Supercritical fluid extraction (SFE)

Supercritical fluid extraction (SFE) has been widely used to extract active compounds from plant matrices, especially food ingredients, supplements, and plant protection products. It is an environmentally friendly and efficient technique (PEREIRA; MEIRELES, 2010). The supercritical state is reached when the temperature and pressure are increased above their critical value, as shown in Figure 5, which presents the phase diagram for CO_2. The supercritical fluid has characteristics of gases and liquids. Compared to liquid solvents, they have several advantages, such as (1) the dissolution power of a supercritical solvent depends on its density, which is highly adjustable by changing pressure or/and temperature; (2) the supercritical fluid has a higher diffusion coefficient, lower viscosity and surface tension than a liquid solvent, leading to more favorable mass transfer (SUWAL; MARCINIAK, 2018; WANG; WELLER, 2006).

Supercritical CO_2 can be considered an ideal supercritical fluid thanks to its low critical constant (TC=31.1ºC, Pc=7.38MPa), inert nature, non-toxic, non-explosive, inexpensive, readily available, easy removal, and suitable extraction capacity due to its greater penetration power, being supercritical CO_2 an excellent solvent for the extraction of nonpolar analytes. Furthermore, CO_2 is also Generally Recognized as a Safe (GRAS) solvent and leaves no residue after extraction simply by depressurization at room temperature (CHEMAT *et al.*, 2019).

Figure 5 – Phase diagram of a pure substance.



Source: (ŽELJKO KNEZ *et al.*, 2019)

However, due to its low polarity, supercritical CO_2 is a nonpolar solvent and is less effective for extracting polar compounds from plant matrices. Therefore, in low concentrations, other solvents (e.g., methanol and ethanol) can be used as modifiers, known as co-solvents, promoting changes in solvent properties and increasing extraction efficiency (CHEMAT *et al.*, 2019; SUWAL; MARCINIAK, 2018). The other disadvantage of this method is the device's high primary cost and inability to handle it (AZWANIDA, 2015; PANNU et al., 2018).

Several compounds can be used as supercritical fluids, although carbon dioxide is the most commonly used solvent, as listed in **Table 4**, which shows the physicochemical properties of some supercritical fluids.

Table 4- Physicochemical properties of some solvents used in supercritical extraction

Supercritical fluids	Critical Temperature (T_c) C°	Critical Pressure(P_c) (bar)	Crítical Density ρ(g/ml)
Carbon Dioxide	31,2	73,9	0,470
Etano	32,4	48,8	0,200
Eteno	10,1	51,2	0,200
Methanol	-34,4	80,9	0,272
n-Pentano	−76.5	33.3	0.237
Water	101.1	217.6	0.322

Source: Adapted from (SILVA; ROCHA-SANTOS; DUARTE, 2016)

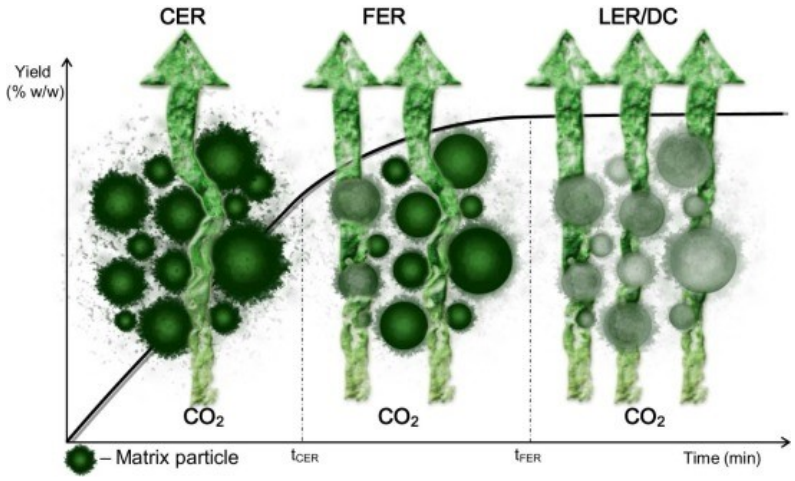
Supercritical extraction occurs in two stages: the solubilization of chemical compounds in the solid matrix and their separation in the supercritical solvent. The solvent flows through the packed bed during extraction, solubilizing the compounds present in the matrix. After the solvent leaves the extractor that transports the solubilized compounds, the extract becomes solvent-free by pressure, reduction, and/or temperature increase (SILVA; ROCHA-SANTOS; DUARTE, 2016).

The solvating power of the supercritical fluid is directly related to pressure and temperature; in addition, the processing time is usually one of the main issues when choosing extraction parameters. Given this, knowing the best conditions involves the study of the global yield and/or solubility and kinetic parameters (PEREIRA; MEIRELES, 2010).

Global yield isotherms provide a framework for analyzing the effects of temperature and pressure on extraction. The SFE kinetics can be represented by the global extraction curve, which is determined by considering the mass of solute extracted as a function of process time or solvent consumed (HERRERO *et al.*, 2013; PEREIRA; MEIRELES, 2010).

The extraction curves can be divided into three periods, controlled by different mass transfer mechanisms (MENDIOLA *et al.*, 2013; MEZZOMO; MARTÍNEZ; FERREIRA, 2009; SOVOVÁ, 1994), as shown in Figure 6.

Figure 6- The three different extraction periods



Source: (SILVA; ROCHA-SANTOS; DUARTE, 2016)

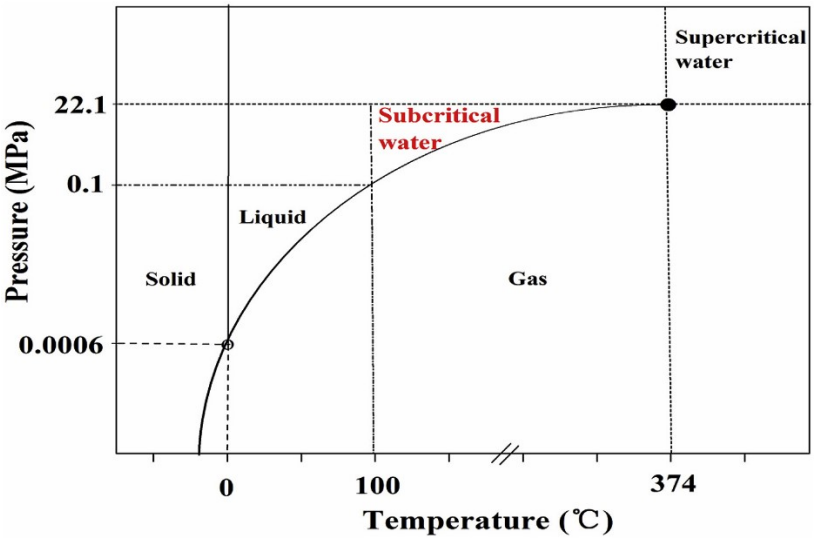
CER is the period of Constant Extraction Rate at the initial stage of the curve when the solute is easily accessible to the outer surface of the particles, and convection is the dominant mass transfer mechanism. Subsequently, FER (Falling Extraction Rate) is when the extraction rate drops, when the diffusion mechanism starts, operating in conjunction with convection, due to faults in the outer solute layer of the surface. In the end, LER/DC is the period of Low Extraction Rate (LER) or Controlled by Diffusion (DC), when the mass transfer occurs mainly by diffusion in the bed and inside the solid particles, as the outer layer of solute disappears, leading to a low extraction rate at the end of the curve.

Industrial activities generate a wide variety of by-products that generally have no commercial value. Various matrices (seeds, fruits, leaves, flowers, rhizomes, roots, fruit peels, and tree branches) can be used to extract high-value compounds and provide enormous benefits from an environmental and economic point of view. In this sense, SFE has been widely used to add value to agricultural and food by-products(SILVA; ROCHA-SANTOS; DUARTE, 2016; XU *et al.*, 2017).

2.3.3 Subcritical water extraction (SWE)

Subcritical water extraction (SWE), also known as extraction by superheated water or Pressurized Hot Water Extraction (PHWE), is an eco-friendly process by which water is kept in its liquid state at using high temperature, that is hot water above boiling point to high temperature-pressure conditions just below its critical point (374 °C, 1 to 22.1 MPa) (GIL-CHÁVEZ *et al.*, 2013; HERRERO; CIFUENTES; IBAÑEZ, 2006; ZHANG *et al.*, 2020). **Figure 7** shows the physical state of water at different temperatures and pressure.

Figure 7- Physical state of water at different temperatures and pressure.



Source: (ZHANG *et al.*, 2020)

The temperature applied during the subcritical water extraction process has an important impact on extraction efficiency and selectivity. As a result, physical advantages are achieved, including high diffusion, low viscosity, and low surface tension. In addition, increases in steam pressures and rapid thermal desorption of target compounds from the matrices can also increase extraction efficiency (ZAKARIA; KAMAL, 2016). Especially under high temperatures and high pressure, subcritical water can change solvents' polarity and dielectric constant, contributing to better extraction, improving the mass transfer efficiency of extracts, and maintaining its biological activities with a high application perspective (ZHANG *et al.*, 2020).

The following five steps can generally describe the process of subcritical water extraction of solid and semi-solid samples (GILBERT-LÓPEZ *et al.*, 2017; KNEZ *et al.*, 2018; ZHANG *et al.*, 2020): 1) Humidification of the solid matrix in the extractor; 2) Initial desorption of analytes from the matrix (including breaking of chemical bonds); 3) Solving the analyte in the extraction solvent; 4) Diffusion of the analyte out of the matrix; and 5) Diffusion through the thick solvent layer around the matrix.

The extraction involves thermodynamic and kinetic aspects. Thus, three interrelated factors influence extraction efficiency: solubility, mass transfer, and matrix effect. Different parameters limited the distinct steps of SWE, such as the selection of temperature, pressure, time, and flow, among others, used in the extraction (GILBERT-LÓPEZ *et al.*, 2017).

The advantages of using subcritical water extraction over conventional methods are associated with the choice of solvent. Water is an ecologically correct solvent, as it is non-toxic, non-flammable, does not contribute to the greenhouse effect, is cheap, readily available, and does not generate waste or by-products. Water is less corrosive, requiring much lower temperature and pressure than supercritical water extraction. In addition, SWE adopts relatively less sophisticated technology, requiring much lower engineering costs for equipment. SWE extraction can be easily controlled to achieve selective extraction of polar, moderately polar, and nonpolar organics by adjusting the dielectric constant of water using extraction parameters such as temperature, pressure, and co-solvent (CASTRO-PUYANA; MARINA; PLAZA, 2017; KNEZ *et al.*, 2018; YAHYA; ATTAN; WAHAB, 2018). Large amounts of bioactive ingredients such as polysaccharides, proteins, antioxidants, and polyphenols can be extracted using subcritical water (HERRERO; CIFUENTES; IBAÑEZ, 2006; ZHANG *et al.*, 2020).

As a disadvantage of SWE, high temperatures can degrade thermolabile compounds, Maillard reaction, and caramelization can occur, which are not always desirable, and toxic compounds form. Another unfavorable issue is removing water from extracts, which requires additional procedures and energy costs, including evaporation, chemical dehydration or precipitation (CASTRO-PUYANA; MARINA; PLAZA, 2017; GBASHI *et al.*, 2017a).

2.3.4 Microwave-assisted extraction (MAE)

Microwave-assisted extraction (MAE) is one of the most advanced extraction techniques. Microwaves (MW) are non-ionizing electromagnetic radiation from 300 MHz to 300 GHz (VERNÉS; VIAN; CHEMAT, 2020). The technique is efficient because it can heat the matrix internally and externally without a thermal gradient. Resulting in greater extraction efficiency and preserving natural bioactive compounds as less energy and less solvent volume are required. Polyphenols and ionic solutions are composed of molecules with a permanent dipole moment and can strongly absorb microwave energy. They resulted in a rapid rise in temperature and rapid completion of the reaction (GIL-CHÁVEZ et al., 2013; YAHYA; ATTAN; WAHAB, 2018).

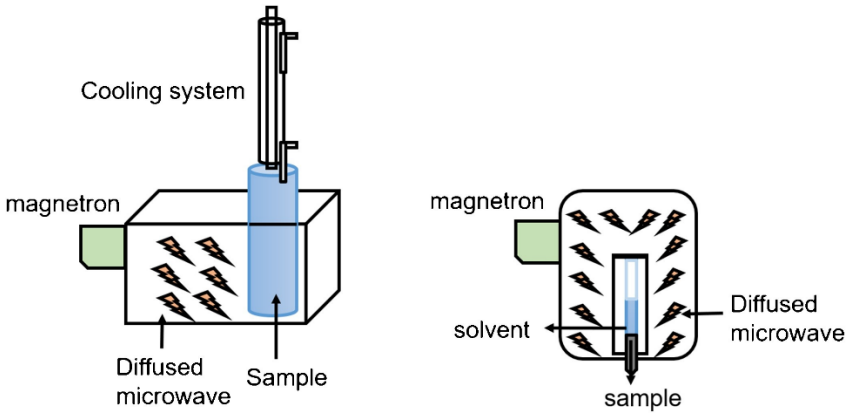
Microwave heating is based on two principles: ionic conduction and dipole rotation (VERNÉS; VIAN; CHEMAT, 2020). Dipole molecules are sensitive to the alternating electric field generated by microwaves, which continually change their direction, causing the molecules to spin rapidly (4.9 × 109 times per second) to align their dipole with that of the electric field.

Ionic conduction refers to the electrophoretic migration of ions in a solution under an electromagnetic field. This displacement is responsible for the friction between the moving ions and the medium, producing heat (MORET *et al.*, 2019).

Unlike traditional heating sources, which require some time to heat the sample core (heat transfer occurs by conduction and/or convection), microwaves act on the entire sample volume (if the medium is homogeneous) or on centres localized heating elements made up of polar molecules contained in the product, allowing rapid heating while keeping the temperature gradient low(MORET *et al.*, 2019). Therefore, microwave energy's effect strongly depends on the dielectric susceptibility of the solvent and the solid matrix of the plant (CHEMAT *et al.*, 2020; LLOMPART *et al.*, 2018; VERNÉS; VIAN; CHEMAT, 2020).

There are two types of Microwave-Assisted Extraction (MAE) systems available on the market: closed (multimode) extraction systems, which operate under controlled pressure and temperature, and open (single-mode) extraction systems, which operate at atmospheric pressure (CHATURVEDI, 2018), as shown in Figure 8, below.

Figure 8-Schematic diagram of open (a) and closed (b) MAE system.



Source: YAHYA; ATTAN; WAHAB (2018)

In multimode systems, microwave radiation is randomly scattered in a cavity, allowing the sample to be irradiated uniformly, while in single-mode systems, microwave radiation is focused in a restricted zone where the sample is more intensely irradiated than in the multimode system(VERNÉS; VIAN; CHEMAT, 2020; WANG; WELLER, 2006).

MAE extraction is considered a robust technique due to the shorter extraction time, smaller volumes of solvent, and higher extraction yields. However, MAE has some disadvantages, including the need for processes such as filtration or centrifugation to remove

solid waste, unlike SFE. In addition, constraints regarding the recovery of nonpolar compounds and elucidation changes in the chemical structure of target compounds must be considered for industrial applications (GIL-CHÁVEZ *et al.*, 2013; YAHYA; ATTAN; WAHAB, 2018).

2.4 CONSIDERATIONS OF THE STATE-OF-THE-ART

The global market for bioactive ingredients will reach US$ 45.5 billion in 2022. Consumers have an increased demand for bioactive ingredients, which help to strengthen the immune system and reduce the risk of developing various chronic diseases such as cancer, diabetes, and Alzheimer's. Additionally, there has been an emerging demand from the coronavirus disease (COVID-19) pandemic for bioactive ingredients to boost the immune system. The market is expected to reach $69.9 billion by 2028, exhibiting a compound annual growth rate (CAGR) of 8.21% between 2022 and 2028 (https://www.imarcgroup.com/bioactive-ingredients-market).

Due to their nutritional composition, tropical and subtropical fruits are recognized as a source of high content of bioactive compounds and health-promoting properties. These beneficial health effects are related to the content of several of these bioactive compounds, mainly flavonoids and non-flavonoid phenolics (SAYAGO-AYERDI *et al.*, 2021).

Nutrient-rich juices obtained from tropical fruits are gaining acceptance owing to the desire of consumers for healthy products whilst experiencing new and exotic flavors, and at the same time, due to the focus of the industry on producing beverages with health claims as a parameter of differentiation(SAYAGO-AYERDI *et al.*, 2021)

The beverage industry constitutes an important sector of the transformation industry, specifically in the segment of functional beverages. The Functional Beverages (FBs) segment includes various types of products, among which energy drinks, sports drinks, and functional bottled water belong to the functional and fortified drinks category, which have recorded remarkable growth in recent years (MALEŠ *et al.*, 2022).

According to available data, despite the outbreak of the COVID-19 pandemic, the global FB market development annual growth rate has increased to a CAGR of 8.07% in 2021 and will reach US$158,280 million in 2023. Moreover, the expected increase in CAGR during the next five years will probably be 7.50% (FUNCTIONAL BEVERAGES GLOBAL MARKET REPORT 2021: COVID-19 GROWTH AND CHANGE TO 2030, 2021).

Consumers are increasingly aware of their health and safety, driving the market for bioactive compounds. The academic community is already aware of this trend, as shown by the increase in research in this area. Using the Scopus database with the keywords: “Bioactive compounds AND extraction AND fruit,” it was possible to find a total of 1.107 publications in the last 5 years, with a growing trend.

The juice agri-food sector generates an enormous volume of waste or by-products (peel/skin, seeds, leaves, tubers, roots, and pomace) that are considered valuable sources of bioactive compounds, containing highly valuable metabolites that have enormous potential for producing sustainable additives, chemicals, biofuels, and energy (CÁDIZ-GURREA *et al.*, 2020).

The search for more sustainable ways of chemicals and food production has been a hot topic in the research area and industry. In particular, high-added value compounds that are still present in by-products of the food processing industry can be employed as food additives and for the formulation of new functional foods, nutra-/pharmaceuticals, cosmeceuticals, and beauty products, as well as for innovative and more sustainable packaging (LI *et al.*, 2022).

These bioactive compounds can be extracted from the by-products using different technologies and can be used to develop valorized products, including functional foods or dietary supplements (PATRA; ABDULLAH; PRADHAN, 2022).

Conventional methods of extracting bioactive compounds can be found in the scientific literature. These techniques have limitations in obtaining solvent-free extracts, presenting disadvantages such as degradation or loss of the target compound, consumption of large volumes of organic solvents, and long extraction times.

Techniques for intensifying extraction processes have been applied because they are more compact, safer, energy-efficient, and environmentally friendly. So, supercritical fluid extraction (SFE), subcritical water (SWE), and microwave-assisted extraction (MAE) have significant advantages, including high selectivity, low viscosity, high diffusivity, and high solvent power. These techniques can be associated with extracting plant compounds to improve the yield and quality of the extracted products.

Combining techniques for processing the same raw material is advantageous since each type of extraction can extract different compounds due to their specificities. SFE with CO_2 uses a solvent that extracts more nonpolar compounds; SWE and MAE extract more polar substances using solvents such as water.

Despite this, few works have been found to extract bioactive compounds using soursop, acerola, or strawberry, especially considering these agro-industrial residues prove the novelty of the present work.

Therefore, these inedible parts can be valued by recovering bioactive compounds by supercritical, subcritical, and microwave-assisted extraction, transforming food by-products into resources, reducing environmental pollution, and transforming a linear economic system into a circular one.

CHAPTER 3 RECOVERY AND VALORIZATION FROM SOURSOP PROCESSING BY-PRODUCTS

This study evaluated the high-pressure extraction, chemical composition, and antioxidant activity of extracts from soursop seeds using different environmentally friendly techniques with green solvents. Based on the literature review, the soursop seeds have a large amount of oil. The SFE was employed as a method to degrease the raw material and then was followed by the SWE technique with polar solvents to access the polar part.

Therefore, extraction methodology combining SFE in the first step and SWE in the second was used to recover extracts with different polarity and biological activity from soursop seed. Therefore, this study hopes to contribute information that can add value to soursop by-products.

The results presented in this chapter were published in the Food Chemistry: X (https://doi.org/10.1016/j.fochx.2021.100164)

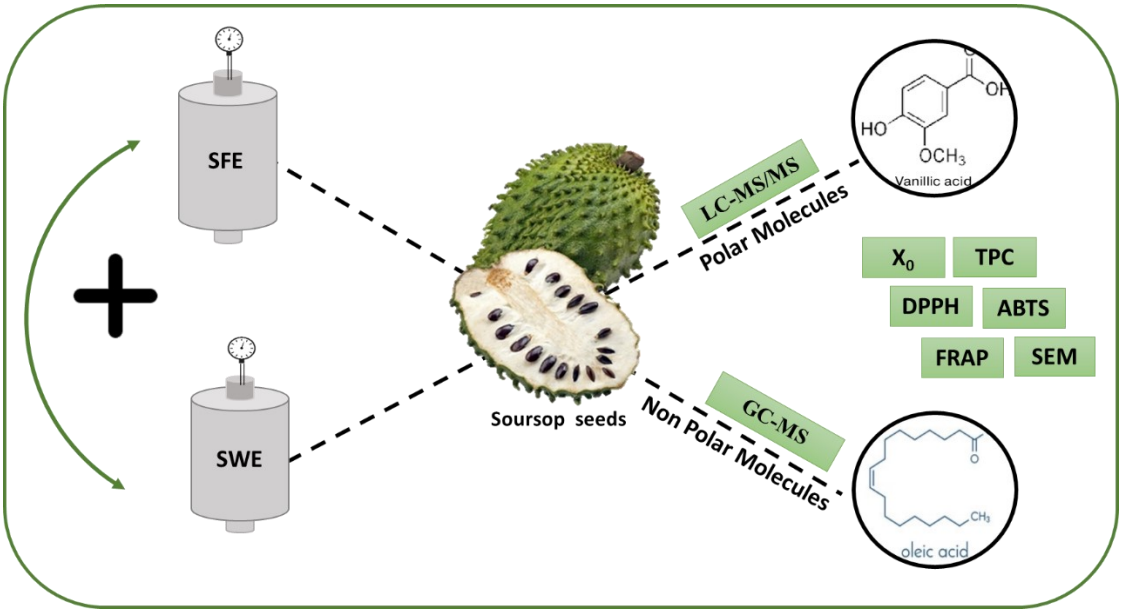
3 INTENSIFIED GREEN-BASED EXTRACTION PROCESS AS A CIRCULAR ECONOMY APPROACH TO RECOVER BIOACTIVE COMPOUNDS FROM SOURSOP SEEDS (*Annona muricata* L.)

Abstract

Soursop (*Annona muricata* L.) seeds, which is a residue obtained from juice agro-industries, were subjected to supercritical fluid extraction (SFE) and subcritical water extraction (SWE) in single or combined mode to extract the potential value-added compounds. Different extraction methods were evaluated in terms of extraction yield, phenolics content, antioxidant activity (DPPH, ABTS, and FRAP), and Maillard reaction products. The extracts were analyzed using SEM, GC-MS, and LC-MS/MS techniques. The temperature and a combination of high-pressure techniques positively affected the overall results (SFE + SWE), affording nonpolar and polar extracts rich in phenolics and antioxidant compounds. SEM analysis showed that the use of SFE caused modifications in the cell wall, and the oil fraction was rich in fatty acids. Twenty-nine compounds associated with soursop seed extracts were detected for the first time using LC-MS/MS, showing the potential of the raw material as well as promoting resource re-utilization in a circular economy.

Keywords: *Annona muricata* seeds, Supercritical fluid extraction, Subcritical water extraction, Phenolic compounds, Antioxidant activity, Principal component analysis

Graphical Abstract



3.1 INTRODUCTION

Tropical fruit production, trade, and consumption have increased significantly in domestic and international markets because of the attractive sensory properties of these fruits and growing recognition of their nutritional constituents such as minerals, fibers, vitamins, secondary phytochemical compounds, and therapeutic values (SILVA *et al.*, 2020b).

Agribusiness is one of Brazil's most important commercial activities, and the country is the third-highest fruit producer worldwide, behind China and India (VIDAL, 2019). Among the various fruit species, the soursop tree (*Annona muricata* L.), a plant native to South America, is widely distributed in the tropical and subtropical regions of the world. It belongs to the *Annonaceae* family and has high economic potential, mainly because its fruit has a pleasant taste and aroma (OLIVEIRA *et al.*, 2016).

Soursop fruit is primarily consumed as fresh fruit, and processed products comprising soursop fruit include juice, nectar, puree, jellies, yogurt, syrups, sweets, ice cream, and other foods (OLIVEIRA *et al.*, 2016)). However, the residues generated during the processing steps, which correspond to 33% of the whole fruit, are commonly not used or discarded, producing a significant amount of waste that causes environmental contamination (AGUILAR-HERNÁNDEZ *et al.*, 2019).

Once food security is ensured, this waste can be valorized in an integrated manner during the industrial application of downstream processes, transforming waste into secondary raw materials and allowing the extraction of value-added compounds via sustainable and green methodologies (CAMPOS *et al.*, 2020).

These approaches are related to circular economy concepts, including the recovery and valorization of waste materials, which allows their reusage and input back into the supply chain, affording economic growth from environmental losses (CAMPOS *et al.*, 2020). Many bioactive and phytochemical compounds have been identified from the organic and aqueous extracts of primary and secondary raw materials of soursop (fruits, bark, leaves, roots, and seeds), with acetogenins being the predominant class of compounds, followed by alkaloids, phenols, flavonoids,carbohydrates, cardiacglycosides, saponins, tannins, phytosterols, terpeno ids, and proteins (ORAK; BAHRISEFIT; SABUDAK, 2019).

Accordingly, the integration of the valorization concept allows the conversion of fruit waste into high-value products with potential applications for human consumption, such as the extraction of specific molecules and the production of extracts with antioxidant activities (CAMPOS *et al.*, 2020). Conventional solvent extraction techniques, such as maceration and Soxhlet (SOX) extraction, are used to obtain the bioactive compounds of inedible soursop parts such as seeds, leaves, and bark (NAM *et al.*, 2017; ORAK; BAHRISEFIT; SABUDAK, 2019).

Novel extraction methods have also attracted the attention of researchers because of their advantages in comparison to the conventional extraction techniques, including faster speed of extraction and reduction in solvent consumption, as well as the possibility of combination with other processes in the biorefinery, thereby reducing or eliminating process residues (HERRERO *et al.*, 2015).

Among these new methods, supercritical fluid extraction (SFE) and subcritical water extraction (SWE) are efficient tools for extracting bioactive components from different natural sources (RODRIGUES *et al.*, 2019). SFE combines liquid-like and gas-like properties, affording high density, solubilization, and solvent diffusivity, thereby increasing mass transport (PEREIRA; MEIRELES, 2010). SWE is an attractive technique because it affords an enhancement in the yield and a decrease in the extraction time, using water in a liquid state below its critical point (T_c = 374 °C and P_c = 22.064 MPa) instead of traditional solvents to recover important phenolic compounds from various agricultural and food by-products (PLAZA; TURNER, 2015; RODRIGUES *et al.*, 2019, 2020b).

This study aims to evaluate the chemical composition and antioxidant activity (AA) of the soursop seed extracts obtained using different techniques. Na extraction method combining SFE in the first step and SWE in the second step was used to obtain biologically active extracts from the soursop seeds. In addition, the results of single-step SWE extraction and SOX technique were compared. Therefore, this study provides information to obtain value-added products from soursop by-products, promoting resource re-utilization and circular economy.

3.2 MATERIAL AND METHODS

3.2.1 Raw material and sample preparation

Soursop seeds, the by-products of fruit pulp processing, were provided by the Tropicássia Polpa de Fruta company, Fortaleza, Brazil. The seeds collected in 2018 were placed in plastic bags, frozen at −18 °C, and transported to the Laboratory of Thermodynamics and Supercritical Technology (LATESC) of the Federal University of Santa Catarina (UFSC). Upon arrival, the raw material was thawed and dried in an air-circulated oven (DeLeo, Porto Alegre/RS, Brazil) for 10 h at 50 °C. The dried material was crushed in a knife mill (DeLeo, Porto Alegre/RS, Brazil) and stored in polyethylene packaging at – 18 °C until use. The raw material presented a mean particle size of 0.53 mm and moisture content of 4.98 ± 0.15% (w/w), determined according to the method reported by (GOMIDE, 1983) and the AOAC method 925.09 (AOAC, 2005a), respectively.

3.2.2 Extraction procedures

3.2.2.1 SFE

The SFE unit and extraction procedure have been previously described in a report by MAZZUTTI et al. (2018). Pure CO_2 (99.9%) delivered at a pressure of up to 0.6 MPa (White Martins, Brazil) was used for SFE. Briefly, 30 g of raw material (dried and milled samples) was placed inside a stainless-steel extraction vessel (internal diameter of 20 mm and height of 440 mm, the volume of 138.2 mL), which the empty space was filled with glass beads and cotton to form a fixed bed. Kinetic evaluation of the overall extraction curve (OEC) for the soursop seeds was performed to determine the extraction time. This assay was performed using

supercritical CO_2 at 20 MPa, solvent flow rate of 0.7 $kg \cdot h^{-1}$, and temperature of 50 °C, where the extract samples were collected at pre-established time intervals. Based on the OEC, the extraction time was fixed at 3.5 h for the SFE assays (as presented in Supplementary material **Fig. S1 Appendix A**). The SFE experiments were carried out in duplicate at temperatures of 40, 50, and 55 °C, pressures of 15, 20, 25, and 30 MPa (CO_2 density 0.653, 0.784, 0.834, and 0.909 g cm^{-3}, respectively), and a constant solvent flow rate of 0.7 kg CO_2 h^{-1}. The kinetic and the SFE experiments conditions were established based on the prior experience of the group (MAZZUTTI *et al.*, 2018). After each experiment, the obtained extracts were collected in amber flasks, weighed, and stored in a domestic freezer at −18 °C.

3.2.2.2 SWE

The SWE assays were performed in a customized unit following the experimental procedure described by RODRIGUES et al. (2019), at least in duplicate. The SWE period was defined based on a kinetic assay performed to obtain the OEC by collecting the extract samples at pre-established period intervals at 10 MPa, 110 °C, and using a solvent flow rate of 4 $mL \cdot min^{-1}$. These conditions were established based on the previous experience of the group (RODRIGUES *et al.*, 2019, 2020b). The extraction time of SWE was established (five minutes) in dynamic mode, the assays were interrupted, and the system was drained to examine the OEC for recovering most of the soluble material, as presented in Fig. S1B of the Supplementary material.

Soursop seeds were subjected to extraction in a fixed extractor vessel in two modes: (i) combined-mode (CM), which involved SFE in the first step followed by SWE in the second step (SFE + SWE); (ii) single-mode (SM), where SWE was performed for comparison with the CM. In CM, soursop seeds were first subjected to SFE at 30 MPa and 40 °C (first step), and the residue of SFE was then subjected to SWE (second step). Briefly, the extraction procedure consisted of placing 5 g of soursop seeds (raw material soursop seeds or residue of SFE step), followed by mixing with 64 g of glass spheres to form a fixed bed of particles inside the AISI 316 stainless-steel extraction vessel (internal diameter of 25 mm and height of 180 mm, the volume of 90 mL).

The CM was performed at 10 MPa using a water flow rate of 4 $mL \cdot min^{-1}$ and temperatures of 70, 90, 110, and 130 °C. In contrast, SM SWE (raw material soursop seeds)

was performed at 10 MPa using a water flow rate of 4 mL·min^{-1}, and temperatures of 70 and 130 °C, at least in duplicate, for comparison with the CM. All experiments were performed using sonicated distilled water pumped directly into the extraction cell packed with dried samples using na HPLC pump. The extracts were collected in glass flasks, rapidly cooled using a cooling fan, and stored in a refrigerator without light for solvent removal by freeze-drying for 24 h (Liotop, model LD101, São Paulo, Brazil).

3.2.2.3 Conventional extraction

Conventional atmosphere-pressure extraction was performed using the SOX technique with hexane (Hex) and ethanol (EtOH) as solvents, following the AOAC method 920.39C (AOAC, 2005a), with assays performed at least in duplicate. The procedure employed 150 mL of solvent recycling over 5 g of crushed dried soursop seeds in a SOX apparatus for six hours at the solvent boiling temperature with an average of 10–15 solvent refluxes. The obtained extracts were stored in amber flasks at −18 °C before analysis. The results, expressed in extraction yield (X_0), represent the mean values ± standard deviation of triplicate experiments.

3.2.2.4 Global extraction yield (X0)

The global extraction yield (X0) was calculated as the percentage of dried mass extracted ($m_{extract}$) relative to the total mass of the raw material on a wet basis (m_{RM}), according to the equation. (1):

$$\mathbf{X_0}(\%) = \frac{\mathbf{m}_{extract}}{m_{RM}} * \mathbf{100} \qquad \text{Equation 1}$$

3.2.2.5 Total phenolic content (TPC)

TPC of the soursop seed extracts was determined using the Folin–Ciocalteu method (KOŞAR; DORMAN; HILTUNEN, 2005). Briefly, a 10 µL aliquot of the extract water solution (10 mg·mL^{-1}) and 600 µL of water was mixed with 50 µL of undiluted Folin–Ciocalteu reagent

(Sigma-Aldrich, USA), a solution of hexavalent phosphomolybdic and phosphotungstic acid complexes. After allowing the solution to stand and stirring it for one minute, 150 µL of 20% Na_2CO_3 (w/v) was added, and the volume was increased to 1 mL using water. The samples were incubated for two hours at 25 °C in the dark. The absorbance was measured at 760 nm using the standard calibration curve of gallic acid. The results were expressed in milligrams of gallic acid equivalent (GAE) per gram of the dry extract, based on triplicate measurements.

3.2.2.6 DPPH free radical scavenging assay

The free radical scavenging capability of the soursop seed extracts was evaluated using the 1,1-diphenyl-2-picrylhydrazyl (DPPH; Sigma-Aldrich, USA) method (MENSOR *et al.*, 2001). Briefly, different extract concentrations were tested (five concentrations for each extract) by mixing 0.3 mM DPPH solution (710 µL) and 290 µL of the extract solution, providing the reaction medium (1 mL). The absorbance at 517 nm was monitored after 30 min in darkness at room temperature and converted into antioxidant activity percentage (AA%). As the mean value of triplicate assays, the results are expressed as EC_{50} values (test concentration required to decrease 50% absorbance compared to the blank solution) in $\mu g \cdot mL^{-1}$ units. The EC_{50} values were calculated based on the linear regression of the AA% curves obtained for all extract concentrations. The AA of the extracts are expressed as antiradical power (ARP), the inverse of EC_{50}, which is used to define the AA of an antioxidant as a reciprocal of EC_{50}.

3.2.2.7 TEAC-ABTS assay

The ABTS assay of the soursop seed extracts was performed according to the method described by RE et al., 1999 with some modifications. Synthetic vitamin E, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; Sigma-Aldrich, USA), was used as an antioxidant standard. First, 7 mM ABTS solution and 2.45 mM potassium persulfate solution were reacted at room temperature for 16 h in darkness to produce the radical ABTS+ (2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)) diammonium salt. The ABTS+ solution was then diluted with 5 mM sodium phosphate (pH 7.4) until an absorbance of 0.70 (±0.05) was achieved. Thirty microliters of the extract (five concentrations) were mixed with 970 µL of ABTS•+ and incubated in the dark for 45 min, followed by absorbance measurement at

734 nm, providing the standard curve (0.25–2 mM). The final data are expressed in micromoles of Trolox equivalent per gram of the dry extract (μmol TE·g^{-1}). The results are expressed as mean ± standard deviation (average of triplicate assays).

3.2.2.8 FRAP assay

The FRAP assay of soursop seed extracts was performed according to the method described by BENZIE; STRAIN, 1996. Briefly, 10 μL (0.1–0.5 mg·mL^{-1}) of the solubilized extract was placed together with 290 μL of the FRAP reagent (0.3 M, pH 3.6 acetate buffer, 10 mM TPTZ (2,4,6-tripyridyl-s-triazine), and 20 mM ferric chloride; Sigma-Aldrich, USA) in a microplate. This solution was kept in the dark at room temperature for 30 min, and the absorbance was measured at 593 nm using a microplate reader (Tecan Infinite M200). The analyses were performed in quintuplicate using a blank for each sample (10 μL of solvent + 290 μL of FRAP). Trolox (Merck, Germany) was used as a reference, and the values were calculated from the standard curve (50–500 μM). The results are expressed as micromoles of Trolox equivalent per gram of the dry extract (μmol TE·g^{-1}).

