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DESENVOLVIMENTO E VALIDAÇÃO DE UM SISTEMA DE EXTRAÇÃO E HIDRÓLISE SEQUENCIAL COM PURIFICAÇÃO E ANÁLISE EM TEMPO REAL PARA PRODUÇÃO DE COMPOSTOS DE MAIOR VALOR AGREGADO

DEVELOPMENT AND VALIDATION OF A SEQUENTIAL EXTRACTION AND HYDROLYSIS SYSTEM WITH REAL-TIME PURIFICATION AND ANALYSIS FOR THE PRODUCTION OF COMPOUNDS WITH HIGHER ADDED VALUE

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DEDICATÓRIA

Dedico

À Deus, à minha família, aos meus professores e amigos.

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RESUMO

Através do desenvolvimento de processos relacionados à economia circular e bioeconomia, esta tese teve como objetivo geral projetar e elaborar um sistema verde de processamento e análise para obtenção de produtos de alto valor agregado a partir de matérias-primas naturais. Por meio de operações automatizadas de extração e hidrólise sequencial acopladas com etapas preparação de amostra e análise que pudessão ser operados de maneira integrada ou individual. Inicialmente, foram realizadas duas revisões bibliográfica utilizando análise bibliométrica longitudinal que avalia todas as publicações sobre um determinado tema aplicando métodos estatísticos e matemáticos para analisar as tendências, perspectivas e tópicos quentes da área de pesquisa. A primeira revisão avaliou o fruto do açaí, os dados mostraram que poucas investigações foram realizadas sobre a recuperação energética da biomassa residual do açaí. Consequentemente, uma análise mais aprofundada para avaliar o potencial de geração de eletricidade foi desenvolvida, indicando que a digestão anaeróbia integrada à hidrólise em água subcrítica pode ser uma forma inovadora de repensar a cadeia produtiva do açaí em um conceito de economia circular. A segunda revisão avaliou as vantagens e limitações da extração de compostos bioativos, técnicas de preparação de amostras, automação e acoplamento com detecção on-line. Destacando as principais técnicas de extração verde para amostras complexas de matrizes vegetais: extração com fluido supercrítico (SFE), extração assistida por ultrassom (UAE), extração assistida por micro-ondas (MAE) e extração líquida pressurizada (PLE). Em seguida foi realizado a montagem do sistema integrado de extração e hidrólise sequencial, juntamente com purificação in-line por meio de extração em fase sólida (SPE) e análise on-line por cromatografia líquida de alta eficiência (HPLC), bem como monitoramento de pH in-line. A validação do sistema de extração e hidrólise capaz de instantaneamente caracterizar quimicamente seus produtos foi efetuada e uma patente foi publicada no Instituto Nacional de Propriedade Industrial (INPI) com o número de registro BR132021018722-0. Posteriormente foi dado início as pesquisas experimentais. A primeira consistiu em um estudo que teve como objetivo extrair antocianinas da polpa de açaí liofilizada e semi-desengordurada utilizando o sistema patenteado. Parâmetros críticos que afetam a eficiência da extração de líquido pressurizado (PLE) e a purificação usando SPE com o adsorvente PoraPakTM Rxn foram investigados e otimizados pela metodologia de superfície de resposta (RSM). As condições ótimas foram 71 °C e água a pH 2 para extração; e 30°C e etanol a 50% para purificação. O método de extração desenvolvido forneceu recuperação de antocianina semelhante a outras técnicas, e o acoplamento in-line com SPE produziu um extrato 5 vezes mais concentrado que o PLE sozinho. O segundo estudo experimental teve o objetivo de avaliar a recuperação de antocianinas, açúcares e ácidos orgânicos do bagaço de uva preta seca (DBGP) utilizando o sistema patenteado. As condições usadas foram baseadas na experiência do grupo de pesquisa com amostras semelhantes e informações da literatura. Doze picos de compostos antociânicos foram identificados e o rendimento de extração foi de 47,60 \pm 0,69 miligrama equivalente de cianidina-3-glicosídeo por grama de DBGP (mgC3G.gDBGP⁻¹). Por meio da hidrólise sequencial, foram identificados e quantificados 7 ácidos orgânicos (ácido tartárico, ácido cítrico, ácido succínico, ácido lático, ácido acético, ácido propiônico e ácido isobutírico) e 5 açúcares (celobiose, glicose, manose, frutose e arabinose). O ácido tartárico representou 53% dos ácidos orgânicos totais e a manose 52% dos açúcares totais. Portanto, pode-se concluir que o sistema desenvolvido se mostrou viável para realizar extração e hidrólise sequencial com purificação por SPE in-line e análise por HPLC on-line de diferentes classes de compostos naturais.

Palavras-chave: *Polpa de açaí, Bagaço de Uva, Compostos Bioativos, Açúcares Fermentescíveis, Ácidos Orgânicos, Processos Integrados.*

ABSTRACT

Through the development of processes related to the circular economy and bioeconomy, this thesis had the general objective of designing and elaborating a green processing and analysis system to obtain high-value-added products from natural raw materials. Through automated sequential extraction and hydrolysis operations coupled with sample preparation and analysis steps that could be operated in an integrated or individual manner. Initially, two literature reviews were performed using longitudinal bibliometric analysis that evaluates all publications on a given topic by applying statistical and mathematical methods to analyze trends, perspectives, and hot topics in the research area. The first review evaluated the açaí fruit, and the data showed that few investigations were carried out on the energy recovery of the açaí residual biomass. Consequently, a more in-depth analysis to assess the potential for electricity generation was developed, indicating that anaerobic digestion integrated with hydrolysis in subcritical water can be an innovative way to rethink the açaí production chain in a circular economy concept. The second review evaluated the advantages and limitations of extracting bioactive compounds, sample preparation techniques, automation, and coupling with online detection. Highlighting the main green extraction techniques for complex samples of plant matrices: supercritical fluid extraction (SFE), ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE) and pressurized liquid extraction (PLE). Then, the assembly of the integrated sequential extraction and hydrolysis system was carried out, together with in-line purification through solid phase extraction (SPE) and on-line analysis by highperformance liquid chromatography (HPLC), as well as monitoring of in-line pH. The validation of the extraction and hydrolysis system capable of instantly chemically characterizing its products was carried out, and a patent was published at the National Institute of Industrial Property (INPI) with registration number BR132021018722-0. Subsequently, experimental research began. The first consisted of a study that aimed to extract anthocyanins from freeze-dried and semi-defatted acaí pulp using the patented system. Critical parameters affecting the efficiency of pressurized liquid extraction (PLE) and purification using SPE with PoraPakTM Rxn adsorbent were investigated and optimized by response surface methodology (RSM). The optimal conditions were 71 °C and water at pH 2 for extraction; and 30°C and 50% ethanol for purification. The extraction method developed provided similar anthocyanin recovery to other techniques, and in-line coupling with SPE produced an extract 5 times more concentrated than PLE alone. The second experimental study aimed to evaluate the recovery of anthocyanins, sugars, and organic acids from dried black grape pomace (DBGP) using the

patented system. The conditions used were based on the research group's experience with similar samples and information from the literature. Twelve peaks of anthocyanin compounds were identified, and the extraction yield was 47.60 ± 0.69 milligram equivalent of cyanidin-3-glucoside per gram of DBGP (mgC3G.gDBGP⁻¹). Through sequential hydrolysis, 7 organic acids (tartaric acid, citric acid, succinic acid, lactic acid, acetic acid, propionic acid, and isobutyric acid) and 5 sugars (cellobiose, glucose, mannose, fructose, and arabinose) were identified and quantified. Tartaric acid represented 53% of the total organic acids, and mannose 52% of the total sugars. Therefore, it can be concluded that the developed system proved to be viable for performing sequential extraction and hydrolysis with in-line SPE purification and on-line HPLC analysis of different classes of natural compounds.

Keywords: Açaí pulp, Grape Pomace, Bioactive Compounds, Fermentable Sugars, Organic Acid, Integrated Processes.

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CHAPTER 1 – General Introduction, Objectives, and Thesis Structure

1.1 GENERAL INTRODUCTION

Bioactive compounds are intensely sought after by the food, pharmaceutical, and cosmetic industries, due to the physiological, nutritional, and medicinal benefits that promote human health (PLAZA & TURNER, 2015). Efforts are being made to obtain these compounds from natural sources, such as plants, animals, and microorganisms, as well as to replace the use of synthetic products (CARREIRA-CASAIS et al., 2021). The main classes of natural bioactive are phenolic compounds, glucosinolates, carotenoids, phytosterols, alkaloids, and terpenes (ROSTAGNO & PRADO, 2013). The chemical complexity and diversity of these compounds are not readily achieved in human-synthesized compounds (FERNANDES et al., 2019).

These natural compounds from plants are products of secondary metabolism. Their distribution and concentration are influenced by several factors, such as variety, climate, location, year of cultivation, and agricultural practices, among others (WIANOWSKA & GIL, 2019). Additionally, depending on the type and intensity, processing can cause transformation and/or degradation of these compounds, altering their properties and/or decreasing their activity. However, in many cases, a large amount still remains viable after processing, although they are commonly underused or even discarded as agro-industrial waste (SRIVASTAVA et al., 2021).

The extraction of bioactive compounds has been carried out from different plant matrices. However, obtaining these from food industry residues has a great advantage for providing greater use of natural resources and minimizing impacts on the environment by reducing the disposal of waste (ALARA et al., 2021). In addition to being an alternative to containing forest deforestation, through the valorization of underused resources, thus generating a greater aggregation of value in the production chain (MACIEL-SILVA et al., 2019).

Another alternative that has been the focus of research in all parts of the world is using energy from lignocellulosic residues to produce bioenergy and biofuels, as they are renewable, abundant, and non-food sources. Capable of reducing the drastic climate changes attributed to our excessive dependence on fossil fuels (RAUD et al., 2019). Each country usually focuses on a residue from its local agroindustry; however, Brazil has an immense and diversified agroindustrial production with different residues that can be exploited in each region (OLIVEIRA et al., 2015). Biomass hydrolysis is a crucial step for increasing production efficiency in this process (CANDIDO et al., 2019). It can be performed sequentially with the extraction of bioactive compounds to obtain a better use of the material (LACHOS-PEREZ et al., 2020).

Several green extraction processes have been applied to recover bioactive compounds from a wide range of plants in recent years, alternatives for replacing conventional extraction techniques that use toxic solvents. Among the main, environmentally friendly techniques are supercritical fluid extraction (SFE), microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), and pressurized liquid extraction (PLE) (CHEMAT et al., 2017; MACIEL-SILVA et al., 2022; TÝSKIEWICZ et al., 2018; WANG et al., 2016). The selection of the extraction method and conditions is essential for producing extracts with good yield and functionality. In this context, PLE is one of the most efficient methods to recover bioactive compounds from vegetable matrices through green solvents such as ethanol and water (SOUZA et al., 2021).

The PLE method applies high pressure to allow solvents to remain liquid even at temperatures above the normal boiling point. Furthermore, the higher pressure improves the solvent targeting the pores of the raw materials and increases the solubility of the target compounds (WIANOWSKA & GIL, 2019). Higher temperatures are used to obtain better extraction yields, as temperature increases mass transfer and extraction rate, primarily due to the improved ability to solubilize the extract, break solute-matrix bonds, decrease solvent viscosity, and reduce the surface tension (VIGANÓ et al., 2022).

When using only water as a solvent, PLE can also be called pressurized hot water extraction (PHWE) or hot compressed water extraction (HCWE) (HOFF & PIZZOLATO, 2018; PLAZA & TURNER, 2015). Additionally, under more severe temperature and pressure conditions, water acts as an alternative solvent for biomass hydrolysis (COCERO et al., 2018). When the process that occurs under conditions above the critical point of water (Tc 374 °C and Pc 22.1 MPa) is called supercritical water hydrolysis (SCWH), and when water is kept in the liquid state by pressure at a higher temperature than its natural boiling point of 100 °C and lower than its critical temperature of 374 °C the process is defined as subcritical water hydrolysis (SubCWH) (THIRUVENKADAM et al., 2015). In these situations, water has low viscosity and high diffusivity that facilitates its penetration into the complex structure of the lignocellulosic

matrix, in addition to a low dielectric constant, similar to non-polar organic solvents, enabling the solubility of organic compounds (ZAKARIA & KAMAL, 2016).

However, depending on the concentration of analyte present in the sample, using these techniques, a large volume of solvent is required to obtain a high yield of extraction and hydrolysis. To overcome this challenge, in-line coupling, that is, connection directly to the flow with solid phase extraction (SPE) can be performed (VIGANÓ et al., 2021). SPE is widely used to remove unwanted compounds, and separate and concentrate complex samples, allowing a high level of automation and eliminating post-extraction and post-hydrolysis steps such as centrifugation, filtration, concentration, and detoxification (HOFF & PIZZOLATO, 2018). Thus, it is possible to couple to the analytical process tools such as capillary electrophoresis (CE), supercritical fluid chromatography (SFC), gas chromatography (GC), and highperformance liquid chromatography (HPLC), enabling the on-line analysis of target compounds simultaneously with extraction and hydrolysis in a closed system (CLAVIJO et al., 2015; Liang & Zhou, 2019). This coupling occurs through direct contact between the flow system and sensing equipment components, usually through a flow bypass. From the point of view of analytical chemistry, the coupling of these techniques can bring additional benefits, including greater sensitivity, reliability and speed to obtain results (FRENZEL & MARKEVICIUTE, 2017; MEJÍA-CARMONA et al., 2019). In addition to avoiding some problems associated with traditional analytical techniques, such as sample loss and contamination at each stage of preparation (SAMMANI et al., 2019). Additionally, the coupling can reduce product characterization time and allow quick decision-making in industrial processes, which avoids product losses such as reprocessing (VIGANÓ et al., 2021).

In this context, the use of green technologies and solvents to obtain bioactive compounds and precursors of bioenergy from natural resources is a circular economy alternative to contain the advance of environmental problems, promoting better use of vegetable matrices, reducing the waste disposal and generating products with high added value. Thus, this work aims to promote the development of more efficient processes for extraction and hydrolysis, with greater purity, in less time and generating less impact on the environment, through the development of a sequential extraction and hydrolysis system coupled with in-line SPE purification and on-line HPLC analysis.