3.2.2.9 Analysis of the final Maillard reaction products (MRPs)

The products of the Maillard reaction (melanoidin formation) were estimated by the darkening intensity of the soursop seed extracts obtained by SWE extraction (SM and CM) according to the methodologies reported by SAMARAS et al. (2005); PLAZA et al. (2010), with some modifications. The extracts obtained by SWE under different conditions were diluted, starting with a concentration of 1 mg·mL^{-1} in water and DMSO (70:30, v/v) solution, which was filtered with a hydrophobic PTFE syringe filter (25-mm diameter and 0.45-μm pores). Absorbance at 420 nm was measured using a cuvette with a light path of 10 mm to determine the Maillard reaction degree. The samples were diluted when necessary to obtain an absorbance reading of < 1.5 arbitrary units. The analysis was performed in duplicate, and the results are expressed as absorbance ± standard deviation.

3.2.2.10 Volatile compound analysis by gas chromatography-mass spectrometry (GC-MS)

The extract samples obtained by SFE at 30 MPa and 40 °C (based on the high yields and AA; Sections 3.2 and 3.3) and SOX extraction with hexane were selected for GC-MS analysis. The samples were subjected to methylation fractionation reaction (FAME) to assist the analysis of the compounds by GC-MS (O'FALLON *et al.*, 2007). The identification and relative quantification of the volatile compounds in the soursop seed extract were performed using a gas chromatography system equipped with a mass spectrophotometer (GC-MS, model 7890 A, mass detector 5975C, Agilent Technologies, USA), attached to an HP-5MS column (30 m × 0.32 mm (internal diameter) with a film thickness of 0.25 µm, Agilent Technologies, USA), following the method described by MAZZUTTI et al. 2018. Helium was used as a carrier gas with a flow rate of 4 $mL \cdot min^{-1}$, split ratio of 5:1, injector temperature of 250 °C, and Thermal Aux 2 (MSD Transfer Line). The column temperature was increased from 60 °C to 230 °C at a rate of 3 $°C \cdot min^{-1}$, in a total time of 55.56 min, and a quadrupole detector temperature of 150 °C was used. The major components of the selected extracts were identified by comparing the compounds' mass spectra and retention times to those available in the NIST 11 Mass Spectral Library.

3.2.2.11 Identification and quantification of phenolic compounds by LC-ESI-MS/MS

3.2.2.11.1 Sample preparation

The samples were prepared according to the protocol described by SCHULZ et al. (2015), with some modifications. Briefly, defatted soursop seed extracts were subjected to acid hydrolysis at 85 °C using 5 mL of methanol and 5 mL of hydrochloric acid for 30 min. The solution pH was adjusted to 2 using 6 $mol \cdot L^{-1}$ sodium hydroxide solution. Next, the acidified solution was partitioned with 10 mL of diethyl ether by centrifugation at 3000 × g for 10 min. This process was repeated twice for each sample. The supernatants were then combined in a round-bottom flask. The solvent was removed using a rotary evaporator at 40 °C until dryness. The dried sample was then resuspended in 1 mL of chromatographic grade methanol and diluted 10 times with methanol: water (30:70, v/v) mixture for injection into the LC-ESI-MS/MS system.

3.2.2.11.2 LC-ESI-MS/MS analysis

The identification and quantification of 41 phenolic compounds were performed using a high-performance liquid chromatography (HPLC) system (1200 Series, Agilent Technologies, Waldbronn-BW, Germany), following the methodology described by SCHULZ et al. (2015). A Synergi column (4.0 μm, 2.0 × 150 mm d.i.; Phenomenex, Torrance-CA, USA) was used for HPLC separation by employing gradient elution conditions. The mobile phases were composed of methanol: water (95:5 %, v/v) – A and an aqueous formic acid solution (0.1 %, v/v) – B. The separation was carried out at 30 °C using a segmented elution gradient of 10% A for 0–5 min, 10–90% A for 5–7 min, 90% A for 7–10 min, and 10% A for 10–17 min. The column was conditioned for five minutes between the analyses using the mobile phase employed at the beginning of the separation. The flow rate was 250 μL·min^{-1}, and the injection sample volume was 10 μL.

The LC system was coupled to a hybrid triple quadrupole/linear ion trap mass spectrometer (Q Trap 3200 Applied Biosystems/MDS Sciex, Concord-ON, Canada). The mass spectrometer was operated in negative electrospray ionization mode (TurboIonSpray Applied Biosystems/MDS Sciex, Concord-ON, Canada). The MS/MS parameters were as follows: capillary needle maintained at 4500 V; curtain gas pressure of 7 × 10^{-2} MPa; a temperature of 400 °C; gas 1 and gas 2 pressure of 3 × 10-1 MPa; CAD gas: medium. Analyst software (version 1.6.2) was used to control the LC-ESI-MS/MS system and analyze data. The mass spectrometer parameters of each phenolic compound were obtained based on the data reported by SCHULZ et al.(2015). Quantitative data for phenolic compounds were obtained from calibration curves constructed using the standards.

3.2.2.12 Scanning electron microscopy (SEM) analysis

SEM analysis (JEOL JSM 6390 L V, Musashino, Akishima, Japan) was performed at the Central Laboratory of Electronic Microscopy (Federal University of Santa Catarina, Florianópolis, SC, Brazil), with operation at 5 kV and × 300 magnification. Two samples were analyzed: (A) raw material soursop seeds before the SFE procedure and (B) solid residue of soursop seeds after SFE at 30 MPa and 40 °C. Before scanning, the samples were coated with a thin gold layer using a sputter coater.

3.2.2.13 Statistical analysis

All data are expressed as mean ± standard deviation (SD) based on triplicate measurements. One-way analysis of variance (ANOVA) for the extraction yield, TPC, AA, and MRP assays was conducted using the Statistica software (Statsoft Inc., USA), while the Tukey test was used to evaluate significant differences ($p < 0.05$). Principal component analysis (PCA) was performed according to the method described by ZIELINSKI et al. (2014) using the Origin Lab Software. Before PCA and HCA analysis, all variables were auto-scaled (transformation into z-scores) to standardize the statistical importance of all responses. Then, the PCA data indicated the patterns in the dataset that showed the interrelationships between the recovery of phenolic compounds and SM and CM extraction parameters through a projection in a bidimensional scatter plot.

3.3 RESULTS AND DISCUSSION

3.3.1Extraction kinetics

A kinetic study of SFE and SWE was performed to determine the process extraction time. **Fig. S1A** of the supplementary material shows the overall extraction curves (OEC) obtained by the SFE of raw material soursop seeds with pure CO_2 at 20 MPa, 50 °C, and 0.7 $kg \cdot h^{-1}$ and SWE of the residual material of SFE with distilled water at 110 °C, 10 MPa, and 4 $mL \cdot min^{-1}$. The extraction curves indicate different periods: constant extraction rate (CER) period, controlled by convection and characterized by the extraction of the easily accessible solute; falling extraction rate (FER) period, combining convection and diffusion mechanisms due to the partial exhaustion of surface solute; diffusion-controlled rate (DIF) period, where the solute from the particle surface is depleted (PEREIRA; MEIRELES, 2010). Then, for the SFE considering the OEC, the extraction time was fixed at 210 min, allowing the recovery of > 88% of the extractable material up to the DIF period. An analogous extraction curve was obtained for SWE, where > 62% of the total extracted mass was accumulated in the first five minutes of the process (fixed time for SWE). According to Viganó and Martinez (2015), the pressurized liquid extraction (PLE)/SWE process can be divided into two stages. The first stage corresponds

to the extraction controlled by solubility (CER), and the diffusion-controlled solutes into the solvent (DIF) represent the second stage, as shown in **Fig. S1B** of the supplementary material-Appendix A. As previously reported by our research group (RODRIGUES *et al.*, 2019, 2020b), after an established time for SWE (five minutes), representing the first linear part of the OEC, the process is controlled by the convection mechanism because of the recovery of the easily accessible extract on the particle surface (VIGANÓ; MARTINEZ, 2015). The assays were interrupted, and the system was drained employing a dynamic model. This drain step allowed the recovery of soluble compounds inside the vessel and particles, combining CER and DIF fractions, representing the convection and diffusion mass transfer mechanisms, respectively (VIGANÓ *et al.*, 2016). This SWE methodology decreased the solvent use and process time, affording a high yield (VIGANÓ *et al.*, 2016).

3.3.2 Global extraction yield and TPC

Table 5 shows the data for global extraction yield (X0, w.b.), TPC values obtained by different methods (SFE, SWE, and SOX), and process settings for soursop seeds extraction. The SFE data in **Table 5** are affected by variations in pressure and temperature, with X0 ranging from 3.6% to 16.4%. This increase can be explained by changes in the CO_2 density and solvation power of the solvent (PEREIRA; MEIRELES, 2010).

Corroborating these results, Dorado; Hurtado-Benavides; Martínez-Correa (2016); and Santos et al.(2018) reported extraction yields of 6.9% and 12.9%, respectively, for soursop seeds using SFE under different conditions (20 MPa, 40 °C, 0.12 kg $CO_2 \cdot h^{-1}$, and 145 min and 38.1 MPa, 49.8 °C, 1.8Kg $CO_2 \cdot h^{-1}$ and 150 min, respectively).

In SWE, a constant pressure of 10 MPa was employed because the pressure had a negligible effect in comparison to temperature on the solvent characteristics and, therefore, on process selectivity and efficiency (RODRIGUES *et al.*, 2019). This is because water is relatively incompressible at temperatures below 300 °C. No significant effect of pressure on the physical properties of the liquid state was observed (PLAZA; TURNER, 2015).

Table 5. Global extraction yield (X0), total phenolics content (TPC), and antioxidant activity, evaluated by DPPH, ARP, ABTS, and FRAP assays, of soursop seed extracts obtained by different extraction techniques in single-mode (SM) and combined mode (CM).

	Extraction method	X_0 (%)	TPC (mg GAE·g^{-1})	DPPH EC_{50} (µg·mL^{-1})	ARP[1]	ABTS (µmol TE·g^{-1} extract)	FRAP (µmol TE·g^{-1} extract)
SM	SFE 15 MPa/55 ºC	3.6 ± 0.4^{i}	6.0 ± 0.5^{g}	>2500	<4.0x10^{-4}	2.5±0.9^{g}	2.3±0.5^{g}
	SFE 20 MPa/50 ºC	13.9 ± 0.2^{d,e}	13.5 ± 0.3^{e,f}	>2500	<4.0x10^{-4}	3.3±1.5^{g}	7.8±0.5^{f}
	SFE 25 MPa/50 ºC	15.3 ± 0.2^{c,d}	15.4 ± 0.5^{e}	>2500	<4.0x10^{-4}	3.9±0.8^{g}	8.4±0.2^{f}
	SFE 30 MPa/40 ºC	16.4 ± 0.0^{c}	20.9 ± 0.5^{d}	>2500	<4.0x10^{-4}	4.6±1.1^{g}	8.6±0.4^{f}
	SOX EtOH	21.2 ± 0.3^{b}	15.6 ± 0.9^{e}	>2500	<4.0x10^{-4}	3.8±0.5^{g}	5.5±1.4^{f,g}
	SOX Hex	23.5 ± 1.4^{a}	21.9 ± 1.8^{d}	>2500	<4.0x10^{-4}	2.0±0.1^{g}	5.2±0.0^{f,g}
	SWE 70 ºC	11.6 ± 0.0^{h}	11.4 ± 0.7^{f}	>2500	<4.0x10^{-4}	246.5±5.2^{e}	41.3±0.3^{e}
	SWE 130 ºC	11.8 ± 0.1^{h}	72.2 ± 0.3^{b}	2337±69^{b}	4.3x10^{-4}	523.2±3.9^{b}	152.5±4.6^{b}
CM*	SWE 70 ºC	12.1 ± 0.1^{h}	11.8 ± 0.5^{f}	>2500	<4.0x10^{-4}	148.7±3.9^{f}	30.2±0.3^{d}
	SWE 90 ºC	12.2 ± 0.5^{h}	13.8 ± 0.2^{e,f}	>2500	<4.0x10^{-4}	282.6±4.0^{d}	44.2±0.8^{e}
	SWE 110 ºC	12.3 ± 0.3^{f,g}	33.4 ± 2.0^{c}	>2500	<4.0x10^{-4}	323.9±2.3^{c}	59.6±0.8^{c}
	SWE 130 ºC	13.6 ± 0.2^{e,f}	77.3 ± 0.4^{a}	1264±31^{a}	7.9x10^{-4}	587.3±8.8^{a}	162.5±2.9^{a}
	BHT	nd	nd	67 ± 0.3**	149x10^{-4**}	391.9±0.6**	215±2**

* SFE 30 MPa/40 °C (first step) + SWE (second step); SFE: supercritical fluid extraction; SWE: subcritical water extraction; SOX: Soxhlet extraction; EtOH: ethanol; HEX: hexane; GAE: gallic acid. TE: Trolox equivalent. BHT: butyl hydroxytoluene (synthetic antioxidant). **(Battistella Lasta et al., 2019); (1) antiradical power (ARP) inverse of EC_{50}. Superscript letters indicate the statistically different groups ($p < 0.05$) in each column.

In this study, a combined extraction procedure was performed, in which the residual soursop seeds treated with SFE were further subjected to SWE (second step) to afford extracts with polar characteristics and more abundant phenolic compounds. Therefore, SFE at 30 MPa and 40 °C were selected to perform the combination experiments with soursop seeds, as the highest values of X0 and TPC were obtained under these conditions (**Table 5**). In SFE + SWE CM, different compound classes from the same raw material could be obtained, with nonpolar compounds extracted primarily by SFE (30 MPa/40 °C), followed by the use of water under pressure to obtain extracts with polar characteristics. According to Pereira and Meireles, (2010), fractionation can be used in the extraction and/or separation steps to increase the selectivity and recovery of different extracts of the same raw material.

The data presented in **Table 5** show a gradual increase in the yield with an increase in the extraction temperature from 70 to 130 °C in the CM (SFE + SWE) and SM extraction, affording a maximum value of 13.6%, which results in a cumulative global yield of 30% (SFE 30 MPa/40 °C + SWE 130 °C). According to HERRERO et al.(2015), the water temperature affects the extraction efficiency and selectivity in SWE. An increase in temperature results in increased diffusion rate, reduced viscosity, surface tension, and water polarity. Thus, moderately polar and nonpolar materials can be recovered in an aqueous medium at high temperatures (HERRERO *et al.*, 2015).

Additionally, the hexane and ethanol SOX extraction method yielded the highest yields of 23.5% and 21.2%, respectively. This result could be related to the longer extraction time and greater contact of the plant matrix with the solvent. Vegetable seed oils are traditionally obtained through extraction with nonpolar solvents, typically hexane, and other solvents with boiling points of up to 70 °C (BELWAL *et al.*, 2018b). This process generally affords high yields, but a late stage for solvent elimination after extraction is required, demanding high energy; additionally, the solvents are toxic to humans and dangerous to the environment. The novelty is evident because no SWE SM or CM yields are available for comparison purposes to the best of our knowledge.

Furthermore, **Table 5** shows the TPC values obtained for soursop seeds using different extraction techniques, ranging from 6.0 (SFE 15 MPa/55 °C) to 77.3 mg GAE·g^{-1} of dry extract (SFE 30 MPa/40 °C + SWE 130 °C). SFE at 30 MPa and 40 °C afforded the highest TPC value among the SFE experiments and was selected for the CM process. The TPC values obtained in the CM showed a significant increase of approximately six times when the temperature was increased from 70 to 130 °C (**Table 5).** Interestingly, the SWE SM and CM processes were

compared at 130 °C, and a significant difference was observed in the TPC values. This indicates that the combination of extraction processes is a promising method for the efficient separation of polar and apolar fractions of soursop seeds owing to the differences in the solubilization of the plant matrix components, which improves the usage and value-addition of this raw material through the application of sustainable and green methodologies.

For comparison, conventional SOX extraction using ethanol and hexane as polar and nonpolar solvents, respectively, was performed to afford results for TPC (**Table 5)**. These results were in agreement with the data reported in the literature for the TPC of soursop seeds obtained by conventional extraction techniques at room temperature and different organic solvents (8.2 to 78.5 mg GAE·g^{-1} extract) (MORENO; JORGE, 2012; MENEZES et al., 2019). New extraction techniques that can allow energy conservation and reduce the usage of organic solvents can allow the recovery of bioactive compounds from soursop seeds.

3.3.3 Antioxidant activity

A mixture of different antioxidants with different action mechanisms determines the antioxidant capacity of foods; therefore, the AAs of food products must be assessed using various methods to evaluate different mechanisms (SILVA *et al.*, 2020b). **Table 5** shows the AAs of soursop seeds obtained by other techniques, including the DPPH, ABTS, and FRAP methods. As expected, the SFE extracts exhibited low AAs. The goal was to remove nonpolar compounds and evaluate the extracts for AA. The SFE extracts afforded high EC50 values or low AA capacities (ARP values) in the DPPH/ARP assay (**Table 5**). PINTO et al. (2018) also reported that the *Annona muricata* seed oil showed a low AA *in vitro*. These results could be explained by the nonpolar characteristics of carbon dioxide that did not favor the solubilization of phenolic compounds of intermediate to high polarities (MAZZUTTI *et al.*, 2018).

Furthermore, an increase in the AA was observed during analysis by all methods when the extraction temperature was increased for the extracts obtained by SWE, and the best values were obtained at the highest temperature (130 °C). Accordingly, the CM technique at 130 °C afforded the best AA value (significant results or relatively close to those for the synthetic antioxidant, BHT), probably because of the degreasing step used for the raw material sample, which allowed greater access to the compounds that were protected by the lipid layer in the soursop seeds. In the SWE technique, an increase in the extraction temperature changed the physical properties of water, allowing the recovery of different compounds, increasing the

AAs of the compounds present in the extracts, or forming new compounds, for example, through the Maillard reaction. Plaza et al. (2010b) reported that the compounds formed by Maillard reaction, caramelization, and thermo-oxidation could increase the AAs of the extracts obtained with SWE at temperatures above 100 °C.

The AA values for the extracts obtained by SFE and SOX with hexane were not significantly different ($p > 0.05$) in the TEAC-ABTS assay, which was slightly more reactive than the extracts obtained by SWE. The best results were afforded for the extracts obtained by SWE SM and CM at 130 °C (523.2 and 587.3 μmol TE·g^{-1} of dry extract, respectively), and a significant difference ($p > 0.05$) was observed.

Based on the comparison of the methods used for determining the AAs, **Table 5** shows that the best AA values (closer or greater than those for BHT) are obtained by the ABTS method, which is generally used to evaluate the AAs of hydrophilic compounds. All AA methods have been validated in the literature; however, the global values are not always similar, owing to the affinity of the antioxidant compounds and the reaction rate of each method (RODRIGUES *et al.*, 2019).

According to the literature, considerable variations and differences in the AA data of Annonaceae seeds are observed using different methods. For the DPPH method, the EC_{50} values vary from 40 to 724.1 μg·mL^{-1}, while those for the ABTS and FRAP methods are 0.8 –905 μmol TE·g^{-1} of dry extract and 194.9 μmol TE·g^{-1} of dry extract, respectively, for different conventional extraction techniques employed at atmospheric pressure using organic solvents (BENITES *et al.*, 2015; ORAK; BAHRISEFIT; SABUDAK, 2019). The results obtained using the SWE method were superior to those reported in the literature for the AAs of the soursop seeds. For example, PINTO et al.(2018) obtained extracts using methanol and chloroform as solvents with a value of 40.2 μmol TE·g^{-1} of dry extract for the ABTS assay. In a cold extraction study using chloroform, methanol, and water in a 2:1:0.8 (v/v/v) ratio, SILVA; JORGE (2014) reported a value of 0.8 μmol TE·g^{-1} of dry extract for the ABTS assay. Therefore, the SM and CM SWE can be used as an alternative to SOX with hexane or ethanol, providing superior or similar extracts in a significantly shorter period. Additionally, as shown in **Table 5**, the highest ARP values are obtained by SM SWE at 130 °C (4.3×10^{-4}) and CM SWE at 130 °C (7.9×10^{-4}) in comparison to other conditions for soursop seeds.

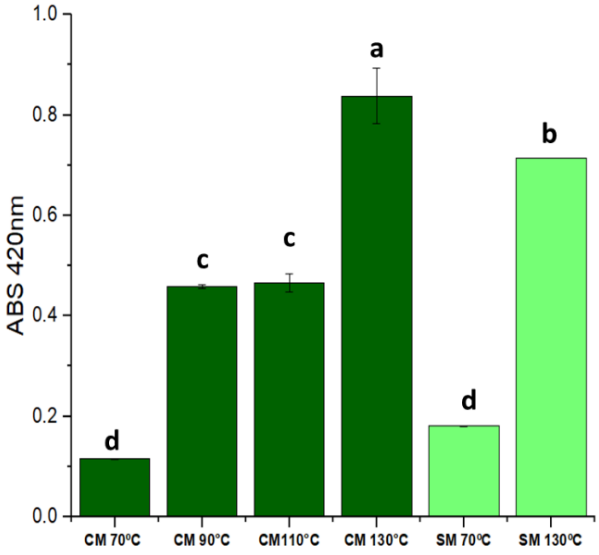
Thus, the AA of soursop seed extracts is dependent on several conditions related to the origin of the raw material, differences in cultivation, variety of species, as well as the extraction

technique, solvent characteristics, and extraction time and temperature that affect the selectivities of the extracted compounds (SILVA *et al.*, 2020b).

3.3.4 Analysis of the final MRPs of SWE extracts

Maillard reaction is a non-enzymatic reaction employed in food thermal processing of reducing sugars and amino acids, peptides, or proteins, which affords a complex matrix of compounds called the MRPs (PLAZA *et al.*, 2010b). The soursop seed extracts obtained by SWE were analyzed spectrophotometrically at 420 nm to determine the thermal effect during extraction and monitor the progress of the Maillard reaction (**Fig. 9**).

Figure 9 - Effect of subcritical water extraction (SWE) temperature in single-mode (SM) and combined mode (CM) on the amounts of Maillard reaction products of soursop seed extracts.



The same letters indicate no significant difference at 5 % ($p < 0.05$). Source: Author

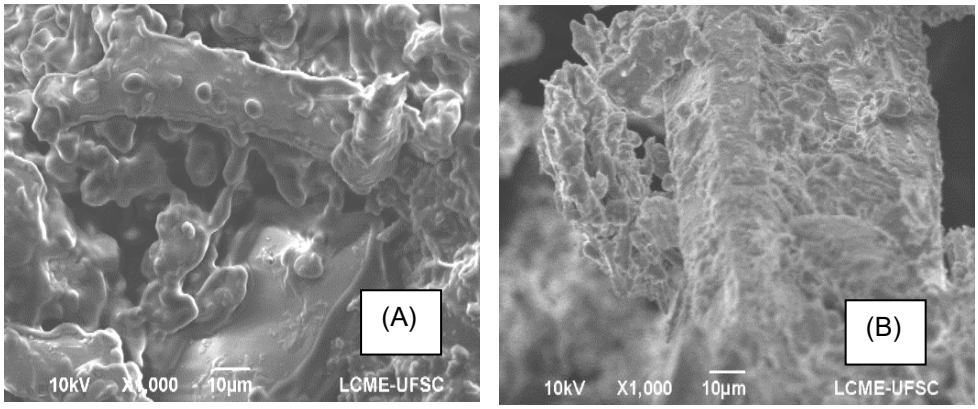
As reported in prior literature, temperature is an important factor affecting the Maillard reaction, which is performed at high temperatures and typically used in SWE (RODRIGUES *et al.*, 2019). The MRPs increased with an increase in the temperature to 130 °C in the SM and CM SWE, affording the highest yields of the extracts with significant values ($p < 0.05$).

Therefore, an increase in temperature increased the reactivity between the sugars and amino groups in the plant matrix, indicating the progression of the Maillard reaction, which led to new antioxidant compounds (PLAZA; TURNER, 2015).

3.3.5 SEM

Fig. 10 shows the SEM data of the dried material soursop seeds material and SFE extraction residue (30 MPa/40 °C). The raw material sample shows tiny oil droplets on its surface (Fig. 10A), whereas the residue material obtained by SFE shows the decreased amount of droplets and an opening in the vegetable matrix surface, allowing high recovery of polar bioactive compounds (Fig. 10B). The SEM images assist in interpreting the results for global extraction yield, TPC, and AA described in previous sections and further confirm that the combined process can allow the recovery of compounds with different phytochemical properties. The pressure applied during SFE causes a rupture of the matrix structure. In addition, the best SWE data obtained for SFE residues are related to the structural modifications caused in the raw material by the first extraction.

Figure 10- Scanning electron microscopy (SEM) images of soursop seeds with a magnification of 1000×: (A) raw material; (B) residue material after supercritical fluid extraction (SFE) at 30 MPa and 40 °C.



Source: Author

3.3.6 Chromatographic analysis

3.3.6.1 GC-MS analysis of volatile compounds of soursop seeds extract

SFE using CO_2 allows the extraction of easily oxidizable or thermosensitive compounds by operating at low temperatures using a non-oxidizing medium. Furthermore, in this process, the compounds are not exposed to light. It's important, due CO_2 is ideal for lipid, greasy, and non-polar substances (such as carotenoids, aromas, and volatile compounds) and limited in its affinity for polar solutes (HERRERO *et al.*, 2015). For this reason, the chemical profile of the volatile fraction was determined to allow the identification of the main compounds present in the soursop seed extracts obtained by SFE at 30 MPa and 40 °C compared to SOX using hexane. Soursop seed oil extracted by SFE contained 27.6% saturated fatty acids and 70.0% unsaturated fatty acids (**Table 6**). Similar fatty acid profiles have also been reported in the literature, with unsaturated fatty acid percentages of 64–75% (DORADO; HURTADO-BENAVIDES; MARTÍNEZ-CORREA, 2016; PINTO *et al.*, 2018). The major compounds identified in terms of the relative area percentage and/or relevance in the extracts were the unsaturated fatty acids, oleic acid (ω9), 10,13-octadecadienoic acid (PUFA), linoleic acid (ω6), palmitoleic acid (ω7), and elaidic acid (a *trans* geometric isomer of oleic acid), as well as saturated fatty acids, palmitic acid, and stearic acid.

The unsaturated fatty acids were predominant in the soursop seeds obtained by SFE, mainly including oleic and linoleic acid as well as essential fatty acids, which regulate various functions including blood pressure, blood clotting, blood lipid levels, immune response, and anti-inflammatory properties, and protect the cardiovascular system (BHARDWAJ; SATPATHY; GUPTA, 2014). These also aid in maintaining integrity and nutrition, strengthening the lipid barrier, hydrating the skin, and other vital functions of the human body (DORADO; HURTADO-BENAVIDES; MARTÍNEZ-CORREA, 2016).

Table 6-Major compounds determined by gas chromatography-mass spectrometry (GC-MS) in the volatile fraction of soursop seeds obtained by supercritical fluid extraction (SFE) at 30 MPa and 40 °C and Soxhlet (SOX) technique using hexane as a solvent.

Compounds	Relative peak area (%)	
	SFE 30 MPa/40 ºC	SOX Hexane
Miristic acid	0.1	0.1
Palmitoleic acid	1.5	1.8
Palmitic acid	21.7	22.6
Linoleic acid	12.3	19.1
10,13-Octadecadienoico acid	24.4	10.9
Elaidic acid	-	38.3
Oleic acid	32.1	-
Stearic acid	5.9	5.6
Methylpalmitic acid	0.7	0.6

* SFE 30 MPa/40 °C (first step); SFE: Supercritical fluid extraction. Source: Author

The SOX method, employing hexane as a solvent for extraction, is one of the most common methods for preparing raw material soursop seeds oil (ORAK; BAHRISEFIT; SABUDAK, 2019). However, using temperatures close to that of the hexane boiling point (69 °C) during extraction can affect the quality of the extracted oil (BELWAL *et al.*, 2018b). During SOX extraction, the isomerization of oleic acid can occur as it is a thermally induced process (CHENG *et al.*, 2018), resulting in the formation of elaidic acid, which is a *trans*-isomer of oleic acid. Trans fatty acids are correlated to coronary diseases and arteriosclerosis (DEBBABI *et al.*, 2017). In contrast, the oil obtained by low-temperature SFE has a significant amount of oleic acid, and the formation of *trans* isomers does not occur, affording a better-quality extract.

Only a few efforts have been directed toward the industrial exploitation of soursop seeds commonly discarded during the processing of commercially harvested fruit juices and pulps (PINTO *et al.*, 2018). Thus, based on the composition of fatty acids, soursop seeds contain essential compounds that can be applied in food industry. Therefore, seeds are a potential source of oil, which should be further investigated to determine their properties; oil extraction from these can be an alternative to the use of commercial fruit seeds waste, which can contribute toward a sustainable and circular economy (CAMPOS *et al.*, 2020; MENEZES *et al.*, 2019).

3.3.6.2 LC-MS/MS analysis

Table 7 shows the phenolic compounds identified and quantified by LC-ESI-MS/MS in soursop seed extracts obtained by different methods. Of the 41 phenolic compounds tested (standards), all 41 were detected, and 23 were quantified in the SWE and SOX ethanol extracts; the concentrations ranged from 0.0465 to 0.2656 mg g^{-1} of extract. Among these, the process that afforded the highest amounts of phenolic compounds was SM SWE at 130 °C (21 compounds quantified), and that which provided the lowest amounts was SOX (11 compounds quantified). The phenolic profiles of soursop seed extracts were predominantly composed of phenolic acids and flavonoids, but other phenolic compound classes were also detected. The major phenolic compounds found in soursop seed extracts were vanillic acid (17), p-coumaric acid (12), 3,4 dihydroxybenzoic acid (1), ellagic acid (7), vanillin (38), kaempferol (26), 4-aminobenzoic acid (2), 4-hydroxymethylbenzoic acid (3), caffeic acid (4), and ferulic acid (8). The extract also contained smaller amounts of quercetin (30), mandelic acid (10), salicylic acid (14), myricetin (27), gallic acid (9), sinapic acid (15), syringaldehyde (37), coniferaldehyde(35), umbelliferone (40), syringic acid (16), epicatechin (21), taxifolin (32), ro smarinic acid (13), and other compounds below the limit of quantification (LOQ).

In both SM and CM SWE, a high temperature (130 °C) favored extracting the highest amounts of total phenolic compounds (**Table 7**). For example, at this temperature, vanillic acid (17) was the main component of the phenolic content of soursop seeds extracts, and the highest amounts of phenolics were detected for the first time in the soursop seeds (*Annona muricata*; 0.0060–0.0635 mg·g^{-1}) with SM and CM SWE. These findings corroborate the TPC and AA data (**Table 5 and 7**, respectively). The profiles of the phenolic compounds obtained for the SOX samples showed the presence of 39 compounds, of which 11 were quantified; *p*-coumaric and vanillic acid were the most abundant compounds with amounts of 0.0270 and 0.0141 mg·g^{-1}, respectively. The low quantity of compounds detected in SOX extracts compared to those in the extracts obtained by SWE could be attributed to the lengthy process time of 360 min for the SOX method compared to five minutes for SWE. Furthermore, these differences in the quantification of phenolic compounds found (**Table 7**) may also be due to the solvent and its solubility characteristic of the desired analyte and their diffusivity in the solvent. In subcritical water medium, the temperature increase resulted in an increase in the diffusion rate and higher solubility of phenolic compounds.

According to Munir et al. (2018), depending on temperature, polar to medium polar compounds can be extracted with high solubility using SWE. Besides that, the effect of the high pressure of the system (at SFE and SWE processes) gives rise to a phenomenon called penetration, increasing the interaction between the matrix and solvent (CHAVES *et al.*, 2020). It is noteworthy that the SWE process, if integrated with the SFE, allows the SFE to weaken cell walls from the solid phase, changing the characteristics of the plant matrix and enabling more excellent solubility of the compounds (FERRO *et al.*, 2019).

Ideally, an extract of high purity and high selectivity should be achieved, which implies that the analyte of interest should have high solubility in the solvent while other compounds should have no or minimal solubility, and when extracting analytes at low concentrations, the rate of extraction is not affected by the analyte concentration but rather by the rate of mass transfer (MUSTAFA; TURNER, 2011). However, measuring the solubility of molecularly complex and very polar solutes in hot pressurized water is difficult due to their high cost and low thermo-stability at high temperatures and pressures (SRINIVAS *et al.*, 2009). Therefore, this result shows the advantages of the SWE technique, including faster and greener characteristics compared to the SOX method (RODRIGUES *et al.*, 2019).

Vanillic acid was also found in high concentrations in the methanol/water (80:20, v/v) extracts of the peel and seeds of Annona cherimola cultivars, '*Campa*' and '*Fino de Jete*,' as reported in a study describing the identification and quantification of phenolics and other polar compounds in the edible part of *Annona cherimola* and its byproducts by HPLC-DAD-ESI-QTOF-MS (GARCÍA-SALAS *et al.*, 2015). Vanillic acid content was 0.0812 and 0.3813 $mg \cdot g^{-1}$ in *'Campa'* and *'Fino de Jete,'* respectively, and it was also higher in *'Fino de Jete'* peel than *in 'Campa'* peel. The concentration of vanillic acid was higher in *'Campa'* seeds than in *'Fino de Jete'* seeds, i.e., 0.132 and 1.0129 mg g^{-1}, respectively.

Table 7 - Antioxidant compounds in soursop seeds extract obtained by single-mode (SM) and combined-mode (CM) subcritical water extraction (SWE) and Soxhlet (SOX) technique using ethanol (EtOH) as a solvent.