1.2 OBJECTIVES

1.2.1 General Objective

Design and elaborate a sequential extraction and hydrolysis system with in-line purification and on-line analysis, aiming to obtain products with high added value from natural raw materials.

1.2.2 Specific objectives

- Develop a review of açaí with bibliometric analysis and energy potential assessment;
- Elaborate a review with bibliometric analysis of green extraction techniques with online detection coupling;
- Assemble, validate and patent an extraction and hydrolysis system coupled with in-line purification and on-line analysis;
- Evaluate the extraction of anthocyanins from açaí (*Euterpe oleracea*) using the new system integrating extraction, purification, and analysis;
- Perform a sequential extraction and hydrolysis process with in-line purification/concentration and on-line analysis using grape pomace (*Vitis vinifera L.*).

1.3 THESIS STRUCTURE

This thesis is divided into chapters. The chapters that present the bibliographic reviews and the experimental results correspond to articles that are published or are being reviewed in scientific journals. The system assembly details are presented in the patent chapter published by the Instituto Nacional de Propriedade Industrial (INPI).

Chapter 1 consists of a general introduction that describes the central theme of the thesis, briefly exposing the most relevant points. In addition to presenting the objectives and thesis structure.

Chapter 2 presents a review that evaluates all research on açaí through longitudinal bibliometric analysis. This study showed that few investigations were carried out on the energy recovery of açaí residual biomass. Consequently, a more in-depth analysis to assess the potential for electricity generation was developed, indicating that the generation of electricity from the burning of biogas produced through anaerobic digestion integrated with the hydrolysis in subcritical water of the totality of açaí seeds generated in Brazil it could supply all the needs of the frozen açaí pulp industry with an additional surplus for sales to the chain. Therefore, assessing electricity generation potential illustrates how technology integration can be an innovative way of rethinking the açaí production chain in a circular economy concept. In addition, the replacement of electricity contributes to the mitigation of greenhouse gases.

This chapter consists of a review article entitled "Sustainable development in the Legal Amazon: energy recovery from açaí seeds" published in the journal "Biofuels, Bioproducts and Biorefining" Vol. 15, n. 4, p.1174–1189, 2021. https://doi.org/10.1002/bbb.2222.

Chapter 3 presents a review that evaluates all publications on extraction processes of bioactive compounds coupled with on-line analysis through a longitudinal bibliometric analysis, applying statistical and mathematical methods to analyze trends, perspectives, and hot topics of this research area. The objective was to discuss the advantages and challenges of extracting bioactive compounds, sample preparation techniques, and coupling with on-line analytical techniques. In addition, relevant green extraction techniques of complex plant matrices coupled with on-line analysis systems are highlighted.

This chapter corresponds to a review article entitled "Green extraction processes for complex samples from vegetable matrices coupled with on-line detection system: A critical review" published in the journal "Molecules" Vol. 27, n. 19, p. 6272, 2022. https://doi.org/10.3390/molecules27196272.

Chapter 4 depicts an integrated subcritical and supercritical water extraction and hydrolysis system coupled with in-line purification through solid-phase extraction and on-line analysis by high-performance liquid chromatography, as well as in-line pH monitoring. To obtain bioactive compounds and fermentable sugars from any raw material containing these compounds, preferably agro-industrial residues. The system comprises two modules, an extraction/hydrolysis module and another analysis module, including a purification system and a liquid chromatography system. The modules can be operated independently or integrated, allowing the chemical characterization of industrial waste in terms of bioactive compounds, sugars and organic acids, in addition to the development of integrated extraction and hydrolysis methods.

This chapter consists of a patent application (BR 132021018722-0) filed at the Instituto Nacional de Propriedade Industrial (INPI) on September 20, 2021 and published on December 6, 2022. Titled Sistema integrado de extração e hidrólise em água subcrítica e supercrítica para obtenção de compostos bioativos e açúcares fermentescíveis.

Chapter 5 contains a study that aimed to extract anthocyanins from freeze-dried and semi-defatted açaí pulp using the equipment from the patent application (BR132021018722-0). Critical parameters affecting the efficiency of pressurized liquid extraction (PLE) and purification using Solid Phase Extraction (SPE) were investigated and optimized by response surface methodology (RSM). The optimal conditions were: 71 °C and pH 2 for extraction; and 30°C and 50% ethanol for purification. The extraction method developed provided similar anthocyanin recovery with other techniques, and in-line coupling with SPE produced an extract 5 times more concentrated than PLE alone. Therefore, the system (PLE-SPE × HPLC-PDA) proved to be a powerful tool for monitoring the extraction process in real-time.

This chapter corresponds to an experimental article entitled "Pressurized liquid extraction coupled in-line with SPE and on-line with HPLC (PLE-SPExHPLC) for the recovery and purification of anthocyanins from SC-CO2 semi-defatted Açaí (Euterpe oleracea)" published in the journal "Food Research International" Vol. 160, p. 111711, 2022. https://doi.org/10.1016/j.foodres.2022.111711.

CHAPTER 1 – General Introduction, Objectives, and Thesis Structure

Chapter 6 presents an article whose objective was to evaluate the recovery of anthocyanins, sugars, and organic acids from dried black grape pomace (DBGP) using the equipment of the patent application (BR132021018722-0). Through the automated system (PHWE/SubCWH \times SPE \times HPLC-PDA/RI) based on the coupling of pressurized hot water extraction (PHWE) and sequential subcritical water hydrolysis (SubCWH) with in-line purification by solid phase extraction (SPE) and on-line analysis by high-performance liquid chromatography (HPLC) using photodiode array (PDA) and refractive index (RI) detectors. Twelve peaks of anthocyanin compounds were identified, and the extraction yield obtained by HPWE was 47.60 \pm 0.69 mgC3G gDBGP⁻¹. Through SubCWH, 7 organic acids (tartaric acid, citric acid, succinic acid, lactic acid, acetic acid, propionic acid, and isobutyric acid) and 5 sugars (cellobiose, glucose, mannose, fructose, and arabinose) were identified and quantified. Lactic acid represented 53% of the total organic acids and mannose 52% of the total sugars. Therefore, the newly developed method proved viable for performing HPWE and SubCWH of DBGP with in-line SPE purification and on-line HPLC analysis of different classes of natural compounds.

This chapter represents the experimental article entitled "An automated system to integrate sequential extraction and hydrolysis with in-line sample preparation and real-time monitoring" submitted to a scientific journal in the field of food engineering.

Chapter 7 presents a general discussion of the main results obtained in the thesis. Chapter 8 presents the main conclusions of the thesis. Chapter 9 presents a memorandum of the doctoral period with the most relevant academic work carried out in parallel with the development of this thesis. Chapter 10 contains all the bibliographic references used in each chapter of the thesis. In chapter 11, Appendices, the supplementary materials of the published articles are presented. Finally, in Chapter 12 Attachments, the authorizations given by the publishers to use the articles published in this thesis are presented.

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CHAPTER 2 – Review (Açaí: Bibliometric Analysis and Assessment of Energy Potential)

SUSTAINABLE DEVELOPMENT IN THE LEGAL AMAZON: ENERGY RECOVERY FROM AÇAÍ SEEDS

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ABSTRACT

The Brazilian açaí's industry reached national and international prominence in the last few years. Its final product, mainly frozen pulp, became worldwide popular, accounting for a high market share, including exports. Nevertheless, the solid by-products arising from the processing do not always find environmentally suitable disposal. This review presents a scientometrics analysis of açaí research in the last 27 years (1993 to 2019), applying a longitudinal bibliometric study. Through data analysis, it can be observed that Brazil and the USA are the countries that mostly contributed to the development of açaí research. Although Brazil has more publications, the USA presents more articles in partnership with 21 countries. The results also showed that few documents addressed renewable energy recovery with the following technologies: combustion, pyrolysis, supercritical water hydrolysis, and anaerobic digestion. Further analysis of the anaerobic digestion of the totality of açaí seeds generated in Brazil indicated that, theoretically, the electric energy from biogas burning could supply the whole frozen pulp industry requirement with an additional surplus for sales to the grid. Hence, electricity replacement contributes to greenhouse gas mitigation. The assessment of the potential electric energy generation illustrates how technological integration can be an innovative way to rethink the açaí's production chain in a circular economy concept. Moreover, technological advances could stimulate sustainable agro-industrial intensification in areas such as the Legal Amazon, considered a hotspot for climate change mitigation.

Keywords: Açaí Seeds, Bibliometric Analysis, Green Energy, Bioeconomy.

2.1 INTRODUCTION

Açaí is a tropical fruit typical in the Brazilian North region, over the Amazon and Tocantins rivers basins and wetlands. Although the extractive (harvesting of native nature, without any change in the environment) is still present in açaí's production systems, management practices are the most dominant in the last 5 years. Managed systems are characterized by controlling açaí's tree growth to avoid the natural competition for light and nutrients with other local species aiming to obtain higher harvesting yields.¹ There have been significant changes in the production of açaí to meet the growing demand from both the domestic and foreign markets, there have been significant changes in the production of aquit trees, there has been an outstanding increase in the production of fruit from cultivated production, commonly referred to as aquif from firm lands or irrigated açaí. Previously to 2015, the production was firmly based on the extractive process. Pará and Amazonas States are the most significant contributors to açaí's production and sales in the last 15 years compared to the other Brazilian states (**Figure 1**).

However, the açaí industry faces the challenge of low fruit yield, where the fresh pulp, acclaimed as a nutraceutical or functional food, corresponds to only 10-15% by weight of the fruit.^{2–4} Primarily, the solid by-product, açaí seed, is destined for combustion, and, sometimes, it does not receive environmentally suitable disposal.^{5,6} The biggest açaí producers are located in the North region of Brazil, in the "Legal Amazon", which is, currently, threatened by the expansion of agricultural frontier for cattle and soybean crops, construction of highways, and new hydroelectric power plants.^{7,8}

The Amazon rainforest is being subject to several anthropogenic interferences intimately related to deforestation, which can result in climate change, wildfires, and modification of the typical vegetation as a mechanism of adaptation to the new condition, including a phenomenon referred to as "savanization".⁹ Land Use Change (LUC) is a major greenhouse gas (GHG) emitter in Brazil,¹⁰ reaching a gross emission of 499 Mton of CO_{2eq} in 2018 from the Amazon deforestation alone, a 3.6% increase from 2017. LUC GHG emissions were mainly pushed by Pará State, which total deforestation reached 21% of its total area until 2018, a total of 267,393 km².^{11,12}

Some measures to avoid the aforementioned ecosystem's side-effects would be to increase carbon dioxide fixation and photorespiration rates by keeping green areas with native



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Figure 1. Historical açaí production sales. (Source: data collection from IBGE, 2019)¹³

vegetation.¹⁴ As açaí is a regional, native tree element of the Amazon rainforest, its production could positively act in the carbon fixation without jeopardizing the ecological dynamics.¹⁵ A sustainable açaí agroindustry applying green technologies for energy recycling would be specially designed to mitigate the deforestation and climate change environmental burdens in the Legal Amazon while contributing to the energy matrix decarbonization.

Due to the growing açaí's market, the issue related to the recovery of solid by-products (seeds) from industrial processing, and the locality aspects associated with a fragile agro producer ecosystem, this work aims to supply scientific information related to the advances in local renewable energy production from the processing of açaí seeds. The scientific research on açaí was reviewed through the bibliometric analysis of the past 27 years to identify the most developed research fields and the possible knowledge gaps on energy recovery. Based on previous technological results from the present research group, an analysis of potential energy generation from açaí seeds is presented. Lastly, the discussion deals with the evolution of açaí's production chain, focusing on emerging technologies, to support greener strategies for a sustainable pathway. The results could be helpful to subsidize public policies towards a circular economy¹⁶, contributing to the development of more sustainable agroindustry in the Legal Amazon.

2.2 METHODS

2.2.1 Bibliometric methods

2.2.1.1 Data collection

The bibliometric analysis retrieved the publications from the Web of Science (WoS) database, from the company Clarivate Analytics, applying the Science Citation Index Expanded (*SCI-EXPANDED*) option. This database is suitable for academic and scientometrics studies since it presents extensive and multidisciplinary bibliographic data from the leading scientific publications.^{17,18}

The bibliometric search was done in June 2020, and the retrieval process is shown in **Figure 2** (**A**). It was used the "*TOPIC*" search field, which searches for words in titles, abstracts, author's keywords, plus keywords defined by the database WoS. The keywords used to collect the documents about the açaí fruit were: *Açaí* and *Assaí*. The Boolean operators "OR" (to perform union of the results of each term) were used to gather all of the available documents,

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regardless of the writing adopted by the authors. A total of 756 documents were found until 2019. After the first step of refining, where only articles and reviews were selected, the number dropped to 607 documents. The secondary refining step filtered the titles and abstracts to establish more accurate documents related to this work research objective. For example, articles in the art area written in languages other than English were disregarded in the WoS database for the keyword *Assaí*. Articles from different areas that used the abbreviation *ACAI* for various purposes were also disregarded since they are not consistent with the research direction herein presented. Ultimately, the final dataset reached 555 selected documents (539 articles and 16 reviews).

2.2.1.2 Data processing

The bibliometric analysis was done using the "ANALYZING RESULTS" tool provided by WoS, from which information on the types of documents, number of publications, year of publication, research areas, journals, most cited articles, and countries were gathered. The support material was prepared using the processor Word 2019 from Microsoft® Office. The graphical illustrations were done using the software Origin 8.0 from OriginLab®. The construction of the bibliometric map based on co-authorship was created in the VOSviewer© 1.6.13 software, which is functional to make a graphical representation of bibliometric maps, facilitating data interpretation.¹⁹ **Figure 2 (B)** illustrates the methodology applied.

2.2.2 Estimation of energy recovery from açaí seeds

Based on experimental results previously obtained by Maciel-Silva et al., (2019)²⁰ for the anaerobic digestion (AD) of açaí seeds without and with subcritical water hydrolysis pretreatment (SH+AD), an estimation of the potential electric energy generation (EG) from biogas burning was done. Subsequently, the greenhouse gas (GHG) avoided emissions from the replacement of the power obtained from the grid by the one obtained from biogas burning was estimated. According to Maciel-Silva et al., (2019)²⁰, the methane contents in the biogas for the two scenarios were 50% of methane for AD arrangement and 81% for SH+AD. Accordingly, for each ton of feedstock (açaí seeds), 1.14 m³ of methane from the biogas was generated for the AD reactor and 54.90 m³ for SH+AD. These experimental yields were assumed to estimate the total volume of methane for the total Brazilian açaí seed production as follows: CHAPTER 2 – Review (Açaí: Bibliometric Analysis and Assessment of Energy Potential)



Figure 2. Methodological synthesis applied to the bibliometric analysis.

(i) açaí fruit production, obtained from Brazilian National Statistics, for the year 2018, accounted for 1.73×10^6 ton;¹³

(ii) from which 90%, $^{2-4}$ corresponds to the seed mass, i.e. 1.56×10^6 ton;

(iii) for AD, the methane volume could reach 3.55×10^6 Nm³/year, and for SH+AD, 105.49×10^6 Nm³/year.

For the biogas burning in a stationary engine, it was assumed conversion of 85% of the methane into electric energy (with an efficiency of 38%). The potential of electric energy generation (EG) from biogas burning was obtained according to the theoretical **Equation 1**, as follows:

$$EG = Q_{CH4} \times LCV_{CH4} \times \eta_e \times \eta_a \times F_c \times C$$
 (Eq. 1)

Where, EG: Electricity generation (MWh/year), Q_{CH4} : Volume of methane (m³/year), LCV_{CH4} : Low calorific value of methane (35.59 MJ/Nm³), η_e : Motor efficiency (38%), η_g : Generation efficiency (85%), F_c : Correction factor for energy losses, and *C*: Conversion from MJ to MWh.

The potential avoidance of GHG emissions, considering the replacement of the electric energy from the Brazilian National grid by the potential generation from biogas burning, was estimated from **Equation 2**:

$$A_{GHG} = 0.07398 \times EG \times t \tag{Eq. 2}$$

Where, 0.07398 is the average emission factor for the generation of electric energy in the National Interconnected System of Brazil1 (tCO_{2eq}/MWh) relative to 2018,²¹ *EG* (MWh/year) is the potential of electric energy generation previously calculated, and *t*: Operating time in hours (20h in 364 days per year).

The emissions from the biogas burning in stationary engines (BB_{GHG}) were estimated according to the emission factor from the Intergovernmental Panel on Climate Change (IPCC) model for a Tier 1 level for the Energy sector,²² which is 0.19656 tCO2eq/MWh (**Equation 3**). Finally, the net emissions (Net_{GHG}) were estimated following **Equation 4**.

$$BB_{GHG} = 0.19656 \times EG \times t \tag{Eq. 3}$$

¹ It accounts for the average generation emissions, taking into account all the plants that are generating energy, and does not include ecosystem disruption.

$$Net_{GHG} = BB_{GHG} - A_{GHG}$$
 (Eq. 4)

2.3 BIBLIOMETRIC EVALUATION

2.3.1 Scientific development

Research on açaí has proven to be worldwide relevant, covering 45 different countries in the total publication period (1993 to 2019). **Figure 3** presents the evolution of the publications and the main research areas. **Figure 3** (**A**) focuses on annual publications and **Figure 3** (**B**) on the total ones. It is possible to observe the growth of publications in the main areas of *Food Science Technology, Chemistry, Agriculture, Nutrition Dietetics, Pharmacology, Biochemistry,* and *Environmental Science Ecologic.* The annual publications showed significant growth since 2008, reaching more than 20 articles per year. In 2019, 78 studies were published, and the total reached 555 publications.

The *Food Science Technology* area is the leadership since 2008 for both annual publications and total publications. These results can be attributed to, the variety of phenolic compounds present in the açaí fruit, which places it as a "superfruit".²³ Also, the fruit is composed of substances related to antioxidant, anti-inflammatory, anticarcinogenic, antimicrobial, analgesic, and vasodilator properties.^{24,25} Due to the dissemination of these benefits, scientifically addressed, the consumption of açaí has increased.^{26,27} Probably, this explains the increase in scientific production in other areas related to açaí, such as *Chemistry, Agriculture*, and *Nutrition Dietetics*. Recently, the *Environmental Science Ecologic* area started to emerge due to growing environmental concerns. Several works present açaí seed as the central research issue.^{28–30}

In association with the environmental research area, waste management and energy/biochemicals recovery technologies studies arose in the last years. The main works addressing açaí seed as raw material to develop routes to take advantage of this by-product energy potential, follows: (i) extraction and evaluation of bioactive compounds,^{2,31,32} (ii) hydrolysis to obtain sugars by hydrothermal and enzymatic treatment,^{33–35} (iii) evaluation of physical properties to improve energetic attributes for combustion and fuel applications,^{4,36,37} (iv) açaí seed gasification,⁵ (v) activated carbon production,²⁸ (vi) pyrolysis⁶, and (vii) super supercritical water hydrolysis followed by anaerobic digestion to improve biogas and methane production.²⁰



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Figure 3. Evolution of the number of publications and main research areas. (A) Annual Publications, and (B) Total Publications.

2.3.2 International collaboration

The evaluation of the international collaboration in publishing was carried out in the VOSviewer© software to understand the partnership between the countries involved in açaí research. For this, the type analysis of co-authorship and the number of cumulative publications were chosen as thresholds. For the design of the clusters, the "association strength" method was used for the normalization. **Figure 4** shows the bibliometric network, in which the size of the marker is proportional to the accumulated publications. The thickness and colors of the connection lines indicate the cluster belonging and relationship strength.

A total of 45 countries that published in scientific journals using açaí as research subject were identified. The 2 top countries are Brazil and the USA, with 409 (74% of the total 555 documents) and 109 (20%) articles, respectively. Both countries present outstanding higher publishing in comparison to the 3rd most productive country, Spain - with only 19 publications. Brazil is the largest producer of the açaí fruit³⁸; therefore, Brazilian researchers are expected to be the leaders in the field. Other publishing countries located in the Amazon rainforest large area are Colombia and French Guiana (possibly because they are also açaí producers). The USA stands out in publications for being the main consumer market outside Brazil.³⁹ Another significant international consumer market is Europe.²³ Coincidentally, the whole American continent and Europe presents 7 countries in the top 15.

Among the top 15 most productive countries, Brazil is the only one in partnership with all of the countries in the network, followed by the USA (without connections with only three countries). Despite several collaborative studies between Brazil and the USA, the bibliometric map did not characterize them in the same cluster because the cluster grouping considers the affinity/proximity between all the items analyzed.^{40,41} Regarding the geographical division, there is no direct relationship of territorial proximity clusters grouping. It is noteworthy to observe that, although açaí is a local product, there is substantial international scientific attention on it.


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Figure 4. Collaboration map among the top 15 most productive countries.

2.3.3 Journals with more publications and most cited articles

All of the articles found until 2019 were published in 276 different journals, which illustrates the diversity in this research field and the broad interest in this fruit from multiple perspectives. One hundred and seventy-eight (178) journals have only one publication, 64.5% of the total number of scientific journals, and 32% of the publications. According to Bradford's law, this distorted distribution is expected in the bibliometric analysis since there are journals dedicated to specific topics.⁴²

Table 1 presents the top 10 journals ranked by the number of publications, responsible for approximately 25% of publications. The *Food Chemistry* journal is the first, with more than 6% of total publications, possibly because of its high Impact Factor (6,306), followed by the *Journal of Agricultural and Food Chemistry* (Impact Factor 4.192, 23 records). Finally, there is a tie between *Food Research International's* (Impact Factor 4.972, 18 records) and *Revista Brasileira de Fruticultura* (Impact Factor 0.614, 18 records).

Table 1 reveals that 70% of the most productive journals belong to the American continent, with 4 from Brazil and 3 from the USA, corroborating that these countries have the largest number of publications. Nevertheless, Brazilian journals have the smallest Impact Factors. Thereat, the journals most sought by the authors are from Europe.

Table 2 presents the top 10 most cited açaí documents until 2019, all of them experimental articles. Only 1 of them was not published in one of the top 10 most productive journals (**Table 1**).

The work with the highest number of citations (380) is focused on the physicochemical properties of açaí powder produced by spray drying, which approach dealt with technological process conditions.⁴³ In general, it is observed that all the most cited articles were published before 2011, and the most recent article is from 2010.⁴⁴

2.3.4 Keyword analysis

The keyword analysis can identify the most popular subjects covered by the research field.⁴⁵ The top 15 most frequently used keywords (**Table 3**) were compared to identify trends and possible knowledge gaps in the subject.¹⁷

| N. | Rank | Journals | Country | Publishing company | Impact Factor | Records | % Publications |
|----|------|--|-------------|--------------------|----------------|---------|----------------|
| 1 | 1° | Food Chemistry | England | Elsevier | 6.306 | 34 | 6.126 |
| 2 | 2° | Journal of Agricultural and Food Chemistry | USA | ACS 4.192 | | 23 | 4.144 |
| 3 | 3° | Food Research International | USA | Elsevier | Elsevier 4.972 | | 3.243 |
| 4 | 3° | Revista Brasileira de Fruticultura | Brazil | SBF | 0.614 | 18 | 3.243 |
| 5 | 4° | Journal of Functional Foods | Netherlands | Elsevier | 3.701 | 9 | 1.622 |
| 6 | 5° | PLOS ONE | USA | PLOS | 2.740 | 8 | 1.441 |
| 7 | 6° | Ciência e Tecnologia de Alimentos | Brazil | SBCTA | 0.625 | 7 | 1.261 |
| 8 | 6° | Food Science and Technology | Brazil | SBCTA | 1.443 | 7 | 1.261 |
| 9 | 6° | Journal of Food Engineering | England | Elsevier | 4.499 | 7 | 1.261 |
| 10 | 6° | Pesquisa Agropecuaria Brasileira | Brazil | Embrapa | 0.644 | 7 | 1.261 |

Table 1: Top 10 journals ranked by publication accounting.

ACS - American Chemical Society; SBF - Sociedade Brasileira de Fruticultura, PLOS - Public Library of Science; SBCTA - Sociedade Brasileira de Ciência e Tecnologia de Alimentos; Embrapa - Empresa Brasileira de Pesquisa Agropecuária; % publications - Percentage of total number of publications. The impact factor measures the average number of citations received in a particular year by papers published in the journal during the two preceding years. Journal Impact Factor (Clarivate Analytics, 2020).⁴⁶

Table 2: Top 10 articles ranked by number citations.

| Rank | Title | Journals | Authors | Year | Citations | Ref. |
|------|--|--|---------------------------------|------|-----------|------|
| 1 | Influence of process conditions on the physicochemical properties of açai (Euterpe oleraceae Mart.) powder produced by spray drying | Journal of Food Engineering | Tonon, R.V. et al. | 2008 | 380 | 43 |
| 2 | Comparison of antioxidant potency of commonly consumed Polyphenol-Rich beverages in the united states | Journal of Agricultural and Food Chemistry | Seeram, N.P. et al. | 2008 | 347 | 47 |
| 3 | Antioxidant activity of dietary fruits, vegetables, and commercial frozen fruit pulps | Journal of Agricultural and Food Chemistry | Hassimotto, N.M.A. et al. | 2005 | 245 | 48 |
| 4 | Anthocyanin stability and antioxidant activity of spray-dried açai (Euterpe oleracea Mart.) juice produced with different carrier agents | Food Research International | Tonon, R.V. et al. | 2010 | 221 | 44 |
| 5 | Antioxidant capacity and other bioactivities of the freeze- dried Amazonian palm berry, Euterpe oleraceae Mart. (Açai) | Journal of Agricultural and Food Chemistry | Schauss, A.G. et al. | 2006 | 212 | 49 |
| 6 | Phytochemical and nutrient composition of the freeze-dried Amazonian palm berry, Euterpe oleraceae Mart. (Açai) | Journal of Agricultural and Food Chemistry | Schauss, A.G. et al. | 2006 | 191 | 50 |
| 7 | Phytochemical composition and pigment stability of açai (Euterpe oleracea Mart.) | Journal of Agricultural and Food Chemistry | Del Pozo- Insfran, D. et al. | 2004 | 140 | 51 |
| 8 | In vitro and in vivo antioxidant and anti-inflammatory capacities of an antioxidant-rich fruit and berry juice blend. Results of a pilot and randomized, double-blinded, placebo- controlled, crossover study | Journal of Agricultural and Food Chemistry | Jensen, G.S. et al. | 2008 | 128 | 52 |
| 9 | Pharmacokinetics of anthocyanins and antioxidant effects after the consumption of anthocyanin-rich açai juice and pulp (Euterpe oleracea Mart.) in human healthy volunteers | Journal of Agricultural and Food Chemistry | Mertens-Talcott, S.U. et al. | 2008 | 126 | 53 |
| 10 | Total oxidant scavenging capacities of Euterpe oleracea Mart. (Açai) fruits | International Journal of Food Sciences and Nutrition | Lichtenthaler, R. et al. | 2005 | 124 | 54 |

| Rank | Author Keywords | Occurrences | Rank | keywords plus | Occurrences |
|------|------------------------|-------------|------|------------------------|-------------|
| 1 | Açai | 139 | 1 | Anthocyanins | 62 |
| 2 | Euterpe oleracea | 85 | 2 | Fruit | 61 |
| 3 | Anthocyanins | 57 | 3 | Amazonian palm berry | 60 |
| 4 | Antioxidant | 44 | 4 | Antioxidant | 55 |
| 5 | Euterpe oleracea Mart. | 39 | 5 | Euterpe-oleracea Mart. | 48 |
| 6 | Amazon | 30 | 6 | Capacity | 47 |
| 7 | Antioxidant activity | 26 | 7 | Oxidative stress | 44 |
| 8 | Oxidative stress | 25 | 8 | In-vitro | 40 |
| 9 | Polyphenols | 22 | 9 | Extract | 36 |
| 10 | Inflammation | 18 | 10 | Mart. | 35 |
| 11 | Arecaceae | 16 | 10 | Stability | 35 |
| 12 | Açai berry | 15 | 12 | Juice | 32 |
| 13 | Phenolic compouds | 14 | 13 | Antioxidant capacity | 28 |
| 14 | Bioactive compounds | 11 | 14 | Pulp | 24 |
| 15 | Açai pulp | 10 | 15 | Polyphenols | 23 |

Table 3. Top 15 most frequently used keywords (Author Keywords and Keywords Plus).