	Phenolic Compound	**SWE SM 70 °C (mg·g^{-1})**	**SWE SM 130 °C (mg·g^{-1})**	**SWE CM 70 °C (mg·g^{-1})**	**SWE CM 130 °C (mg·g^{-1})**	**SOX EtOH (mg·g^{-1})**
	Phenolic acid					
1	3,4 Dihydroxybenzoic acid	0.0021±0.0	0.0374±0.0025	0.0033±0.0003	0.0474±0.0051	0.0032±0.0007
2	4- Aminobenzoic acid*	< LOQ	0.0194±0.0026	< LOQ	0.0220±0.0058	<LOQ
3	4-Hydroxymethylbenzoic acid*	< LOQ	0.0135±0.015	< LOQ	0.0267±0.0028	0.0005±0.0003
4	Caffeic acid	0.0015±0.0001	0.0152±0.0010	0.0017±0.0003	0.0109±0.0015	0.0011±0.0
5	Cinnamic acid	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
6	Chlorogenic acid	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
7	Ellagic acid*	0.0043±0.0005	0.0382±0.0024	0.0018±0.0	0.0080±0.0014	0.0044±0.0010
8	Ferulic acid*	0.0052±0.0018	0.0073±0.0011	0.0038±0.0005	0.0074±0.0010	0.0031±0.0003
9	Gallic acid	< LOQ	0.0014±0.0002	< LOQ	0.0016±0.0001	<LOQ
10	Mandelic acid*	0.0012±0.0002	0.0039±0.0005	0.0014±0.0	0.0056±0.0001	0.0013±0.0002
11	*p*-Anisic acid*	<LOQ	< LOQ	<LOQ	<LOQ	<LOQ
12	*p*-Coumaric acid	0.0182±0.0036	0.0189±0.0028	0.0166±0.0017	0.0243±0.0046	0.0270±0.0058
13	Rosmarinic acid*	<LOQ	<LOQ	<LOQ	0.0001±0.0	<LOQ
14	Salicylic acid*	0.0024±0.0	0.0028±0.0002	0.0001±0.0	0.0033±0.0004	<LOQ
15	Sinapic acid*	nd	0.0004±0.0001	0.0006±0.0002	0.0014±0.0	<LOQ
16	Syringic acid	<LOQ	0.0017±0.0	< LOQ	<LOQ	<LOQ
17	Vanillic acid*	0.0060±0.0010[a]	0.0400±0.0047	0.0086±0.0006	0.0635±0.0068	0.0141±0.0019
	Flavonoid					
18	Apeginin*	<LOQ	nd	<LOQ	<LOQ	<LOQ
19	Catechin	<LOQ	nd	nd	nd	<LOQ
20	Chrysin*	<LOQ	<LOQ	nd	<LOQ	<LOQ
21	Epicatechin*	<LOQ	0.0005±0.0	0.0001±0.0	<LOQ	<LOQ
22	Eriodictyol*	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
23	Fustin*	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
24	Galagngina*	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
25	Hispidulin*	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
26	Kaempferol*	0.0411±0.0065	<LOQ	0.0025±0.0007	<LOQ	<LOQ
27	Myricetin*	0.0008±0.0	0.0011±0.0	0.0009±0.0	0.0026±0.0001	0.0012±0.0

28	Naringenin*	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
29	Pinocembrin*	<LOQ	<LOQ	< LOQ	nd	nd
30	Quercetin	0.0117±0.0011	0.0006±0.0001	0.0051±0.0007	0.0028±0.0002	0.0039±0.0006
31	Rutin	<LOQ	<LOQ	< LOQ	<LOQ	<LOQ
32	Taxifolin*	<LOQ	0.0004±0.0001	< LOQ	<LOQ	<LOQ
33	Vitexina*	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
	Stilbene					
34	Resveratrol	nd	nd	nd	<LOQ	nd
	Phenolic Aldehyde					
35	Coniferaldehyde*	<LOQ	0.0005±0.0001	< LOQ	0.0014±0.0002	<LOQ
36	Sinapaldehyde*	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
37	Syringaldehyde*	<LOQ	0.0022±0.0008	<LOQ	<LOQ	<LOQ
38	Vanillin	0.0003±0.0001	0.0152±0.0025	< LOQ	0.0357±0.0071	0.0008±0.0004
	Coumarin					
39	Scopoletin*	<LOQ	nd	< LOQ	nd	<LOQ
40	Umbelliferone*	<LOQ	0.0008±0.0001	< LOQ	0.0009±0.0003	<LOQ
	Phenolic Diterpene					
41	Carnosol*	<LOQ	<LOQ	< LOQ	<LOQ	<LOQ
Total Phenolic Content (mg g^{-1})		**0.0948**	**0.2214**	**0.0465**	**0.2656**	**0.0606**

< LOQ, not quantifiable. nd: not detected; * reported for the first time. Source: Author

Other phenolic compounds, such as 3,4 dihydroxybenzoic acids (1), caffeic acid (4), cinnamic acid (5), chlorogenic acid (6), ferulic acid (8), gallic acid (9), *p*-coumaric acid (12), syringic acid (16), catechin (19), epicatechin (21), kaempferol (26), naringenin (28), quercetin (30), rutin (31), taxifolin (32), and vanillin (38), are known to contribute to the AAs of the soursop seed, pulp, and leaf extracts obtained using different techniques and solvents (AGUILAR-HERNÁNDEZ *et al.*, 2019; MENEZES *et al.*, 2019). These were also detected in the soursop extracts obtained in the present study.

The identified compounds, caffeic acid (4), cinnamic acid (5), ferulic acid (8), and syringic acid (16), have been reported to have therapeutic applications in preventing diabetes, cancer, and cerebral ischemia, as well as possess antioxidant, antimicrobial, anti-inflammatory, antitumor, antiendotoxic, neuro and hepatoprotective activities (CHEEMANAPALLI *et al.*, 2018; NAM *et al.*, 2017; PEPERIDOU *et al.*, 2017). Several factors can affect the extraction characteristics, including the extraction and analytical method, time of harvest, maturity, variety, climate and soil conditions, sun exposure, location of the fruits on the plant, and post-harvest handling (MENEZES *et al.*, 2019; NAM *et al.*, 2017). Additionally, as shown in **Table 7,** 29 phenolic compounds have been detected for the first time, 16 of which are quantified in the extracts obtained by SWE of the soursop seeds (*Annona muricata*) by LC-ESI-MS/MS analysis, while six compounds are quantified in the SOX ethanol extracts for the first time.

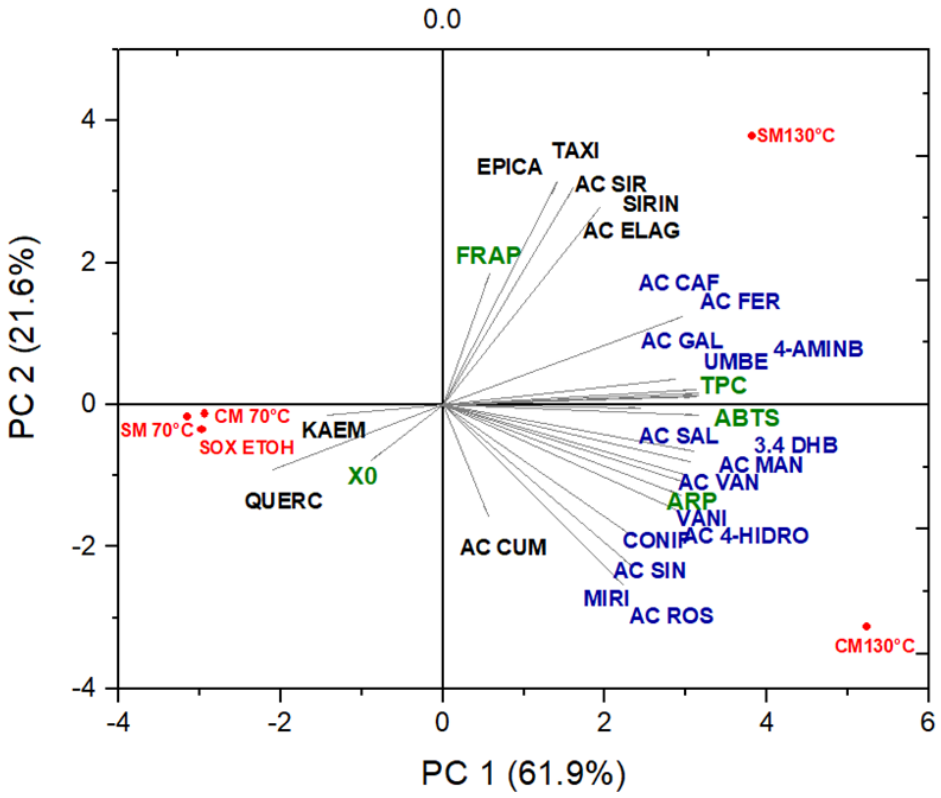
The scarcity of studies describing the recovery of phenolic compounds from soursop seeds by SWE makes the information presented herein important for value addition to the raw material. Additionally, it has not been extensively explored as an alternative greener technique compared to the traditional methods. Therefore, information regarding the chemical profile and AA is valuable for its applications in food or nutraceutical industries.

3.3.7 Principal component analysis (PCA)

PCA was applied to correlate the results obtained for X0, TPC, ABTS, ARP, FRAP, and the compounds identified by LC-MS/MS with the performance of the SWE (SM and CM) and SOX extraction methods. Multivariate treatment of the sample data allowed the reduction of the variables to two principal components. **Fig. 11** shows a two-dimensional graph with 83.5% experimental data variability. Principal component 1 (PC1) accounted for up to 61.9% of the total variance, and PC2 for 21.6% of the explained variance determined by eigenvalues

of > 1. As shown in **Fig. 11**, the extracts obtained by SWE at 130 °C in the SM or CM, TPC, ARP, ABTS, and FRAP data, and most phenolic compounds are positively correlated with the PC1 and PC2 axis. The SWE (SM and CM) extracts obtained at 70 °C, and quercetin and kaempferol contents are negatively correlated with the PC2 axis. In addition, SOX and X0 are negatively correlated.

Figure 11- Principal component analysis (PCA) of subcritical water extraction (SWE) in single-mode (SM) and combined mode (CM) at 70 °C and 130 °C and using the Soxhlet technique (SOX) with ethanol (EtOH) as a solvent, based on the global extraction yield (X0), total phenolics content (TPC), antiradical power (ARP), ABTS, FRAP assays, and phenolic compounds scores for the biplot of the first two PCs.



Compounds: 3,4 DHB (3,4-dihydroxybenzoic acids); 4-AMINB (4-aminobenzoic acid); AC 4-HIDRO (4-hydroxymethylbenzoic acid); AC CAF (caffeic acid); AC ELAG (ellagic acid); AC FER (ferulic acid); AC GAL (gallic acid); AC MAN (mandelic acid); AC CUM (*p*-coumaric acid); AC ROS (rosmarinic acid); AC SAL (salicylic acid); AC SIN (sinapic acid); AC SIR (syringic acid); AC VAN (vanillic acid); EPICA (epicatechin); KAEM (kaempferol); MIRI (myricetin); QUERC (quercetin); TAXI (taxifolin); CONIF (coniferaldehyde); SIRIN (sinapaldehyde); VANI (vanillin); UMBE (umbelliferone). Source: Author

Fig. 11 also shows a higher number of phenolic compounds on the right side of the graph, near SM and CM at 130 °C, forming a group with 15 phenolic compounds with higher TPC, ARP, and ABTS data, as well as a second group containing eight phenolic compounds, X0, and FRAP lower data, according to HCA analysis. The PCA revealed that SOX and SWE at 70 °C (SM and CM) showed a few phenolic compounds recovered on the left side of the graph but a high concentration of quercetin (0.0117 $mg.g^{-1}$) and kaempferol (0.0411 $mg.g^{-1}$). PCA Biplot shows more influence of CM extraction at 130 °C than the SM in recovery phenolic compounds. These data are consistent with the results of the previous studies describing the AA and phenolic compounds identified from soursop seed extracts (**Table 5, Table 7)**. Therefore, PCA is a suitable technique to determine similarities among the phenolic compound compositions of the extracts obtained by SWE and SOX methods. The PCA scatter plot is significant once all samples are projected in a two-dimensional graph, and comparisons between the samples are performed based on the response variables used in the study.

3.4 CONCLUSION

This study shows that a combination of green extraction techniques allows the collection of different fractions from soursop seeds. In the SWE method, X0, TPC, AA, and MRPs were positively correlated with temperature for a combination of high-pressure techniques, SFE + SWE. SEM analysis showed that the SFE step allowed lipid removal and caused the raw material cell wall rupture, which afforded a high recovery of polar bioactive compounds of the SFE residue when the CM SFE + SWE method was used. The soursop seeds oil extracted by SFE was mainly composed of unsaturated fatty acids, oleic and linoleic acids, and saturated fatty acids such as palmitic and stearic acids. Furthermore, the soursop seeds extract obtained from SWE, and SFE residue under different conditions showed the presence of vanillic acid, 3,4 dihydroxybenzoic acids, *p*-coumaric acid, vanillin, and caffeic acid as the most abundant phenolic compounds.

Moreover, 29 phenolic compounds were detected for the first time from soursop seed extracts. PCA analysis was a valuable tool for correlating phenolic compounds and AA with the extraction and technique temperature to obtain the soursop seeds extracts. Thus, nonpolar and polar extracts with phenolic and antioxidant compounds could be obtained using sequential high-pressure extraction steps, showing the potential valorization of the soursop industrial by-product from the cosmetic, pharmaceutical, and food industries. Then, the results found in this

work are important information to the circular economy approach by recycling food waste derived from the agri-food production chain, using green-based extraction techniques to recover bioactive compounds, encouraging a perspective zero waste.

3.5 ACKNOWLEDGMENTS

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CHAPTER 4 - RECOVERY AND VALORIZATION FROM ACEROLA PROCESSING BY-PRODUCTS

This study used an untargeted metabolomics approach to compare the chemical profile of acerola *(Malpighia emarginata* DC.) pomace extracts and the effect of raw material drying pretreatment before subcritical water extraction (SWE) at 70 to 130°C on the overall extraction yield, phenolic content, and *in vitro* antioxidant activity (DPPH, TEAC and, FRAP).

Here, since the SWE solvent is water, it was verified whether the drying step could be eliminated and whether this would have an influence on the extraction of bioactive compounds. In addition, as the amount of lipids here was low, the strategy was to use a polar technique and solvents. The SWE results were compared with those obtained through the Soxhlet method.

The results presented in this chapter were published in the Journal of Food Chemistry (https://doi.org/10.1016/j.foodchem.2022.133718)

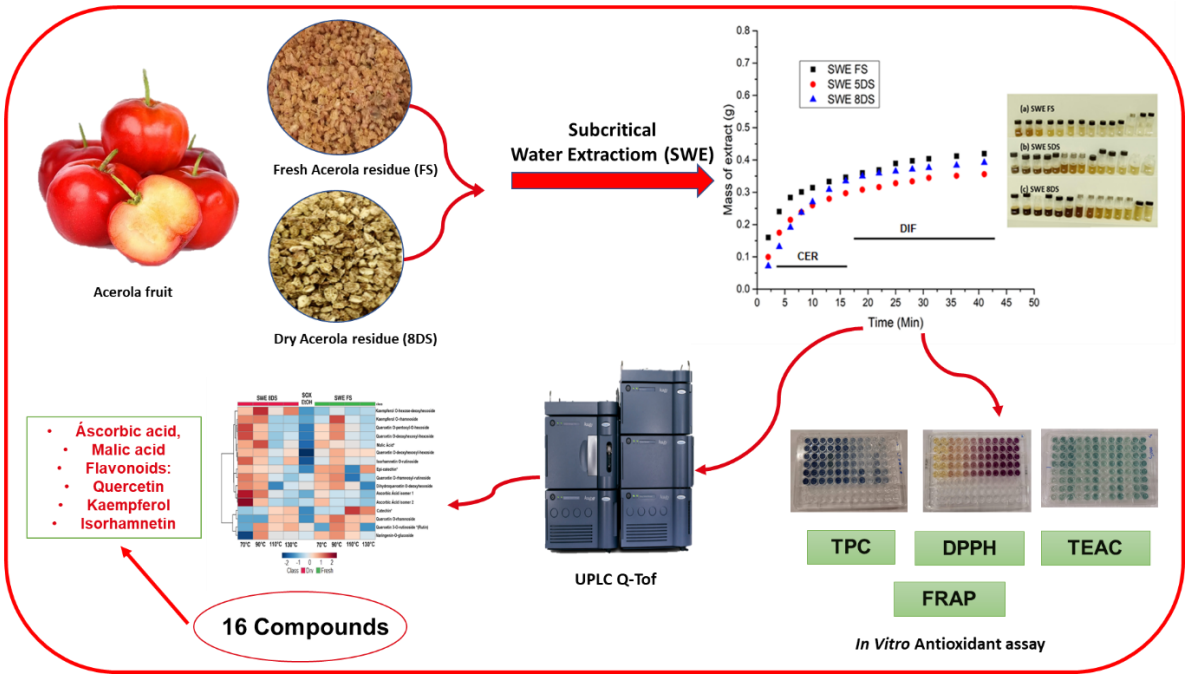
4 UNTARGETED METABOLOMIC PROFILE OF RECOVERED BIOACTIVE COMPOUNDS BY SUBCRITICAL WATER EXTRACTION OF ACEROLA (*Malpighia emarginata* DC.) POMACE

Abstract

The untargeted metabolomics approach was used to compare the chemical profile of acerola *(Malpighia emarginata* DC.) pomace extracts and the effect of raw material drying pretreatment before subcritical water extraction (SWE) at different temperatures on the extraction yield, phenolic content, and *in vitro* antioxidant activity. The SWE results were compared with those obtained through the Soxhlet. Overall, the findings suggest that the SWE saves time (15 min) and solvent for extracting valuable components versus the Soxhlet (6 h). The temperature increase affects the extraction yield (23.9 to 33.4%), phenolic content (119.1 to 362 $mgGAEg^{-1}$), and antioxidant activity varied significantly (for fresh and dry samples), being the SWE values higher than Soxhlet. The most abundant compounds detected by UPLC-ESI-QTOF-MS were ascorbic acid, kaempferol, quercetin, and isorhamnetin. The investigation of different moisture contents in the SWE had promising outcomes owing to eliminating a drying operation, saving time and energy, and obtaining highly concentrated phenolic-rich from discarded acerola by-products.

Keywords: *Malpighia emarginata* DC by-product, subcritical water extraction, phenolic compounds, antioxidant activity, heatmap analysis.

Graphical abstract



4.1 INTRODUCTION

The global acerola market is expected to increase to 17.5 billion dollars by 2026, owing to the rising global demand for natural bioactive-rich fruits and derivatives (SILVA *et al.*, 2020a). Brazil is the largest producer of acerola globally (60.966 tons/year), especially the northeastern region, which holds approximately 78% of the country's production (IBGE, 2017). Acerola fruit is typically processed into puree, juice, or juice concentrates, which generate large amounts of by-products, such as peels, seeds, and pomace.

The disposal of these by-products has adverse social and economic impacts (SILVA; DUARTE; BARROZO, 2019). It represents approximately 20% (w/w; Fresh Weight) of the processed weight (RAJHA *et al.*, 2014). These valuable by-products include high amounts of phenolic acids, flavonoids, Vitamin C, and other bioactive compounds, often with values higher than those found in the eatable portions of the fruit, and can be obtained in abundance from local agro-industries (NOGUEIRA *et al.*, 2019; REZENDE; NOGUEIRA; NARAIN, 2017; SILVA; DUARTE; BARROZO, 2019). Moreover, natural phenolic compounds possess antioxidants and anti-inflammatory, antimicrobial, and antiviral properties (ALVAREZ-SUAREZ *et al.*, 2017; BATAGLION *et al.*, 2015; POLETTO *et al.*, 2020). These bioactive

properties play an essential and crucial role because their antiviral activity capacity has been explored as an alternative to boost the immune system against various viruses (GALANAKIS *et al.*, 2020). Therefore, owing to their composition, acerola by-products have attracted significant attention as food and pharmaceutical supplements, supporting these by-products due to the growing global interest in environmentally friendly technologies (SILVA; DUARTE; BARROZO, 2019).

Extraction is the first step to obtaining bioactive materials from plants, and several methods have been reported to extract these compounds from agroindustrial wastes (REZENDE; NOGUEIRA; NARAIN, 2017). Conventional extraction approaches, such as maceration and Soxhlet extraction (SOX) with solvents, possess disadvantages, such as long processing time, high solvent waste rate, and hazardous solvent residues (ZHANG *et al.*, 2020). However, subcritical water extraction (SWE) is advantageous due to its safe and efficient operation, as well as environmental protection. In SWE, water at a temperature above its boiling point (100 °C at 0.1 MPa) but below its critical point (374 °C, 22.1 MPa) and at pressures sufficiently high to maintain the condensed state is used as the extractant (RODRIGUES *et al.*, 2019). In addition, SWE is advantageous due to non-toxic solvent water, short extraction times, high extraction rate, and highly tunable selectivity, which improve the quality of extracts and have application potential in dietary supplements to boost the immune system.

However, the extraction of acerola by-product extracts through the SWE technique has not been reported in detail. Furthermore, few studies have considered the effect of drying as a raw material pretreatment before SWE. For example, Náthia-Neves et al.(2017) used the wet raw material of genipap (*Genipa americana* L.) in the extraction of iridoids and phenolics by PLE. Machado et al. (2015) studied the PLE extraction of bioactive compounds from blackberry (*Rubus fruticosus* L.) fresh residues. Also, Rajha et al.(2014) used accelerated solvent extraction at different temperatures, and Monrad et al.(2012) used semicontinuous extraction using subcritical water to compare the effect of the drying process on the recovery phenolics from grape pomace. Higher yields of polyphenolics were observed using fresh rather than dried pomace.

To the best of our knowledge, this is the first study to evaluate the influence of moisture content (fresh or dry) and extraction temperatures on the SWE of acerola by-products and the possible improvement in the recovery of the antioxidant compound.

This comparison is interesting because the fresh waste is perishable, and treatments, such as enzymatic inactivation, pH reduction, and oxygen removal are required to avoid degradation (MONRAD *et al.*, 2012). Although moisture in fresh waste usually is undesirable in the extraction process, SWE is done in an aqueous medium at high temperatures and pressure, then is possible avoiding the drying step, which is an expensive pretreatment step. Herein, the chemical profile of acerola pomace extract (fresh and dry) was investigated using an untargeted metabolomics approach, expanding the human understanding of the bioactive compound in acerola by-products. This study is hopeful for industrial purposes, whereas utilizing the acerola wet pomace in the SWE eliminates a preliminary drying step, saving time and energy.

The valorization of those by-products reduces waste and permits the purification of added-value products. The recovered bioactive compounds can be further used as functional ingredients for foods and the cosmeceuticals and pharmaceuticals industry. Moreover, the results obtained through SWE were compared with those obtained by the Soxhlet technique to confirm the process's suitability.

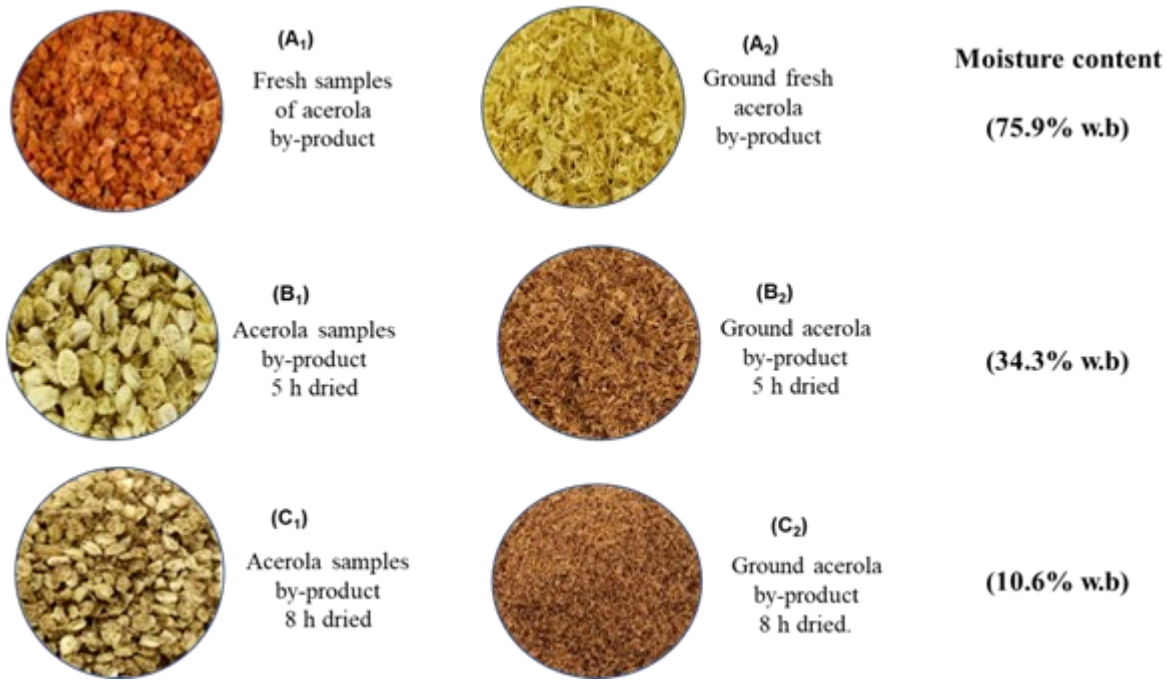
4.2 MATERIAL AND METHODS

4.2.1 Raw material

Ripe acerola (*Malpighia emarginata* DC.) by-product (seeds and pomace) from fruit pulp/juice processing was provided by a company located at the coordinates (3°44'48.8 " S; 38°31'07.3" W) Ceará/Brazil. The by-product was then taken to the Laboratory of Thermodynamics and Supercritical Technology (LATESC-UFSC) and stored at -18 °C. The sample was separated into three portions according to moisture content (w/w; wet basis (w.b)), determined according to the 925.09 AOAC method (AOAC, 2005a), based on our group's experience and as reported previously by Silva et al., (2020a): (i) Fresh samples (SWE FS) with a moisture content of 75.9 ± 0.3% w.b (1.2 g average dry mass); (ii) samples dried at 40 °C for 5 h (SWE 5DS) with air circulation (DeLeo, Porto Alegre/R.S. - Brazil) and moisture content of 34.3 ± 1.2% w.b (3.3 g average dry mass); and (iii) samples dried at 40 °C for 8 h (SWE 8DS) with air circulation and moisture content of 10.6 ± 0.1% w.b. (4.5 g average dry mass). Due to the high moisture content, a domestic blender was used to ground the fresh portions. All dried portions were grounded in a knife mill (DeLeo, Porto Alegre/R.S. - Brazil). The ground

samples (**Figure 12**) were packed in impermeable plastic bags and stored at -18 °C for further extraction assays. The samples with different moisture content were used to evaluate the possible reduction in cost associated with the drying step (a pre-extraction process of the acerola by-product).

Figure 12- (A_1) Fresh samples of acerola by-product (75.9% w.b), (A_2) Ground fresh acerola by-product (SWE FS); (B_1) Acerola by-product samples 5 h dried (34.3% w.b), (B_2) Ground acerola by-product 5 h dried (SWE 5DS); (C_1) Acerola by-product samples 8 h dried (10.6% w.b), (C_2) Ground acerola by-product 8 h dried (SWE 8DS).



Source: Author

4.2.2 Subcritical water extraction

The SWE assays were performed in a customized unit following an experimental procedure described by Rodrigues et al. (2019) in triplicate. First, the SWE period was defined from kinetic assays with SWE FS, SWE 5DS, and SWE 8DS acerola by-product samples (section 4.3.1), performed to obtain the overall extraction curve (OEC) by collecting extracts at pre-established period intervals, conducted at 10 MPa, 110 °C, and solvent flow rate 4 mL·min-1. From the kinetic assays, the acerola by-product samples selected for use in the second step (different extraction methods) were defined based on the higher OEC extraction yields (Figure

13, Section 4.3.2). The authors did not evaluate acerola by-product samples through SWE that were dried for 5 h (34.3% w.b).

The extraction time of SWE was established (15 min) in a dynamic mode, assays were interrupted, and the system drained, thereby exploring the OEC to recover most of the soluble material, as presented in Section 4.3.1.

In the extraction procedure, 5.0 g of acerola by-product samples were placed along with 64 g of glass spheres to form a fixed bed of particles inside the AISI 316 stainless steel extraction vessel (internal diameter of 25 mm, height of 180 mm, 90 mL). The extraction parameters were defined according to Monrad et al., 2012 and our group's previous experience. The SWE was conducted at 10 MPa, a water flow rate of 4 mL min^{-1}, and temperatures of 70, 90, 110, and 130 °C for fresh acerola by-products (75.9% w.b) (SWE FS) and acerola by-products dried for 8 h (10.6% w.b) (SWE 8DS). All experiments were performed in triplicates using sonicated degassed distilled water pumped directly into the extraction cell packed with samples through the HPLC pump (Waters, model 515, USA). The extracts, collected in glass flasks and rapidly cooled by a cooling fan, were stored under refrigeration and without light for further solvent removal by freeze dryer for 24 h (Liotop, model LD101, São Paulo, Brazil).

4.2.3 Conventional extraction

The Soxhlet (SOX) technique was performed with pure ethanol (99.8%) as a solvent following the method 920.39C from AOAC (AOAC, 2005a). The procedure (n = 3, mean values ± SD) consisted of recycling the solvent (150 mL) over 5.0 g of acerola by-products (fresh samples with a moisture content of 75.9% w.b) in a Soxhlet apparatus for 6 h at the solvent boiling temperature. The obtained extracts were then stored in amber flasks at -18 °C for further analysis.

4.2.4 Global extraction yield (X_0)

The global extraction yield (X_0) was calculated as the ratio of mass extracted (M_{Ext}) relative to the mass of the raw material used to feed the system on a dry basis (F), according to Eq. (2) proposed by Náthia-Neves et al., 2017:

$$\boldsymbol{X_0}(\%) = \left(\frac{\boldsymbol{M_{Ext}}}{\boldsymbol{F}}\right) * \mathbf{100} \qquad \text{Equation 2}$$

4.2.5 Total phenolic content (TPC)

TPC was determined through the Folin–Ciocalteu method (KOŞAR; DORMAN; HILTUNEN, 2005). First, 600 µL of water and 10 µL of samples (10 mg mL^{-1}) were mixed with 50 µL of undiluted Folin–Ciocalteu reagent (Sigma Aldrich, USA). After 1 min, 150 µL of Na_2CO_3 (20 % (*w/v*)) and 190 µL of distilled water were added. The mixture was stirred and kept at room temperature for 2 h in the dark; the absorbance was measured at λ = 760 nm against a blank. TPC results (n = 3, mean values ± SD) were expressed as milligram of gallic acid equivalent (GAE) per gram of dried extract from a gallic acid (Sigma Aldrich, >98%) standard curve (from 0.03 to 2.00 mg mL^{-1}).

4.2.6 Antioxidant activity

The antioxidant activity of the acerola by-product extracts was determined using the ABTS, DPPH, and ferric reducing antioxidant power (FRAP) methods. Herein, the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method was performed according to Brand-Williams, Cuvelier, and Berset. (1995). Briefly, 50 µL of diluted acerola by-product extracts was added into 195 µL of 125 µmol L^{-1} methanol: water (1:1 v/v) DPPH solution; the absorbance was measured at λ = 517 nm after 30 min at 25 °C in the absence of light. Trolox (Merck, Germany) was used as a reference, and the values were calculated from the standard curve (50 to 500 µM). The results (n=3, mean values ± SD) are expressed as µmoL of equivalent Trolox per gram of dry extract (µmol TE g^{-1}).

ABTS (2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) method was performed according to RE et al., 1999 with some modifications. The $ABTS^{\bullet+}$ solution was diluted with 5 mM sodium phosphate (pH 7.4) until the absorbance reached 0.7 (± 0.05). Thereafter, 30 µL extract (5 concentrations) was mixed with 970 µL $ABTS^{\bullet+}$, incubated in the dark for 45 min, and measured at λ = 734 nm. A Trolox (Sigma Aldrich, 97%) standard curve was used as a reference (from 50 to 500 µM). Results (n = 3, mean values ± SD) were expressed as millimole of Trolox equivalent (TE) per gram of dry extract.

The FRAP assay was performed according to the Benzie and Strain (1996) method. First, 10 µL (1 to 0.25 mg mL^{-1}) of solubilized extracts were mixed with 290 µL of FRAP reagent (0.3 M, pH 3.6 acetate buffer, 10 mM TPTZ, and 20 mM ferric chloride) in a microplate.

The absorbance was measured at λ = 593 nm after 30 min at 25 °C in the absence of light. Analysis was conducted in triplicate (mean values ± SD) with a blank for each sample (10 μL of solvent + 290 μL of FRAP). Trolox was used as a reference and calculated values from the standard curve (50 to 500 μM). The triplicate results were expressed as a micromole of Trolox equivalent per gram of dry extract (μmol TE g^{-1}).

4.2.7 Chemical profile by UPLC-QTOF-MS

4.2.7.1 Sample preparation

The chemical profile analysis was performed using the acerola by-product extracts of SWE FS and SWE 8DS at 70, 90, 110, and 130 °C and SOX. First, 1 mg of the extracts were suspended in 2 mL of 50% water/acetonitrile solution. The mixture was then sonicated in an ultrasound bath until total dissolution and filtrated in 0.22 μm PTFE (polytetrafluoroethylene) syringe filters, and 5 μL was injected into the Acquity UPLC system (Waters Co., Milford, MA, USA).

4.2.7.2 UPLC-QTOF-MSE analysis and metabolite identification

The UPLC-ESI-QTOF-MSE analysis was carried out on an Acquity UPLC-QTOF-MS (Xevo™, Waters®, Milford, MA, USA) system with an electrospray ionization (ESI) source. The method is detailed in the Supplementary Material (SI). The equipment was controlled by MassLynx 4.1 (Waters® Corporation) software. The chromatographic runs followed the protocols suggested by Da Silva et al. (2017). Compounds were tentatively identified by considering the respective *m*/*z* values, fragmentation profile, botanical family, and literature reports.

4.2.8 Chemometric analysis of the UPLC-QTOF-MS dataset

Data collection (peak list) was carried out after MS peak integration for all UPLC-QTOF-MS^E data obtained using MassLynx (Waters Corporation). The relative contribution of the peak area was calculated based on the total abundance of peak ions in the samples since the relative amplitude of the peak measurements provides the relative abundance of the isotopic forms in the chromatograms. The relative peak intensity is a dimensionless quantity and

corresponds to the values of the area below the peak obtained in the list of peaks (GBASHI *et al.*, 2017b).

For data analysis, a data matrix containing the detected peaks was generated using data pairs of mass data (*m/z*) and retention time (t_R) as identifiers for each peak (t_R-*m/z*), as well as the intensity of the peaks, names of the samples, and groups they belong to (SWE FS and SWE 8DS), where each SWE temperature extraction consists of three replicates. First, the chromatograms (ranging from 0.87 to 6.00 min) were pre-processed using MassLynx version 4.1. The resulting data matrix was submitted to MetaboAnalyst software and saved in base peak intensity (BPI) as CSV comma (*.csv) files. Then, an unsupervised chemometric analysis was performed, which does not require prior knowledge of the sample's nature. The ion intensities were autoscaled (mean-centered and divided by the standard deviation of each variable) to compare the individual characteristics in a better manner. Next, a heatmap analysis was created in MetaboAnalyst 5.0 (available at https://www.metaboanalyst.ca/). To analyze the dataset, Ward's algorithm and Pearson correlation were used for the HCA and Heatmap tools, respectively. The application of chemometric methods was limited to the metabolites tentatively identified between 0 and 5.22 min.

4.2.9 Statistical analysis

The one-way ANOVA followed by posthoc Tukey's HSD test at the $p < 0.05$ level was applied to all extraction procedures and *in vitro* analysis results using software Statistica version 7.1 (Stat-Soft Inc., Tulsa, OK, USA). The peak areas of the compounds obtained from their chromatograms are staggered according to auto-scaling. Chemometric analysis was performed using MetaboAnalyst 5.0 (www.metaboanalyst.ca) based on the non-normalized correlation matrices of normalized data.

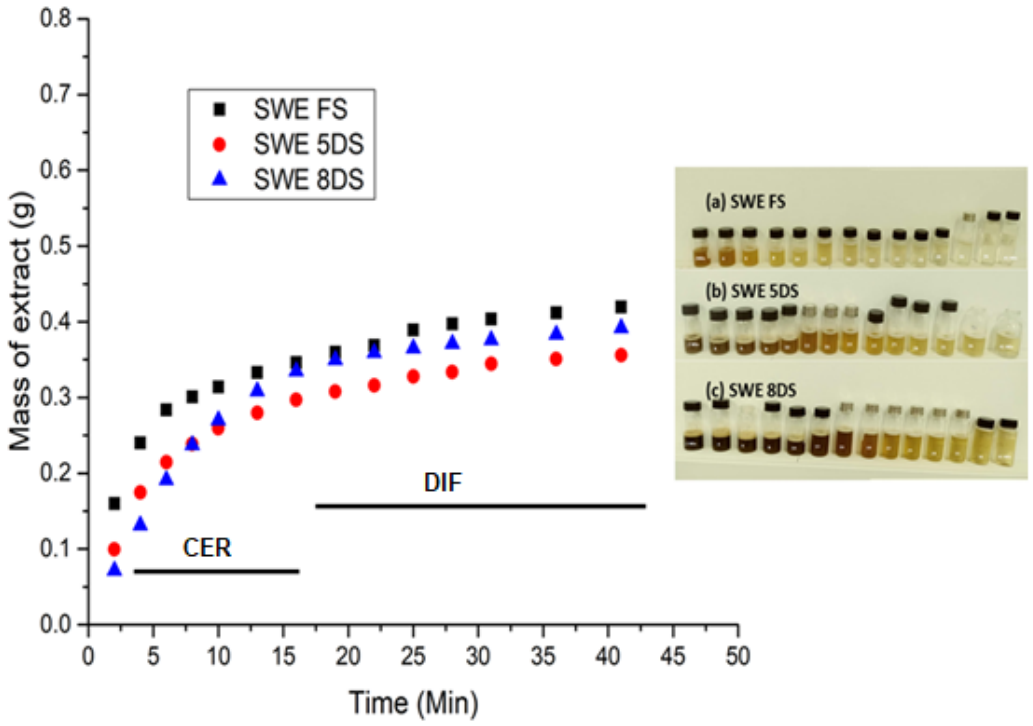
4.3 RESULTS AND DISCUSSION

4.3.1 Extraction kinetics

A kinetic study of SWE was performed to define the process extraction time and the influence of the moisture content on the extraction processes. **Figure 13** shows the OEC, which measures the accumulated mass of extract as a function of extraction time, carried out at 110 °C, 10 MPa, with a flow rate of 4 mL min^{-1} for fresh acerola samples (SWE FS; 75.9 % w. b), as well as those dried for 5 h (SWE 5DS; 34.3 % w.b) and 8 h (SWE 8DS; 10.6 % w.b).