Italics used for words present in the two columns of the table.

In the past 27 years, 3016 keywords were used to characterize açaí researches, with 1622 "authors' keywords" and 1676 "keywords plus", which suggests the existence of a great diversity of research focuses. Corroborating, in the frequency analysis, approximately 75% of the keywords were used in only one publication. According to Chuang et al., 2007,⁵⁵, many unique keywords probably indicate a lack of continuity and a significant disparity in the research focus.

Table 3 shows that the most used term was "açaí", which is the popular name of the fruit; another most used word, either by the authors or by the WoS, is "Anthocyanins", the main bioactive compound in açaí, important due to the sensory attributes from the violet color and biological properties.⁵⁶ The presence of these substances is mainly linked to antioxidant, anti-inflammatory, anti-proliferative, and cardio-protective activities.^{26,27} Finally, it is observed that, in general, the terms most used refer to the bioactive properties of açaí.

None keywords related to solid waste management, energy recovery, and environment were found in the top 15 rank. It can be inferred that, despite international research, local and regional sustainable development presents a knowledge gap.

Some patents with keywords related to açaí fruit describe methods for preparing the extract and assessing its composition,⁵⁷ food supplements with high antioxidant capacity⁵⁸, and anti-obesity properties production.⁵⁹ A patent that deals with the industrial extraction of açaí peel with nine distinct stages (Reception; Bleaching; Pre-cleaning; Sanitization; Extraction of the epicarp; Separation; Epicarp refining; Microbiological treatment; and Filling) was also identified.⁶⁰ Finally, some other patents are related to the process for obtaining hydro-alcoholic extracts from açaí, lyophilization and/or spray-drying of the hydro-alcoholic extract; production of fiber panels based on açaí seed.^{61,62}

2.4 POTENTIAL ENERGY RECOVERY FROM AÇAÍ SEEDS BY AD AND SH+AD

The açaí fruits have a globular and rounded shape, with a diameter of 1 to 1.6 cm and an average weight of 0.8 to 2.3 g, presenting a very thin epicarp.⁶³ Therefore, 85-95% of the total fruit mass is commonly disposed of as waste without further energy recovery, generating several environmental side-effects.^{3,64}

The main by-product of açaí processing industry, the açaí seed, is a small solid endosperm connected to an integument that at its maturity contains 44.5% of cellulose, 22.1%

of hemicellulose, and 22.5% lignin (in the dry basis).^{20,34} Despite the possibility of generating thermal energy (biomass burning in boilers), producing a fertilizer or raw material for natural crafts, these applications are not sufficient to absorb the large amount of waste generated.⁶⁵

From the energy perspective, combustion, gasification, pyrolysis, and anaerobic digestion were the only technologies previously assessed to support energy recovery alternatives for açaí waste management.^{66–68} Virmond et al., (2012)⁶⁷ performed characterization of several agro-industrial wastes aiming to evaluate potential application in biofuels by thermochemical processes, placed the açaí seed among those with the lowest Lower Calorific Value (LHV), the highest ash content and the lowest volatile material content, features less interesting for thermochemical processes compared to the other wastes. Finally, açaí seeds' N and S concentrations (S was the highest among the wastes analyzed), indicated a greater possibility of pollutant emissions during combustion, especially sulfur oxides (SOx). Despite this, another study was carried out by Bufalino et al., (2018)³⁶ who investigated the influence of the separation of fibers and seeds from the açaí pulping on the energy attributes. Both seeds and fibers presented close magnitude for calorific value, fixed carbon and volatile solids. However, the thermogravimetric analysis revealed different initial degradation temperatures and mass loss rates. In short, the authors concluded that the separation of the components is recommended, and a drying process must be carried out before the combustion due to its high moisture content to optimize the process.

Additionally, Itai et al., (2014)⁶⁸ evaluated the use of açaí seeds in gasification systems for energy generation, using a cylindrical downdraft gasifier, and applied an equilibrium model. The model predictions were satisfactory, confirming its applicability for preliminary biomass gasification assessments. Notwithstanding, Teixeira et al., (2013)⁶⁶ carried out a theoretical açaí seed gasification techno-economic study to obtain electricity in the Amazon region; and pointed out the need for complementary cooling systems that negatively influence the economic indicators. They also identified that the Brazilian sales prices of electricity do not justify the investment in gasification, since the electricity market should reach high prices always above 150 USD / MWh, values only observed in the drought period in Brazil.⁶⁹

In contrast, anaerobic digestion (AD), a well-known and commercially available technology, could also be adopted for açaí's solid waste treatment (including wastewater) as a strategy to a more environmentally friendly route while producing green energy (biogas).⁷⁰ In

comparison to other waste energy recovery technologies, AD has some advantages, such as quick installation, easy operation, low investment costs, and good profitability indicators.^{71–73} Nevertheless, for low biodegradability materials, like lignocellulosic ones, it is necessary to carry out pre-treatments to make the substrate quickly accessible to AD to obtain better biogas and methane yields.^{74,75} Maciel-Silva et al., (2019)²⁰ developed an integrated system for açaí seeds pre-treatment with Subcritical Water Hydrolysis (SH) (Efficient green hydrolysis technology)^{76,77} followed by AD. Their results reported successful biogas and methane yields from the lignocellulosic açaí seeds for the biological process when the SH is integrated for solid waste treatment. Although SH was evaluated in experimental conditions, the energetic potential from açaí seeds could be better explored in future pilot and industrial scales.

The electric energy generated by AD can be considered renewable, and its main use could be for the açaí production chain self-consumption (frozen pulp industrial processing). Such an energy conversion could contribute to the renewability of the regional and national energy matrix while stimulating the development of clean mechanisms and the adoption of the circular economy concept.⁷⁸ Despite the electric energy potential generation may be overestimated for (i) assuming the experimental biogas yield, (ii) experimental methane content, and (iii) considering the conversion of the entire production of seeds into methane by AD (with and without pre-treatment) for the estimations, the results show an optimistic scenario for replacing electricity for açaí's production chain. For scale-up purposes, biogas yield and methane contents could be different from the experimental data, anyway for the results obtained here, the conversion of the totality of açaí seeds by AD could generate 4.86×10^3 MWh/year and 233.89 MWh/year for the SH+AD scenario (**Figure 5**).

Even if the replacement of the electric energy production from the whole Pará State (total electric energy production of 5.25×10^7 MWh in the year 2018^{79}) is a small fraction of the full state demand, the locally generated energy could be enough to supply the total electric energy requirement for the frozen açaí pulp industry (for the whole Brazilian açaí fruit production – **Figure 6**).

In addition, an electric energy surplus of 2,609 MWh/year would be available to sales to the grid. For the theoretical best scenario (SH+AD) an electric energy surplus of 2.32×10^5 MWh/year would be generated for sales to the grid. Moreover, assuming the average annual electric energy price (for trade in the Brazilian regulated market) of 60 USD/MWh,⁶⁹ the açaí

industry could obtain a revenue of more than 13 million USD/year for electric energy surplus - additionally to the frozen pulp sales annual revenue. Conservatively assuming that the amount of açaí seed from de-pulping is not entirely destined to the AD or SH+AD processes, four (4) scenarios were assessed to evaluate its potential for electric energy generation and surpluses (**Figure 6**). While the electric energy surplus does not occur when a small amount of açaí seeds (25% of the whole national production) is converted by AD, SH+AD still present outstanding revenue for the electric energy sales.



Figure 5. Electricity generation from açaí seed energy recovery and GHG emissions avoidance.

The net avoided GHG emissions (Net_{GHG}) were conservatively calculated, and express the difference of the emissions from biogas burning in stationary engines (BB_{GHG}) and the avoided emissions by the replacement from the national energy mix by the potential electricity generation from açaí seeds (A_{GHG}), i.e. it indicates the potential reduction of GHG by the usage of biogas from biomass conversion instead of other energy sources. The estimations here presented are limited to the replacement of the power obtained from the grid by locally produced biogas burning. BB_{GHG} emissions for the whole energy produced by AD was estimated in 955 tonCO_{2eq}/year, and for SH+AD, 45,974 tonCO_{2eq}/year. The A_{GHG}, respectively to AD and SH+AD, was estimated as 359 and 17,257 tonCO_{2eq}/year. Therefore, Net_{GHG} from the energy recovered by AD system would account for 596 tonCO_{2eq}/year, and from SH+AD, 28,717 tonCO_{2eq}/year (**Figure 5**).



CHAPTER 2 – Review (Açaí: Bibliometric Analysis and Assessment of Energy Potential)

Figure 6. Scenarios for açaí seed energy recovery and electric energy surpluses. (A) Anaerobic Digestion (AD) and (B) Subcritical Hydrolysis pre-treatment + Anaerobic Digestion (SH+AD)

Although hydroelectric energy, the primary source of the electric energy production in the North region (97% of the total energy production)⁷⁹, is considered highly renewable, and it is dominant in the Brazilian electricity generation (63.5% of the total), the share of biomass is still inexpressive accounting for only 8.3%.⁸⁰ Moreover, the downstream GHG emissions for hydropower plant construction, transmission, and distribution lines are not usually addressed in its environmental assessments.⁸¹ Brazil presents a huge underexploited renewable energy generation. The energy demand in the Amazon could be met by local renewable sources, that do not rely exclusively on new hydropower facilities that are intimately associated with ecosystem disturbance (deforestation and fauna disruption) for its construction.⁹

As a renewable source, the use of biogas contributes to the non-dependence on fossil energy. In addition to decarbonization,⁸² the adoption of AD could enable decentralized energy generation systems in an optimal biogas production network,⁷¹ with a consequent decrease in transmission and distributions infrastructures dependence, which are scarce in the Amazon region despite governmental programs to expand the power network to the whole population located in the remote regions.⁸³ More than an opportunity to strengthen local nutrients/energy recycling, it could improve the local community involvement and foster economic development. Furthermore, a decentralized biogas system could be helpful to face water scarcity and severe and intense droughts related to climate change.⁸⁴ Finally, besides the biogas burning for electric energy generation, after purified and converted into biomethane, the biogas could replace natural gas for cooking or vehicular fuel.^{85,86} The replacement of natural gas by biomethane has an outstanding potential to lower GHG emissions and to help Brazil to meet its carbon budgets accordingly to the commitments established in the 21st United Nations Climate Change Conference (COP 21). In 2019, the National Interconnected System of Brazil of Brazil was responsible for 34,700 tonCO_{2eq} emissions (3% more than in 2018), of which 24,700 (59.7% of the total) correspond to natural gas, which alone had an increase of 9.6% compared to 2018 in the accounted emissions.⁸⁰

2.5 OVERVIEW OF AÇAÍ PRODUCTIVE CHAIN AND SUSTAINABILITY

From the bibliometric analysis results and patent search, it was possible to identify and quantify the researches related to energy recovery from açaí waste. The few documents addressing the issue evince the incipience in this research field (see **section 2.3.1**). Along with the estimations of the electric energy replacement from açaí seeds conversion by AD with and

without pre-treatment, an alternative development mechanism towards a more beneficial and environmentally friendly agro-industrial activity was outlined. A favorable scenario to address the environmental concerns associated with the Legal Amazon agricultural expansion could flourish from the results here discussed. Legal Amazon is under the influence of several modifying agents, likewise deforestation for large-scale soybean crops, cattle farming, wood extraction, forest burning - both due to anthropogenic or natural causes, mining, highways expansion, hydroelectric facilities, and urban population growth.⁸⁷ According to the Brazilian Greenhouse Gas Emissions Analysis,¹¹ the Amazonian states have very high per capita emissions due to deforestation. Reducing deforestation is an ongoing challenge; nevertheless, several agricultural technologies could support a competitive production model while addressing a significant economic development associated with the preservation of natural resources in diverse Brazilian agricultural areas.^{88,89}

Due to Legal Amazon complexity, several knowledge gaps associated with the agricultural systems' biodiversity, and to resources sustainable exploitation represent an undergoing global daring task.^{84,90} A solution could be to find a way to reconcile the economy and the environment without the need for a production inflection point.^{91,92} The concept of sustainable intensification suggests new land-use strategies such as continuous increases in productivity per unit of arable land to reduce the deforestation.^{89,93}

Açaí family farming communities, which usually adopt agroecological production systems, are attempting to intensify the production to keep up with the growing market. Notwithstanding, a successful intensification requires robust governmental policies and incentives. The açaí familiar production results in a high level of tree species biodiversity loss without ecological and economic planning.⁹⁴ The use of the own natural resources to boost production, associated with digital and biological innovations, would be strategies for public and private investments to achieve a so-called "sustainable technological development" in the Amazon region.⁹ Yet, the mega Amazonian biodiversity and its insertion in the production scenario require research in different areas, scales, and sectors of the economy. Despite the 500 years of Amazon exploration, only a few commodities are traded in Brazil and abroad (rubber, Brazilian nuts, açaí, and "guaraná"), and more than 10,000 varieties remain with latent potential.⁹⁵

For açaí agro-industrial high added-value products and growing exports, a technological alternative with more investment in residues' reuse could contribute to preserving native vegetation. Also, adding value to waste favors the establishment of a circular economy. New energy sources applied in integrative production models closes cycles - from the raw material to the waste recovery. In the concept of a circular economy, the productive chain sustainability would be maximized while reducing anthropic negative side-effects.⁹⁶ Brazil could achieve competitive advantages while fulfills the daring GHG emissions mitigation and reducing deforestation goals. A strategy adopted by the Brazilian government to reduce impacts on the cultivation and production of açaí in the state of Pará aimed to standardize the production density in 200 stem/ha as a good management practice in the region.⁹⁷ In addition to the standardization of crop density, land-sparing strategies could be adopted to avoid the risks associated with extractive activities. Land spare can be an aggressive strategy to keep the native plant community and its beneficial effects on the ecosystem. At the same time, economic aspects are addressed in favor of local or regional development.⁹⁸ A land-sparing strategy can support increasing yields, reducing farmland area, and offset GHG emissions while actively restoring the natural habitat, even considering that its conceptual and analytical strengths and limitations remain little explored and widely contested or misunderstood.⁹⁹ Indeed, for the acaí case, improved management of the existing resources could lead to a reduced-impact agroecosystem with more extensive conservation when compared to a land-sparing strategy.¹⁰⁰ Moreover, for the Legal Amazon, the circular economy paradigm encompasses the recognition of the economic importance of natural resources and the wide adoption of integrated and innovative technologies. Nevertheless, to cope with science, technology, and innovation, an institutional governance model is essential to policy-making and to better establish mandatory regulatory frameworks for the new agricultural frontier in Legal Amazon,⁹ avoiding the risk of converting the larger tropical rainforest of the world in a wide and non-diversified crop area of açaí, soybeans and pasture for cattle.

2.6 CONCLUSION

Açaí growing market has become of great interest to future investments. Extractive production is seen by many researchers as a sustainable alternative, preventing Legal Amazon deforestation. However, the agricultural frontier expansion in the Legal Amazon also offers development opportunities related to energy recovery from açaí waste through the integration

of technologies likewise herein discussed. The assessment of the scientific knowledge related to this approach revealed that açaí waste recovery research is still incipient. Moreover, most of the investigations on açaí's energy recovery occur in a regionalized way, mainly in Brazilian universities and research institutions. In the last five years, the research on açaí waste energy recovery focused on converting it into renewable energy. The technology integration of subcritical water hydrolysis followed by anaerobic digestion can be an innovative way to rethink the açaí production chain towards a circular economy. Furthermore, this sort of technological advance could stimulate sustainable agro-industrial intensification in areas such as the Legal Amazon, considered hotspots for biodiversity preservation and mitigation of the risks associated with climate change. Future research should address a comprehensive Life Cycle Analysis to support decision-making processes related to investments on clean and affordable energy and climate change mitigation for the açaí industry.

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CHAPTER 3 – Review (Extraction processes with on-line detection)

GREEN EXTRACTION PROCESSES FOR COMPLEX SAMPLES FROM VEGETABLE MATRICES COUPLED WITH ON-LINE DETECTION SYSTEM: A CRITICAL REVIEW

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ABSTRACT

The detection of analytes in complex organic matrices requires a series of analytical steps to obtain a reliable analysis. Sample preparation can be the most time-consuming, prolonged, and error-prone step, reducing the reliability of the investigation. This review aims to discuss the advantages and limitations of extracting bioactive compounds, sample preparation techniques, automation, and coupling with on-line detection. This review also evaluates all publications on this topic through a longitudinal bibliometric analysis, applying statistical and mathematical methods to analyze the trends, perspectives, and hot topics of this research area. Furthermore, state-of-the-art green extraction techniques for complex samples from vegetable matrices coupled with analysis systems are presented. Among the extraction techniques for liquid samples, solid-phase extraction was the most common for combined systems in the scientific literature. In contrast, for on-line extraction systems applied for solid samples, supercritical fluid extraction, ultrasound-assisted extraction, microwave-assisted extraction, and pressurized liquid extraction were the most frequent green extraction techniques.

Keywords: Sample Preparation; Green Extraction Techniques; On-Line Coupling; Fully Automated Analysis; Bibliometric Analysis.



CHAPTER 3 – Review (Extraction processes with on-line detection)

3.1 INTRODUCTION

The extraction of bioactive compounds from natural substrates for food, pharmaceutical, and cosmetic applications can be advantageous for producing high value-added products and for nutrients recycling from industrial byproducts and waste [1–3], as a circular economy strategy [4–6]. Furthermore, the current extraction techniques coupled with on-line detection can be considered more environmentally friendly than conventional techniques [7–9].

Currently, most extraction techniques are operated off-line to the analysis system [10,11]. Nevertheless, these processes could be integrated into an on-line system for several cases, where the entire analytical procedure takes place in a closed complex—usually automated. On-line systems could notably provide manifold benefits, including shorter analysis time, better reproducibility, improved detection limits, sensitivity, and results reliability. In addition to avoiding some problems associated with the traditional approach, and its consequent experimental error propagation, on-line sample preparation systems tend to be more productive and less prone to accuracy and precision errors [12].

The sample preparation step aims to isolate the target analytes and to improve the selectivity, precision, and repeatability of the analysis. This step generally includes extraction, concentration, and cleaning procedures for impure and complex samples [13,14]. Meanwhile, these procedures could represent a significant disadvantage, since individual preparation steps substantially increase the risk of sample loss and possible sample contamination, leading to reduced analysis reliability [7,14].

When considering sample preparation and chromatographic analysis, the first is the most time-consuming, tedious, and error-prone step [13], commonly considered by researchers as the bottleneck of the whole analytical procedure [10]. The analysis automation potentially improves the measurement precision, sensitivity, and accuracy, also reducing the consumption of organic solvents [7].

Accordingly, several techniques based on dynamic systems were developed to meet the imminent demand for fastness, sensitivity, repeatability, and reliability [15]. The main available green technologies with coupling capability for on-line detection systems are solid-phase extraction (SPE), supercritical fluid extraction (SFE), liquid–liquid extraction (LLE), microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), pressurized

liquid extraction (PLE), accelerated solvent extraction (ASE), supercritical water (SCW), and subcritical water (SubCW) [9,10,14,16–19].

The above-mentioned technologies are more efficient than conventional technologies (maceration, decoction, and Soxhlet extraction) with respect to many aspects, such as lower time demand, higher selectivity, and lower solvents and reagents consumption[12]. These modern technologies are commonly coupled with liquid chromatography (LC), gas chromatography (GC), supercritical fluid chromatography (SFC), and capillary electrophoresis (CE) [11,20]. The SPE and LLE are best suited for liquid samples, as long as the others are usually applied to solid samples [7,15].

Automated flow-based sample treatment systems and separation techniques appear to be a trend in analytical chemistry coupling systems, provided that remarkable assertiveness of analysis results could be achieved [10–12]. Moreover, coupled systems are likely to increase the analytical throughputs and efficiency in diversified and easier ways to use on-line systems [21] in a wide range of applications; for instance, for lipophilic samples using SFE with separation based on carbon dioxide (CO₂) [9,22], and food drinks with juices, beer, coffee, tea, and water, mainly using the SPE technique [11].

In this context, this article presents a systematic review of extraction systems coupled with on-line analysis systems for solid matrices from vegetable origin. The prevailing publications on extraction processes associated with on-line analysis techniques from 1990 to 2021 were collected and examined. In addition, to evaluate the documents on this topic, statistical and mathematical methods were applied to assess the timeline evolution and future trends of this research field through a longitudinal bibliometric analysis. The results of this review allow the identification of the current state-of-the-art coupling systems for green bioactive compound extraction.

The extraction technologies can be coupled with detection techniques in various ways, depending on the degree of software/hardware automation and human intervention [23]. Accordingly, **Figure 1** illustrates the coupling forms, classifying them as off-line, at-line, in-line, and on-line.



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Figure 1. Classification of coupling forms with the detection system.

The off-line coupling approach includes manual injection of the pre-processed sample into the analysis equipment [24]. This procedure is appropriate for a small number of samples, when conventional methods are expected to be sufficient and there is no need for automated methods [13]. The advantage of carrying out separated processes is a kind of increased flexibility. For LC analysis, it is possible to minimize interferences and undesirable effects related to dirt samples, which may cause bandwidth expansion, overlapping peaks, co-elution with empty volume, and peak asymmetry. Nevertheless, this procedure presents the lowest degree of automation, requiring more time and labor, as well as a risk of sample loss or contamination, which would deliver a lower analysis sensitivity and precision [12].

For at-line coupling, the analyzer is disconnected, yet close to the process [25]. Generally, at-line coupling procedures include a programmable robotic station to link the flow system to the separation or detection instrument. Usually, the pre-treated sample is delivered from the flow system to a vial or to the injector of the analytical instrument [24]. It enables automation and allows for performing a parallel analysis to the flow system with a high precision level, little labor requirement, and low analysis time. Nevertheless, the risk of sample loss or contamination is considered high for this approach [13].

In-line coupling involves complete and close integration of the detection or separation instrument into the flow system [23]. The analyzer is equipped with probes inserted into the process stream, and the analysis is performed in situ [25]. The in-line procedure is entirely

automated, promoting more injections while the process is still in progress, which allows a faster response in the feedback cycle. It is possible to implement an automatic periodic cleaning ("self-cleaning") routine. However, a significant disadvantage is its inability to manipulate samples in parallel because the overall analytical process is restricted to the integrated flow device. Therefore, it does not apply to many methods [12].

The on-line coupling strategy implies direct contact between the flow system and the components of the detection equipment, usually through a flow bypass [11,21]. The analyte from the sample processed in the flow system is transported to an interface and introduced into the pressure-driven separation system in CE [26], mobile phase in LC [14,20] and carrier gas in GC [27]. This configuration enables sequential analysis and high automation capacity, requiring little labor. It is subject to a low possibility of sample loss or contamination, promoting high precision and sensitivity in the analyses. In turn, it requires sophisticated equipment and software, representing high installation costs [12,13]. Similar to in-line methods, a "self-cleaning" routine is possible for on-line methods.

Among other requirements, the robustness of an analysis system under specific process conditions represents an important issue. The demand for in-process analysis equipment is high; the limits of accuracy and detection are often lower than state-of-the-art laboratory equipment. However, the analyses used are typically adapted to the process-specific requirements, leading to highly specialized equipment incorporated into sophisticated control circuits that would allow quality control in real time [23].

Moreover, various technologies are capable of promoting the extraction of bioactive compounds, obtaining a large variety of biochemical products, in addition to platform chemicals. Most of them are viable for foods, pharmaceuticals, and cosmetics [28]. Frequently using these technologies is a relevant strategy for developing a circular economy, since its exceptional advantage in valorizing agro-industrial waste is notable [29]. Furthermore, many current extraction technologies enable on-line or in-line detection system coupling, which is considered more environmentally friendly than conventional technologies [14]. A common trend in extraction technology is to replace the use of toxic reagents with reagents obtained from renewable sources, as well as the integration of analytical processes and operations, saving energy, and reducing the use of reagents [30].

3.2 MATERIALS AND METHODS

3.2.1 Data Collection

The bibliometric analysis retrieved publications from the Web of Science (WoS) core collection, from Clarivate Analytics, using the Science Citation Index Expanded (SCI-Expanded) option. The WoS database presents extensive and multidisciplinary coverage of bibliographic data from leading scientific publications. Consequently, it is a primary information source for academic and bibliometric studies [31,32].

The keywords used to identify the articles in the area of interest were based on a scientific literature search. The terms selected to collect information related to the extraction processes were "solid-phase extraction", "liquid-liquid extraction", "supercritical fluid extraction", "pressurized liquid extraction", "supercritical water", "subcritical water", "accelerated solvent extraction", "superheated water extraction", "microwave-assisted extraction" and "ultrasound-assisted extraction". The terms used for the detection techniques were "chromatography", "HPLC", "LC", "GC", "UV-detection", and "supercritical fluid chromatography". Finally, to assess the integration of processes and detection techniques into an on-line system, the keywords selected were "on-line" and "in-line".

The bibliometric research was carried out on January 13, 2022, using the TOPIC search field, which combines words in titles, abstracts, author keywords, and keywords defined by the WoS database (keywords plus). The strategy of placing the terms in quotation marks was used to limit the search to publications that contained those terms in that exact word order. The Boolean operators OR (union of terms in groups) and AND (providing an intersection between groups) were used to select the final dataset. A total of 2,633 documents published from 1990 to 2021 were found. Furthermore, a refining step was performed to choose only articles and reviews, for which 2,353 files were obtained (2,146 articles and 207 reviews). The **Supplementary material** illustrates the methodology synthesis applied for the data collection and bibliometric analysis.

3.2.2 Data Processing

The bibliometric analysis was performed using the "Analyzing Results" tool provided by WoS. The number of publications, year of publication, number of citations, types of documents, research areas, and countries were gathered. The graphic illustrations were generated using Origin 8.0 software from OriginLab®. The construction of bibliometric maps based on co-authorship was created using the VOSviewer© 1.6.13 software, providing the graphical representation of bibliometric maps to facilitate data interpretation [33].

3.3 RESULTS AND DISCUSSION

The bibliometric analysis broadly presented all works related to "*on-line*" and "*in-line*" coupling systems for: *i*) extraction techniques with analysis systems with or without sample purification/cleaning techniques; *ii*) extraction techniques with sample purification/cleaning techniques, and *iii*) sequential analyses. A detailed bibliometric analysis results description and discussion follows in the next sections.

3.3.1 Scientific Development of On-line and In-line Coupling

The scientific literature data collection regarding on-line and in-line coupling is proven to be relevant worldwide, covering 67 different countries in the total publication period assessed. **Figure 2** shows the evolution of the number of publications and highlights the main research areas.

Figure 2A emphasizes the advancements in annual publications, while **Figure 2B** focuses on the total breakthrough of the works performed. The publishing started in 1990 with only one document, and rapid growth in the following years is observed. The publishing peak occurred in 2007, with 123 studies. Since 1995, studies in this area reached the mark of more than 60 annual publications, exposing the broad scope and the particular importance of this area for the international scientific community. From 2005, more than 80 yearly publications were identified, except for the years 2020 and 2021, in which only 69 and 48 papers were found, respectively (a decrease may be associated with the COVID-19 pandemic).