Viganó and Martinez (2015) reported that the pressurized liquid extraction kinetics curves could be characterized by two different extraction periods. The first is a constant extraction rate (CER), where the solubility controls the extraction. The second period is the diffusion-controlled rate (DIF) period, which is controlled by the diffusion of solutes in the solvent. The global extraction curves (**Figure 13**) show that different mechanisms control mass transfer. In the initial period, i.e., approximately 15 min, the extraction yield increases with time due to the high solubility of solutes in the matrix surface representing a convection-controlled or solubility-controlled zone in the CER stage. Then, the DIF period combines a convection and diffusion mechanism (desorption-controlled zone), initiating the recovery of the compounds within the particle (16 to 41 min). In this step, it is possible to observe the reduction in the mass of the extract concerning time, which can be verified by the discoloration of all the acerola by-product extracts (**Figure 13** detail).

Figure 13 – Overall extraction curves obtained by SWE of acerola by-product at 110 ºC, 10 MPa, and 4 mL min^{-1} separated into three portions according to moisture content (w.b). In detail, the discoloration of extracts: (A) SWE FS (75.9 % w.b), (B) SWE 5DS (34.3 % w.b), and (C) SWE 8DS (10.6 % w.b).



CER: Constant Extraction Rate period. DIF: diffusion-controlled rate period. Source: Author

According to **Figure 13**, the highest values of accumulated mass over time presented in the OEC were obtained for the SWE FS and SWE 8DS processes (higher and lower moisture content, respectively), considering the same calculation basis (d.b). Despite the behavior of the SWE 5DS OEC showing accumulated mass values close to the SWE 8DS OEC, we chose not to continue the comparative study of extractions with the dry samples for 5 h, as this curve presented the lowest accumulated mass values. Thus, the research proceeded with the comparison between the extraction process using dry samples for an extended period (8 h) and with low moisture content (10.6% w.b) (which is frequently found in several extraction studies) and fresh samples without drying step (75.9% w.b). This comparison aims to evaluate the possibility of acerola pomace being used with high moisture content, eliminate the costs associated with drying, as well as to obtain acerola by-product extracts with good biochemical quality. These acerola by-products may contain 75-80% water, limiting their storage, transport, and shelf life (SILVA; DUARTE; BARROZO, 2019).

Pereira and Meireles, 2010 reported that 50 to 90% of the total extract is obtained during the CER stage. Therefore, in many situations, extraction ends shortly after the CER period, except when a specific compound is not very soluble in the solvent or is located in cell structures that are very difficult to access by the solvent. In this context, considering the OEC shown in **Figure 13**, more than 80% of the extract was recovered in the first 15 min of the process. This time was used for obtaining the X_0 in dynamic mode; after this period, the tests were interrupted, and the system drained. This drainage allows the recovery of the remaining soluble compounds inside the extraction vessel.

4.3.2 Global extraction yield (X_0) and TPC

Table 8 shows the results of X_0 and TPC for acerola by-product extracts obtained by SWE compared to the conventional technique (SOX) using an intermediate polarity solvent (ethanol). The extraction temperature of 130 °C presented the highest yields for both samples. According to the literature, the viscosity and surface tension of the water decrease with increasing temperature, which facilitates the solvent penetrating the plant matrix, accelerates the mass transfer rate and leads to higher extraction efficiency (NÁTHIA-NEVES *et al.*, 2017). In addition, during SWE at high temperatures, Van der Waals bonds, hydrogen bonds, and dipole-dipole molecular bonds break among the compounds and the plant matrix, which reduces the energy activation required for its desorption (GBASHI *et al.*, 2017a). However, elevated temperature can also cause degradation of the thermally labile analyte and neoformation of antioxidant compounds (ZHANG *et al.*, 2020).

Regarding the influence of the different moisture contents of raw materials on the SWE process, **Table 8** shows that the differences among the results were not more than 9.7% (compared to each corresponding temperature). Therefore, these results indicate that it is possible to use fresh acerola by-product samples in SWE techniques to obtain yields close to those of dry samples, eliminating the drying step during sample pre-preparation and reducing the time and energy used in all processes. A simplified yield analysis could demonstrate the feasibility of SWE extraction. Considering that 40% of the fruit weight is discarded during agro-industrial processing (REZENDE; NOGUEIRA; NARAIN, 2017), the yield analysis of SWE at 70 to 130 °C showed that it could recover 5.7 to 11.2 kg of TPC-rich extracts from 100 kg of acerola residue. These values could be considered remarkable when the yields from FS and 8DS were compared.

The SOX ethanol extraction yielded 21.6%, which was statistically similar ($p > 0.05$) to those obtained by SWE FS with the same moisture content (75.9% w.b). However, SWE was 24 times longer than SOX extraction (6 h SOX versus 15 min SWE).

Náthia-Neves et al. (2017) also conducted an extraction study with fresh ground samples (68 to 75%, wet basis) of genipap fruit (mesocarp, seeds, bark, endocarp, and combinations) to recover phenolic and other bioactive compounds by pressurized liquid extraction (PLE) with ethanol (2 MPa, 50 and 80 °C in static extraction for 5 min) and obtained promising results with an X_0 variation of 10.4 to 44%, expressed in the dry basis. However, the authors also mentioned that the high-water content of raw materials used in the extractions might have increased bed compaction, which reduced the extraction efficiency at high pressures.

TPC analysis (**Table 8**) indicates that many phenolic compounds remain in the fruit waste. For fresh and dry samples (8 h; 40 °C), the SWE at 70 °C had high TPC values, followed by a considerable reduction at 90 °C. The hypothesis for these results is that at 70 °C, the ascorbic acid degradation in acerola by-product extracts was lower than that at higher temperatures (ascorbic acid has low stability at high temperatures), influenced the results detected by the method of TPC. These results could be verified in the heatmap after the UPLC-MS analysis (discussed in **Section 3.4.1**). The second hypothesis concerning the drying process could have caused the degradation of a non-negligible amount of compounds, thus giving a select quantity of phenolic compounds for fresh pomace, which manifests higher TPC activity even at low temperatures. Another reason for this higher TPC observed for wet pomace might be their water content. The contact time with its high water content can be considered maceration, as a pseudo-solvent has already improved the extraction process prior to SWE.

To demonstrate these findings, in comparison to the other temperatures used in the SWE FS and 8DS, we show the heatmap (Fig.14) and Fig. S1 from supplementary material that clearly shows the enhancement of the phenolic compounds intensities as a function of temperature. Rajha et al. (2014) reported a similar trend from wet and dried grape pomace at 45°C used as a pretreatment for the accelerated solvent extraction process (ASE) conducted from 40 to 140°C.

Furthermore, with an increase in the SWE temperature from 90 to 130 °C, the TPC values increased significantly ($p < 0.05$) (both samples). Subcritical water can extract phenolic compounds more than other organic solvents. The solubility of the compounds can increase as the water extraction temperature increases owing to the decrease in water polarity and

weakening of hydrogen bonds (results in water with a lower dielectric constant) (ZHANG *et al.*, 2020). Moreover, the possibility of some reactions occurring at high temperatures during the extraction process may cause the neoformation of antioxidant compounds derived from Maillard and caramelization reactions when proteins/amino acids and reducing sugars were present in the natural samples (HERRERO *et al.*, 2015), enhancing TPC results at high temperature. According to Monrad et al. (2012) and Rajha et al. (2014), optimal extraction temperatures of phenolic compounds from different sources vary between 100 and 180 °C. The TPC of fresh and dry pomace extracts comparison at 70 and 130 °C was conducted. At 70°C, SWE FS was statistically similar ($p < 0.05$) to SWE 8DS pomace extract, while at 130°C, the amelioration of the TPC maintained the same proportion of magnitude despite presenting a statistical difference between SWE FS and SWE 8DS.

Considering that temperature could affect the hydration of the product, the dry acerola pomace phenolic compounds extractability probably increased when the temperature increased. The hydration increases during SWE, and in this specific case, the product could become less compact and more suitable for solid-liquid extraction (NÁTHIA-NEVES *et al.*, 2017; RAJHA *et al.*, 2014). Overall, the phenolic compounds extracted showed a similar trend in the fresh pomace extracts to those of dry pomace, diminishing and rising until tested maximum values of 130 °C. This observation seems to correlate to the drying pretreatment process and possibly caused damage to the food, altering the products' physical and biochemical characteristics and color and texture. Degradation of nutritional substances and aroma compounds also occurs (RAJHA *et al.*, 2014).

The drying operation is a well-known process to obtain dry products with a long shelf life. Long periods of sample exposure to the heat (40 °C/8 h) also promoted a nonenzymatic degradation and confirmed browning due to heating and increased acidity, which usually occurs due to the degradation products monosaccharides (LARROSA; OTERO, 2021). As shown in **Figure 12 C2**, there was a change in the color of the sample after drying.

Table 8– Global extraction yield (X_0), total phenolics content (TPC), and antioxidant activity evaluated by DPPH, ABTS, and FRAP methods of acerola by-product extracts obtained by SWE fresh samples (SWE FS) (75.9% w.b) and SWE 8 h dried samples (SWE 8DS) (10.6% w.b) at 10 MPa, 4 ml min^{-1}, and different temperatures and Soxhlet technique (SOX) with ethanol (EtOH) as a solvent.

Extraction Technique	Temperature	X_0 (%)	TPC (mg GAE g^{-1})	DPPH (µmol TE g^{-1}ext)	TEAC (µmolTE g ext^{-1})	FRAP (µmolTE g ext^{-1})
SWE FS	70 ºC	23.9±2.4^{c,d}	348.3±6.3^{a,b}	340.8±1.3^{e}	1040.6±48.0^{c}	571.8±9.3^{c}
	90 ºC	24.8±0.6^{c,d}	119.1±5.1^{g}	315.8±0.8^{f}	1587.0±84.1^{a,b}	388.3±9.8^{e}
	110 ºC	26.9±0.9^{b,c}	300.8±5.1^{e}	386.9±5.4^{d}	1476±79.8^{b}	452.7±1.1^{d}
	130 ºC	33.4±0.7^{a}	325.8±7.0^{c,d}	312.9±1.6^{f}	1833.1±72.5^{a}	545.1±19.3^{c}
SWE 8DS	70 ºC	24.5±0.02^{c,d}	335.1±2.0^{b,c}	454.8±1.7^{b}	1050.8±28.8^{c}	856.4±16.8^{a}
	90 ºC	24.9±0.6^{c,d}	270.1±5.1^{f}	437.6±5.3^{c}	1675.0±144.8^{a,b}	647.9±26.6^{b}
	110 ºC	29.8±2.0^{a,b}	317.0±2.9^{d}	471.5±1.5^{a}	1682.6±166.4^{a,b}	810.6±38.2^{a}
	130 ºC	30.3±0.3^{a,b}	362.0±6.0^{a}	469.9±4.4^{a}	1810.1±37.2^{a}	698.0±13.8^{b}
SOX EtOH*	-	21.6±3.4^{d}	292.6±5.5^{e}	264.4±0.6^{g}	600.8±27.0^{d}	321.0±9.8^{f}

*SOX EtOH: fresh samples; GAE: Galic acid; TE: TROLOX Equivalent. Values are mean ± standard deviation. In each column, other superscript letters mean groups statistically different ($p < 0.05$). Source: Author

Nogueira et al. (2019) cited that some fruit residues may contain elevated amounts of phenolics, sometimes with higher values than the edible parts of the fruit. In addition to phenolics, ascorbic acid can be recovered by SWE (see Section 3.4), and the cake from the extractor vessel could be explored to obtain other compounds, such as oligosaccharides, owing to its rich composition in dietary fibers. The proximal composition of acerola by-products has been reported by Sancho et al. (2015) and Carmo, Nazareno, and Rufino (2018).

Náthia-Neves et al. (2017) also conducted an extraction study with fresh ground samples (68 to 75%, wet basis) of parts of the genipap fruit (mesocarp, seeds, bark, endocarp, and combinations) to recover phenolic and other bioactive compounds by pressurized liquid extraction (PLE) with ethanol (2 MPa, 50 and 80 °C in static extraction for 5 min) and obtained promising results with an X_0 variation of 10.4 to 44%, expressed in the dry basis. However, the authors also mentioned that the high-water content of raw materials used in the extractions might have increased bed compaction, which reduced the extraction efficiency at high pressures.

We compared our results with those of conventional extractions reported in the literature, and the comparison suggested that the SWE processes recover a significant amount of TPC from acerola by-product extracts. For example, Silva; Duarte; Barrozo,(2019) studied multiple drying stage equipment to remove the moisture of acerola pomace (77.5%–10.7% wet basis) and found higher TPC (12.4 mg GAE g^{-1}) content at milder conditions (80 °C and 1.50 $m.s^{-1}$) than TPC (8.8 mg GAE g^{-1}) with higher temperatures (150 °C and 3.0 $m.s^{-1}$).

The SWE is environmentally safe because it generally uses recognized safe (GRAS) solvents, where the solvent quantity is much smaller than that in traditional techniques, and the extract is less toxic (GALLEGO; BUENO; HERRERO, 2019). Therefore, SWE is considered an attractive extraction process.

4.3.3 Antioxidant activity

The antioxidant activity (AA) of acerola by-product extract was evaluated based on the DPPH free radical capture assay, Trolox equivalent antioxidant capacity (TEAC) assay (ABTS), and FRAP to reflect different potential antioxidant mechanisms. The antioxidant power results from the ability of the compound or extract to prevent the oxidation of the substrate in low concentration, preventing or delaying the self-oxidation reaction. DPPH and ABTS methods are radical-free color solutions and have the same action mechanism, a hydrogen atom donor, and the reduced form of the radical generated followed by loss of color.

By contrast, FRAP is characterized by electron transferability, which reduces iron ions in the presence of antioxidant compounds (FRAIGE *et al.*, 2018).

Table 8 shows the AA values of the extracts obtained with SWE FS and SWE 8DS at different temperatures. As seen by the TPC values, there is a significant reduction in AA when the temperature increased from 70 to 90 °C, except for the ABTS method. This result is probably associated with the ascorbic acid degradation in acerola by-product extracts when the temperature increases. The increase in the SWE temperature from 90 to 130 °C tends to increase the AA owing to the increased solubility and extraction efficiency of the phenolic compounds and the newly-formed phenolic compounds and/or partially degraded phenolic compounds from high temperatures that may result in higher antioxidant activities than their precursors (CO *et al.*, 2012).

This trend hypothesizes that the AA of acerola by-product extracts depends on several factors, such as the presence of phenolic compounds, ascorbic acid content, and other species of radical scavengers, as well as the possible synergistic effect of different constituents (CORREIA *et al.*, 2012; MEZADRI *et al.*, 2008).

When comparing the AA of fresh and dry pomace extracts at all studied temperatures (**Table 8**), the results (DPPH, ABTS, and FRAP) appeared to be divided into two temperature ranges. In the first temperature range, from 70 to 90 °C, the antioxidant activity decreased according to the previously discussed hypothesis regarding ascorbic acid degradation and the synergistic effect of partially degraded phenolic compounds that can probably result in the loss of bioactive molecules and thus leading to an overall lower radical scavenging activity. A similar trend was observed by Rajha et al.(2014) with ASE for wet and dry (60, 100, and 140 °C) grape pomace in the temperature range of 40 to 140 °C, and under the following conditions: 10 MPa, 15 min, 70% ethanol/water. The decrease in TPC and AA in this range was remarkable for SWE FS , but was only slight for SWE 8DS, which was expected. Other possible reasons may be attributed to the degradation of light-sensitive phenolic compounds since the fresh sample was exposed to light and UV radiation during the juice production process. Degradation caused by oxidation is expected when drying is performed in a conventional dryer due to the higher oxygen content (SOKAČ *et al.*, 2022).

Rezende, Nogueira and Narain (2017) reported that acerola residues are rich in anthocyanins, phenolic compounds, and total flavonoids. Therefore, anthocyanin and other polyphenolic compounds may degrade depending on various factors, including polyphenol oxidase activity, organic acid content, sugar concentration, and pH. Polyphenol oxidase can

cause a remarkable reduction in the phenolic constituents during long thermal treatments, affecting the measured TPC values (VASHISTH; SINGH; PEGG, 2011).

On the other hand, in the second temperature range of 110 and 130 °C (**Table 8**) where the AA effect of the dried pomace extracts became more important when compared to the fresh extracts according to the aforementioned hypothesis regarding temperature and drying time effects on the activity and stability of bioactive compounds through chemical and enzymatic degradation, volatilization, and/or thermal decomposition (DUZZIONI *et al.*, 2013). The drying pre-treatment (40 °C for 8 h) could destroy some of the endogenous antioxidants and positively affect the overall free radical scavenging activity properties of the extract which is only manifested at high temperatures. As a part of their resistance to the drying pretreatment, phenolic compounds in plants act as metabolic intermediates and usually accumulate in vacuoles thus appearing to be less accessible. This can explain why the dry pomace bioactivity was obtained after SWE at 110 °C and 130 °C where the phenolic compounds were liberated by breaking the cellular constituents (DUZZIONI *et al.*, 2013). This effect is not observed at low temperatures, during which fresh pomace extracts have better scavenging activity because of the poor extractability of these active compounds at low temperatures (RAJHA *et al.*, 2014).

Generally, our results clearly show that SWE extraction at 130 °C provides the highest phenolic compounds yield for fresh (325 mg GAE g^{-1}) and dry (362 mg GAE g^{-1}) acerola pomace extracts. At this same temperature, dry pomace extract demonstrated a better DPPH free radical scavenging activity (469.9 µmol TE g^{-1}ext) than the fresh extract (312,9 µmol TE g^{-1}ext). Due to the drying process effect on acerola pomace and the high temperature (130°C) extraction process, a specific combination of highly bioactive polyphenols (catechin, epicatechin, quercetin, kaempferol and others detected by UPLC- item 3.4) was extracted, leading to a positive synergism and a better radical scavenging capacity.

All the AA values of the SWE process were higher than those obtained by SOX ethanol (conventional extraction). In addition to the influence of the temperature, this result can be attributed to the high pressures in the SWE, which modified the configuration of tissues, forcing water to permeate areas of the matrix (pores) where solvents at lower pressures usually cannot, resulting in the solubilization of more bioactive compounds (GBASHI *et al.*, 2017a).

Some acerola AA recovery for different extraction techniques and conditions have been reported in the literature for dry acerola by-products. For example, conventional methods report values for dry (10% w.b) acerola by-products to extract of ABTS = 1,445.10 µmol TEAC g^{-1} for the hydroethanolic extract (CAETANO *et al.*, 2011) and FRAP = 351.00 µmol TE g^{-1},

DPPH = 226.86 μmols TE g^{-1} for methanol: water extraction for dried acerola (5.17% w.b) (BIANCHI *et al.*, 2019). Rezende; Nogueira; Narain (2017) found ABTS = 149 μmol TE g^{-1}, DPPH = 170 μmol TE g^{-1}, and FRAP = 272 μmol TE g^{-1} for ultrasound-assisted extraction of fresh acerola by-products in optimized condition (ethanol, by-product ratio, and extraction time). These results were lower than those found in this study for acerola by-product extracts (**Table 8**). Therefore, the SWE with fresh or dried samples can be considered a rapid, environmentally friendly technique which effectively combines temperature and pressure to recover acerola by-products of phytochemicals.

Acerola pomaces are renewable plant resources with precious phenolic compounds rendering them industrially attractive. To date, no studies have reported the use of fresh or dried acerola pomace for SWE. Therefore, SWE with fresh by-products would be an option to process in-house, with a shorter time frame that avoids bioactive compound degradation. High extraction efficiencies for phenolics and AA were realized using the SWE method relative to convention extraction methods (i.e., Soxhlet and maceration) and obtained using organic solvents. According to Monrad et al.(2012), it is unnecessary to add drying and pretreatment steps (such as inactivating enzymes, lowering pH, and removing oxygen) to prevent sample degradation to recover the polyphenolics and AA from acerola pomace by semicontinuous SWE.

In our study, we observed that fresh acerola residue (without previous drying step) subjected to the SWE resulted in an excellent recovery of extracts with phenolic compounds and AA compatible with the SWE extracts obtained from dry samples (SWE 8DS). These findings for fresh pomace are significant because they allowed the direct extraction of crude pomace without the need for a drying step in the pomace extraction process. This is a significant energy-saving advantage by eliminating a step in the process chain.

4.3.4 Chemical profile by UPLC-QTOF-MSE and Multivariate data analysis

UPLC-ESI-QTOF-MSE assessed the metabolic profile of SWE FS, 8DS, and SOX. The representative BPI chromatograms in negative mode (ESI^-) of the samples at different extraction temperatures are shown in **Figure S2** (**Appendix B -Supplementary material**). **Table 9** summarizes the UPLC-QTOF-MSE chromatographic list and the MS data for the experimentally identified compounds, including retention time (t_R), experimental and calculated *m/z* values for the molecular formula, and error fragments obtained by MS/MS, as

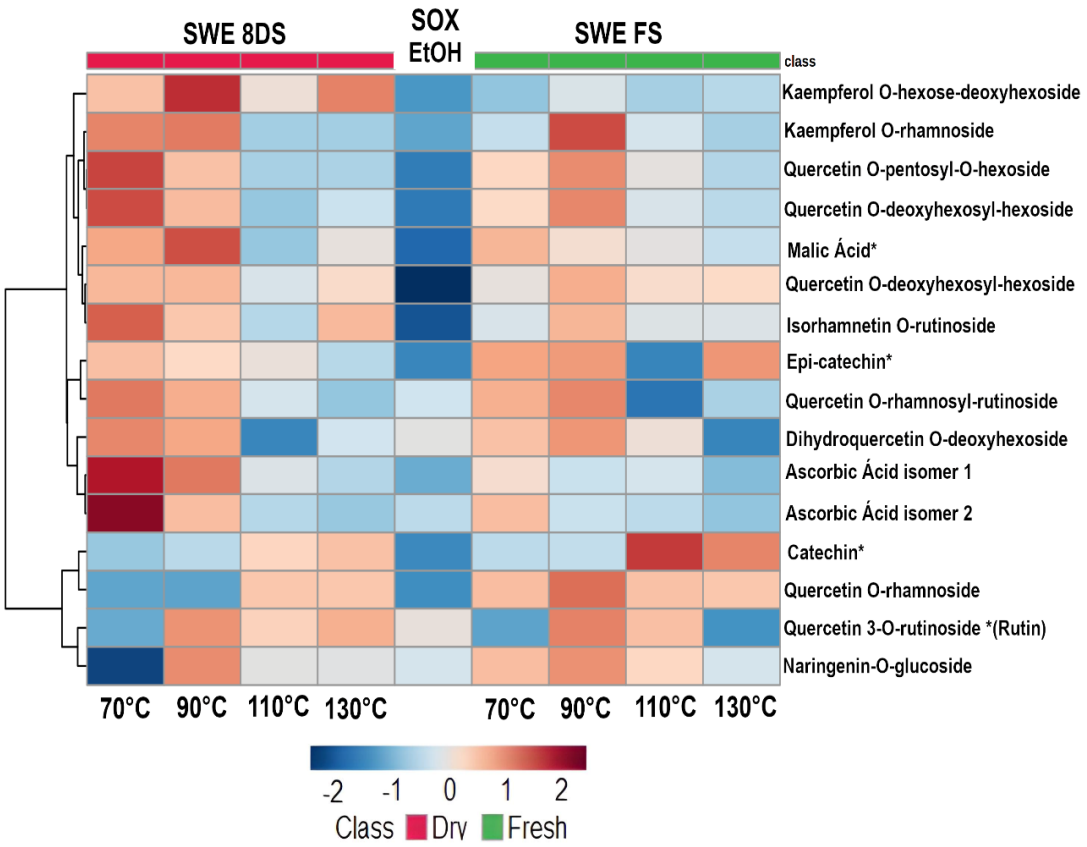
well as the proposed compound for each peak. Sixteen metabolites were tentatively identified as malic acid (1), ascorbic acid (2-3), and phenolic compounds (4-16) for acerola by-product extracts submitted to SWE for fresh and 8 h dried samples at 70, 90,110, and 130 °C and by SOX with ethanol, highlighting their potential reuse. Considering the relationship between the extraction temperature and the metabolites present in the extracts, the chromatograms observed in this study for SWE FS and SWE 8DS at 70 °C to 130 °C and SOX ethanol (**Figure S2**) were quite similar. **Figure S2** also shows the intensity of the peaks as the change in extraction temperature, with ascorbic acid, quercetin, and kaempferol derivatives primarily present in SWE FS and SWE 8DS extracts at different temperatures.

Grape pomace extract obtained by ASE in the 40–140 °C temperature range under the following conditions: 10 MPa and ethanol/water (70%) for wet and dry (60, 100, and 140 °C) samples, was reported by Rajha et al.(2014); the extract also showed a tendency to enhance the yield of phenolic compounds with temperature, which was also observed in SWE FS and SWE 8DS acerola pomace.

Xu et al.(2020) analyzed the extract of acerola fruits (*M. emarginata*) in different stages of maturation and identified 133 metabolites according to the MS/MS fragments compared to internal database standards and found many antioxidant compounds, such as flavonoids, are more predominant in ripe than in immature fruit. However, few studies have a metabolomic approach to acerola residues, especially for ripe fruit residue.

The heatmap in **Figure 14** indicates the influence of moisture and temperature on the extraction into the molecules identified in acerola by-product extracts. For example, the highest intensities were found for ascorbic acid isomers by SWE 8DS process at 70 °C, with subsequent intensity reduction as temperature increases. This trend was observed for almost all 16 compounds identified, with greater intensity for the SWE 8DS process. This result is possibly associated with the bioactive compound degradation (ascorbic acid and flavonoids) in acerola by-product extracts when the temperature increases. This behavior shown by ascorbic acid isomers from the SWE process corroborates the results found in **Table 8**.

Figure 14 - Heatmap with hierarchical clustering of the main metabolites obtained for SWE FS, 8DS, and SOX of acerola by-products at different temperatures.



*Samples are in columns, and variables are in rows. The colors vary from deep blue to dark red to indicate relative abundance values (mean of n=3 for each SWE and SOX extraction time).
Source: Author

Quercetin (quercetin *O*-rhamnoside; quercetin pentosyl-*O*-hexoside) and kaempferol (kaempferol *O*-rhamnoside) proved to be the most abundant among the aglycone identified in the acerola by-product extracts, being more intense in the fresh sample at 90 °C than in the dry sample processed by SWE. Compared with the conventional method (SOX ethanol), the SWE could recover compounds with greater intensity. The flavonoids intensity of catechin (110 and 130 °C) and Epi-catechin (70, 90, and 130 °C) in SWE FS was higher than those in the SWE 8DS, which indicated that fresh samples were more suitable for flavonoids extraction than 8 h dry samples. Other glycosylated flavonoids, such as quercetin *O*-deoxyhexosyl-hexoside,

dihydroquercetin *O*-deoxyhexoside, isorhamnetin *O*-rutinoside, and naringenin-*O*-glucoside, for example, had the optimum extraction temperatures between 70 and 90 °C, SWE 8DS.

An overall interpretation of the heatmap (**Fig. 14**) and chromatograms (**Fig. S2 - Appendix B**) intensity by comparing fresh and dry pomace extracts to flavonoid molecules revealed that these were by far more present in the extracts, especially those obtained at 90 °C from the wet and dry pomace.

Marques et al.(2016); Alvarez-Suarez et al.(2017) also reported catechin, epicatechin, quercetin, and flavonoid derivatives, including the glycosides of quercetin, dihydroquercetin, methyl quercetin and kaempferol as the main phenolic compounds of acerola pomace.

The quantity and composition of phenolic compounds also depend on the ripening stage, cultivation type, and the harvesting season. For example, the phenolic content in the mature fruits collected in the dry season was higher than those collected in the rainy season (RIGHETTO; NETTO; CARRARO, 2005; XU *et al.*, 2020).

Analyzing the impact of drying/not drying raw materials for SWE by observing the chemical profile and AA is complex because of the various compounds present. For example, it is known that factors such as high temperatures, oxidizing conditions, and the ripening stage can lead to significant differences in the antioxidant content in ascorbic acid (NÓBREGA *et al.*, 2015). This work confirmed that the acerola by-products had high phenols, flavonoids, ascorbic acid, and other antioxidant compounds. Some of which are in higher content than those in the edible parts of the fruit (BATAGLION *et al.*, 2015). Moreover, the bioactive compounds of acerola by-products are natural antimicrobial metabolites capable of inhibiting phytopathogenic fungi growth and have high AA, resistance against oxidative stress, antiproliferative activity in colon cancer cells, and other biological effects related to the prevention of common disorders (NOGUEIRA *et al.*, 2019).

Thus, the SWE technique minimizes environmental impact and is an advantageous alternative to recovering bioactive acerola by-product constituents. The study of different moisture conditions for the SWE process showed that the dry sample material is not a prerequisite step before the extraction process. This can be an advantage when using highly perishable fresh products and saves energy, and can bring an economic and environmental positive impact on acerola processing.

Table 9 - Chromatographic and mass spectrometry data of acerola by-product extracts processed by SWE of fresh samples obtained by UPLC-QTOF-MS in negative ion mode.

Peak nº.	t_R (min)	$[M-H]^-$ Observed	$[M-H]^-$ Calculated	Product Ions (MS/MS)	Empirical Formula	Ppm (error)	Tentative Identification
1	0.87	133.0145	133.0137	-	$C_4H_5O_5$	6.0	Malic Acid*
2	1.08	175.0239	175.0243	115.0002, 87.0066	$C_6H_7O_6$	2.3	Ascorbic Acid isomer 1[(1)]
3	2.57	175.0238	175.0243	115.0078	$C_6H_7O_6$	-2.9	Ascorbic Acid isomer 2[(1)]
4	3.29	289.0723	289.0712	-	$C_{15}H_{13}O_6$	3.8	Catechin*
5	3.64	289.0721	289.0712	-	$C_{15}H_{13}O_6$	3.1	Epi-catechin*
6	3.85	595.1307	595.1299	463.0804, 301.0302, 300.021	$C_{26}H_{27}O_{16}$	1.3	Quercetin *O*-pentosyl-*O*-hexoside[(2)]
7	4.09	609.1437	609.1456	301.0372, 300.0397	$C_{27}H_{29}O_{16}$	-3.1	Quercetin 3-*O*-rutinoside *(Rutin)
8	4.15	755.2056	755.2035	609.1501, 301.0398, 300.011	$C_{33}H_{39}O_{20}$	2.8	Quercetin O-rhamnosyl-rutinoside[(3)]
9	4.27	609.1443	609.1456	463.0895, 301.01 e 300	$C_{27}H_{29}O_{16}$	-2.1	Quercetin *O*-deoxyhexosyl-hexoside[(4)]
10	4.31	463.0881	463.0877	301.0354, 300.0288	$C_{21}H_{19}O_{12}$	0.9	Quercetin *O*-hexoside[(5)]
11	4.35	593.1500	593.1506	285.0596	$C_{27}H_{29}O_{15}$	-1.0	Kaempferol *O*-hexose-deoxyhexoside[(5)]
12	4.42	449.1089	449.1084	303.0390, 285.0416, 151.0014	$C_{21}H_{21}O_{11}$	1.1	Dihydroquercetin *O*-deoxyhexoside[(5)]
13	4.51	623.1600	623.1612	315.0474, 314.0426	$C_{28}H_{31}O_{16}$	-1.2	Isorhamnetin *O*-rutinoside[(6)]
14	4.69	447.0928	447.0927	301.0312, 300.0155	$C_{21}H_{19}O_{11}$	0.2	Quercetin *O*-rhamnoside[(3)]
15	4.90	433.1152	433.1135	271.0556	$C_{21}H_{21}O_{10}$	3.9	Naringenin-*O*-glucoside[(7)]
16	5.13	431.0972	431.0978	285.0331, 255.1908,	$C_{21}H_{19}O_{10}$	-1.4	Kaempferol *O*-rhamnoside[(5)]

***Comparison by the standard. (t_R): Retention Time;** (1) Wabaidur, Alothman, & Khan (2013); (2)Fraige et al. (2018);(3)Abu-reidah, Arráez-román, Lozano-sánchez, Segura-carretero, & Fernández-gutiérrez (2012);(4) da Silva, Rodrigues, Mercadante, & Rosso (2014);(5)Alvarez-Suarez et al.(2017);(6)Chen et al. (2015);(7)Tauchen et al. (2016). Source: Author

4.4 CONCLUSIONS

Nontarget metabolomic profiling was performed to find bioactive compounds in acerola agroindustrial by-products. Compared with the conventional approach, the SWE extraction efficiency with fresh and dry samples was higher, with its products possessing a better radical scavenging activity. The UPLC-ESI-QTOF-MSE tentatively identified malic acid, ascorbic acid, and phenolic compounds.

The drying pretreatment of the raw material and the extraction temperature significantly affected the extraction yields, TPC, and antioxidant activity of the resulting compounds. The process performed at 90 °C seemed to harm the SWE extraction of polyphenols from fresh and dry acerola pomace. On the other hand, in the 110–130 °C temperature range, the polyphenol extracts from dry pomace exhibited a slightly higher radical scavenging activity due to a specific polyphenol mixture and an apparent synergistic effect between different phenolic compounds.

Furthermore, the extracted phenolic diversity and relative abundance were directly related to the pretreatment of acerola pomace (wet or dry) and the extraction temperature.

There was no direct correlation between the phenolic content and the free radical scavenging activity. It can be concluded that combining different kinds of flavonoid molecules, ascorbic acid, and newlyformed phenolic compounds induces a synergistic effect, which is the basis of the free radical scavenging activity highlighted in this study.

The strategy of studying different moisture contents in the SWE technique was promising, presenting results similar to the process with a drying step before extraction. Moreover, using water as a solvent, the residue from the extraction process can be further used to recover other compounds of interest, which is an advantage because eliminating the drying operation saves time and energy costs.

The feasibility of the SWE technique permits continuous and direct extraction of fresh acerola pomace to prepare highly concentrated and bioactive phenolic extracts using an environmentally friendly solvent, especially since medium-pressure solvent extraction technology is available on an industrial scale. It is also compatible with the food industry for the valorization of this agriculturally derived waste stream.

This work offers a practical alternative to produce valuable products from discarded acerola by-products and obtain natural products that can be used as food supplements or

ingredients, active compounds, and cosmetics in the pharmaceutical industry, potentially increasing the economic profit while decreasing the environmental waste.

4.5 ACKNOWLEDGMENTS

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CHAPTER 5: RECOVERY AND VALORIZATION FROM STRAWBERRY (*Fragaria x ananassa*) PROCESSING BY-PRODUCTS

This study investigated the recovery of bioactive compounds (total phenol content and antioxidant activity), extraction yield (X_0), and the chemical profile by ultra-performance liquid chromatography coupled to mass spectroscopy (UPLC-MS) from strawberry pomace (70 to 130°C) in a single and/or a sequential extraction approach as follows: (1) Microwave-assisted extraction (MAE); (2) Supercritical pretreatment (SP) of raw material with a rapid depressurization rate, followed by MAE of the SP residue (SP+MAE); (3) Subcritical water extraction (SWE), all using water as a solvent; and compared to (4) Soxhlet (SOX) extraction with ethanol. Moreover, the antiproliferative activity against 4T1, B16F10, SF295, and Sk-MEL-28- tumor cell lines were tested.