In the 32 years of publications in this field, a total of 2353 papers were produced, arranged in six main research areas according to the Web of Science categories: "Chemistry Analytical", "Biochemical Research Methods", "Food Science Technology", "Spectroscopy", "Environmental Sciences", and "Pharmacology Pharmacy". These categories played an essential role in the development of this subject. Since the beginning of publications, the Chemistry Analytical area led the ranking, followed by Biochemical Research Methods.



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Figure 2. Evolution of the number of publications and main research areas. (A) Annual publications and (B) Total publications.

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The other areas of the top six have a similar number of papers, and they change positions in the ranking over the years. The *Food Science Technology* area currently occupies the third position, with 158 articles. Fourth, it is the *Spectroscopy* area with 146. *Environmental Sciences* is in fifth with 145, and in sixth is *Pharmacology Pharmacy* with 143. The *Environmental Sciences* area is currently in evidence due to increased environmental concerns.

3.3.2 International Collaboration

Research partnerships between the leading countries in the on-line and in-line coupling areas were evaluated. The evaluation of international collaboration was carried out through the bibliometric analysis applied to co-authorship and the number of cumulative publications with chosen thresholds to understand how the connections were established. **Figure 3** shows a bibliometric network in which the size of the marker is proportional to the number of accumulated publications, as well as the thickness of the lines that make the connections between the stamps, and the colors indicate to which cluster the item belongs. Furthermore, it is noteworthy that the number of subjects was limited to display only the top 30 countries.

The bibliometric map presented in Figure 3 shows six clusters among the thirty most productive countries. The red-colored cluster has the largest number of countries (corresponding to 10 items). The clusters with the least number of items are the orange and purple ones, both with only two countries. The group responsible for the smallest number of publications is purple, accounting for 72 articles. The cluster with the most considerable incidence (red) accounts for 970 articles, and the second-largest, the yellow one, presents 560 reports. Although there are two clusters with six countries and the yellow one contains only four, the last is placed in the second position in the number of publications containing Spain, which is the most productive country.

Spain, China, and the USA significantly contributed to this research field, with 19.29%, 17.30%, and 11.81% of the scientific recordings, respectively. These countries published a considerable number of scientific articles collaboratively with other countries. However, when analyzing the number of international collaborations, the rank varied as follows: the USA started as the leader, having published in partnership with 32 countries, followed by Spain, with 31 countries; the third country is Germany, which collaborated with 24 countries. Finally, China dropped to the fourth position, with 23 international collaborations.



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Figure 3. Collaboration map among the top 30 most productive countries.
3.3.3 Journals with More Publications and Most Cited Articles

All of the articles found in the research area of extraction with on-line detection systems were published in 282 different journals, which illustrates the diversity of publications and broad interest in this research field from multiple perspectives. Notwithstanding, most journals (approximately 91%) published fewer than 20 articles, accounting for only 25 journals publishing 20 or more reports. Otherwise, this set is responsible for approximately 71% of the publications. Consequently, it can be inferred that those 25 journals are the most chosen for this research area. According to Bradford's law, this distorted distribution is expected in the bibliometric analysis, since journals are dedicated to specific topics [34] and about 500 to 1000 journals are needed to cover 95% of the "significant" literature published in a given field [35]. Accordingly, **Table 1** presents the ten most active journals responsible for 53% of the publications.

From **Table 1**, it can be observed that the *Journal of Chromatography A* leads the ranking with approximately 16% of the total publications, more than double the publications in *Talanta*, the second journal in the ranking. Both journals belong to the company *Elsevier*, which also corresponds to the third, fourth, and eighth journals in the ranking. This publisher is responsible for the four journals with the most significant number of publications and accounted for five journals in the top ten. Among the top ten journals, the other publishing company is ACS Publications, responsible for the fifth ranking position, with the most significant impact factor (6.986). Springer, responsible for two journals, tied for the sixth position in the ranking. Finally, the Wiley Online Library is responsible for the ninth and tenth positions.

The analysis of the ten most cited articles until 2021 (**Table 2**) revealed no direct relationship between citations in a specific publication and the most active journals in the area. The most cited article was published by *Chemosphere*, which is not among the top ten journals, occupying the 23rd position in the ranking and accounting for 18 publications. On the other hand, the *Journal of Chromatography A*, the journal's ranking leader (**Table 1**), was the only journal with more than one publication among the top ten most cited articles.

Table 1. Top 10 journals ranked by number of publications.

| Rank | Journals | Country | Publishing Company | Impact Factor | Records | % of 2353 |
|------|---|-------------|--------------------|---------------|---------|-----------|
| 1 | Journal of Chromatography A | Netherlands | Elsevier | 4.759 | 374 | 15.895 |
| 2 | Talanta | England | Elsevier | 6.057 | 145 | 6.162 |
| 3 | Journal of Chromatography B | Netherlands | Elsevier | 3.205 | 144 | 6.120 |
| 4 | Analytica Chimica Acta | Netherlands | Elsevier | 6.558 | 133 | 5.652 |
| 5 | Analytical Chemistry | USA | ACS Publications | 6.986 | 88 | 3.740 |
| 6 | Analytical and Bioanalytical Chemistry | Germany | Springer | 4.157 | 77 | 3.272 |
| 6 | Chromatographia | Germany | Springer | 2.044 | 77 | 3.272 |
| 8 | Journal of Pharmaceutical and Biomedical Analysis | Netherlands | Elsevier | 3.935 | 72 | 3.060 |
| 9 | Electrophoresis | Germany | WILEY | 3.535 | 66 | 2.805 |
| 10 | Journal of Separation Science | Germany | WILEY | 3.645 | 61 | 2.592 |

The impact factor measures the average number of citations received in a particular year by papers published in the journal during the two preceding years. Journal impact factor (Clarivate Analytics, 2021) [36].

Table 2. Top 10 articles ranked by number of citations.

| Rank | Title | Journals | Citations | Article Type | References |
|------|--|---|-----------|------------------|------------|
| 1 | Phosphorus flame retardants: properties, production, environmental occurrence, toxicity and analysis | Chemosphere | 1424 | Review article | [37] |
| 2 | Solid-phase extraction: method development, sorbents, and coupling with liquid chromatography | Journal of Chromatography A | 835 | Review article | [38] |
| 3 | Green Analytical Chemistry | Trends in Analytical Chemistry | 594 | Review article | [39] |
| 4 | Metal-Organic Frameworks for Analytical Chemistry: From Sample Collection to Chromatographic Separation | Accounts of Chemical Research | 548 | Review article | [40] |
| 5 | Supercritical fluid extraction in herbal and natural product studies - a practical review | Talanta | 434 | Review article | [41] |
| 6 | Supercritical fluid extraction in plant essential and volatile oil analysis | Journal of Chromatography A | 396 | Review article | [42] |
| 7 | Recent advances in high-throughput quantitative bioanalysis by LC-MS/MS | Journal of Pharmaceutical and Biomedical Analysis | 373 | Review article | [43] |
| 8 | A general approach to desalting oligosaccharides released from glycoproteins | Glycoconjugate Journal | 365 | Research article | e [44] |
| 9 | Automated online column-switching HPLC-MS/MS method with peak focusing for the determination of nine environmental phenols in urine | Analytical Chemistry | 324 | Research article | e [45] |
| 10 | Serum concentrations of 11 polyfluoroalkyl compounds in the US population: Data from the National Health and Nutrition Examination Survey (NHANES) 1999-2000 | Environmental Science & Technology | 317 | Research article | e [46] |

The work presenting the highest number of citations is a critical review focused on phosphorus flame retardants [37], which dealt with properties, production, environmental occurrence, toxicity, and analysis issues. This large variety of issues may explain why different researchers from different areas cited it.

Finally, five of the most cited articles [37,39,40,44] do not belong to the top ten journals in terms of publications. In addition to the first in the ranking and the third most cited article [39], which was published by *Trends in Analytical Chemistry*, the journal presents 34 publications in the on-line coupling field and ranks thirteenth. The other articles are the fourth [40] and the eighth [44] on the list, belonging to the *Accounts of Chemical Research* and *Glycoconjugate Journal*, respectively. Both journals have only one article in this field of study. Finally, the 10th position [46] belongs to *Environmental Science & Technology*. This journals

Beyond the fifth, sixth, and seventh, the first three most cited articles belong to Elsevier journals, totalizing six reports from the list. The fourth, ninth, and tenth articles belong to ACS Publications, and the eighth article is from *Springer*. The top five publishers in numbers of published articles are Elsevier, Wiley, Springer, ACS Publications, and the Royal Society of Chemistry, with 1204, 290, 183, 141, and 102 articles, respectively. Another critical detail in **Table 2** is that most of the included works are reviews, and only three documents refer to research articles (eighth, ninth, and tenth).

3.3.4 Keyword Analysis

The keywords are essential sources of information because they provide evidence for the issues and central themes in a publication [47]. The terms used as keywords can be used to investigate the research trajectory, to analyze trends in a research area, and to investigate the existing knowledge gaps in a certain scientific field [31]. Therefore, the 30 main authors' keywords and keywords plus (provided by WoS, based on a unique special algorithm to increase the power of cited reference search) of the research field analyzed in this review are highlighted in **Table 3**.

The examination of the keywords in the 32 years of publication revealed that 8,626 terms were already used to characterize searches about on-line and in-line coupling (with 4,679 author keywords and 4,927 keywords plus), suggesting the existence of a great diversity of

research themes. Another argument reinforces this hypothesis: the VOSviewer software frequency analysis revealed that 6,042 keywords were used only once. According to Chuang, Huang, and Ho [48], many unique keywords probably indicate a lack of continuity and a significant disparity in the research focus.

Table 3 shows that the term most used was "*Solid-phase extraction*", either when chosen by the authors or the WoS. In addition to this term, the two columns present eighteen (18) repeated keywords. It is also possible to observe that the keywords plus have more occurrences than the authors' keywords. Furthermore, 77.4% of the terms in the authors' keywords appeared in only one article, and that number was 64.3% for keywords plus.

It is possible to consider three main groups of keywords in the study area: samples, process techniques, and analysis techniques.

The main terms related to the sample group are "Urine", "Plasma", "Human plasma", "Pesticides", "drugs", "residues", "Waste-water", "Water", and "Water samples", suggesting that automation using on-line and in-line coupling is more advanced in the area of health, water treatment, and food products.

For process techniques, there are general terms, such as "Sample preparation", "Preconcentration", "On-line pre-concentration", "Extraction", "On-line extraction", "Separation", and "Microextraction". In addition, specific terms, such as "Solid-phase extraction", "On-line solid-phase extraction", "On-line SPE", and "Supercritical fluid extraction" are present. Currently, SPE is one of the most used techniques for the extraction and/or concentration of complex samples, allowing analytes in deficient concentrations to be detected by methods such as high-performance LC, GC, and CE [11,20].

In general, the use of the word "on-line" is more performed by the authors. Moreover, for the keywords plus, the term appeared disassociated with the process technique. For plus keywords, the analysis of the association between them, using VOSviewer software, showed that the term "Solid-phase extraction" appeared highly associated with "High-performance liquid chromatography". For the author keywords, the term "Solid-phase extraction" appeared more associated with "Capillary electrophoresis".

| Table 3. Top 30 most | frequent keywords | (author keywords | and keywords plus). |
|----------------------|-------------------|------------------|---------------------|
|----------------------|-------------------|------------------|---------------------|

| Rank | Author Keywords | Occurrences | Rank | Keywords Plus | Occurrences |
|------|--|-------------|------|--|-------------|
| 1 | Solid-phase extraction | 266 | 1 | Solid-phase extraction | 671 |
| 2 | On-line solid-phase extraction | 159 | 2 | High performance liquid-chromatography | 414 |
| 3 | Mass spectrometry | 117 | 3 | Liquid-chromatography | 286 |
| 4 | Capillary electrophoresis | 102 | 4 | Mass-spectrometry | 275 |
| 5 | Pesticides | 96 | 5 | Tandem mass-spectrometry | 214 |
| 6 | Liquid chromatography | 87 | 6 | Samples | 213 |
| 7 | On-line SPE | 82 | 7 | Gas-Chromatography | 209 |
| 8 | LC-MS/MS | 77 | 8 | Separation | 193 |
| 8 | HPLC | 77 | 9 | Water | 172 |
| 8 | Supercritical fluid extraction | 77 | 10 | Metabolites | 144 |
| 8 | Water analysis | 77 | 11 | Chromatography | 135 |
| 12 | Sample preparation | 74 | 12 | Human plasma | 134 |
| 13 | Column Switching | 63 | 13 | Urine | 114 |
| 14 | Urine | 62 | 14 | Preconcentration | 112 |
| 15 | High performance liquid chromatography | 57 | 15 | Microextraction | 111 |

| 16 | Environmental analysis | 54 | 16 | Pesticides | 106 |
|----|------------------------------|----|----|----------------------------------|-----|
| 17 | Automation | 45 | 17 | Polycyclic aromatic-hydrocarbons | 103 |
| 17 | Column liquid chromatography | 45 | 18 | plasma | 102 |
| 19 | On-line preconcentration | 42 | 19 | Identification | 100 |
| 20 | Preconcentration | 40 | 20 | HPLC | 97 |
| 21 | Gas chromatography | 36 | 21 | Extraction | 88 |
| 22 | Water | 35 | 22 | Sample preparation | 85 |
| 23 | LC-MS | 32 | 23 | Capillary-electrophoresis | 83 |
| 24 | On-line extraction | 31 | 23 | Quantification | 83 |
| 25 | Water samples | 30 | 25 | Supercritical-fluid extraction | 82 |
| 26 | Extraction | 29 | 26 | Water samples | 74 |
| 26 | Plasma | 29 | 26 | Waste-water | 74 |
| 26 | Monolithic column | 29 | 28 | On-line | 72 |
| 29 | Human plasma | 28 | 28 | Residues | 72 |
| 29 | Tandem mass spectrometry | 28 | 30 | drugs | 70 |

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Italics used for words present in the two columns of the table.