The strategy for recovering phenolic compounds from strawberry pomace was to expose the matrix compounds with low lipid content (low-yield SFE), through rapid depressurization of the SFE followed by rupture of the achenes + fibrous part, making the solutes more accessible through the use of polar solvents combined with high-pressure extraction techniques

5 GREEN-BASED TECHNIQUES APPROACH TO RECOVER ANTIOXIDANT COMPOUNDS AND ASSESSMENT OF ANTIPROLIFERATIVE ACTIVITY OF STRAWBERRY (*FRAGARIA X ANANASSA*) POMACE FROM JUICE AGROINDUSTRY

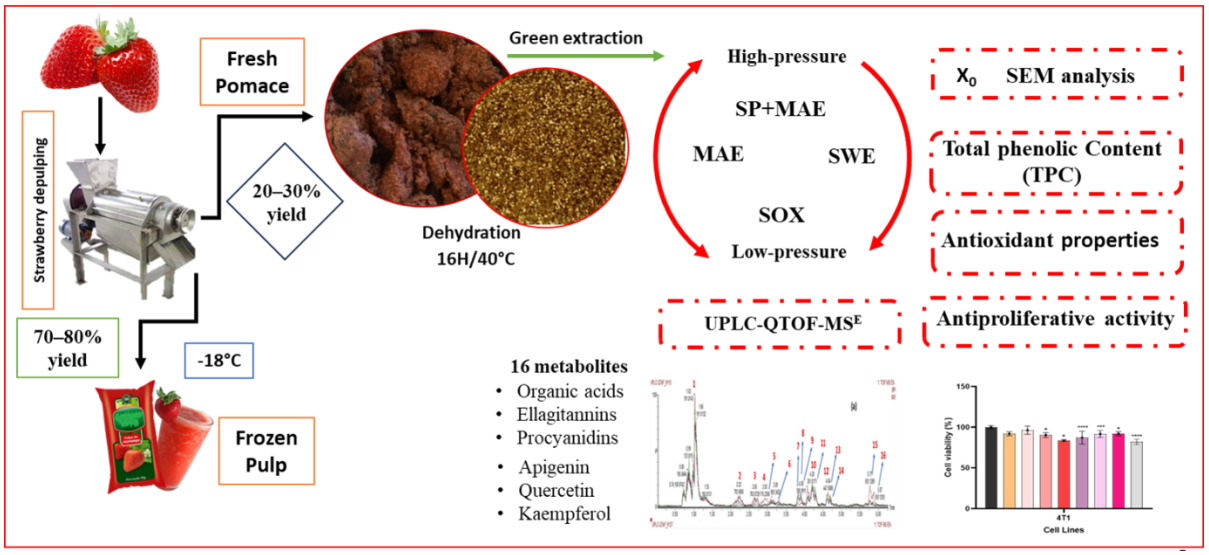
Abstract

The strawberry industrial juicing process produces a large volume of pomace rich in bioactive phytochemicals. This study investigated the recovery of bioactive compounds (total phenol content and antioxidant activity), extraction yield (X_0), and the chemical profile by ultra-performance liquid chromatography coupled to mass spectroscopy (UPLC-MS) from strawberry pomace (70 to 130°C) in a single and/or a sequential extraction approach as follows: (1) Microwave-assisted extraction (MAE); (2) Supercritical pretreatment (SP) of raw material with a rapid depressurization rate, followed by MAE of the SP residue (SP+MAE); (3) Subcritical water extraction (SWE), all using water as a solvent; and compared to (4) Soxhlet

(SOX) extraction with ethanol. Moreover, the antiproliferative activity against 4T1, B16F10, SF295, and Sk-MEL-28- tumor cell lines was tested. UPLC-MS identified 16 metabolites, including organic acid, ellagitannins, procyanidins, and other phenolic compounds. The best results of the X0 (24.65%) and TPC (269,10 mg GAE g-1) were achieved with SWE 130°C extract. The SWE 130 ºC (0,44 EC_{50} mg mL^{-1}), SP+MAE 130 ºC (1205,16 µmolTE g^{-1}), and MAE130 ºC (1010,99 µmolTE g^{-1}) extract possess a strong antioxidant capacity in DPPH, TEAC, and FRAP scavenging. The SP+MAE 130° extract at 50 µg mL^{-1} showed 81.2% cell viability for the SF295 cell line. The results revealed significant advantages compared to conventional extraction methods, and more research is needed to evaluate the antiproliferative activity of strawberry pomace extract.

Keywords: Supercritical CO_2; SWE; MAE; Depressurization rate; UPLC-MS

Graphical abstract



5.1 INTRODUCTION

Strawberry (*Fragaria x ananassa*) is one of the most consumed red fruits globally, and the data show that world strawberry production is around 9 million tons per year, and South America produces about 386 thousand tons (FAOSTAT, 2021). Although Brazil is not on the world's most extensive producers list, the production was around 219 thousand tons, with a cultivation expansion in Brazil's northeastern region, producing 2.857,00 tons (IBGE, 2020).

Strawberries are a great source of valuable ingredients, including vitamins, minerals, and bioactive compounds. Due to their nutritional and sensory characteristics, food products derived from or flavored with strawberries are well accepted (FELIX *et al.*, 2018; KO *et al.*, 2017). Nevertheless, fresh strawberries are highly perishable because of this rapid softening and microbial spoilage, which can decrease the shelf-life of berries (AABY *et al.*, 2018). So, a significant amount of harvested strawberries are processed into juice, purées, wine, jams, and other products (MILLER; FEUCHT; SCHMID, 2019; TERIBIA; BUV; LOEY, 2021).

During the industrial juicing process, a large volume of agro-waste is formed by the achenes (seeds), stems, and fibrous parts named strawberry pomace (STRUCK *et al.*, 2016). This strawberry pomace represents 10–15% of the initial fruit weight and is usually recycled as animal feed, composted, used for biogas production, or discarded, triggering severe environmental problems (GRZELAK-BŁASZCZYK *et al.*, 2017; PUKALSKIENĖ *et al.*, 2021; SÓJKA *et al.*, 2013).

Recently, a great interest in strawberry pomace has arisen to produce bioactive compounds due to their high content of phytochemicals, with phenolic compounds being the main compounds detected, represented by flavonoids, cinnamic acid conjugates, ellagitannins, ellagic acid conjugates, and anthocyanins, a natural dye with antioxidant capacity (FELIX *et al.*, 2018; REYNOSO-CAMACHO *et al.*, 2021; SAPONJAC *et al.*, 2015). Several studies have reported anti-inflammatory, anti-mutagenic, antibacterial, antiviral activity, and antiproliferative activities of strawberry extracts, which have been associated with preventing various diseases (ARIZA *et al.*, 2018; MEYERS *et al.*, 2003; PUKALSKIENĖ *et al.*, 2021).

Conventional extraction methods (maceration, Soxhlet) are widely used to obtain antioxidant compounds from plant matrices. However, these methods have technical, scientific, and economic impediments such as high energy cost and process time, low selectivity, relatively low yields, residual solvent impurities, and thermal degradation (CUBERO-CARDOSO *et al.*, 2020; EKEZIE; SUN; CHENG, 2017). On the other hand, alternative

solvents and innovative technologies such as Microwave-Assisted Extraction (MAE), Supercritical Fluid Extraction (SFE), Pressurized Liquid Extraction (PLE), and Subcritical Water Extraction (SWE) and their combination have been experienced in the development of greener technologies for recovery bioactive compounds from natural sources (CUBERO-CARDOSO *et al.*, 2020; FENG *et al.*, 2022; STRUCK *et al.*, 2016).

Among these greener extraction technologies, the SWE uses heated water at temperatures below its critical value (374 °C) but above 100 °C under sufficient pressure (1 - 22.1 MPa) to maintain its liquid state (PLAZA; TURNER, 2015). SWE is advantageous due to increasing temperature, the dielectric constant, viscosity, and surface tension of water will continuously decrease, and its diffusivity improves, enhancing the solubility of phytochemicals and their transfer from the solid matrix in a shorter time (MESQUITA *et al.*, 2021; PLAZA; TURNER, 2015; PUKALSKIENĖ *et al.*, 2021). The MAE process is an efficient and ecologically correct technology to extract phytochemicals from complex matrices, such as plants and fruits (ZHAO *et al.*, 2018). In a typical MAE procedure, microwave irradiation penetrates a material, interacts with polar compounds, and produces heating through ionic conduction or dipole rotation, resulting in cell walls rupturing and accelerating the release of compounds into the solvent (EKEZIE; SUN; CHENG, 2017; FENG *et al.*, 2022). Unlike conventional extraction techniques, MAE requires a shorter time and less organic solvent consumption to produce a higher yield (ZHAO *et al.*, 2018).

According to the Global Cancer Registry results, breast cancer is the second leading cause of cancer death in women, followed by lung cancer, killing more women yearly. A woman's chance of dying from breast cancer is about 1 in 39 (about 2.5%). Melanoma is a skin cancer that is the most common, and an estimated 97,610 new melanomas will be diagnosed in 2023. Melanoma is one of the most aggressive and chemoresistant human skin cancers. Also, Glioma is a common type of tumor originating in the brain. About 33% of all brain tumors are gliomas, originating in the glial cells surrounding and supporting neurons in the brain, including astrocytes, oligodendrocytes, and ependymal cells. (https://www.cancer.org/).

However, the literature review of strawberry pomace extracts' chemical composition and bioactivities is relatively scarce, mainly concerning antiproliferative activity, using non-conventional extraction techniques as an environmentally friendly method (ELİK; YANIK; GÖĞÜŞ, 2017; LIN *et al.*, 2020; PUKALSKIENĖ *et al.*, 2021). In this way, in the present study, the effects of high-pressure pretreatment methods on the sample structure with

subsequent sequential green extractions, as environmentally friendly practices, are investigated to recover bioactive compounds from strawberry pomace.

To our knowledge, this work offers a pioneering overview to develop new strategies to produce strawberry pomace extracts by taking *in vitro* activity evaluation (total phenol content and antioxidant activity) and the chemical profile by ultra-performance liquid chromatography coupled to mass spectroscopy (UPLC-MS) as a response. Moreover, the antiproliferative activity against 4T1(mouse mammary tumor cells), B16F10 (murine melanoma), SF295(human glioblastoma cells), and Sk-MEL-28 (human melanoma cells) - tumor cell lines were tested to evaluate the antiproliferative activity of strawberry pomace extracts obtained by different methods attempting to explore the *in vitro* bioactivity.

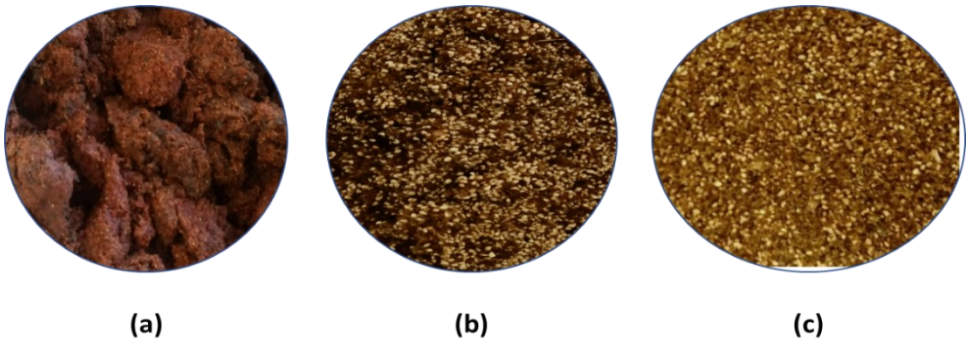
The TPC, antioxidant activity, chemical profile, and antiproliferative activity findings suggest that the strawberry extracts might have antioxidant and antiproliferative activities (PUKALSKIENĖ *et al.*, 2021; RODRIGUES *et al.*, 2020a; ZHANG *et al.*, 2008).

5.2. MATERIAL AND METHODS

5.2.1 Raw material

The by-product of fruit pulp processing, the strawberry pomace, was provided by the Tropicássia Polpa de Fruta company, Fortaleza, Brazil (3°44’48.8 “S; 38°31’07.3 “W). The strawberry pomace, composed of achenes, stems, and fibrous residual pulp parts, were placed in plastic bags, frozen at -18 °C, and transported to the Laboratory of Thermodynamics Supercritical Technology (LATESC) of the Federal University of Santa Catarina (UFSC). Upon arrival, the raw material was thawed and dried in an air circulation oven (DeLeo, Porto Alegre/R.S. - Brazil) for 16 h at 40 ºC presenting a moisture content of 11.07 ± 0,14 % (w.b.) determined according to AOAC method nº 925.09 (AOAC, 2005a). The dried material was crushed in a Willey knife mill (DeLeo, Porto Alegre/R.S. - Brazil) and stored in polyethylene packaging at −18 °C until use. **Figure 15** presents the material *in nature*, dried and crushed dried.

Figure 15-Strawberry pulp pomace material *in nature* (a), dried (b), and crushed dried (c).



Source: Author

5.2.2 Extraction procedures

The extraction procedures were performed using dried and milled *Fragaria x ananassa* pomace in four assay groups for evaluation of a single and/or a sequential extraction approach, as follows: (1) Microwave-assisted extraction (MAE), performed with dry raw material; (2) Supercritical pretreatment (SP) of raw material with a rapid depressurization rate, followed by MAE of the SP residue (SP+MAE); (3) Subcritical water extraction (SWE), performed with dry raw material; and (4) Soxhlet (SOX) extraction with ethanol, performed with dry raw material. All assays were performed in duplicate.

5.2.2.1 MAE

MAE was performed in a microwave reactor (Monowave 300 from Anton Paar GmbH) equipped with a single 850 W magnetron following the extraction procedure described by Mazzutti, Salvador Ferreira, Herrero, & Ibañez (2017) with modifications. Briefly, 30 mL extraction vessels were filled with 1 g of dried material, 20 ml of distilled water (1:20 dried material: solvent ratio), and a small magnetic stirrer. The vessel was placed in the microwave for the extraction process at 70, 90, 110, and 130 °C, under magnetic stirring at 1000 rpm for 10 min (based on the previous kinetic assay), and then the collected extract samples were cooled down to 55 ºC and filtered. The solvent was removed from the solution by freeze-drying

(Liotop, model LD101, São Paulo, Brazil), and the extracts were stored in an amber flask at -18 °C for further analysis.

5.2.2.2 Supercritical pretreatment (SP) + MAE

In this process, a Supercritical Fluid Extraction (SFE) unit previously described by Mazzutti et al. (2018) was used to perform a high-pressure pretreatment with CO_2 followed by a rapid depressurization rate. Firstly, the SP procedure was performed by loading 60 g of dried raw material into the extraction vessel, followed by pressurization to 30 MPa with CO_2 (99.95%, White Martins, Brazil) at 40 °C for 30 min (static compression). After the fixed time, a depressurization rate of 1.97 x 10^{-3} kg CO_2 h^{-1} allowed the pressure to decrease until atmospheric pressure. The procedure conditions were selected based on previous research group work (FERRO *et al.*, 2019). Finally, the pretreated dried raw material was collected from the extraction vessel and stored in a freezer (-18 ºC) for subsequent extraction with MAE (SP+MAE), according to the procedure described in item 5.1.2.1

5.2.2.3 SWE

The SWE assays were performed in a customized unit previously described by Rodrigues, Mazzutti, Vitali, Micke, & Ferreira (2019). The SWE period was defined based on a kinetic assay to obtain the overall extraction curve (OEC) by collecting the extract samples at pre-established period intervals at 10 MPa, 110 °C, and using 5 g of dried raw material and a solvent flow rate of 4 mL·min^{-1}. These conditions were established based on the group's previous experience (MESQUITA *et al.*, 2021; RODRIGUES *et al.*, 2019). From OEC, the extraction time of SWE was set (15 min) in a dynamic mode (1:18 dried raw material: solvent ratio), the assays were interrupted, and the system drained, exploring the OEC to recover most of the soluble material as presented in **section 3.1**. Then, the extraction procedure consisted of placing 5 g of dried raw material, followed by mixing it with 64 g of glass spheres to form a fixed bed of particles inside the AISI 316 stainless-steel extraction vessel (internal diameter of 25 mm and height of 180 mm, the volume of 90 mL). The SWE procedure was performed at 10 MPa, fixed flow of 4 mL $min^{-1,}$ and temperatures of 70, 90, 110, and 130 ºC. The remaining solvent in the extract was removed by freeze-drying for 48 h and stored in amber flasks at −18 ◦C for further analysis.

5.2.2.4 Soxhlet extraction (SOX)

A conventional atmospheric pressure extraction was carried out using the Soxhlet technique (SOX) with ethanol as solvent, performed according to the 920.39C method (AOAC, 2005a). The procedure consisted of 150 mL of solvent recycling over 5 g of dried raw material (1:30 dried material: solvent ratio) in a Soxhlet apparatus for 6 h at the solvent boiling point. Recovered extracts were filtered, and a rotary vacuum evaporator removed the solvent at 40 ºC. Extracts were stored in amber flasks at −18 ºC for further analysis.

5.2.3 Global extraction yield (X_0)

The global extraction yield (X_0) was calculated as the percentage of mass extracted ($m_{Extract}$) relative to the total mass of raw material on a wet basis (m_{RM}), according to **Equation (1)**:

$$\boldsymbol{X_0}(\%) = \frac{\boldsymbol{m_{Extract}}}{\boldsymbol{m_{RM}}} * \mathbf{100} \qquad \textbf{(1)}$$

5.2.4 Total phenolic content (TPC)

With modifications, the TPC of the strawberry pomace extracts was determined by Folin–the Ciocalteu method (KOŞAR; DORMAN; HILTUNEN, 2005). Shortly, 600 µL water and 10 µL aliquot of the extract water solution (10 mg mL^{-1}) were mixed with a 50 µL undiluted Folin–Ciocalteu reagent (Sigma-Aldrich, USA). After 1min, 150 µL of Na_2CO_3, 20% (*w/v*) was added, and the volume was increased to 1 mL using distilled water. The mixture was stirred and kept at room temperature for 2 h in the dark. The absorbance was measured at λ=760 nm (FEMTO, 800 XI, São Paulo, SP, Brasil) using the gallic acid standard calibration curve. TPC results were expressed as mg of gallic acid equivalent (GAE) per g of dried extract based on triplicate measurements.

5.2.5 DPPH free radical scavenging assay

The free radical scavenging capability of the strawberry pomace extracts was evaluated using the 1,1-diphenyl-2-picrylhydrazyl (DPPH; Sigma-Aldrich, USA) method (MENSOR *et al.*, 2001). Briefly, different extract concentrations were tested (five

concentrations for each extract) by mixing 0.3 mM DPPH solution (710 µL) and 290 µL of the extract solution, providing the reaction medium (1 mL). The absorbance at 517 nm was measured in a microplate reader (Tecan Infinite M200) monitored after 30 minutes in darkness at room temperature and converted into an antioxidant activity percentage (AA%). As the mean value ± standard deviation of triplicate assays, the results are expressed as EC_{50} values (test concentration required to decrease 50% absorbance compared to the blank solution) in $\mu g \cdot mL^{-1}$ units. The EC_{50} values were calculated based on the linear regression of the AA curves obtained for all extract concentrations.

5.2.6 TEAC-ABTS assay

The ABTS assay of the strawberry pomace extracts was performed according to the method described by Re et al. (1999) with some modifications. Synthetic vitamin E, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; Sigma-Aldrich, USA), was used as an antioxidant standard. First, 7 mM ABTS solution and 2.45 mM potassium persulfate solution were reacted at room temperature for 16 h in darkness to produce the radical $ABTS^{+}$ (2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)) diammonium salt. The $ABTS^{+}$ solution was diluted with 5 mM sodium phosphate (pH 7.4) until an absorbance of 0.70 (± 0.05) was achieved. Thirty microliters of the extract (five concentrations) were mixed with 970 µL of $ABTS^{\bullet+}$ and incubated in the dark for 45 min, followed by absorbance measurement at 734 nm, providing the standard curve (0.25–2 mM). The final data are expressed in micromoles of Trolox equivalent per gram of the dry extract ($\mu mol\ TE \cdot g^{-1}$). The results are expressed as mean ± standard deviation.

5.2.7 FRAP assay

The FRAP assay of strawberry pomace extracts was performed according to the method described by Benzie & Strain (1996). Briefly, 10 µL (0.1–0.5 $mg \cdot mL^{-1}$) of the solubilized extract was placed together with 290 µL of the FRAP reagent (0.3 M, pH 3.6 acetate buffer, 10 mM TPTZ (2,4,6-tripyridyl-s-triazine), and 20 mM ferric chloride; Sigma-Aldrich, USA) in a microplate. This solution was kept in the dark at room temperature for 30 min, and the absorbance was measured at 593 nm using a microplate reader (Tecan Infinite M200). The

analyses were performed in quintuplicate using a blank for each sample (10 μL of solvent + 290 μL of FRAP). Trolox (Merck, Germany) was used as a reference, and the values were calculated from the standard curve (50–500 μM). The results are expressed as micromoles of Trolox equivalent per gram of the dry extract (μmol TE·g^{-1}).

5.2.8 Scanning electron microscopy (SEM) analysis

SEM analysis (JEOL JSM 6390 LV, Musashino, Akishima, Japan) was performed at the Central Laboratory of Electronic Microscopy at UFSC, operating at 50 kV, with an 80 and 500x magnification. Strawberry pomace samples were analyzed as follows: (a) dry raw material sample, (b) dry solid material after SP (30 MPa, 40 °C,30min static mode), (c) dry solid material after SWE process (10 MPa, 110 °C, 15 min), (d) dry solid material after MAE process at 110 ºC (10 min) and (e) dry solid material after SP+MAE (110 ºC,10 min). Before scanning, the samples were coated with a thin gold layer by a sputter coater.

5.2.9 Chemical profile by UPLC-QTOF-MS

5.2.9.1 Sample preparation

Qualitative analyses of chemical compounds were performed using strawberry pomace extracts of SWE, MAE, SP+MAE at 70, 90, 110, and 130 ºC and SOX technique. First, 1 mg of extracts were suspended in 2 mL 50% water/acetonitrile solution. Then, the mixture was sonicated in an ultrasound bath until total dissolution and filtrated in 0.22 μm PTFE (polytetrafluoroethylene) syringe filters, and 5 μL was injected into the Acquity UPLC system (Waters Co., Milford, MA, USA).

5.2.9.2 UPLC-QTOF-MS^E analysis and metabolite identification

The UPLC-ESI-QTOF-MSE analysis was accomplished on an Acquity UPLC-QTOF-MS (Xevo™, Waters®, Milford, MA, USA) system with an electrospray ionization (ESI) source.

The method is detailed in **Supplementary Material (Appendix B)**. The equipment was controlled by Masslynx 4.1 (Waters® Corporation) software. The chromatographic runs followed the protocol suggested by Da Silva et al.(2017). The organic compounds were identified considering the respective *m*/*z* values, fragmentation profile, botanical family, and literature reports.

Structural elucidation and metabolite identification were based on MS and MS/MS fragmentation, and a presumed identification of compounds was performed, comparing data with the KNApSAcK Core System database SciFinder, PubChem, and ChemSpider. A putative identification was provided, including molecular formula and MS^E fragments. Also, chemical identification was based on chemotaxonomy (family, genus, and species)

5.2.10 Antiproliferative activity

5.2.10.1 Culture of cell lines

Cell lines 4T1 (mouse mammary tumor cells), B16F10 (murine melanoma), SF295(human glioblastoma cells), Sk-MEL-28 (human melanoma cells), and non-tumoral cell lines HUVEC (human umbilical vein endothelium cells), were provided from the Cell Bank of Grupo de Estudos de Interações entre Micro e Macromoléculas (GEIMM) of Federal University of Santa Catarina, Santa Catarina, Brazil and also available at American Type Culture Collection (ATCC), USA.

The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) medium supplemented with 10% fetal bovine serum (FBS). HUVEC, SF-295, and 4T1 cells were cultured in RPMI-1640 medium supplemented with 10%. All cell cultures were supplemented with 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, and 10 mM HEPES, maintained at 37 °C, pH 7.4 in a 5% CO_2, 95% humidified atmosphere, and passaged every 2 days. To evaluate the influence of concentration and time on cytotoxicity, a density of 1 x 10^5 cells well was seeded in a 96-well plate in 100 μL of culture medium and allowed to adhere overnight (24hs).

5.2.10.2 Cell treatment

At the beginning of the experiment, the number of viable cells was estimated by trypan blue exclusion assay in a Neubauer chamber.

The cells were exposed to different strawberry pomace extracts of MAE 130 °C, SP+MAE 130 °C, SWE 130 °C, and SOX technique, chosen due to high yields and better results on phenolic content, antioxidant activity, and chemical profile. Firstly, a stock solution of 5 mg mL^{-1} of the extracts was prepared using water, stirred slowly, and taken to the ultrasonic bath for dissolution. An aliquot was removed and diluted directly in the DMEM culture medium, obtaining 50 µg mL^{-1} and 100 µg mL^{-1} cell concentration, and exposed for 24 h (37 °C, 5% CO_2, 95% humidity). After incubation, 10µl of Alamar Blue was applied to each well and left in contact for 2 h (AHMED; GOGAL; WALSH, 1994).

5.2.10.3 Cell viability assessment: resazurin reduction assay

Evaluation of the cell viability was carried out by the Alamar blue (resazurin) assay (O'BRIEN *et al.*, 2000).

The results were measured with an Elisa fluorimeter reader using excitation/emission 530/590 nm and expressed in percentage as the mean ± standard deviation (sd). Cell viability (%) was determined using **Equation 3**:

$$Cell\ Viability(\%) = \left(\frac{RFU\ treated}{RFU\ Control}\right) * 100 \qquad \textbf{(3)}$$

Where $RFU_{treated}$ = relative fluorescence unit of the wells treated with the samples;

$RFU_{Control}$= relative fluorescence unit of untreated wells.

Results were expressed as mean ± standard deviation (SD) of three independent experiments (replicates on different days) and analyzed by GraphPad Prism® software. Analysis of variance (ANOVA) was followed by post hoc Dunett's test. A value of $P < 0.05$ was considered to be statistically significant.

5.3 RESULTS AND DISCUSSION

5.3.1Extraction kinetics of SWE

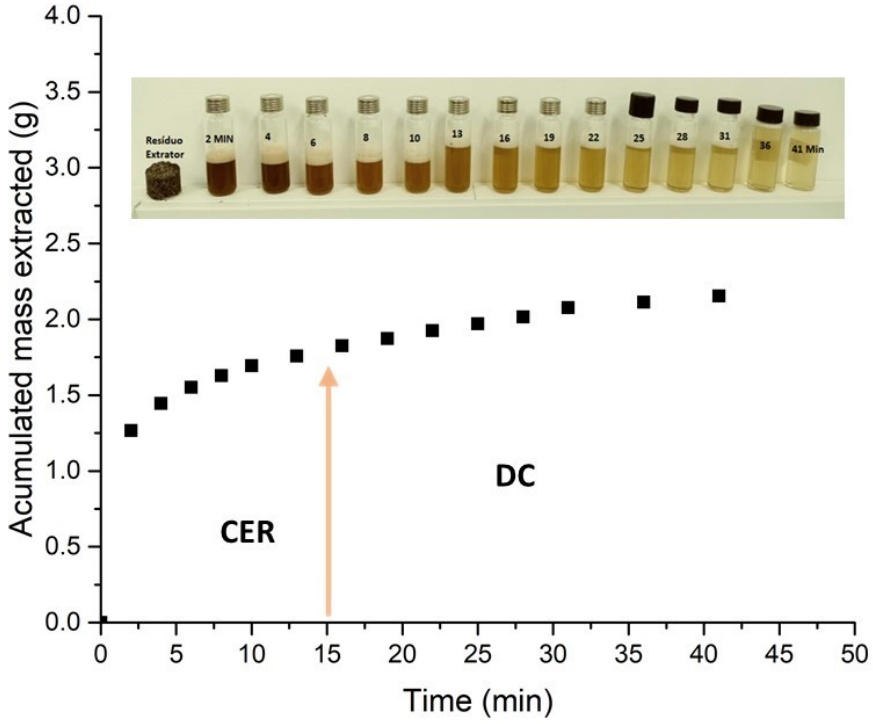
The Overall Extraction Curve (OEC) for evaluating the kinetic behavior of the strawberry pomace extraction by SWE was conducted at 110°C, flow rate 4mL min^{-1} and 10MPa, since the effect of pressure on recovery of most substances is usually negligible, and this variable has less influence compared to temperature on solvent properties (MUSTAFA; TURNER, 2011).

The SWE extraction process is related to an SFE analogous mass transfer mechanism based on classical period descriptions. In addition to that, the analysis of the kinetics behavior for SWE from strawberry pomace identified the following mass-transfer mechanisms: (1) constant extraction rate (CER), where the solubility controls the extraction, and (2) diffusion-controlled (DC), which is controlled by the diffusion of solutes in the solvent (MUSTAFA; TURNER, 2011; VIGANÓ *et al.*, 2016). The OEC (**Figure 16**) shows that different mechanisms control mass transfer during extraction. Firstly, the mass transfer is dominated by convection, characterized by the constant extraction rate of the solute available on the surface of the particles. In this period, approximately 15 min, it recovered 84.8% of the total yield.

Pereira & Meireles (2010) reported that 50 to 90% of the total extract is obtained during the CER period. Then, in the diffusion-controlled period (DC), the mass transfer is controlled by combining convection and diffusion mechanisms due to partial exhaustion of the surface solute. Consequently, the extraction rate for each subsequent fraction decreased with time. This period is characterized by a reduction in the extraction yield (15.2%) in 16 to 41 min. It is possible to observe the decline in the mass extract with the decrease of solute available on the particle's surface, which can be verified by the discoloration of all the strawberry pomace extracts, as highlighted in **Figure 16** (**detail**).

In addition to that, the extraction time was fixed at 15 min for all assays in a dynamic process because it represents the beginning of the DC period, where the solvent has already recovered most of the total solute amount, always performing drainage (with nitrogen gas) to recover the remaining extract in the extraction vessel at the end of each cycle. This result confirms that the SWE process has a high bioactive compound recovery rate and relatively low extraction time.

Figure 16- SWE kinetic curve of strawberry pomace residue; In detail, the concentration of SWE kinetics solutions before solvent recovery.



Source: Author

5.3.2 Global extraction yield (X_0) and total phenolic content (TPC)

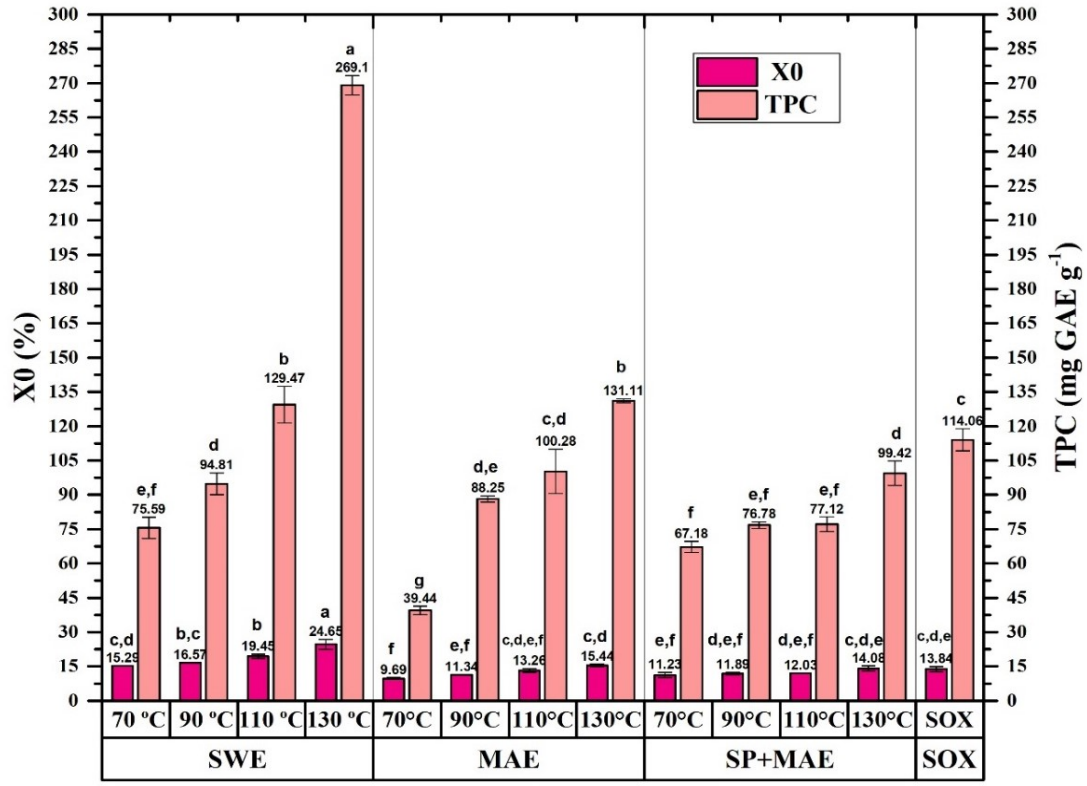
The results of the global extraction yield (X_0) obtained by MAE, SP+MAE, SWE, and SOX of the strawberry pomace extracts at different temperatures are shown in **Figure 17**. The MAE, SP+MAE, and SWE yields data increased with the rising extraction temperature of 70, 90,110, and 130 ºC, reaching 9.69 to 15.44% for MAE, 11.23 to 14.08% for SP+MAE, and 15.29 to 24.65% for SWE, all using water as solvent. The conventional extraction methods yielded 13.84%, showing no significant differences ($p < 0.05$) among all yields of SP+MAE processes, three of the MAE and two of the SWE at different temperatures. However, it should be noted that the Soxhlet technique (with ethanol) has longer process times and greater solvent use than innovative technologies. Then, the highest and most significant extraction yields found for the SWE at 110 and 130 °C can be explained mainly by the extraction time and temperature combined with the high-pressure process. This trend was also verified in the TPC results (**Figure 17**), with higher values for the SWE process at 110 and 130 ºC.

SWE is a technology that provides higher pressure and/or temperature above the boiling point of water and keeps it in a liquid state (MAZZUTTI *et al.*, 2017b). Such properties allow effective mass transfer and higher solubility of more hydrophobic compounds (PLAZA; TURNER, 2015). Thus, the rising temperature associated with high pressure facilitates the extraction process, speeding up intermolecular interactions and molecular motion and increasing the solubility of the solute and the diffusion coefficient (PLAZA; TURNER, 2017; RODRIGUES *et al.*, 2020b). Furthermore, due to their properties as a high dielectric constant, it favors the extraction of polar substances, such as phenolic compounds. So, the use of temperatures between 110 and 130 ºC promoted an increase in the solubility of phenolic compounds, as does the breaking of chemical bonds, increasing molecular diffusion rates and mass transfer, enhancing the solubility of solute into solution, providing better solubilization of medium-polarity phenolics (MAZZUTTI *et al.*, 2017b; RODRIGUES *et al.*, 2020b). Consequently, other phenolic compounds can be produced, and their hydroxyl groups will also react with the Folin-Ciocalteu reagent, causing a significant increase in absorbance (VÁZQUEZ-ESPINOSA *et al.*, 2018). As with the X_0 results, it was observed in **Figure 17** that the pretreatment with supercritical CO_2 (SP+MAE) did not increase the TPC results compared to the isolated MAE process. Conversely, SOX extraction showed TPC values above 100 mg GAE g^{-1}, comparable to the highest TPC values found for the SWE and MAE processes, mainly due to the lengthy process time (6 h) with solvent recycling.

There are few studies on SWE/PLE and MAE techniques using strawberry pulp or pomace, and until now, none on SP+MAE integration. For example, Elİk, Yanik, & Göğüş (2017), in optimization of MAE (100-300 W, extraction time of 2-16 min and solid: liquid ratio of 1:5-1:25 g/mL) with ethanol of organic strawberry crushed fruits, reported yield ranged from 6.90 to 9.69%, and TPC values ranging among 16.59 and 21.58 mg GAE g^{-1}. Pukalskienė et al.(2021) recovered PLE with ethanol and water (10.3 MPa at 90 °C and 110 °C) with yields of 28.6 % and 24.9 %, respectively, while for TPC, values of 29.6 and 21.5 mg GAE g^{-1}, smaller than those found in this study at the same temperature. For conventional extraction, the TPC values for strawberry pomace ranged from 2.64 to 41.55 mg GAE g^{-1} in different conditions of extraction (AABY; SKREDE; WROLSTAD, 2005; SAPONJAC *et al.*, 2015).

MAE and SP+MAE results suggest a relation between power and temperature in the recovery of phenolic compounds. So, reductions and degradation probably occurred after reaching an optimum microwave power, thus decreasing the number of compounds in the solution (LIN *et al.*, 2020).

Figure 17 -Global extraction yield (X_0) and total phenolics content (TPC) of strawberry pomace extract obtained by Subcritical Water Extraction (SWE), Microwave-assisted extraction (MAE), pretreatment with supercritical CO_2 followed by fast depressurization, and subsequent MAE (SP+MAE) at different temperatures and Soxhlet technique (SOX) with ethanol as a solvent.

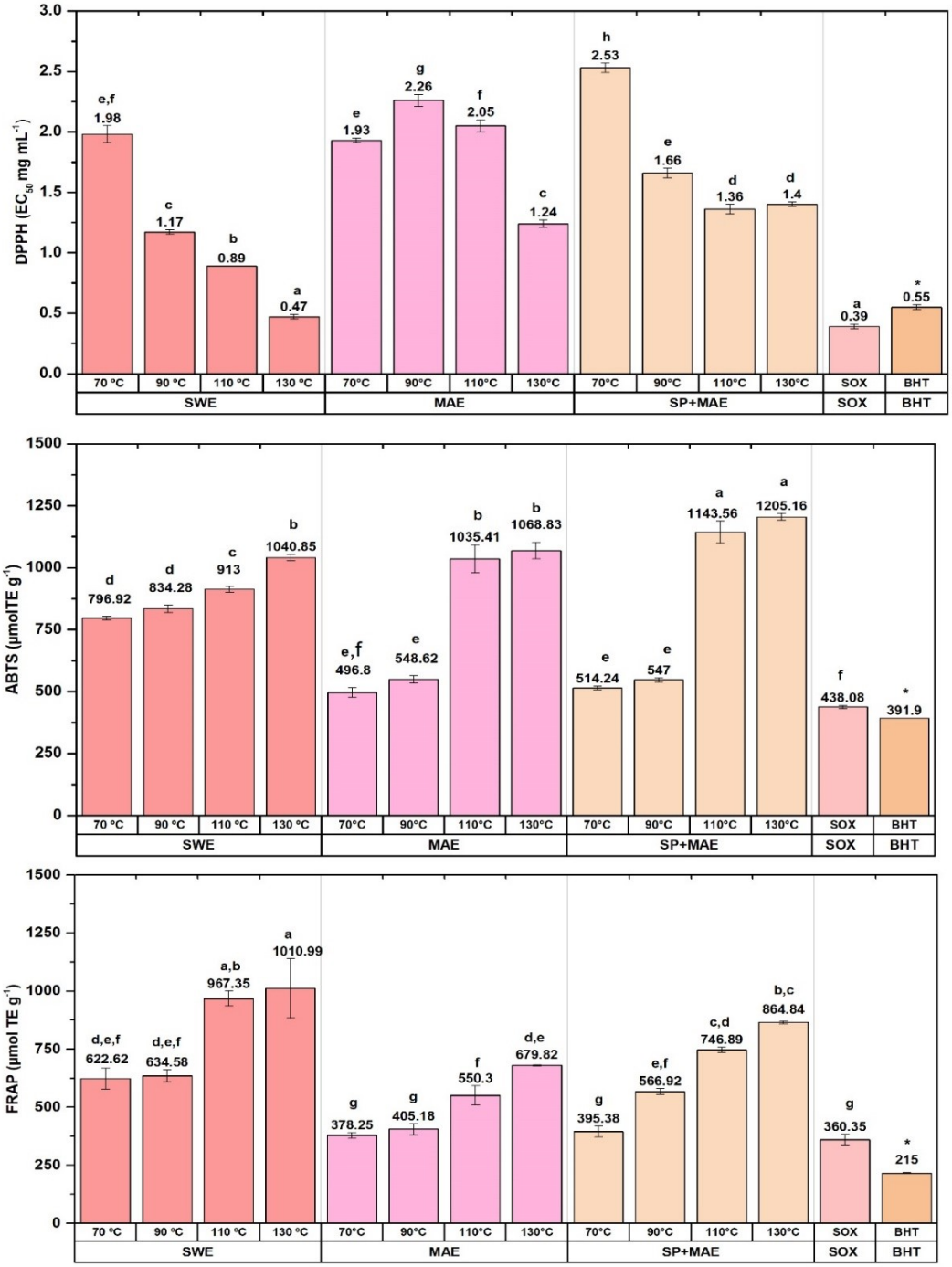


*In each bar/column, other superscript letters mean groups statistically different ($p < 0.05$) in each bar/column. Source: Author

5.3.3 Antioxidant activity (AA)

The antioxidant capacity of strawberry pomace extracts obtained by SWE, MAE, SP+MAE, and SOX was evaluated by different assays that detect the antioxidant activity and are widely used *in vitro*. **Figure 18** shows the results of the DPPH free radical scavenging, TEAC radical scavenging (ABTS), and the ferric reducing antioxidant power (FRAP) tests, which reflect the different potential antioxidant mechanisms of strawberry pomace extracts compared to the synthetic product BHT, used as a standard.

Figure 18 – Antioxidant activity evaluated by DPPH, ABTS, and FRAP methods of strawberry pomace extract obtained by Subcritical Water Extraction (SWE), Microwave-assisted extraction (MAE), pretreatment with supercritical CO_2 followed by fast depressurization, and subsequent MAE (SP+MAE) at different temperatures and Soxhlet technique (SOX) with ethanol as a solvent.



(*) Butylhydroxytoluene (Standart synthetic antioxidant) results from Lasta, Lentz, Mezzomo, & Ferreira (2019). Other superscript letter mean groups were statistically different ($p < 0.05$).
Source: Author

The DPPH analysis is considered a broad-spectrum methodology and is expressed in EC_{50} (mg mL^{-1}), representing the concentration necessary for an antioxidant to eliminate 50% of the initial DPPH concentration; thus, low values indicate high activity. It is observed in **Figure 18** that there is a gradual decrease in EC_{50} values with increasing extraction temperature with values ranging from 2.53 to 0.47 mg mL^{-1} g from SWE, MAE, on a single approach and to SP +MAE integration approach. The SWE 130 ºC and SOX techniques were the best EC_{50} results found, less than necessary to inhibit BHT used as a standard synthetic antioxidant.

This result reinforces one of the advantages of using the SWE technique: the shorter extraction time of bioactive compounds than SOX extraction. This better performance is due to the breaking of hydrogen bonds in the water as the temperature and pressure increase, thus altering its properties (ZHANG *et al.*, 2020).

Considering MAE and SP+MAE, EC_{50} results were better for MAE in a single mode than in combined mode (SP+MAE) and followed the trend of rising values as temperature increased. Also, in the extraction methods performed at high temperatures (SWE, MAE, and SP+MAE) a proportional relation between temperature and the responses DPPH, ABTS, and FRAP were obtained. The temperatures used mainly in SWE possibly increase the recovery of phenolic compounds and other antioxidant substances from the raw material, and may also be able to produce new compounds with antioxidant characteristics. The high-temperature levels, used at certain extraction conditions, can induce the formation of new bioactive substances, named neoantioxidants, for instance, through Maillard reaction, caramelization and/or thermooxidation (REBELATTO *et al.*, 2020).

The scavenging of radicals determined by TEAC (ABTS assay) ranged from 438.08 to 1205.16 μmol TE g^{-1}, and the antioxidant potential measured by FRAP ranged from 360.35 to 1010.99 μmol TE g^{-1} (**Figure 18**). In general, the extraction temperature influenced the recovery of phenolic compounds and the antioxidant activity of strawberry pomace subjected to SWE, MAE, and SP+MAE extraction, possibly due to the increase in the solubility of compounds affected by the rise in the extraction temperature, increasing the mass transfer (PLAZA *et al.*, 2010a). In this study, all ABTS and FRAP values were higher or similar to SOX ethanol and the synthetic antioxidant BHT found by Lasta et al.(2019).

For instance, considering MAE's extractions, TEAC-ABTS and FRAP results were better for SP+MAE (combined mode) than for single mode (MAE), it was observed at temperatures above 90°C. Probably, for these two AA potential analysis assays, the results were influenced by the rapid depressurization rate of the SP+MAE process that helped in the cellular

rupture, allowing the release of the non-polar fraction contained inside the seed (achenes). Consequently, favoring the extraction of polar compounds in the fibrous part and achene surface occurred in the second stage (MAE), enhancing or interfering with the AA potential. This trend was also observed in the study of Ferro et al.(2019) for antioxidant recovery of *Sida rhombifolia* leaves using pretreatment with supercritical CO_2, fast depressurization rate (5.55×10^{-4} kg CO_2 s^{-1}; 37.5 MPa min^{-1}), followed by PLE process (ethanol: water (70:30 v/v), 80 ºC, 10 MPa, 30 min, and flow rate of 2 mL min^{-1}), obtaining higher values of AA by FRAP assay compared to recovered extracts without depressurization rate.

In summary, different operating conditions of extraction may explain the differences in the results observed in **Figure 18** (i.e., ratio solvent: sample, time, static or dynamic mode) besides the mechanism of action for scavenging free radicals methods used to determine the antioxidant activities.

Furthermore, the results found in **Figure 18** are better or in agreement with previous studies of AA of strawberry pulp and pomace extracts by different techniques and solvents. The literature reported values for EC_{50} DPPH ranging from 0.11 mg mL^{-1} (pomace) to 3.77 mg mL^{-1} (fruit extract), ABTS scavenging activity ranging from 7.17(pomace) to 103.3 (Fruit extract) µmol TE g^{-1} of extract, and finally, FRAP values ranging from 17.7 (flesh freeze-dried) to 474 µmol TE g^{-1} extract (freeze-dried achenes). The values mentioned above were found by Aaby et al.(2005), De Souza et al.(2014), Saponjac et al.(2015), and Zhu et al.(2015) using conventional extraction (70% aqueous acetone, sonicated for 10 min), and methanol/water (50:50, v/v) for 1 hour. Emerging extraction methods (PLE and MAE) also were applied for the recovery AA of strawberry extracts, and the results ranged from 1.31 to 1.67 mg mL^{-1} for EC_{50}, from 291.6 to 391.9 mg TE g^{-1} for ABTS, and from 197.8 to 215.3 µmol TE g^{-1} for FRAP (ELİK; YANIK; GÖĞÜŞ, 2017; PUKALSKIENĖ *et al.*, 2021). The variability of the antioxidant activity values for strawberry extracts might be related to the different operating conditions of extraction, solvents, and pretreatments applied to the samples, vintage, type, and variety of material.

Thus, the results found in this study suggest that strawberry pomace extracts from agro-industrial production can be considered a potential source of bioactive phenolic compounds with high natural antioxidant capacity recovered by promising techniques such as SWE, MAE, and SP + MAE. Furthermore, these extracts can be technologically attractive as valuable nutraceutical components in foods, pharmaceutical preparations, or cosmetics.

5.3.4 Scanning electron microscopy (SEM) analysis

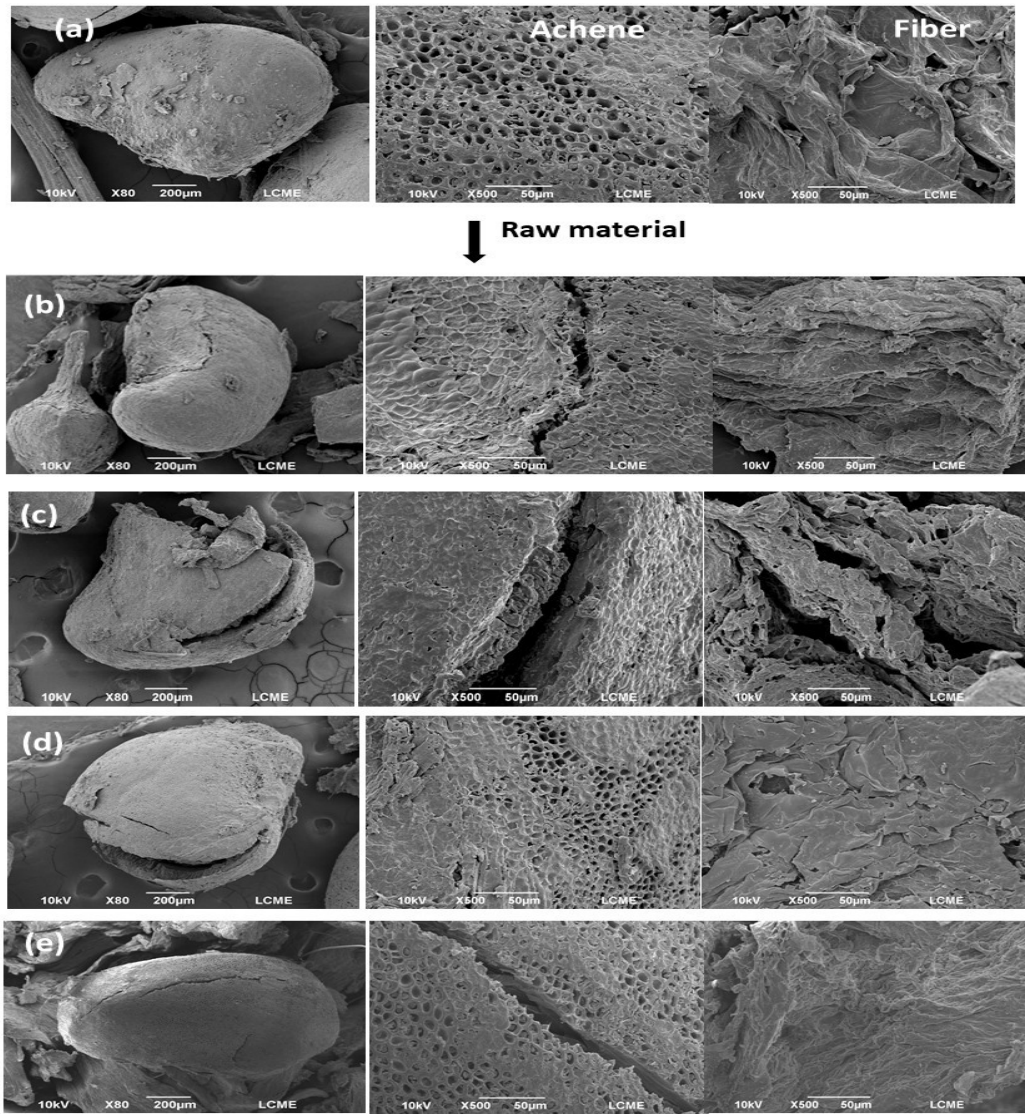
Figure 19 shows the images obtained by SEM for different strawberry pomace materials with 80x and 500x magnification to identify the structural impact of supercritical CO_2 pretreatment, SWE, and MAE extraction at a cellular scale. **Figure 19** (a) represents the pomace raw material (with no treatment), revealing intact morphology, compact structure without any disruption, cracks, or fragments, and consisting of the pulp and achene parts. However, when supercritical pretreatment (SP) with subsequent rapid depressurization was applied, a slight crack in the achene cell wall could be observed (**Figure 19** (b)). Then, when rapid depressurization occurs, CO_2 reaches its gaseous state with the maximum volume change, causing cell wall rupture in processed samples (UQUICHE; ANTILAF; MILLAO, 2016). Also, it can be seen that there was a change in the morphology of the cell wall (increased number of pores), which suggests a slight increase in the surface area.

On the other hand, in **Figure 19** (c), the sample of SWE extractor residue, 'it is observed that the structure material after SWE agglomerated and presented holes in the wall without destroying the wall structure fibrous part. The achenes presented disruption, cracks, or fragments causing morphological changes and released a complex mixture of phytochemicals contributing to scavenging activity. In **Figure 19** (d), the morphology of the residue material after the MAE process had a rough surface with the formation of alveoli (magnification 80 and 500x), changes on the surface of the achene, and the presence of less turgid, apparently more dehydrated pulp (500x magnification) compared with Figures (a) and (b). This fact is related to microwave irradiation that suddenly increases cell temperature and internal pressure due to the vaporization of the water from the plant matrix, causing the rupture of cell tissue. Besides, dehydration of cell tissue caused by microwave radiation can make plant tissue more fragile and easily ruptured during extraction (UQUICHE; JERÉZ; ORTÍZ, 2008). In contrast, prolonged exposure to microwave irradiation could decrease sample moisture content, directly influencing the amount of phytochemicals from the achenes and fibrous parts extracted from the strawberry pomace (ĐURĐEVIĆ *et al.*, 2017).

The effect of supercritical CO_2 pretreatment followed by MAE (SP+MAE) on the cell morphology was confirmed by SEM analysis (**Figure 19** (e)). A slight rupture of the cell wall is observed, causing a physical change in strawberry pomace due to existing high pressure in the supercritical phase, increased pores due to CO_2 high diffusibility, and improved surface areaSo as described earlier, the MAE effect would help break down the achene and dehydrate

cell tissue by microwave radiation, resulting in decreased cell pore and permeability compared to SWE and MAE in a single mode. Indeed, cell disruption was ineffective in this combination of processes (SP+MAE). Supposedly, it may allow chemical compounds, such as fats, proteins, phenolics, and polysaccharides, to exude into the surrounding solvent resulting in good but not overall better enhancement recovery of bioactive compounds and antioxidant activity than SWE or MAE in a single-step. The difference in the range of the values was reflected in yield, TPC, and antioxidant activities as assessed by the DPPH, ABTS, and FRAP.

Figure 19- SEM analysis images of strawberry pomace with a magnification of 80x and 500x: a) raw dry sample; (b) after pretreatment with supercritical CO_2 and rapid depressurization (SP at 40 °C, 30MPa,30 min); (c) after SWE process; (d) after MAE process and (e) after pretreatment with supercritical CO_2, rapid depressurization and MAE (SP+MAE).



Source: Author

5.3.5 Chemical profile by UPLC-QTOF-MS^E

The strawberries' pomace of the agro-industrial process has been an excellent source of bioactive compounds with great antioxidant potential. Studies have shown that phenolic compounds are better preserved in pomace than in juice (AABY *et al.*, 2007; AABY; SKREDE; WROLSTAD, 2005; PUKALSKIENĖ *et al.*, 2021). The chemical profile of strawberry pomace extracts was evaluated by untargeted UPLC-QTOF-MS^E analysis as this technique provides high detection selectivity and a wide range of determination.

The results obtained in our study support the bioactive potential of strawberry pomace extracts. The UPLC-MS dataset indicated that strawberry pomace has organic acids, ellagitannins, procyanidins, and other phenolic compounds. Phenolic compounds play an essential role in cancer prevention and treatment by exhibiting various physiological properties such as antioxidant, anti-mutagenic, and anti-inflammatory (AABY *et al.*, 2007). **Table 10** summarizes the putatively assigned metabolites identified in strawberry pomace extracts, experimental mass *m*/*z,* Q-TOF-MS/MS fragmentation patterns, retention time (Rt), masses of negative ionization mode $[M - H]^-$, molecular formula, and based on the data provided in literature databases.

Overall, 16 metabolites were identified, including organic and phenolic acids. The UPLC–MS chromatograms of SWE, MAE, SP+MAE, and SOX strawberry pomace extracts are shown in **Figure 20**. The UPLC analysis revealed that strawberry pomace extraction at different temperatures and solvents yielded the same compounds with differing intensity amounts when extracted with SWE, MAE SP+MAE, and SOX. This behavior demonstrates the tendency observed in TPC and antioxidant activity (Figures 17 and 18) that rises with the rising extraction temperature.

The citric acid (peak 1) was identified by integrating extracted ion chromatograms of the namely *m*/*z* values 191.0197, found in different intensities in all analyzed extracts, and they were eluted together with other organic acids not reported in this study. Citric acid is related to maturity, contributing to the sweetness of strawberries, making the sugar-acid ratio an essential indicator of fruit quality (MARKOVINOVI *et al.*, 2022). Organic acids, among them, citric acid, were found by Pukalskienė et al. (2021) in PLE aqueous at 110 °C and in a solid-liquid aqueous extract of strawberry pomace composed of pulp, stalk, and seeds were air-dried at 40 ºC for 48 h, similar to used in this study.

In **Figure 20**, the compounds 2,3, 7, 8, and 9 were identified as Bis-HHDP-glucose (peak 2), Galloyl-HHDP-glucose (peak 3), ellagic acid-O- rutinoside (peak 7), ellagic acid-O-rhamnoside (peak 8), and ellagic acid (peak 9). These metabolites were classified as Galloylglucoses and ellagitannins (HANHINEVA *et al.*, 2008). The ellagic acid derivatives, comprising ellagic acid, ellagic acid glycosides, and ellagitannins, the most abundant hydrolyzable tannins, represent an essential group of phenolic compounds in strawberries, being a primary dietary source of ellagic acid-containing compounds, also present together with raspberries and blackberries (AABY *et al.*, 2007; GIAMPIERI; ALVAREZ-SUAREZ; BATTINO, 2014). Ellagic acid is a natural phenolic antioxidant and an antiviral compound found in numerous fruits and vegetables (KIM *et al.*, 2016).

Ellagitannins yield ellagic acid upon hydrolysis of the hexahydroxydiphenic acid (HHDP) group and consist of a polyol core, typically glucose, which is esterified with HHDP acid, in some cases, gallic acid (AABY *et al.*, 2012; AABY; EKEBERG; SKREDE, 2007; HANHINEVA *et al.*, 2008). Ellagitannins are the most abundant hydrolyzable tannins found in strawberry fruit. They may influence the shelf life of strawberries owing to their antioxidative and antimicrobial properties. Besides, antioxidative, anticarcinogenic, anti-tumorigenic, anti-mutagenic, anti-diabetic, antiproliferative, antibacterial, and antimycotic properties have been reported. Then, strawberry consumption is associated with preventing chronic diseases (GIAMPIERI; ALVAREZ-SUAREZ; BATTINO, 2014; HANHINEVA *et al.*, 2008; MARKOVINOVI *et al.*, 2022).

Villamil-Galindo, Van de Velde, & Piagentini (2020) studied the ultrasound-assisted extraction of strawberry var ‘Festival’ by-products (sepal, peduncle, stems, etc.) with various solvent types (water, ethanol 80%, methanol 80%, and acetone 80%) and formic acid concentration (0 and 0.5%). Then hydrolyzable tannins were detected, ellagic acid derivatives, flavonoids, especially dimer of galloyl-bis-HHDP-glucose. Additionally, ellagic acid pentoside, free ellagic acid , and the flavonols quercetin-3-*O*-glucuronide and kaempferol-3-*O*-glucuronide.

Different from Pukalskienė et al. (2021), in our study, ellagitannins were found for the first time in the form of acid (HHDP) group (peak 2 and 3) from strawberry pomace extracts recovered by high-pressure methods at the single (SWE, MAE) and combined mode SP+MAE using water as a solvent and also detected ellagic acid derivatives such as ellagic acid glycosides (peak 7,8, and 9) in SWE, MAE, SP+MAE, and SOX strawberry pomace extracts. According

to **Figure 20**, the amount of these compounds rises with the extraction techniques and temperature, being more intense at SWE 130 °C.

Strawberries also contain high concentrations of condensed tannins, so proanthocyanidins (condensed tannins) occur widely as end products of the flavonoid pathway, and plants produce them to protect against predation and have been reported from strawberry fruit and leaves (AABY *et al.*, 2012; D'URSO *et al.*, 2016; HANHINEVA *et al.*, 2008). Then a series of procyanidins derived from the fragmentation of flavan oligomers, peaks 4 and 6, represent procyanidin B trimer and procyanidin B Dimer, respectively. They presented, namely, *m*/*z* values 865.1978 and 577.1372, respectively, found in different intensities in all analyzed extracts and were identified based on accurate mass and fragmentation MS/MS spectrum showed a most abundant peak at 289 m/z.

To the best of our knowledge, the result of the present study first reports a series of procyanidins from strawberry pomace extracts recovered by high-pressure methods using green solvents. This metabolite from SWE, MAE, and SP+MAE samples can be correlated with the high antioxidant capacity of these extracts (Sections 5.2.2 and 5.2.3); once proanthocyanidins are highly antioxidative molecules contribute to the health-beneficial effect of a flavonoid-rich diet (HANHINEVA *et al.*, 2008).

Flavonoids glycosides compounds were tentatively identified as apigenin-*O*-glucoside (peak 5), isoquercitrin (peak 10), quercetin-*O*-glucuronide (peak 11), kaempferol-*O*-hexoside (peak 12), kaempferol-*O*- glucuronide (peak 13), kaempferol hexoside (peak 14), quercetin (peak 15) and kaempferol (peak 16). Also, according to **Figure 20**, the SWE extract (sample (c)) shows remarkably high peak intensity to peaks 15(quercetin) and 16 (kaempferol), and SOX shows high intensity compared to other samples. While MAE (sample (a)) shows low intensity and SP+MAE (sample (b)) shows intermediate peak intensity compared to other samples. Quercetin and kaempferol and their derivatives are the significant flavonols found in strawberry pomace extracts (**Figure 20** (a to d)). These metabolites follow the TPC trend presented in item 5.2.2. As shown in **Figure 20**, the peak intensity of these compounds varies according to the increase in temperature and the extraction techniques used. This trend could be explained due to the viscosity and surface tension of the solvents decreasing at higher temperatures, which might increase the encroachment of the solvent into the matrix and a faster dissolution. Moreover, the mass transfer rate is increased, thus resulting in higher TPC yields in SWE (PUKALSKIENĖ *et al.*, 2021).

Some flavonoid glycosides are first time reported from strawberry pomace extracts recovered by high-pressure methods using green solvents like isoquercitrin (peak 10) and kaempferol-*O*-hexoside besides quercetin (peak 15) and kaempferol (peak 16). Primarily strawberries have been described as a good source of flavonols, and their bioactive compounds are already known to have potent bioactivity such as antioxidant, antiproliferative, and cardiovascular potential (AABY *et al.*, 2012; D'URSO *et al.*, 2016; PUKALSKIENĖ *et al.*, 2021).

The strawberry pomace in our study is composed of achenes, stems, and fibrous residual pulp parts. Several studies reported the influence of achenes on phenolic compounds and antioxidant activity. They concluded that most phenolic compounds are contained in the achenes and the receptacles of strawberry fruits, and when strawberry fruits are processed into juice, the phenolic compounds remain bound to the cell wall material (AABY *et al.*, 2007; MARKOVINOVI *et al.*, 2022; OSZMIAŃSKI; WOJDYŁO, 2009; SÓJKA *et al.*, 2013). So, strawberry pomace is rich in bioactive compounds, including phenolic acids, flavonoids, and tannins, which could be great potential in producing functional foods used as nutraceuticals or natural antioxidants.

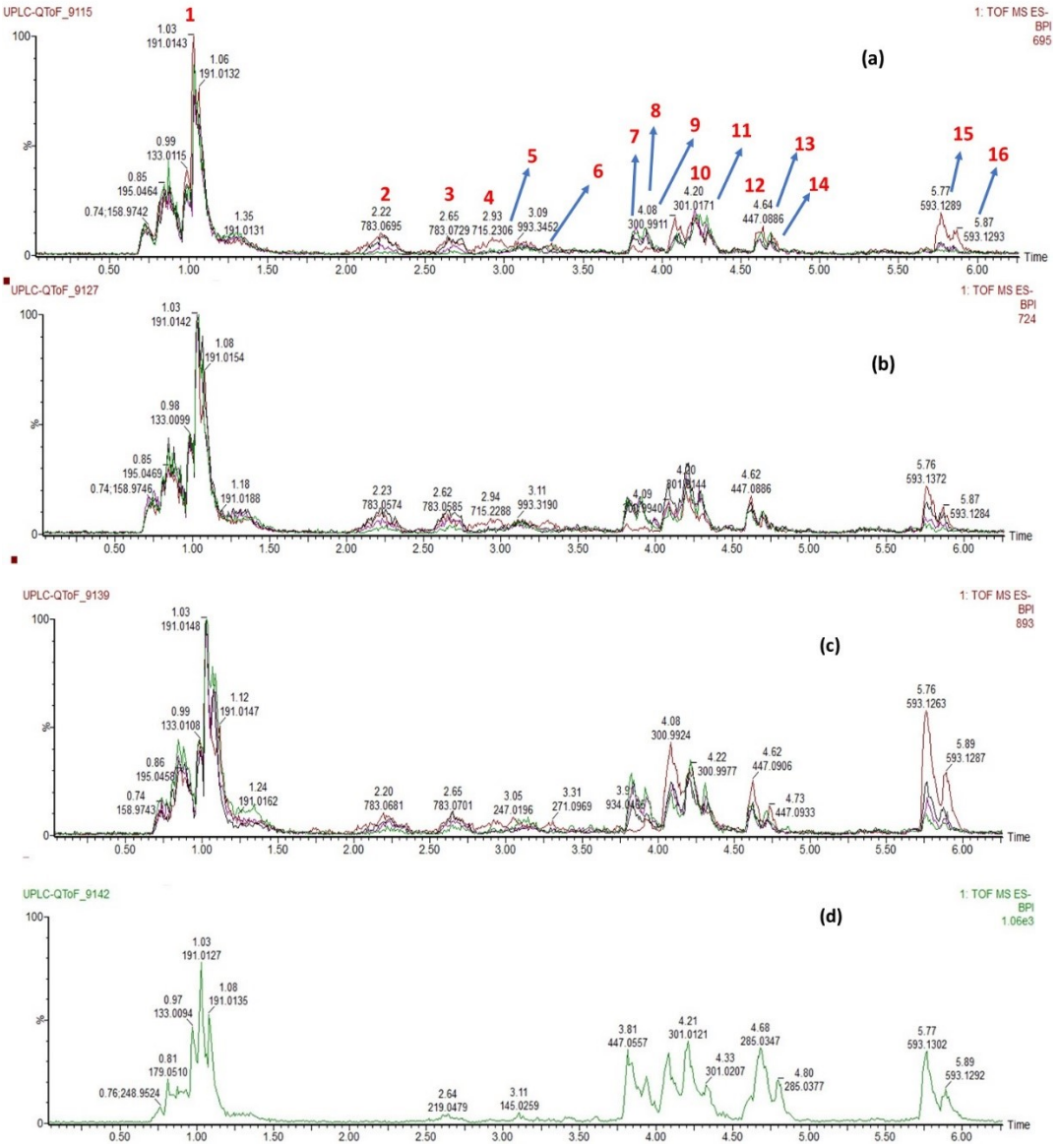
Even though some differences were found in the intensity of the chromatographic peaks, in general, the profile of the SWE, MAE, and SP+MAE water extract samples was very similar, showing that compressed fluids efficiently provided extracts with bioactive compounds.

Table 10- Chromatographic and mass spectrometry data of strawberry pomace extracts obtained by UPLC-QTOF-MS in negative ion mode.

Peak N°.	t_R (min)	$[M-H]^-$ Observed	$[M-H]^-$ Calculated	Product Ions (MS/MS)	Empirical Formula	Ppm (error)	Putative Name	References
1	1.03	191.0197	191.0192	111.0377	$C_6H_7O_7$	2.6	Citric Acid	(ELSADIG KARAR; KUHNERT, 2016)(PUKALSKIENĖ *al.*, 2021)
2	2.24	783.0714	783.0681	300.9928	$C_{34}H_{23}O_{22}$	0.6	Bis-HHDP-glucose	(HANHINEVA *et al.*, 2008)
3	2.65	633.0725	633.0728	463.0341, 300.9922	$C_{27}H_{21}O_{18}$	-0.5	Galloyl-HHDP-glucose	(KIM *et al.*, 2016) ;(HANHINEVA *et al.*, 2008)
4	2.93	865.1978	865.1980	577.1438, 425.0956, 407.0833, 289.0750	$C_{45}H_{37}O_{18}$	-0.2	Procyanidin B trimer	(HANHINEVA *et al.*, 2008)
5	3.12	431.0964	431.0978	269.0456	$C_{21}H_{19}O_{10}$	0.2	Apigenin-*O*- glucoside	(Elsadig Karar & Kuhnert, 2016);(OLATE-GALLEGOS *e al.*, 2019); (PUKALSKIENĖ *et al.*, 2021)
6	3.14	577.1372	577.1346	289.0788	$C_{30}H_{25}O_{12}$	4.5	Procyanidin B Dimer	(D'Urso et al., 2016)10
7	3.81	609.1087	609.1092	301.0087	$C_{26}H_{25}O_{17}$	-0.8	Ellagic acid-*O*- rutinoside	(JIA *et al.*, 2018)14
8	3.90	447.0558	447.0564	301.0116	$C_{20}H_{15}O_{12}$	-1.3	Ellagic acid-*O*- rhamnoside	(D'URSO *et al.*, 2016)10
9	4.09	300.9974	300.9984	229.0124, 257.0038	$C_{14}H_5O_8$	-3.3	Ellagic acid*	(HANHINEVA *et al.*, 2008)4
10	4.20	463.0866	463.0877	301.0295	$C_{21}H_{19}O_{12}$	-2.4	Isoquercitrin*	(D'URSO *et al.*, 2016)10
11	4.23	477.0666	477.0669	301.0276, 300.0310	$C_{21}H_{17}O_{13}$	-0.6	Quercetin-*O*- glucoronide	(D'URSO *et al.*, 2016)10
12	4.62	447.0911	447.0927	285.0348	$C_{21}H_{19}O_{11}$	-3.6	Kaempferol-*O*-hexoside	(HANHINEVA *et al.*, 2008)4
13	4.69	461.0724	461.0720	285.0348	$C_{21}H_{17}O_{12}$	0.9	Kaempferol-*O*- glucuronide	(HANHINEVA *et al.*, 2008);(PUKALSKIENĖ *et al.*, 2021
14	4.73	447.0925	447.0927	285.0364	$C_{21}H_{19}O_{11}$	-0.4	Kaempferol hexoside	(PUKALSKIENĖ *et al.*, 2021);(D'URSO *et al.*, 2016)
15	5.81	301.0357	301.0348	-	$C_{15}H_9O_7$	3.0	Quercetin*	(ELSADIG KARAR; KUHNERT, 2016)23
16	6.67	285.0407	285.0399	255.1712	$C_{15}H_9O_6$	2.8	Kaempferol	(REYNOSO-CAMACHO *et al.*, 2021)

***Comparison by the standard. (t_R): Retention Time.** Source: Author

Figure 20 - Chromatograms acquired in UPLC-QTOF-MS/MS in negative ionization mode ([M-H]$^-$) for the strawberry pomace extracts processed at 70, 90, 110, and 130 °C and SOX ethanol by the following extraction methods: (a) Overlaid chromatograms for MAE with water as a solvent, (b) Overlaid chromatograms for SP+MAE with water as a solvent, (c) Overlaid chromatograms for SWE with water as a solvent and,(d) SOX with ethanol as a solvent.



Peak Legend: Citric Acid (1), Bis-HHDP-glucose (2), Citric acid (3), Galloyl-HHDP-glucose (4), Procyanidin B trimer (5), Apigenin-*O*- glucoside (6), Procyanidin B Dimer (7), Ellagic acid-*O*- rutinoside (8), Ellagic acid-*O*- rhamnoside (9), Ellagic acid (10), Isoquercetrin (11), Quercetin-*O*-glucuronide (12), Kaempferol-*O*- glucuronide(13), Kaempferol hexoside (14), Quercetin (15) and Kaempferol (16). Source:Author

5.3.6 Antiproliferative activity

Berry extracts have been widely explored due to their high polyphenol content, providing a robust antioxidant capacity. Mainly, strawberry has several bioactive phenolic compounds, especially flavonoids, anthocyanins, and ellagitannins, with proven biological activities, such as antioxidant, antiproliferative, and cardiovascular potential (AMATORI *et al.*, 2016; PUKALSKIENĖ *et al.*, 2021). Antiproliferative assays were performed using confluent and non-differentiated **4T1** (mouse mammary tumor cells), **SF295** (human glioblastoma cells), **Sk-MEL-28** (human melanoma cells), and **B16-F10** (murine melanoma) cells, to characterize the antiproliferative activity of strawberry pomace extracts obtained by different methods to attempt to explore the *in vitro* bioactivity. As shown in **Figure 21**, the aqueous strawberry pomace extract recovered by high-pressure techniques was tested at 50, 100 µg mL^{-1} concentration and obtained a range value from 81.2 to 107.1%. Also, SOX with ethanol extracts ranged from 82.1% to 117.3% of cell viability, showing few or no antiproliferative effects.