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The main terms of the analysis techniques group are "Mass spectrometry", "Capillary electrophoresis", "Liquid chromatography", "Tandem mass spectrometry", "Chromatography", "Identification", and "Quantification". It was found that among the keywords of this group, there is an extensive use of synonyms, such as "Liquid chromatography", "High-performance liquid chromatography", "Column liquid chromatography", "HPLC", "LC-MS", and "LC-MS/MS". The two last terms mean LC coupled to mass spectrometry, with one or two detectors, also known as tandem mass spectrometry.

3.4 INNOVATIVE EXTRACTION TECHNIQUES SUITABLE FOR ON-LINE COUPLING

From the bibliometric analysis results, it was possible to collect, to review, and to assess state-of-the-art on-line coupling systems. The main findings are further scrutinized and critically analyzed. Several extraction systems can be coupled on-line with analysis systems using liquid and solid samples. Liquid samples are more straightforward to operate than solid samples, considering that on-line sample preparation techniques are broadly based on continuous flow approaches (which are especially suitable for liquids).

Among the extraction techniques for liquid samples, SPE is the most common in combined systems [7,13]. In SPE, the compounds dissolved or suspended in a liquid mixture are extracted using an interface coupled or not with solid extraction techniques and chromatography [49]. Otherwise, the solid matrix analytes are first extracted through polar or nonpolar solvents before the analysis. The most common on-line extraction systems for solid samples are SFE, UAE, MAE, and PLE. The coupling is usually performed with the aid of multiport valves. In cases where the extracts have a high content of soluble solids or a high concentration of impurities, SPE is used for in-line purification [50]. **Figure 4** shows a simplified diagram of the primary extraction and analysis techniques for on-line coupling.

On-line extraction systems can be carried out in either static or dynamic mode. In the context of transferring the extract to the analytical system, the dynamic mode is the most common, and the analytes are removed continuously from the sample to the fluid (solvent). Furthermore, as the sample is continually exposed to the solvent, the transfer of analytes from the sample matrix to the solvent is enhanced [13]. Therefore, for solid samples, low flow rates and extract volumes of less than 1–2 mL must be kept. Otherwise, the extract must be concentrated before transferring it to the analytical system.



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Figure 4. Simplified diagram of on-line coupling among the main extraction and analysis techniques.

Another option is in-line SPE coupling. Several works in the literature demonstrated that SPE considerably improves concentration and sensitivity when diluted samples are analyzed and makes it possible to analyze complex matrices by extracting solids, enabling simple coupling [51]. The solvent (or fluid) is continuously washed through the SPE extractor at a constant flow rate for a specified period.

Furthermore, the solvent (or fluid) must be compatible if the analytical system is chromatographic (mobile phase for LC and carrier gas for GC). It is essential to highlight that the solvent (or fluid) must be sufficiently volatile and preferably nonpolar when coupled with the GC. On the other hand, the extracts should preferably be a weak strength eluent for coupling with the LC system [7,13]. The following sections will delve into extraction systems coupled on-line with analytical techniques for vegetable origin solid matrices.

3.4.1 Supercritical Fluid Extraction

SFE is an attractive, green, novel technique that uses supercritical fluids (typically carbon dioxide). Due to its selectivity in adjusting the temperature or pressure, it is possible to obtain extracts with a low concentration of undesired compounds. Moreover, with the addition of a modifier to CO_2 , termed cosolvent, the efficiency of the extraction of more polar analytes is possible and a wide variety of compounds of different polarity can be recovered. In this regard, it is essential to highlight that SFE is hardly matrix-dependent, and therefore, the chosen chromatographic technique will depend on the sample and analytes chemical composition [52,53].

SFE is well-suited for on-line coupling systems (LC, GC, and SFC), considering that CO₂ becomes gaseous upon depressurization and it is easily removed from a flow system. Additionally, SFE produces clean extracts with minimal residual organic solvents and usually does not require additional analytes pre-concentration before the chromatographic analysis [7,52]. In general, on-line coupling systems employ intermediate trapping, multiport valves, and one or more pumps for the dynamic extraction or for transferring the extract to the chromatographic system. As mentioned above, on-line coupling systems can be carried out in either static, dynamic, or as a combination of both modes. **Table 4** presents the different SFE on-line coupling systems, conditions (feed, pressure, and temperature), static and dynamic modes of different samples, and detectors.

On-line SFE coupled to LC is technically a challenge due to the incompatibility of the technique that produces a large volume of gas (normally CO₂) while using liquid as a mobile phase. Therefore, a crucial requirement is to keep a gas-free mobile phase to avoid bubbles that promote an unstable pump performance and detector sensitivity loss [57,61–63]. Hamada et al. [55], studied the coupling system SFE-SPE-LC-MS for on-line extraction and trap columns in green, yellow, and red peppers, presenting excellent results with the concentration column with a six-port valve. The interfaces based on a split/splitless injector, an on-column injector, or a programmable temperature vaporizer were less common in on-line SFE coupled to LC than in SFE coupled to SFC [27,64–67].

Zhang et al. [16] presented a coupling system SFE-SFC with a photodiode array detector (PDA) for the rapid determination of oleanolic acid and ursolic acid in *Chaenomelis Fructus* within 40 min (10.79 mg/g dry plant, Rs = 2.36). Additionally, coupling the SFE-SFC system with the PDA detector, compared with other off-line methods obtained, higher extraction yields for all four aromatic constituents of vanilla beans. The direct on-line extraction and determination by SFE with chromatography and mass spectrometry were studied for apocarotenoids and carotenoids compound extraction. The results show a very fast extraction yield at 80 °C, in less than 17 min for monoesters and diesters carotenoids [59]; and apocarotenoids and apocarotenoids fatty acid esters were detected in yellow tamarillo through conventional and sub-2-micron particle C30 columns [56].

Although the on-line SFE-GC system might seem simple, the flow rate of the CO₂ increases after depressurization, and the volume of the gas is significant, the only coupling is found at the output of the system, not through an interface valve. Handling such a high flow rate and a large gas volume requires careful selection of the interfacing technique, if pressurized. Finally, SFC is an analytical technique that uses supercritical fluid as a mobile phase. Although SFC has some advantages, such as high resolution and high throughput, its development and application are not widely used as GC and LC, due to some aspects related to instrumental particularities and a complex handling of diverse operational variables, which can lead to a complicated optimization process [58,68–71].

Table 4. Comprehensive report of SFE systems coupled with on-line analysis systems for solid matrices from vegetable origin.

| Sample | Compounds of Interest | Coupled Techniques | Solvents | SFE Conditions (F; P; T) | SFE Method (Static mode) | SFE Method (Dynamic mode) | SFC/LC Method | Detector | References |
|--|---|-------------------------|--|---------------------------------------|-----------------------------|------------------------------|--|----------------|------------|
| Chilli Peppers | Carotenoids and apocarotenoids | SFE-SFC- QqQ/MS | CO ₂ (A) CH ₃ OH (B) | 2.0 mL/min; 150 bars; 80 °C | 0–3 min, 10% B | 3–4 min, 0% B | 4–6 min, 0% B; 6–21 min, 0–80% B; 21–22 min, 80–100% B; 22- 24, 100% B | APCI-MS | [54] |
| Green, yellow, and red bell peppers | Capsaicin | SFE-SPE- LC-MS | CO ₂ (A) CH ₃ OH (B) | 5.0 mL/min; 15 MPa; 50 °C | 0–4 min, 5% B | 4–8 min, 5% B | 0 min, 45% B; 0–10 min, 80% B; 10–12 min, 100% B; 12–13.5 min, 100% B; 15.5– 13.6 min, 45% B | ESI-MS | [55] |
| Yellow tamarillo fruits | Apocarotenoids and carotenoids | SFE-SFC- QqQ/MS | CO ₂ (A) CH ₃ OH (B) | 2.0 mL/min; 150 bars; 80 °C | 0–3 min, 5% B | 3–4 min, 10% B | 4–6 min, 0% B; 6– 14 min, 0–40% B; 14– 16 min, 40% B | APCI-MS | [56] |
| Chaenomelis Fructus | Oleanoic acid and ursolic acid | SFE-SFC | CO ₂ (A) CH ₃ OH (B) | 5.0 mL/min; 15 MPa; 35 °C | 0–1 min, 20% B | 1–8 min, 5% B | 0-10 min, 5–10% B; 10–14 min, 10% B; 14– 16 min, 10–40% B; 16– 20 min, 40% B | PDA | [16] |
| Microalgae | Carotenoids, chlorophyll A, ergosterol, and total lipids | SFE- UV/Vis- ELSD | CO ₂ (A) CH ₃ CH ₂ OH (B) | 1.5 mL/min; 15–30 MPa; 40–60 °C | - | - | - | UV/Vis ELSD | [57] |

| Vanilla beans | Aromatic constituents | SFE-SFC | CO ₂ (A) CH ₃ OH (B) | 2.0 mL/min 10–20 MPa; 35–55 °C | - | - | 0–13 min, 2–10% B; 13–17 min, 10–15% B; 17–18 min, 15% B | PDA | [58] |
|----------------------|-----------------------|--------------------|--|--------------------------------------|----------------|---------------|--|---------|------|
| Red Habanero peppers | Carotenoids | SFE-SFC- QqQ/MS | CO ₂ (A) CH ₃ OH (B) | 3.0 mL/min; 150 bar; 40–80 °C | 0–3 min, 10% B | 3–4 min, 0% B | 4–6 min, 0% B; 6–14 min, 0–40% B; 14–16 min, 40% B | APCI-MS | [59] |
| Linseed | Lipids | SFE-ELSD | CO ₂ (A) CH ₃ CH ₂ OH (B) | 1.5 mL/min 30 MPa; 80 °C | - | - | - | ELSD | [60] |

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3.4.2 Ultrasound-Assisted Extraction

Ultrasound is an intensification extraction technique widely used to improve the removal of analytes from a solid sample matrix. UAE is widely applied due to faster extractions, high reproducibility, simplification of manipulation and processing, and higher purity of the final product, allowing lower consumption of solvents and energy compared to conventional extraction techniques. Only five works approaching UAE systems coupled on-line with GC were identified [72–76]. **Table 5** presents UAE coupled systems' operational conditions (retention time, pressure, and temperature) for different samples.

Extraction of materials and purification of the extract can be simultaneously achieved, thereby reducing the "liquid-liquid" purification steps. The process also enables continuous feeding, slag discharge, and liquid discharge, which improves the extraction [81]. Falkova et al. [78] studied an automated flow batch-based determination of anthraquinones with on-line ultrasound-assisted surfactant-mediated extraction by one sequential injection and another stepwise injection. The results show sampling frequencies of 12 h⁻¹ for SIA and 6 h⁻¹ for stepwise injection, respectively. Similar studies with continuous UAE incorporated into an on-line flow injection manifold were used for the determination of zinc in meat samples by flame atomic absorption spectrometry, and the flow injection methodology allowed a sampling frequency of 80 samples per hour [82].

The cited works presented on-line combinations in dynamic mode: *i*) a predetermined volume of solvent flows through the solid matrix that is static and fixed in the extractor, termed a semi-continuous process, and *ii*) solvent and the solid matrix continuously flow through an ultrasonic bath, termed a continuous process.

Dynamic extraction (semi-continuous or continuous) is favorable in many aspects: the analytes are removed as soon as they are transferred from the solid matrix to the solvent, and the continuous exposure to fresh solvent enhances the transfer of analytes from the sample matrix to the solvent.

Table 5. Comprehensive report of UAE systems coupled with on-line analysis systems for solid matrices from vegetable origin.

| Sample | Compounds of Interest | Coupled Techniques | Solvents for UAE | Mobile Phase for LC | UAE Conditions | Reference |
|--|--------------------------|---------------------------------|--|--|--|-----------|
| Porous fungus, P. vaninii | Phytochemicals | UAE and on- line extraction | n-Hexane–ethyl acetate–acetonitrile– water (5.5:2.5:5.0:0.4, v/v/v) was used as the solvent system | elution procedure: 0–20 min, 45%–80% (acetonitrile), 65%–10% (water); flow rate: 0.4 mL/min | 500 W, 170 mL/min 40 °C | [77] |
| Frangula alnus (cortex) and Rubia tinctorum (roots and rhizomes) | Anthraquinones | UAE + spectrophotome tric | Triton X-100 | Non-applicable | 325 W, 35 kHz at 75 °C for 10 min | [78] |
| Rhodiola rosea | Rhodiosin | UAE+SPE coupled UPLC | Ethanol:water (95%) | ACN-formic acid (0.1%, v/v): 0–8 min, 5–40%, acetonitrile; 8–15 min, 40–100% acetonitrile. The flow rate of 0.3 mL/min | 60 kHz, 360 W on the scale of 0–100 | [79] |
| <i>Scutellaria baicalensis</i> Georgi | Flavonoids | UAE-HPLC | Ethanol:water (60%) | ACN-water with 0.1% phosphoric acid. The gradient conditions was 0–15 min, 20–30% ACN; 16–20 min, 30–50% ACN; 21–28 min, 50–20% CAN. The flow rate of 1 mL/min | 40 kHz, 150 W | [72] |
| basil (Ocimum basilicum L.), oregano (Origanum vulgare L.), rosemary (Rosmarinus officinalis L.), sage (Salvia officinalis L.), spearmint (Mentha spicata L.) and thyme (Thymus vulgaris). | Phenolic acids | UAE+SPE coupled on HPLC | Ethanol:water (60%) | 0 min 5% methanol, 2 min 5% methanol, 6 min 25% methanol, 13 min 40% methanol, 26 min 40% methanol. The flow rate of 1 mL/min | Flow rate 0.25 mL/min, temperature 45 °C and extraction time 15 min. | [73] |

| Textile fragments | Formaldehyde | UAE coupled on -HPLC | Water | 4 mmol L^{-1} sodium dihydrogen phosphate in 50% ACN at a flow rate of 1.0 mL min ⁻¹ . | 40 kHz. 100 and 800 W, 80 °C. | [74] |
|-------------------|------------------|-------------------------|-------|---|----------------------------------|------|
| Colistin in feed. | colistin A and B | UAE coupled with HPLC | - | - | - | [80] |

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3.4.3 Microwave-Assisted Extraction

MAE is a well-established green extraction technique widely acknowledged as rapid, easy to operate, and efficient for solid and complex matrices [83,84]. The microwave energy heats the solvent in contact with the matrix, solubilizing the analytes. The solvent or even the sample must be dielectric. Therefore, the dielectric constant is one of the critical parameters associated with MAE. Due to this fact, it is crucial to select solvent mixtures to alter the dielectric constant until suitable characteristics are achieved for the extracted sample. Good extraction efficiencies are observed in the scientific literature. Several of them are similar to or higher than classical techniques, such as Soxhlet, SFE, or PLE [83]. Moreover, MAE solvent consumption and extraction time are lower than classical techniques, facilitating on-line analysis [84]. **Table 6** presents MAE coupled techniques, operational conditions, methods (static mode and dynamic mode) for different samples, and composts of interest.