The high intensity of individual phytochemicals and increased antioxidant capacity of the SWE, MAE, SP+MAE at 130 °C, and SOX with ethanol extracts may be responsible for the inhibitory effects against cancer cell growth (**Figure 21, Figures 17 and 18**).

Treatment of 4T1 mouse mammary tumor cells with SP+MAE 130°C and Soxhlet with ethanol at 50 and 100µg.mL^{-1} reduced cell growth by about 8 to 12.8 % and 18 to 16%, respectively, while the treatment with MAE 130°C at 100µg.mL^{-1} and SWE 130°C at 50µg.mL^{-1} reduced cell growth by about 10 and 8.3%, statistically significantly different from the control group, as shown in Figure 21.

SF295 human glioblastoma cells treated with SP+MAE 130°C at 50µg.mL^{-1} significantly differed from the control group and reduced cell growth by 18.8%, affecting cell proliferation. Also, Sk-MEL-28**,** human melanoma cell lines, was treated with soxhlet with ethanol at 50 and 100µg.mL^{-1} reduced the cell growth at 9.4 and 8%, respectively, and were statistically significantly different from the control group. Moreover, the B16-F10 murine melanoma cell lines, only the ones treated with SWE 130°C, MAE 130°C at 100µg.mL^{-1} and with SOX with ethanol at 50µg.mL^{-1} extracts reduced cell growth by 6.6%, 8.3%, and 9% differing in the control group ($p < 0.05$).

This study's findings were similar to those of Pukalskienė et al. (2021) for an aqueous PLE extract against HT29 cell lines that were not active, most likely due to the higher content of secondary metabolites with anticarcinogenic properties. In addition, the extraction

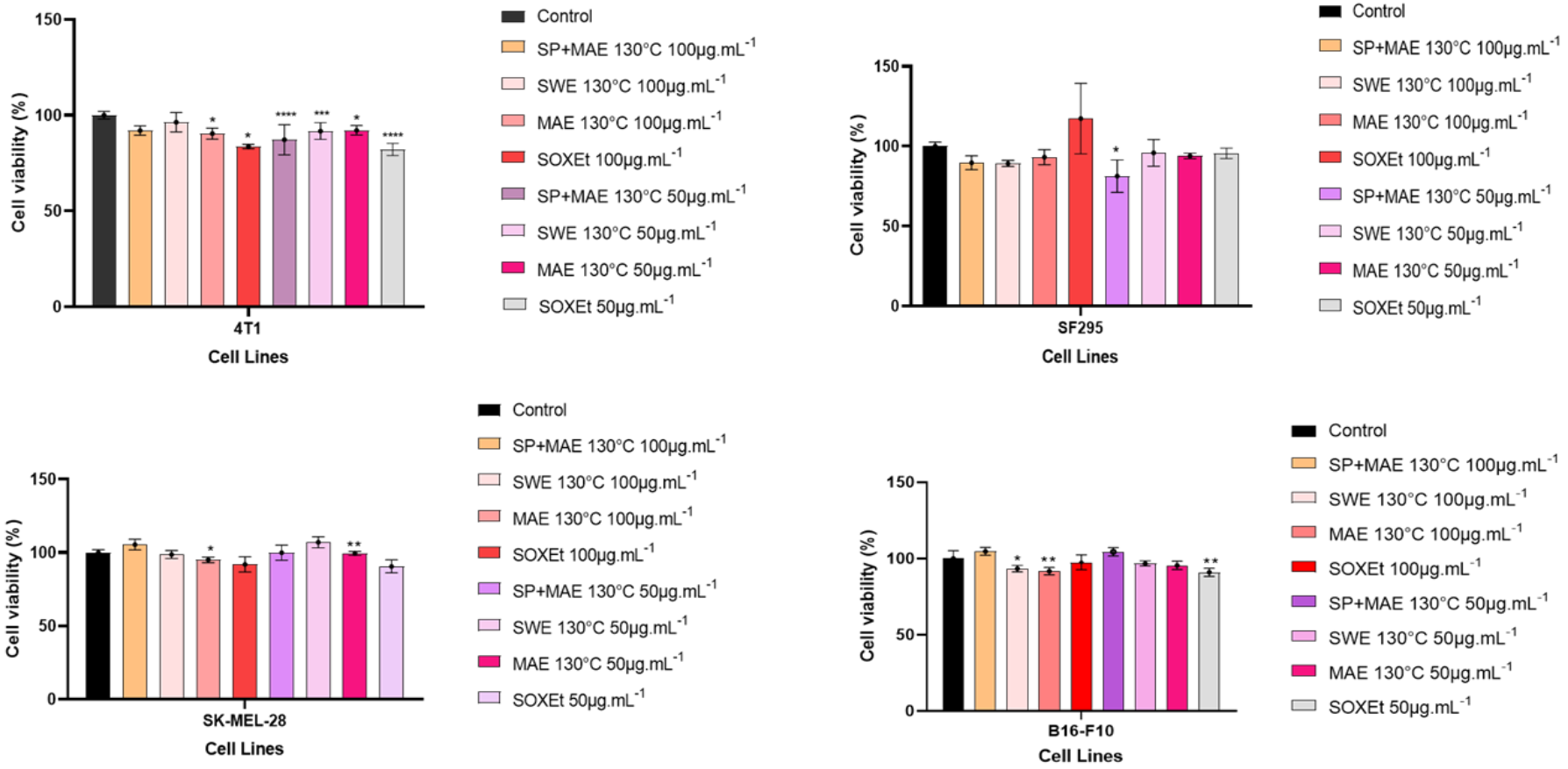
temperature at 130 °C in our study case of the pressurized pomace extraction possibly was too high. This factor may affect the stability of the quercetin compound, indicating that glucuronidation may decrease the cytotoxic effect of quercetin *in vitro* (WU *et al.*, 2018). For instance, some well-known anticancer ellagitannins compounds, quercetin, and kaempferol derivatives present in pressurized and SOX extract may play an essential role in the antiproliferative activity (AABY; EKEBERG; SKREDE, 2007; GIAMPIERI *et al.*, 2015).

Fruit phenolics may exhibit antagonistic and additive/synergistic activities and interactions with other phenolics and phytochemicals in a particular fruit (MEYERS *et al.*, 2003). Different studies on extracts of strawberries, raspberries, and other fruits and berries, did not find any correlations between the content of some phytochemicals and the inhibition of cancer cell proliferation (AMATORI *et al.*, 2016). However, many natural products used for cancer treatment occur at deficient levels in plant tissues, and due to the compositional complexity of secondary plant metabolites, antioxidant capacity values measured by *in vitro* methods do not always correlate with their activities *in vivo* (LUCIOLI *et al.*, 2019; PUKALSKIENĖ *et al.*, 2021). In this way, healthy strawberry effects have been attributed to the synergic activities of various nutrients and phytochemicals; however, the effect of strawberry bioactive compounds on cancer is not yet fully elucidated (AMATORI *et al.*, 2016).

Lucioli et al. (2019); Meyers et al.(2003); Zhang, Seeram, Lee, Feng, & Heber (2008) reported a reduction of proliferation in HEp-2, colon adenocarcinoma HT-29 cell line, colorectal adenocarcinoma Caco-2, human oral (CAL-27, KB), colon (HT29, HCT-116), prostate (LNCaP, DU145) cancer cells and HepG2 human liver cancer cell lines tested by strawberry fruit extract at atmospheric extraction.

Amatori et al. (2016) studied polyphenol-rich methanolic strawberry extract in breast cancer using cell lines MCF-7 (human breast cancer) and A17 (murine mammary carcinoma), and the polyphenol-rich strawberry extract was shown to be in the time- and dose-dependent manner. Recently, Forni et al. (2016) showed that blueberry and strawberry fruit extracts and cell suspension cultures of *Fragaria x ananassa* Duch (cv. Don) remarkably reduced proliferation in B16-F10 melanoma murine cells decreased murine melanoma cell proliferation. Although this is a preliminary study, we can glimpse the future use of strawberry pomace extracts in cancer prevention, and further investigations are needed to clarify the roles of the different strawberry phytochemicals against cancer cells.

Figure 21- Antiproliferative activity of strawberry pomace extract (50 and 100 µg/mL concentrations) against 4T1, SF295, SK-Mel-28, and B16-F10 cancer lines cells after 24 h of incubation at 37°C.



The graph shows the mean ± SD of three independent experiments. * $p < 0.05$, as compared with control. Source: Author

5.4 CONCLUSION

A high-pressure extraction approach using SWE, MAE, and combinations of processes (SP+MAE) could lead to a promising strategy for effectively valorizing strawberry by-product pomace available in abundance and cheap to produce bioactive compounds as sources of functional ingredients. The extraction yield and recovery of the antioxidant compounds were affected by temperature, time, ratio sample: solvent, and extraction technique. The extracts obtained by SWE at 130 °C provided the highest TPC and antioxidant capacity. The UPLC-MS chemical profile identified 16 metabolites, including organic acids, ellagitannins, procyanidins, and flavonoids, mainly apigenin, quercetin, kaempferol, and its derivatives differing in peak intensity when extracted with SWE, MAE, SP+MAE, and SOX.

Concerning antiproliferative activity, the best results were achieved against the 4T1 mouse mammary tumor cells treated with strawberry pomace extracts SP+MAE 130°C (12.8%) and Soxhlet with ethanol (16.3%) at 50μg.mL^{-1}, respectively, and to SF295 human glioblastoma cells treated with SP+MAE 130°C at 50μg.mL^{-1}, which significantly differed from the control group and reduced cell growth by 18.8%, affecting cell proliferation.

Although more research is needed to evaluate the antiproliferative activity of strawberry pomace extract obtained by innovative extraction methods using green solvents, we can glimpse the future use of strawberry pomace extracts in cancer prevention. In this study, the emergent extraction processes show significant advantages compared to conventional extraction methods. These approaches are environmentally friendly due to the reduced time and less organic solvent consumption.

5.5 ACKNOWLEDGMENTS

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CHAPTER 6: SCREENING OF POTENTIAL ANTIPROLIFERATIVE ACTIVITY OF EXTRACTS OBTAINED BY HIGH-PRESSURE

This study investigated the potential antiproliferative activity of the recovered bioactive compounds from soursop, acerola, and strawberry agri-food by-products extracts (70 to 130°C) in a single and/or a sequential extraction approach as follows: (1) Supercritical fluid extraction (SFE), (2) Supercritical pretreatment (SP) of raw material with a rapid depressurization rate, followed by MAE of the SP residue (SP+MAE), (3) Subcritical water extraction (SWE), (4) Microwave-assisted extraction (MAE), all using water as a solvent; and compared to (5) Soxhlet (SOX) extraction with hexane and ethanol. Moreover, the antiproliferative activity against 4T1(mouse mammary tumour cells), B16F10(murine melanoma), SF295(human glioblastoma cells), Sk-MEL-28 (human melanoma cells), and PC3 (human prostate cancer) tumour cell lines were tested.

The results presented in this chapter will be submitted to a high-impact factor journal.

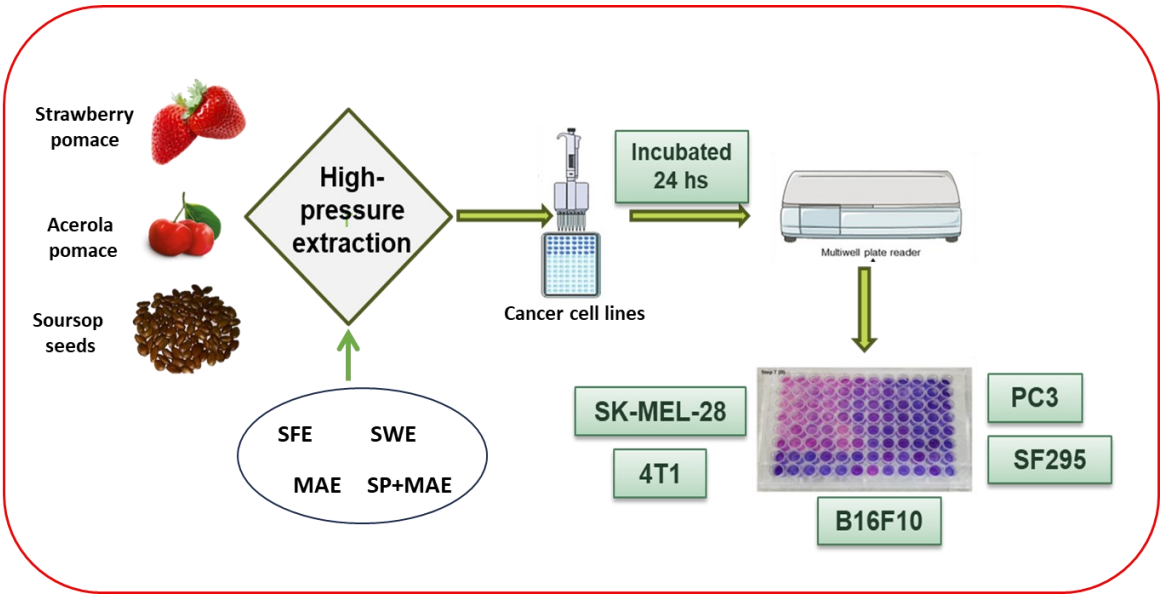
6 SCREENING OF POTENTIAL ANTIPROLIFERATIVE ACTIVITY OF EXTRACTS OBTAINED BY HIGH-PRESSURE EXTRACTS AS AGRI-FOOD BY-PRODUCTS VALORIZATION

Abstract

The industrial juicing process produces a large volume of pomace rich in bioactive phytochemicals. This study aimed to investigate the potential antiproliferative effects through single-concentration initial screening tests (50 and 100 $\mu g.mL^{-1}$) of SFE, SWE, MAE, single or combined extraction, and SOX ethanolic extract from soursop, acerola, and strawberry by-product against 4T1(mouse mammary tumour cells), B16F10 (murine melanoma), SF295 (human glioblastoma cells), Sk-MEL-28 (human melanoma cells) and PC3 (human prostate cancer) tumour cell lines associating with the chemical profile of the extracts. The results revealed that the antiproliferative activity (Alamar blue assay) showed low potential on cell growth reduction, achieving a maximum of 31% on 4T1 cell line (SWE 130°C soursop seed extract), 18 % on SF-295 cell line (SP+MAE 130°C strawberry pomace extract), 26% on SK-MEL-28 cell line (SWE 130°C soursop seed extract), 35% (SFE 30MPa 40°C and SWE 130°C soursop seed extract) on B16F10 cell line, and 9,44% (SWE at 90°C Acerola pomace

extract) on PC3 cell lines. However, UPLC and HPLC demonstrated significant bioactive compounds such as ascorbic acid and phenolics exhibiting antiproliferative activity in agreement with recent reviews highlighting this activity.

Graphical abstract



6.1 INTRODUCTION

Consumption of tropical fruits has demonstrated numerous beneficial health effects related to the high content of bioactive compounds present in these fruits. Beneficial health properties of bioactive compounds include antioxidant, antiproliferative, anti-inflammatory, neuroprotective, antihypertensive, hypo-cholesterolemic, and hypoglycemic properties (ALBUQUERQUE *et al.*, 2019; SAYAGO-AYERDI *et al.*, 2021).

Tropical fruits are consumed as fresh foods or processed products (i.e., juices, nectar, puree, desserts, compotes, marmalades, sauces, syrups, snacks, jellies, flours, and wines), peels, seeds, and pomaces being the main by-products of industrial processing. Depending on the species, tropical fruits' by-products can encompass a substantial amount of peel (10%–66%) and seed (1%–22%), which are usually disposed of as wastes (ALVAREZ-RIVERA *et al.*, 2020).

In contrast, these residues are a natural source of nutrients and different bioactive molecules. These biocompounds have great potential to be used as functional food ingredients or for application as phytochemical pharmaceutical substances for the prevention or treatment of human diseases (ALBUQUERQUE *et al.*, 2019; PATRA; ABDULLAH; PRADHAN, 2022).

Phenolic compounds are known to be widely present in fruits, and their by-products, and they are the most important group of natural antioxidants in the diet (LIZÁRRAGA-VELÁZQUEZ *et al.*, 2020). Studies have shown that fruits' seeds, peels, and pomace may have a higher concentration of phenolic compounds than the edible portion (CARVALHO GUALBERTO *et al.*, 2021).

Agri-food residues such as soursop seeds, acerola, and strawberry pomace are rich in bioactive compounds, mainly polyphenols and flavonoids (AABY; EKEBERG; SKREDE, 2007; CORIA-TÉLLEZ *et al.*, 2018; MESQUITA *et al.*, 2021, 2022; SÓJKA *et al.*, 2013; XU *et al.*, 2020).

These residues are generated during the production of juices/pulps throughout the year, in greater quantities during the harvest period for these fruits. In this way, studying the use of pressurized fluid extraction techniques (SFE, SWE, MAE) alone or in combination with environmentally friendly techniques using green solvents, can be a useful alternative to encourage the circularity of these by-products. rich in bioactive compounds and antioxidants. Therefore, the use of SFE can be useful in the extraction of seed oils (apolar compounds) while SWE and MAE can extract more polar compounds.

Considering the bioactive potential of fruit by-products, developing green valorization strategies for recovering valuable active ingredients from plant residues can be an exciting and unique opportunity for the juice processing industry to deliver a value-added product to the market with health-promoting properties. Thus, strategies based on efficient extraction solvent selection and new green extraction processes can help to fulfill the United Nations' responsible consumption and production goal (SDG 12) related to agriculture-friendly practices. Contributing to the efficient management of natural resources (e.g., soil and water), reducing food waste and waste production, and being a natural and safe source of polyphenols, they are inexhaustible, low-cost, and sustainable resources (MACHADO *et al.*, 2021).

Recently, Supercritical fluid extraction (SFE), Subcritical water extraction (SWE), and microwave-assisted extraction (MAE) and their combinations have been used, before or during the extraction process, to enhance the recovery of phenolic compounds from several food by-

products as intensification strategy (SÁNCHEZ-CAMARGO *et al.*, 2021). As Chemat et al. (2020) pointed out, these extraction technologies are considered sustainable techniques since they can complete processes in shorter times with high reproducibility and simplified manipulation, resulting in a higher quality of the final products.

Cancer is the primary cause of death worldwide, and researchers are working to develop more therapeutic components for cancer treatment with fewer side effects. Plants are the primary sources of pharmacologically active molecules used for therapeutic purposes (CARVALHO; CONTE-JUNIOR, 2021; SALEHI *et al.*, 2020). Brazilian plant species are potential candidates to develop novel drugs to treat cancer diseases, highlighting fatty acids, polyphenols, diterpenes, and new drugs against human breast, lung, colon, colorectal, brain, and skin cancer. Polyphenols are compounds in Brazilian native food related to several chemoprevention modes of action with high selectivity, without cytotoxicity to normal cells (CARVALHO; CONTE-JUNIOR, 2021).

Some studies report evidence that soursop (*Annona muricata*), acerola (*Malpighia emarginata*), and strawberry (*Fragaria spp*) has extensive traditional use, and considerable evidence has been developed that they may be a helpful therapeutic agent in the battle against certain cancers.

Gavamukulya et al.(2017), Qazi et al.(2018), and Leite et al.(2020) studied the *in vitro* effect of the extracts from the fruit, bark, seeds, roots, and leaves of soursop (*Annona muricata*), which contained numerous bioactive substances responsible for the observed antitumoral, antioxidant, and cytotoxic to some cancer cells. The cited substances were polyketides, acetogenins, alkaloids, terpenes, tannins, phenolics, flavonoids, and oils.

Although acerola (*Malpighia emarginata*) has been investigated for its various biological activities, extracts from different fruit parts of acerola showed potent antioxidant and antiproliferative activity, among others (ALVAREZ-SUAREZ *et al.*, 2017; BELWAL *et al.*, 2018a; MOTOHASHI *et al.*, 2004b). The main bioactive compounds mentioned in the literature in acerola by-products, rutin, and quercetin, are flavonoids that benefit biological activities (MISKINIS; NASCIMENTO; COLUSSI, 2023), but studies related to biological properties and applications are still scarce.

Lately, it was reported that extracts from strawberry (*Fragaria X ananassa*) fruits remarkably reduced proliferation in B16-F10 melanoma murine cells (FORNI *et al.*, 2016), breast cancer cell line A17 (AMATORI *et al.*, 2016) and human colon cancer HCT116 cells (HUANG *et al.*, 2022).

Properties of soursop, acerola, and strawberries have been reported, and lots of bibliographic information mainly involves chemical composition and antioxidant activity. However, scarce information about SFE, SWE, and MAE extracts on potential biological activities such as antiproliferative and anti-cancer are available.

This study aimed to investigate the potential antiproliferative effects of SFE, SWE, MAE, single or combined extraction, and SOX extract from soursop, acerola, and strawberry by-product in 4T1(mouse mammary tumour cells), B16F10 (murine melanoma), SF295 (human glioblastoma cells), Sk-MEL-28 (human melanoma cells) and PC3 (human prostate cancer) tumour cell lines associating with the chemical profile of the extracts.

6.2. MATERIAL AND METHODS

6.2.1 Raw material and sample preparation

Soursop seeds, acerola, and strawberry pomaces- by-products of fruit pulp processing, were provided by the Tropicássia Polpa de Fruta company, Fortaleza, Brazil (3°44'48.8 "S; 38°31'07.3 "W). The raw material collected was placed in plastic bags, frozen at −18 °C, and transported to the Laboratory of Thermodynamics and Supercritical Technology (LATESC) of the Federal University of Santa Catarina (UFSC). Upon arrival, the raw material was thawed and dried in an air-circulated oven (DeLeo, Porto Alegre/RS, Brazil) at 40 °C. The dried soursop seeds, acerola, and strawberry pomace were crushed in a knife mill (DeLeo, Porto Alegre/RS, Brazil) and presented moisture content of 4.98 ± 0.15% (w.b), 10.6 ± 0.1% w.b. and 11.07 ± 0,14 % (w.b.) respectively, determined according to AOAC method 925.09 (AOAC, 2005). The dried material was crushed in a Willey knife mill (DeLeo, Porto Alegre/R.S. - Brazil) and stored in polyethylene packaging at −18 °C until use.

6.2.2 Extraction procedures

The extraction procedures were performed, at least in duplicate, using dried and milled soursop seed, acerola, and strawberry pomace in ten assay groups for evaluation of a single and/or a sequential extraction approach, as follows:

Treatment	Fruit	Code	Extraction technique
1	**Soursop**	(G5)	supercritical fluid extraction (SFE) at 30 MPa at 40°C
2		(G11)	Subcritical water extraction (SWE) at 10MPa at 130°C
3		(G9)	SFE + SWE 130°C
4		(G12)	Soxhlet (SOX)
5	**Acerola**	(A6)	Subcritical water extraction (SWE) at 10MPa at 90°C
6		(A1)	Soxhlet (SOX) extraction with ethanol
7	**Strawberry**	(M12)	Subcritical water extraction (SWE) at 10MPa at 130°C
8		(M4)	Microwave-assisted extraction (MAE) at 130°C
9		(M8)	Supercritical pretreatment (SP) of raw material with a rapid depressurization rate, followed by MAE of the SP residue (SP+MAE) at 130°C
10		(M1)	Soxhlet (SOX) extraction with ethanol

6.2.2.1 Supercritical Fluid Extraction (SFE)

The SFE unit and extraction procedure have been previously described in a report by Mazzutti et al. (2018). Pure CO_2 (99.9%) delivered at a pressure of up to 0.6 MPa (White Martins, Brazil) was used for SFE. Briefly, 30 g of soursop seeds raw material was placed inside a stainless-steel extraction vessel (internal diameter of 20 mm and height of 440 mm, a volume of 138.2 mL), and the space was filled with glass beads and cotton to form a fixed bed. Mesquita et al.(2021) achieved the soursop seed extraction parameters previously. The SFE experiments were carried out in duplicate at temperatures of 40 °C, pressures of 30 MPa (CO_2 density 0.909 g cm^{-3}), and a constant solvent flow rate of 0.7 kg CO_2 h^{-1}. After each experiment, the

obtained extracts were collected in amber flasks, weighed, and stored in a domestic freezer at −18 °C.

6.2.2.2 Subcritical Water Extraction (SWE)

The SWE assays were performed in a customized unit following the experimental procedure described by Rodrigues et al. (2019), at least in duplicate. Mesquita et al.(2022) defined the SWE extraction conditions previously and established at 10 MPa, 90°C to acerola pomace and 130 °C soursop seeds and strawberry pomace for 15 min and using a solvent flow rate of 4 $mL \cdot min^{-1}$.

SFE + SWE 130°C: The soursop seeds were first subjected to SFE (item 6.2.2.1) at 30 MPa and 40 °C (first step), and the residue of SFE was then subjected to SWE (second step) at 130°C.

6.2.2.3 Microwave-assisted extraction (MAE)

MAE was performed in a microwave reactor (Monowave 300 from Anton Paar GmbH) equipped with a single 850 W magnetron following the extraction procedure described by Mazzutti, Salvador Ferreira, Herrero, & Ibañez (2017) with modifications. Briefly, 30 mL extraction vessels were filled with 1 g of strawberry pomace dried material, 20 ml of distilled water (1:20 dried material: solvent ratio), and a small magnetic stirrer. The vessel was placed in the microwave for the extraction process at 130 °C, under magnetic stirring at 1000 rpm for 10 min, and then the collected extract samples were cooled down to 55 ºC and filtered. The solvent was removed from the solution by freeze-drying (Liotop, model LD101, São Paulo, Brazil), and the extracts were stored in an amber flask at -18 °C for further analysis.

SP+MAE 130°C: Supercritical Fluid Extraction (SP) unit (item 6.2.2.1) performed high-pressure pretreatment with CO_2 followed by a rapid depressurization rate. Firstly, the SP procedure was performed by loading 60 g of dried raw material into the extraction vessel, followed by pressurization to 30 MPa with CO_2 (99.95%, White Martins, Brazil) at 40 °C for 30 min (static compression). After the fixed time, a depressurization rate of 1.97×10^{-3} kg CO_2 h^{-1} allowed the pressure to decrease until atmospheric pressure. The procedure conditions were selected based on previous research group work (FERRO *et al.*, 2019). Finally, the pretreated dried raw material was collected from the extraction vessel and subsequently extracted with MAE at 130°C, from now on called SP+MAE, according to the procedure described above.

6.2.2.4 Conventional extraction

A conventional atmospheric pressure extraction was carried out using the Soxhlet technique (SOX) with ethanol (EtOH) as a solvent, performed according to the 920.39C method (AOAC, 2005a). The procedure involved 150 mL of solvent recycling over 5 g of dried raw material in a Soxhlet apparatus for 6 h at the solvent boiling point. Recovered extracts were filtered, and a rotary vacuum evaporator removed the solvent at 40 ºC. Extracts were stored in amber flasks at −18 ºC for further analysis.

6.2.3 Total phenolic content (TPC)

TPC of the soursop seed extracts, acerola, and strawberry pomace extracts was determined using the Folin–Ciocalteu method (KOŞAR; DORMAN; HILTUNEN, 2005).

The results were cited in Chapter 3 (section 3.3.2) for soursop seeds and cited by MESQUITA et al.(2021) in the Journal of Food Chemistry: X, Chapter 4 (section 4.3.2) for acerola pomace extracts and cited by MESQUITA et al.(2022) in the Journal of Food Chemistry and chapter 5 (section 5.2.2) for strawberry pomace extracts of this document.

6.2.4 Antioxidant activity

The antioxidant activity of the soursop seeds extracts, acerola and strawberry pomace extracts were determined using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay according to MENSOR et al. (2001), ABTS (2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) assay according to RE et al.1999 with some modifications, and ferric-reducing antioxidant power (FRAP) assay was performed using the Benzie and Strain (1996) method.

The results were reported in this document in Chapter 3 (Section 3.3.3) for soursop seeds and cited by MESQUITA et al.(2021) in the Journal of Food Chemistry: X, chapter 4 (Section 4.2.6) for acerola pomace extracts and cited by MESQUITA et al.(2022) in the Journal of Food Chemistry, and lastly at chapter 5 (section 5.2.3) for strawberry pomace extracts.

6.2.5 Chemical profile

The chemical profile of soursop seeds extract was achieved by the identification and quantification of phenolic compounds by LC-ESI-MS/MS as described in this document in chapter 3 item 3.2.2.11; the results were reported in section 3.3.6.2 and cited by MESQUITA et al.(2021) in Journal of Food Chemistry: X.

The chemical profile of acerola and strawberry pomace was obtained by UPLC-QTOF-MS as described in Chapter 4, item 4.2.7 for acerola pomace extracts and Chapter 5, item 5.2.5. for strawberry pomace extract. The results of the acerolas chemical profile were reported by MESQUITA et al.(2022) in the Journal of Food Chemistry, while the strawberry chemical profile results were reported in Chapter 5, item 5.2.5, and will be published in a high-impact journal very soon.

6.2.6 Antiproliferative activity assay

6.2.6.1 Cell culture

B16F10 (murine melanoma), *4T1(* an animal model for stage IV human breast cancer), SK-MEL-28 (human malignant melanoma), SF295 (human Glioblastoma), and PC-3 (human prostate cancer cell line) were provided by GEIMM/UFSC and from the Rio de Janeiro Cell Bank. Cell lines were cultured with Dulbecco's Modified Eagle Medium (DMEM – Sigma® St. Louis, MO, USA) or RPMI-1640 Medium supplemented with 10 % fetal bovine serum (FBS - Gibco® Grand Island, NY, USA), 100 U/mL of penicillin, 100 µg/mL of streptomycin and 10 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (all from Gibco®), and incubated at 37 ◦C under 5% CO2 in a humidified atmosphere. When the cell was in the exponential growth phase (80–90% confluence), it was detached by trypsin-EDTA and subcultured into new sterile culture flasks for further propagation.

6.2.6.2 Antiproliferative activity assay

The antiproliferative activity of the obtained extracts was evaluated by Alamar blue (resazurin) assay (O'Brien et al., 2000). B16F10, 4T1, SF295, SK-MEL-28, and PC-3 cells in

the exponential growth phase (80–90% confluence) were trypsinized, counted, and seeded in 96-well plates at a density of 1.0×10^5. The wells with seeded cells were incubated for 24 h at 37°C to allow cell adhesion. Cells were treated with the vehicle (sterile PBS buffer 7.2 pH) regarded as untreated controls, with 50 and 100 μg/mL of extracts diluted directly in the DMEM culture medium and incubated for 24 h at 37 ° C, with an atmosphere of 5% CO_2 and 95% humidity. After incubation, 10μL of Alamar Blue (0,312 mg/mL; Sigma) was applied to each well and left in contact for 2 hours. The results were measured with an Elisa fluorimeter reader (BioTek Synergy HT)) using excitation wavelength 530-560nm and emission 590nm.

Cell viability (%) determination using equation 3:

$$\boldsymbol{Cell\ Viability}(\%) = \left(\frac{\boldsymbol{RFU\ treated}}{\boldsymbol{RFU\ Control}}\right) * \mathbf{100} \qquad \text{Equation 3}$$

$RFU_{treated}$ = relative fluorescence unit of the wells treated with the samples

$RFU_{Control}$= relative fluorescence unit of untreated wells

The cell viability was expressed as a percentage of live cells relative to controls, and the results are expressed as the average ± standard deviation (SD) of at least three independent tests with a minimum of three replicates analyzed by GraphPad Prim 9.0 (Prim software, Irvine, CA, USA).

Analysis of variance (ANOVA one-way) followed by Dunett's test was applied to evaluate the statistical significance of differences between the study group compared to a control. A p-value < 0.05 was chosen as the criterion for statistical significance.

6.3 RESULTS AND DISCUSSION

Considering the remarkable phenolic composition of the soursop, acerola, and strawberry extracts (**Tables 7, 9, and 10**), the phenolics were determined previously using chromatographic methods. The results revealed that the single-mode extraction method: acerola pomace SWE at 90°C (A6), strawberry pomace SWE at 130°C (M12) and MAE at 130°C (M4), Soursop seed SFE 30MPa 40°C(G5), Soursop seeds SWE 130°C (G11), combined-mode extraction method: strawberry pomace (SP+MAE) at 130°C (M8), Soursop seed SFE + SWE

130°C (G9) and the soxhlet ethanolic extracts of acerola (A1), strawberry (M1) and soursop seeds (G12) had presents significant phenolics compounds.

For soursop seed extracts the main phenolics compounds were vanillic acid, 3,4 dihydroxybenzoic acids, p-coumaric acid, vanillin, and caffeic acid as the most abundant phenolic compounds. Moreover, 29 phenolic compounds were detected for the first time from soursop seed extracts **(Table 7)**. For acerola pomace extract the most abundants proved to be quercetin (quercetin O-rhamnoside; quercetin pentosyl-O-hexoside), kaempferol (kaempferol O-rhamnoside), catechin, epi- catechin, quercetin-O-deoxyhexosyl-hexoside, dihydroquercetin-O-deoxyhexoside, isorhamnetin -O-rutinoside, and naringenin-O-glucoside (**Table 9**). For strawberry pomace extracts, overall 15 phenolics were identified, including ellagitannins, procyanidins, ellagic acid and its glycosides derivatives in addition to galloylglucoses and flavonoids, mainly apigenin, quercetin, kaempferol (**Table 10)**.

These extracts were tested for their antiproliferative effects against B16F10 (murine melanoma), 4T1*(* an animal model for stage IV human breast cancer), SK-MEL-28 (human malignant melanoma), SF-295 (human glioblastoma), and PC-3 (human prostate cancer) cancer cells using the Alamar blue assay, as shown in **Figure 22.** The cell viability was expressed as a percentage of live cells relative to controls.

Several studies have been conducted on the antiproliferative activity of soursop fruit, leaves, and seeds (CHAMCHEU *et al.*, 2018; GAVAMUKULYA; WAMUNYOKOLI; EL-SHEMY, 2017; ILANGO *et al.*, 2022; QAZI *et al.*, 2018), strawberry (FENG *et al.*, 2022; FORNI *et al.*, 2014; LUCIOLI *et al.*, 2019; PUKALSKIENĖ *et al.*, 2021) and a few about acerola (MOTOHASHI et al., 2004).

The screening test evaluated the extract concentration required for a ≥50% reduction in cell growth in order to be considered a potential antitumor substance.

Through single-concentration initial screening tests (50 and 100 $\mu g.mL^{-1}$), we showed that soursop seed extracts, in comparison with acerola and strawberry-studied pomace extracts, achieved the best results comparing all the cell lines, showing cell counts ranging from 86 to 64% compared with the control treatment.

Considering the single-concentration screening test at 50 $\mu g.mL^{-1}$, the soursop seeds extract SFE 30MPa 40°C (G5) resulted in cell viability of 68,62% (31,38% reduction on cell viability) for 4T1 cell lines compared with the control treatment. However, this treatment was not statistically different from the combine-mode (SFE + SWE 130°C-G9) and single-mode (SWE 130°C-G11) extracts that showed 22,01% and 25,79% reduction in cell viability

compared with the control treatment (p>0,05). Similar results could be found for the single-concentration screening test at 100 $\mu g.mL^{-1}$, but only G5 and G11 extracts were statistically similar by the Dunnetts test and showed a 28,05% and 30,86% reduction in cell viability compared with the control treatment. Also, the strawberry pomace (SP+MAE) at 130°C (M8) at 50$\mu g.mL^{-1}$ and the soxhlet ethanolic extracts of acerola and strawberry A1 and M1 at 50 and 100 $\mu g.mL^{-1}$showed a reduction between 18% to 12 % in cell viability compared with the control treatment. Among the studied extracts, the soursop seeds SFE 30MPa 40°C (G5) and SWE 130°C (G11) achieved the best results concerning the cell antiproliferative effect against 4T1 cell lines. Thus, the results of these experiments demonstrated that reduction in cell viability that both cells were inhibited in a dose-dependent manner by various extracts, although their effects differed.

In this regard, Almutairi et al.(2023) studied the *in vitro* antiproliferative efficacy of *Annona muricata* seed and fruit extracts on the human breast carcinoma (MDA-MB-231 and MCF-7) cell lines and human lung carcinoma (A-549) cell lines. The results revealed that only the chloroform fruit extract of *A. muricata* was significantly effective and inhibited the proliferation of MDA- MB-231 and MCF-7 cells dose-dependently. Comparing our results, **Table 6,** about GC-MS analysis (item 3.3.6.1) of the SFE 30MPa 40°C soursop seeds extract, we could find a chemical fingerprint in common with Almutairi et al.(2023). One primary compound identified regarding relevance to both extracts was hexadecanoic acid (palmitic acid)—this fatty acid is a potential contributor to the anticancer properties of the extract.

Kariyil et al.(2021) evaluated the anti-cancer potential of chloroform fraction of methanolic extract of seeds of *Annona muricata* (CMAM) against MDA-MD-231 (epithelial human breast cancer adenocarcinoma cell line), 4TI (mouse mammary tumor cell line), and BT-549 (human breast cancer ductal carcinoma cell line) and Merlín-Lucas et al.(2021) studied the cytotoxic and antitumor properties using 4T1 cells of *Annona muricata* ethanol extracts collected for a year in two different regions of Mexico. In the first study, the plant fraction exhibited a reduction in cell viability with increasing concentrations of CMAM against cell lines like MDA-MD-231, 4TI, and BT-549 and could be used for further purification of anti-cancer compounds according the criteria of the National Cancer Institute, USA- an extract can only be considered for further purification of anti-cancer compounds if the IC_{50} limit is lower than 30µg/mL(KALAIVANI *et al.*, 2011). The second study showed cytotoxic activity against 4T1 closer than doxorubicin, the drug used as a positive control.

Our study also found in high-pressure extracts of acerola (*Malpighia emarginata* DC) pomace, ascorbic acid, quercetin, and kaempferol and their glycosidic flavonoid derivatives (**Table 9**- item 4.3.4), indicating that they may be chemical fingerprints since they were also present in the article by El-Hawary et al.(2020) on the cytotoxic effect of the ethanolic extract of *Malpighia glabra* Linn leaves, which significantly reduced cell viability in breast (MCF-7) and colon cell lines (HCT-116).

Jamshidi-Adegani et al.(2020) studied the anti-cancer effect of leaf and stem extracts of *Acridocarpus Orientalis* (Malpighiaceae family) on 4T1 cell lines. The results demonstrated that leaf extract has selective activity against 4T1 cancer cell lines, enhancing stem cell proliferation. The leaf extract was responsible for inhibiting 4T1 cell proliferation, which is predicted to play an essential role in cancer treatment.

Amatori et al.(2016) describe the biological effects of a polyphenol-rich strawberry extract (PRSE) against breast cancer cell line A17, and the experiments showed that PRSE was able to decrease the cellular viability of A17 cells in a time- and dose-dependent manner.

Breast cancer is a disease present worldwide that is most frequently diagnosed cancer in women (ŁUKASIEWICZ *et al.*, 2021). In this context, in 2020, nearly 2.2 million women worldwide were diagnosed with breast cancer (WHO/B. ANDERSON, 2023).

Studies have linked *A. muricata*-derived compounds to a variety of anticancer effects, including cytotoxicity, induction of apoptosis, necrosis, and inhibition of proliferation on a variety of cancer cell lines, including breast and ovarian cancers (GAVAMUKULYA; WAMUNYOKOLI; EL-SHEMY, 2017; JYOTHI; MALATHI; PATIL, 2023). In this context, phytochemical tests on *A. muricata* revealed the presence of various secondary metabolites, including alkaloids, flavonols, phenolics, essential oils (CORIA-TÉLLEZ et al., 2018; MESQUITA et al., 2021; ODÍLIA et al., 2020), cyclopeptides and annonaceous acetogenin who had been identified as one of the major bioactive compounds of *A. muricata,* with activity on several cancer cell lines (RADY *et al.*, 2018).

For SF-295 cell lines, the strawberry pomace (SP+MAE 130°C, M8) and SFE 30MPa 40°C (G5) at 50 $\mu g.mL^{-1}$ achieved the best performance among the extracts studied, significantly differing from the control. They showed a reduction of 18,77 and 13,24 % in cell viability compared with the control treatment.

The strawberry pomace Soxhlet extraction with ethanol (M1) at 100 $\mu g.mL^{-1}$ did not show cytotoxic effects for the SF-295 cell line and stimulated cell growth as visualized by the increase in cellular viability.

The biological activities of berries are partially attributed to their high content of a diverse range of phytochemicals such as flavonoids (anthocyanins, flavonols, and flavonols), condensed tannins (proanthocyanidins, ellagitannins, and gallotannins, stilbenoids (e.g., resveratrol), phenolic acids (hydroxybenzoic and hydroxy-cinnamic acid derivatives), and lignans (GIAMPIERI *et al.*, 2015; SEERAM *et al.*, 2006).

Da Silva Nunes et al.(2011) studied the cytotoxic effect of acerola fruit extract against SF-295 cell lines, and no sample of acerola showed inhibition of tumor growth. This result was similar to our study, in which no acerola pomace extract sample showed cell growth inhibition.

Cytotoxic effects were shown by roots methanolic extracts of *Potentilla fulgens* L (Family: Rosaceae) at higher concentrations (50 µg/mL & 100 µg/mL) and showed *in-vitro* cytotoxic activity against neuroblastoma (SF- 295) and prostrate (PC-3) human cancer cell lines (BARUA; YASMIN, 2018).

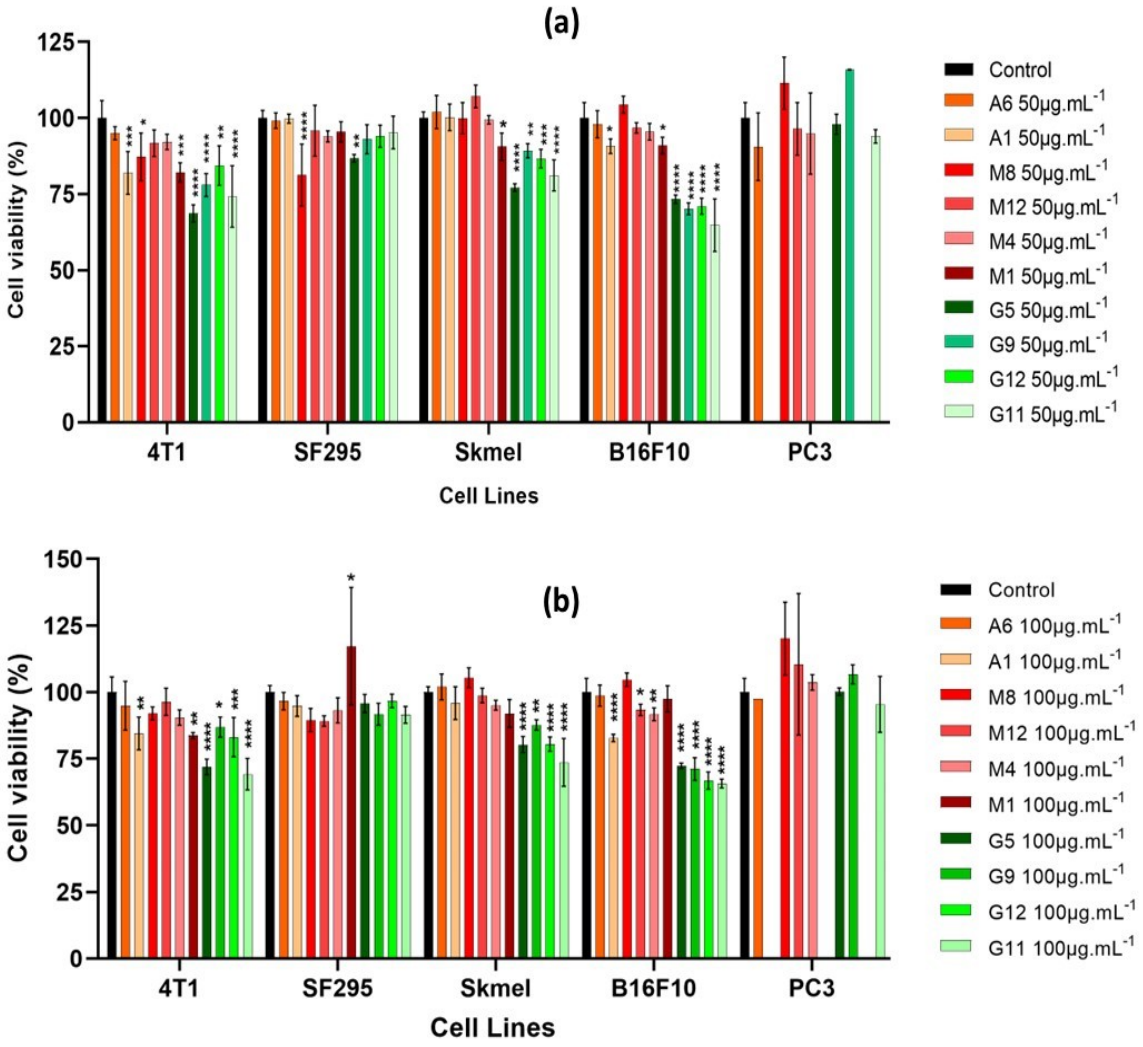
Pinheiro et al.(2016) investigate stem bark ethanolic, methanolic, chloroformic, hexane, and ethyl acetate extracts' phytochemistry and cytotoxic activity from *Anaxagorea dolichocarpa* and *Duguetia chrysocarpa* (Annonaceae family.) against SF-295 human glioma, which is the most common and aggressive glioma, characterized by brain invasion capability. They were very resistant to the current therapies, even under treatment, surgery, and chemotherapy. The ethanolic extracts of *A. dolichocarpa* (65.49%) and the chloroform extract of *D. chrysocarpa (* 63.17%) showed moderate cytotoxicity against the cell culture of SF-295 since extracts showed, in general, low (1 – 50 %) to moderate (51 – 75 %) cell growth inhibition (TECHNICAL COMMITTEE ISO/TC 194, 2009).

Skin cancer results from several mutations in cancer-related genes, including proto-oncogenes and tumor suppressors in skin cells, which cause an imbalance in cell homeostasis and excessive cutaneous cell proliferation. The main skin cancer classes are based on cell type, cutaneous melanoma, and non-melanoma. Melanoma, originating from melanocytes at the deepest layer of the epidermis, has the lowest prevalence rate but the worst prognosis and is responsible for 80% of mortality from skin cancer (GARCÍA-VILLEGAS *et al.*, 2022).

Soursop, acerola, and strawberry extracts were tested against SK-MEL-28 (human malignant melanoma) cell lines at 50 and 100 $\mu g.mL^{-1}$ concentration. The strawberry pomace Soxhlet ethanolic extract (M1) at 50 $\mu g.mL^{-1}$ showed a reduced cell viability of 9.5% compared to the control ($p>0.05$). The soursop seeds extract obtained by pressurized single-mode extraction: SFE 30MPa 40°C (G5), SWE 130°C (G11), and Soxhlet ethanolic extract (G12) showed a reduction of 22.84%, 18.82% and 13.34% at 50 $\mu g.mL^{-1}$ and 19.67%, 26.35% and

19.48% at 100 µg.mL^{-1}, respectively, in cell viability compared with the control treatment (p> 0.05), while pressurized combined-mode extraction - SFE + SWE 130°C (G9) showed a reduction of 10.77% at 50 µg.mL^{-1} and 12,31% at 100 µg.mL^{-1}, respectively, in cell viability compared with the control treatment (p> 0.05).

Figure 22-Antiproliferative activity of soursop seeds extracts, acerola, and strawberry pomace extracts after 24 h of incubation at 37°C: (a) concentration of 50 µg mL^{-1}, and (b) concentration of 100 µg mL.



Legend: C= Control, A6= Acerola pomace SWE at 90°C and A1= Acerola pomace Soxhlet (SOX) extraction with ethanol; M8 = Strawberry pomace (SP+MAE) at 130°C, M12= Strawberry pomace SWE 130°C, M4= Strawberry pomace MAE at 130°C, M1= strawberry pomace Soxhlet (SOX) extraction with ethanol and G5= Soursop seed SFE 30MPa 40°C, G9= Soursop seed SFE + SWE 130°C, G11= Soursop seed SWE 130°C, G12= Soursop seed Soxhlet (SOX) extraction with ethanol. Data expressed as means ± SD, * $p < 0.05$; *** $p < 0.001$: sample vs.Control. Cell lines: 4T1*(* an animal model for stage IV human breast cancer), SF-295 (human Glioblastoma), SK-MEL-28 (human malignant melanoma), B16F10 (murine melanoma), and PC-3 (human prostate cancer). Source: Author

As observed, for the SK-MEL-28 cell line, the extract exerts a dose-dependent manner reduction in cell proliferation after each treatment. The other extracts showed no cell viability reduction and were not significantly different from the control.

Regarding the B16F10 cell line, the Soxhlet ethanolic extracts of acerola pomace (A1) at 50 and 100µg.mL^{-1} showed a reduction of 9,25% and 17,21%, while strawberry pomace (M1) and soursop seed (G12) showed a reduction of 9,02% and 29,01% at 50 µg.mL^{-1} in cell viability compared with the control. On the other hand, the single-concentration screening test at 50 and 100µg.mL^{-1} of soursop seed SFE 30MPa 40°C (G5) extracts and soursop seed SWE 130°C (G11) at single-mode extraction method showed a reduction of 26,68%, 27,63% and 35,24% 34,29%, respectively, on cell viability compared with the control treatment ($p > 0.05$). Also, concerning the single-mode extraction, the strawberry pomace SWE at 130°C (M12) and strawberry pomace MAE at 130°C (M4) showed a reduction of 6,63% and 8,34%, respectively, in cell viability compared with the control treatment ($p > 0.05$). These results were compared with combined-mode extraction of soursop seed SFE + SWE 130°C (G9) at 50 and 100µg.mL^{-1}, respectively, showed a 29,84% and 28,86% reduction in cell viability compared with the control treatment ($p > 0.05$). Analyzing these results, the best answer among the single and combined extraction modes was for soursop seed SWE 130°C (G11) at 50µg.mL$^{-1,}$ although the soursop seed extracts did not differ statistically.

The single-concentration screening test at 50 and 100µg.mL^{-1} for PC-3 cell lines antiproliferative activity (AlamarBlue assay) is presented in **Figure 22** with their respective cell viability percentages, and no sample showed antiproliferative effect for all extracts.

Prostate cancer is the second most common cancer diagnosed globally, after lung cancer, and it is more common in men older than 50. Age and family history are the key contributing factors to this risk factor for prostate cancer (AHMAD *et al.*, 2022).

D'Urso et al.(2016) and Zhu et al.(2015) studied the phytochemical profile and biological activity of ripe and unripe strawberry fruits and their various parts, and Forni et al.(2014) related the anti-cancer treatment of B16-F10 melanoma cells with strawberry extracts affected cell proliferation, reducing cell growth by about 30% after 48 h and 27% after 72 h.

Baby, Antony, and Vijayan (2018) reported in their review paper that several studies have demonstrated the anti-proliferative effect of strawberry crude extracts against the human

colon (HT-29 and HCT- 116), oral (KB and CAL-27), breast cancer (MCF-7) and prostate (LNCaP and DU145) cell lines in a dose-dependent manner.

Respecting the phytochemical composition, our study (**Table 10**) corroborated with the findings mentioned above, where ellagic acid is a typical strawberry fruit compound, in addition to isoquercitrin, quercetin, kaempferol, procyanidin dimer, and trimer, and Galloyl-bis-HHDP-glucose moieties. They show anti-cancer properties *in vitro* and *in vivo* studies, as well as in human intervention trials, and are known to augment the effects of chemotherapeutic agents (BABY; ANTONY; VIJAYAN, 2018; GIAMPIERI *et al.*, 2015).

Da Silva Nunes et al.(2011) reported that tumor lines MDAMDB- 435 (human melanoma) showed inhibition of 29,50% on the percentage of tumor growth at a single dose of 50 μg/ml for the aqueous lyophilized extract of acerola pulp fruit.

In a recent study, other plants belonging to the Malpighiaceae Family demonstrate potential biological activities such as antioxidant, anti-inflammatory, anti-diabetic, anti-microbial, anti-depressant, and cytotoxic properties (ABBAS *et al.*, 2022).

Studies have linked *A. muricata* (bark, fruit, leaves, root, and seeds) aqueous and ethanolic extracts, among others, to a variety of anticancer effects, including cytotoxicity., induction of apoptosis, and inhibition of proliferation on a variety of cancer cell lines, for example, SK- MEL-2 (melanoma cancer), A375 and M14 human melanoma cell line, besides PC-3 (prostate cancer) (AMINIMOGHADAMFAROUJ; NEMATOLLAHI; WIART, 2011; LEITE *et al.*, 2020; RADY *et al.*, 2018; WAHAB *et al.*, 2018).

This preliminary study of the extraction methods, Soxhlet (conventional), and high-pressure extraction (novel) of soursop seeds, acerola, and strawberry pomace and their antiproliferative activity (AlamarBlue assay), achieved by the single-concentration screening test at 50 and 100μg.mL^{-1} demonstrates a low potential cell growth reduction from soursop seeds, acerola, and strawberry pomace extracts. However, UPLC (**Figure 14, Table 9 and Table 10**) and HPLC (**Table 7**) demonstrated significant bioactive compounds such as ascorbic acid (vitamin C) and phenolics- quercetin, kaempferol, catechin, isorhamnetin, vanillic acid, 3,4 dihydroxybenzoic acid, p-coumaric acid, vanillin, caffeic acid, cinnamic acid, chlorogenic acid, ferulic acid, syringic acid, naringenin, rutin, isoquercitrin, dihydroquercetin, taxifolin, gallic acid, epicatechin, procyanidins, ellagic acid, and its glycosides derivatives in addition to galloylglucoses and ellagitannins.

However, some limitations are proper of most cell culture studies. Factors such as cell line specificity, single-concentration test sample (50 and 100μg.mL^{-1}), stability and/or potential

transformation of phenolic compounds, duration of cell exposure to treatment samples to the extract (i.e., 24, 48, or 72hs), whether phenolic compounds are uptaken into cells and in what forms, as well as other potential reactions, should be considered when evaluating cell culture experimental results.

Several recent reviews have highlighted that phenolics may prevent cancer initiation (cytoprotective effect), relapse, or its progression and metastasis to distant organs (cytotoxic effect) generally attributed to their antioxidant activity, while the actual anti-cancer efficacy is due to antioxidant-independent mechanisms, including their pro-oxidant action (GARCÍA-VILLEGAS *et al.*, 2022).

6.4 CONCLUSION

Allegedly, nutritional substances and phytochemicals beneficial for the health and well-being of humans are found in tropical fruits, although mainly in an inedible fraction.

For the first time, we evaluated the antiproliferative activities of the high-pressurized extracts of soursop seeds, acerola, and strawberry pomace using *in vitro* assay. The extracts exhibited antioxidant effects through free radical scavenging activities. The antiproliferative activity (Alamar blue assay) showed low potential for cell growth reduction, achieving a maximum of 31% on 4T1 cell line (SWE 130°C soursop seed extract), 18 % on SF-295 cell line (SP+MAE 130°C strawberry pomace extract), 26% on SK-MEL-28 cell line (SWE 130°C soursop seed extract), 35% (SFE 30MPa 40°C and SWE 130°C soursop seed extract) on B16F10 cell line, and 9,44% (SWE at 90°C Acerola pomace extract) on PC3 cell lines. Among the extracts studied, the soursop seeds extract seemed to have more potential for cell growth reduction than the acerola and strawberry pomace extracts.

The results also provide evidence that agri-food by-product extracts obtained by high-pressure methods are a rich source of active secondary metabolites with antiproliferative activity and thus can guide future phytochemical studies searching for novel anticancer agents.

As previously stated, the soursop seeds (*Annona muricata* L.), acerola (*Malpighia emarginata* DC), and strawberry (*Fragaria x ananassa*) pomaces contain a wide range of bioactive constituents, including vitamin C, polyketides, acetogenins, alkaloids, terpenes, tannins, oils and especially phenolics and flavonoids such as ellagitannins, procyanidins, ellagic

acid, apigenin, quercetin, kaempferol, catechin isorhamnetin, vanillic acid, 3,4 dihydroxybenzoic acid, p-coumaric acid, vanillin, caffeic acid, cinnamic acid, chlorogenic acid, ferulic acid, syringic acid, naringenin, rutin, isoquercitrin, dihydroquercetin, taxifolin, gallic acid, epicatechin, and its glycosides derivatives in addition to galloylglucoses which are responsible for the observed antitumoral, antioxidant, and cytotoxic effect to some cancer cells.

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CHAPTER 7: CONSIDERATIONS AND FUTURE PERSPECTIVES

This chapter summarizes the previous chapters' results and discusses future perspectives.

7 OVERALL CONSIDERATIONS

Soursop seeds, acerola, and strawberry pomace, by-products from juice processing, were well-valorized by high-pressure extraction technologies, encompassing SFE, SWE, and MAE and their combinations compared to conventional bench methods (SOX).

From the results, it is possible to conclude that the selected recovery conditions for bioactive compounds in soursop seeds (Chapter 3), a combination of green extraction techniques, allowed the collection of different fractions from soursop seeds. The single mode (SFE 30 MPa/40 ºC) step allowed lipid removal and caused the raw material cell wall rupture, which afforded a high recovery of polar bioactive compounds of the SFE residue at the Combined-mode (SFE + SWE) method. In the SWE at single and combined mode, X0, TPC, AA, and MRPs were positively correlated with temperature. Regarding chemical composition, the soursop seeds oil extracted by SFE mainly comprises unsaturated fatty acids, oleic and linoleic acids, and saturated fatty acids such as palmitic and stearic acids. Moreover, 29 phenolic compounds were detected by HPLC analysis for the first time from soursop seed extracts, and SWE and SFE residue under different conditions showed the presence of vanillic acid, 3,4 dihy-droxybenzoic acids, p-coumaric acid, vanillin, and caffeic acid as the most abundant phenolic compounds. Thus, the recovered nonpolar and polar extracts with phenolic and antioxidant compounds were obtained using sequential high-pressure extraction steps. This approach encouraged a circular economy by potentially valorizing and recycling food waste from the agri-food production chain, using green-based extraction techniques to recover bioactive compounds for cosmetic, pharmaceutical, and food industries applications encouraging a zero-waste perspective.

Regarding the acerola pomace (chapter 4), we can conclude by the approach of the SWE extraction with fresh and dry pomace as a pretreatment step that the acerola residue subjected to the SWE resulted in a recovery of malic acid, ascorbic acid, and phenolic compounds, mainly quercetin (quercetin O-rhamnoside; quercetin pentosyl-O-hexoside) and kaempferol (kaempferol O-rhamnoside which is compatible with the excellent antioxidant

activity found on SWE extracts obtained from both fresh and dry samples. Also, compared with the conventional approach (SOX), the SWE extraction efficiency with fresh and dry samples was higher. These findings for fresh pomace are significant because they allowed the direct extraction of crude pomace, driving a significant energy-saving advantage by eliminating a step in the process chain.

The strategy of studying different moisture contents in the SWE technique was promising, presenting results similar to the process with a drying step before extraction and presenting phenolic-rich extracts and other bioactive compounds using an environmentally friendly solvent, offering a practical alternative to produce valuable products from discarded acerola by-products that can be used as food supplements or ingredients, active compounds, and cosmetics, and in the pharmaceutical industry, potentially increasing the economic profit while decreasing the environmental waste.

The strawberry pomace extracts (chapter 5) obtained by MAE, SP+MAE, and SWE were compared to SOX. Through the findings of total phenolic content, antioxidant activity *in vitro* analyses, chemical profile by UPLC-MS and antiproliferative activity (Allamar Blue assay) against 4T1(mouse mammary tumor cells), B16F10 (murine melanoma), SF295(human glioblastoma cells), and Sk-MEL-28 (human melanoma cells) suggest that the strawberry extracts from agro-industrial production can be considered a potential source of bioactive phenolic compounds with high natural antioxidant capacity since 16 metabolites were identified, including organic and phenolic acids (ellagic acid, ellagitannins, procyanidins and galloylglucoses) and recovered by promising techniques such as SWE, MAE, and SP + MAE Furthermore, these extracts can be technologically attractive as valuable nutraceutical components in foods, pharmaceutical preparations, or cosmetics.

Regarding antiproliferative activity, the best cell growth reduction results were achieved by SP+MAE 130°C at 100µg.mL^{-1} extract concentration against 4T1 cells (12.8 %), SP+MAE 130°C at 50µg.mL^{-1} for SF295 (18.8%) , soxhlet with ethanol at 50µg.mL^{-1} against SK-MEL-28 (9.4%) and MAE 130°C at 100µg.mL^{-1} (8.3%) against B16-F10. Although the extracts showed a high intensity of individual phytochemicals, they showed low antiproliferative activity. Despite being a preliminary study, we can glimpse the future use of strawberry pomace extracts in cancer prevention, and further investigations are needed to clarify the roles of the different strawberry phytochemicals against cancer cells.

Chapter 6 discusses further high-pressurized soursop seeds, acerola, and strawberry pomace extract biological application through antiproliferative screeening *in vitro* assay against some cancer cell lines considering the extracts' chemical profile.

Notwithstanding soursop seeds, acerola and strawberry pomace extracts obtained by high-pressure methods are a rich source of active secondary metabolites, including vitamin C, polyketides, acetogenins, alkaloids, terpenes, tannins, oils and especially phenolics and flavonoids such as ellagitannins, procyanidins, ellagic acid, apigenin, quercetin, kaempferol, catechin isorhamnetin, vanillic acid, 3,4 dihydroxybenzoic acid, p-coumaric acid, vanillin, caffeic acid, cinnamic acid, chlorogenic acid, ferulic acid, syringic acid, naringenin, rutin, isoquercitrin, dihydroquercetin, taxifolin, gallic acid, epicatechin, and its glycosides derivatives in addition to galloylglucoses, the antiproliferative activity (Alamar blue assay) showed low potential for cell growth reduction, achieving a maximum of 31% on 4T1 cell line (SWE 130°C soursop seed extract), 18 % on SF-295 cell line (SP+MAE 130°C strawberry pomace extract), 26% on SK-MEL-28 cell line (SWE 130°C soursop seed extract), 35% (SFE 30MPa 40°C and SWE 130°C soursop seed extract) on B16F10 cell line, and 9,44% (SWE at 90°C Acerola pomace extract) on PC3 cell lines. Among the extracts studied, the soursop seeds extract seemed to have more potential for cell growth reduction than the acerola and strawberry pomace extracts.

Even if phenolic-rich extracts with allegedly anti-cancer properties, further studies based on *in vitro* assays are required to establish the best conditions that could have antiproliferative effectiveness.

Therefore, in this thesis, our findings demonstrate the feasibility of the high-pressure extractions of soursop seeds, acerola, and strawberry pomaces. These techniques allow continuous extraction of phenolics-rich and other compounds that are highly bioactive using an environmentally friendly solvent in agreement with international efforts in promoting efficient uses of natural resources and fostering innovation by valorizing discarded by-products from juice processing. They can be used as food supplements or ingredients, active compounds, cosmetics, and in the pharmaceutical industry, potentially increasing economic profit while decreasing the waste stream in the environment, reaching the Sustainable Development Goals from the United Nations – SDGs/UN).

7.1 FUTURE PERSPECTIVES

Considering Brazil is the third largest fruit producer in the world, and the polyphenol global market forecast surpasses USD 3.3 billion by 2031 but mainly explores apple, green tea, and grape seeds as phenolics sources and thus, due to the growing demand for sustainable products and foods that promote health benefits, our country has a considerable potential to extract phenolics and others bioactive compounds from agrifood by-products.

To achieve this, studies on the optimization of extraction, isolation, and purification of bioactive compounds from soursop seeds, acerola, and strawberry pomace will be necessary.

In addition, studies related to the biological *in vitro* and *in vivo* application of high-pressure residue extract and their possible bioactivity and bioavailability.

Further experiments are needed to learn about its antitumor activity and mechanisms and to continue with fractionation to isolate the secondary metabolites responsible for the cytotoxic and antitumor properties.

Cancer treatment methods include surgery, radiotherapy, chemotherapy (use of anticancer drugs), and other specialized techniques. On the other hand, Brazil has a myriad of plant species that are phytochemicals rich, such as fruits and their non-edible parts, which highlights an enormous potential to produce novel products with added values bringing insights into the development of novel chemotherapies with lesser side effects and cost, antimicrobials, and food supplements.

Studies on improving the by-products and applications should be conducted to develop processes that promote the production of derivatives with low cost and good nutritional properties, such as feed, additives, cosmetics, or nutraceuticals, and is an environmentally friendly measure that results in a product with added value.

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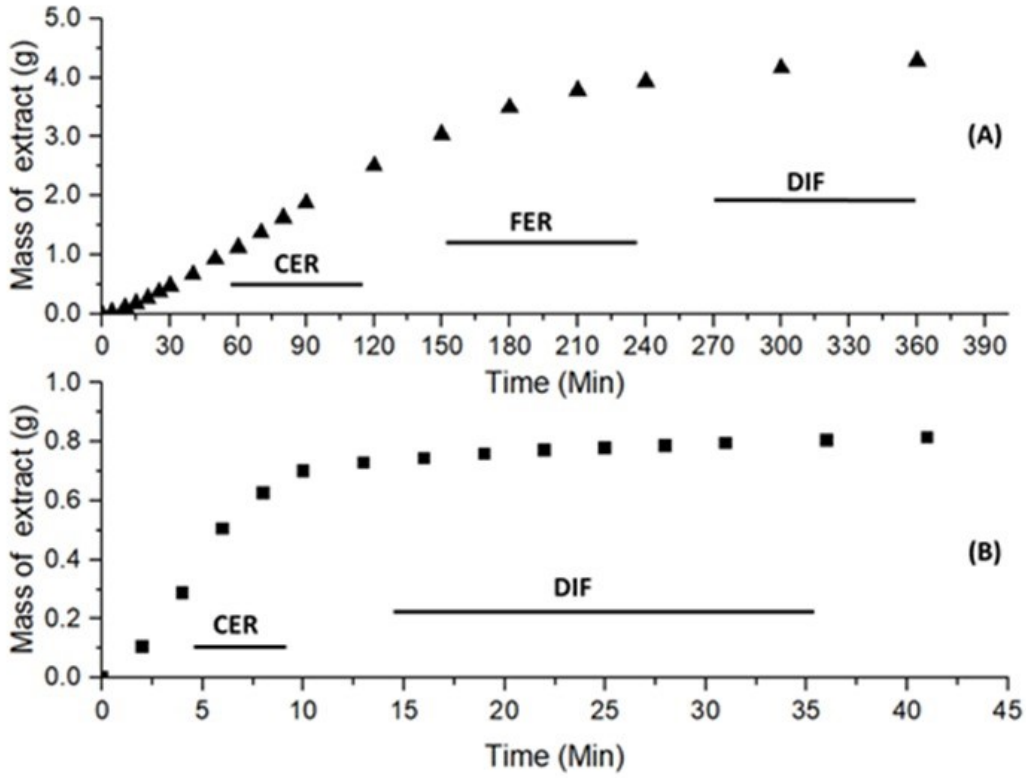
APPENDIX A – SUPPLEMENTARY MATERIAL

Chapter 3

Intensified green-based extraction process as a circular economy approach to recover bioactive compounds from soursop seeds (*Annona muricata* L.)

Figure S1- Kinetics assays for soursop seeds by (A) supercritical fluid extraction (SFE) at 20 MPa, 50 °C, and 0.7 kg CO2·h-1 and (B) subcritical water extraction (SWE) at 10 MPa, 110 °C, and 4 mL·min-1.



CER: Constant extraction rate period; FER: Falling extraction rate period; DIF: diffusion-controlled rate period. Source: Author.

APPENDIX B – SUPPLEMENTARY MATERIAL

Chapter 4

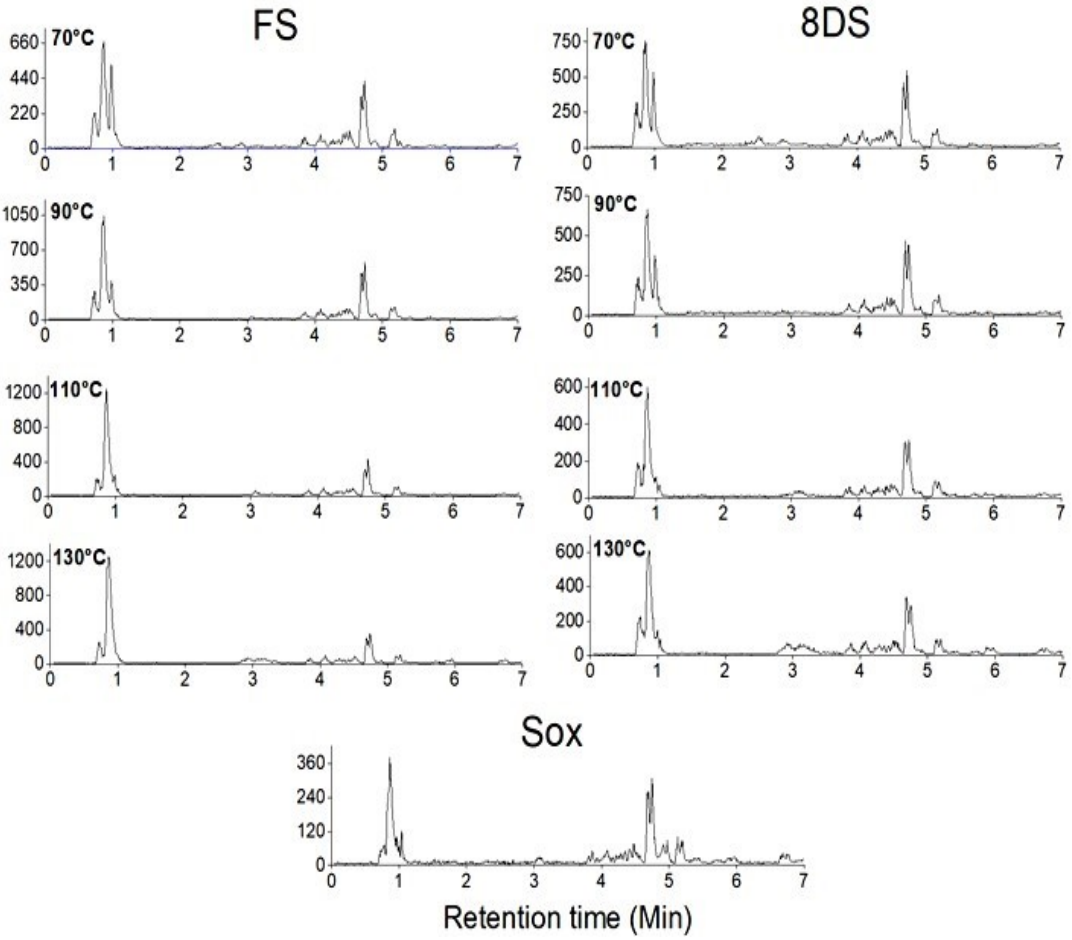
Untargeted metabolomic profile of recovered bioactive compounds by subcritical water extraction of acerola (*Malpighia emarginata* DC.) pomace

UPLC-QTOF-MSE analysis and metabolite identification

Methods. The mobile phase consisted of water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). The gradient was 0–15 min, elution at 2–95 % B; 15.1–17 min, 100% B; and equilibration with 2% B (17.1–19.1 min). A Waters Acquity BEH UPLC column (150 mm × 2.1 mm ID, 1.7 μm) with a flow rate of 0.4 mL min^{-1} maintained at 40 °C—the sample injection volume is 5.0 μL. The MS conditions were as follows: negative ionization mode (ESI); acquisition range: 110–1200 Da; source temperature: 120 °C; desolvation gas temperature: 350 °C; desolvation gas flow: 500 L h^{-1}; extract cone voltage: 50 V; capillary voltage: 2.8 kV; The mode of acquisition was MSE; Leucine enkephalin was used as lock mass (leucine-enkephalin, 0.2 ng μL^{-1}; [M−H] − ion at m/z 556.2771).

Figure S2 – Supplementary material

Figure S2- UPLC-QTOF-MSE chromatograms in negative mode (ESI-) with mass spectra of Subcritical water extraction of fresh samples (FS) at 70°C, 90°C, 110°C and 130 °C; Subcritical water extraction of 8 h dried samples (8DS) at 70°C, 90°C, 110°C and 130 °C, and Soxhlet with ethanol (SOX) acerola (*Malpighia emarginata* DC) byproduct extract.



Source: Author .