Gao et al. [85], studied on-line ionic liquid-based dynamic microwave-assisted extraction with high-performance liquid chromatography (DMAE-HPLC) to determine lipophilic constituents in an herbaceous substrate; the proposed method showed a homogeneous and stable analytes suspension when compared with the off-line ionic liquid-based DMAE and other methods. Additionally, another DMAE-HPLC coupling system showed optimal conditions for extracting Andrographis and Andrographolide for a flow of 1.0 mL/min, microwave power of 80 W, and extraction time of 6 min in 60% of aqueous methanol [87]. Wang et al. [86], studied DMAE coupled on-line with a cleaning up process (silica gel column) to remove chlorophyll from tea; the proposed method presented higher selectivity and sensitivity than conventional extraction methods, such as Soxhlet and LLE. In other studies, the analytes were automatically transferred from the SPE column to the analytical column, with a UV detector at 238 nm [51,87], a PAD detector [49] and a UV detector monitoring at 270 nm [86]. MAE may be a promising on-line coupling technique. Nevertheless, only limited research on food substrates were published [85,89].

| Table 6. Com | prehensive re | port of MAE s | vstems cou | pled with o | n-line anal | vsis systems | s for solid | matrices from | vegetable ori | gin. |
|--------------|---------------|---------------|------------|-------------|-------------|--------------|-------------|---------------|---------------|------|
| | | | J | F | | J | | | | 0 . |

| Sample | Compounds of Interest | Coupled Techniques | Solvents | MAE Conditions | LC/GC Method | Detector | Reference |
|--|--|--|--|-----------------------|--|--|-----------|
| Salvia miltiorrhiza Bunge | Lipophilic constituents (tanshin-one I, cryptotanshinone, and tanshinone IIA) | DMAE- HPLC | [C6MIM]Cl aqueous solution (A), Ethanol (B) | 1.6 mL/min; 180 W | methanol-water (v/v, 81/19) and isocratic elution; 0.5 mL min ⁻¹ ; at 40 °C and injection volume of 20 μL. | Photodiode- array detector (PAD) | [85] |
| Mushroom | Nicotine | HTDMAE- SPE | Water, elution solvent (methanol– ammonia, 95:5, v/v) | 2.0 mL/min; 1000 W | 20 mmol L ⁻¹ ammonium acetate solution (pH = 3) and methanol (80:20, v/v)); 1.0 mL min ⁻¹ , at 30 °C and injection volume of 20 μ L. | UV detector monitoring at 260 nm | [49] |
| Tea | Caffeine | DMAE coupled on- line with clean-up | Ethanol | 1.0 mL/min; 70 W | 30% methanol and 70% water; 1.0 mL min^{-1} . | UV detector monitoring at 270 nm | [86] |
| Andrographis paniculata Nees | Andrographolide and dehydroandrographolide | DMAE- HPLC | Methanol | 1.0 mL/min; 80 W | 65% aqueous methanol; 1.0 mL min ⁻¹ and injection volume of 20 μ L. | Photodiode- array detector (PAD) | [87] |
| Grain samples, including wheat, rice, corn and bean | Organochlorine pesticides | DMAE– SPE–HPLC | 95% acetonitrile | 1.0 mL/min; 80 W | Mobile phase of 75% ACN aqueous solution; 1.0 mL min ⁻¹ . | | [51] |
| Flos Carthami | Safflower yellow | MAE-UV | 60% methanol | 1.0 mL/min; 80 W | | | [88] |

3.4.4 Pressurized Liquid and Subcritical Water Extraction

PLE, also known as accelerated solvent extraction (ASE) or pressurized solvent extraction (PSE), extracts a sample using conventional solvents under high temperature and pressure. When the PLE technique operates with solvent water (or fluid) at different pressures, different terms can be used to define this technique: hot water extraction (HWE), subcritical water extraction (SubCWE), and pressurized hot water extraction (PHWE). PLE technique employs high temperatures and pressures that can increase the solvating power, decrease the viscosity, and increase the diffusion rate [2,90].

Therefore, PLE is a promising, environmentally friendly, and faster extraction technique than conventional solid–liquid extraction techniques, such as Soxhlet extraction. The temperature and pressure parameters improve the solubility, mass transfer, and increase the disruption of the surface equilibrium [2,91]. **Table 7** presents the different PLE studies on vegetal origin solid samples with coupled on-line detection systems, detailing the sample used, analyte, PLE and LC conditions, and type of detection.

Some publications approaching SubCWE on-line with either LC or GC in a dynamic mode were reported, and the samples analyzed were soils, sediments, and wastewater [97–101]. The growing interest in the SubCWE might be associated with the fact that water is environmentally friendly and intimately related to LC. SubCWE systems coupled on-line with LC are frequently applied because the solubility of the analytes extracted in water decreases dramatically when subcritical water is cooled down; trapping analytes before LC analysis is therefore relatively accessible. The analytes are collected either to a solid-phase trap (SP-trap) for LC or GC, or even in a membrane extraction unit for GC. SubCWE-LC coupling with an SP-trap is simpler than SubCWE-GC coupling, since the extract from the SP-trap can be directly eluted to the LC column by an appropriate eluent [100,101]. Therefore, SubCWE successfully shows the potential of this integrated approach. SubCWE is inherently limited to using water, and its properties change in a subcritical state, where water above 300 °C behaves similarly to organic solvents [28].

| Sample | Compounds of Interest | CoupledPLE Conditions TechniquesSolventsLC Method | | Detector | Reference | | |
|---|---|--|---------------------------------------|--|---|--------|------|
| Yerba mate (Ilex paraguariensis) | Alkaloids, phenolic acids and flavonoids | PLE-SPE- HPLC | 2.0 mL/min; 100 bars; 40–80 °C | H ₂ O (A) CH ₃ CN (B) | 1 min, 10% B; 2 min, 20% B; 4 min, 30% B; 5 min, 90% B; 8 min, 10% B | UV-Vis | [92] |
| Strawberry and apple | Herbicide 2-(3- chlorophenoxy) propionic acid (3- CPA) | µPLE-SPE- HPLC | 1.0 mL/min; 30 bars; 150–160 °C | H ₂ O (A) CH ₃ CN (B) | 70% A / 30% B / 0.1% Formic Acid | UV-Vis | [93] |
| Black tea | Gallic ccid, caffeine, and flavonols | PLE-SPE- HPLC | 2.0 mL/min; 100 bars; 40–80 °C | H ₂ O (A) CH ₃ CN (B) | 0 min, (95% A); 1 min, (95% A); 3 min, (90% A); 7 min, (87.5% A); 9 min, (85% A); 10 min, (82% A); 18 min, (77% A); 20 min, (0% A); 22 min, (0% A); 23 min, (95% A) | UV-Vis | [94] |
| Dried root (Polygonum viviparum) | Antioxidants | PLE-HPLC | 1.0 mL/min; 70 °C | H ₂ O (A) CH ₃ CN (B) | 0–5 min, 0% B; 5–6 min, 5% B; 6–21 min, 15% B; 21–30 min, 20% B; 30–35 min, 80% B; 35–37 min, 0% B | PDA | [95] |
| Ginseng of the desert (Cistanche deserticola) | Primary phenylethanoid glycosides | PLE-TFC- HPLC | 2.5 mL/min; 13 MPa; 70 °C | H ₂ O (A) CH ₃ CN (B) | 0–3 min, 10% B; 3–10 min, 10–20% B; 10–25 min, 20–30% B; 25–35 min, 30– 45% B; 35–40 min, 45–60% B; 40–45 min, 60–90% B; 45–48 min, 90% B; 48,1-60 min, 10% B | PDA | [96] |

Table 7. Comprehensive report of PLE systems coupled with on-line analysis systems for solid matrices from vegetable origin.

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The choice of analytical system type is primarily determined by the specific requirements of the sample, i.e., sample composition and complexity, and amount/concentration of analytes. For these reasons, the works with PLE presented on-line coupling only with LC, unlike SubCWE, which had studies using coupling with GC systems. This aspect is probably associated with the high eluent strength of the organic solvents usually applied for PLE. Then, the volumes used are usually significant to efficiently trap the analytes into the SP-trap sorbent [13,93]. Therefore, methods that can effectively couple PLE to chromatography via on-line solid-phase trapping/pre-concentration would be beneficial to explore. Some works were reported using on-line coupling PLE-LC systems [102,103], and those that used solid samples of plant origin are detailed in Table 7.

3.5 FUTURE DIRECTIONS

From this systematic review, it is clear that there is a need to continue the development of green extraction systems coupled with on-line analysis systems for solid matrices of vegetable origin. Through the coupling of more advanced techniques, it is possible to obtain several benefits, including shorter operating time, ease of operation, better reproducibility, improved detection limits, greater sensitivity, and reliability of the results.

A common trend in current extraction and analysis technologies is the search for replacing the use of toxic reagents with green reagents obtained from renewable sources and the integration of extraction processes and analytical operations, saving energy and reducing the use of reagents. For chromatography, the main analysis technique that provides a lot of detailed information, alternative reusable solvents, such as eutectic solvents and ionic liquids, are sought for use as a mobile phase. The development of chromatography can reduce analysis time, improve separations, and obtain more accurate information. This evolution is carried out through the elaboration of more efficient columns, with smaller lengths and smaller filling particles. Additionally, the use of SFC to replace the LC mainly in normal phase and the enhancement of detectors are key points for on-line detection.

On-line detection can be used not only to monitor the process, but also to characterize samples and extracts in real time, providing greater quality and speed in obtaining information. Through the advancement of bioinformatics and artificial intelligence, a greater automation of processes will be possible, making it easier to calibrate equipment to identify certain failures, and allowing for the ability to interrupt or make adjustments to the process that is currently taking place.

In addition, the use of the coupling of these technologies can be a relevant strategy for the development of a circular economy. The benefits of the revalorization of agro-industrial waste are remarkable, and the establishment of biorefineries producing high value-added ingredients makes it possible to take full advantage of vegetable matrices.

3.6 CONCLUSION

Research on the automation and coupling of extraction processes with on-line detection started in 1990. The total number of publications reached the mark of 2353 articles, and since 1995, more than 60 publications are made annually, except in the last year of 2020. Spain published most of these studies, while the USA had the largest network of international partnerships. The most cited articles are primarily reviews, and their volume of information (among other aspects) is one of the reasons for the high number of citations. Advanced extraction techniques for solid matrices on-line systems coupled to chromatography (LC, GC, and SFC) are usually performed in dynamic mode with innovative techniques, such as SFE, UAE, MAE, PLE, and SubCWE. On-line coupling systems employ intermediate trapping, multiport valves, and one or more pumps for the dynamic extraction or transfer of the extract to the chromatographic system. The on-line coupling of an extraction to a chromatographic technique allows the entire analysis to be performed in a closed system. The main advantages are improved sensitivity, minimal sample contamination, and the possibility of a fully automated analytical system. For solid samples, more complex solutions are required for online analysis than for liquid samples. It usually requires some adaptation and optimization, as well as special knowledge of the underlying principles. However, the time invested in optimizing conditions is quickly paid back in terms of more efficient sample throughput, better reproducibility, and improved sensitivity.

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SISTEMA INTEGRADO DE EXTRAÇÃO E HIDRÓLISE EM ÁGUA SUBCRÍTICA E SUPERCRÍTICA PARA OBTENÇÃO DE COMPOSTOS BIOATIVOS E AÇÚCARES FERMENTESCÍVEIS

Certificado de adição da invenção BR 10 2020 017238-7, depositada em 24 de agosto de 2020.

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RESUMO

SISTEMA INTEGRADO DE EXTRAÇÃO E HIDRÓLISE EM ÁGUA SUBCRÍTICA E SUPERCRÍTICA PARA OBTENÇÃO DE COMPOSTOS BIOATIVOS E AÇÚCARES FERMENTESCÍVEIS

O presente certificado de adição se refere a um sistema integrado de extração e hidrólise em água subcrítica e supercrítica com detecção *on-line* para obtenção de compostos bioativos e açúcares fermentescíveis a partir de qualquer matéria-prima contendo esses compostos, mas preferencialmente resíduos agroindustriais. O objetivo direto da tecnologia é conceber um sistema de extração e hidrólise que realize instantaneamente a caracterização química de seus produtos de modo *on-line*. O sistema permite tanto a caracterização química dos resíduos industriais em termos dos compostos bioativos e açúcares, quanto o desenvolvimento de métodos integrados de extração, hidrólise e caracterização. Os compostos bioativos extraídos pelo sistema podem ser aplicados para aumentar a lucratividade da indústria, visto que possuem um alto valor de mercado e são extremamente requisitados no setor alimentício, farmacêutico e de cosméticos.

SISTEMA INTEGRADO DE EXTRAÇÃO E HIDRÓLISE EM ÁGUA SUBCRÍTICA E SUPERCRÍTICA PARA OBTENÇÃO DE COMPOSTOS BIOATIVOS E AÇÚCARES FERMENTESCÍVEIS

Certificado de adição da invenção BR 10 2020 017238-7, depositada em 24 de agosto de 2020.

4.1 CAMPO DE INVENÇÃO

[001] O presente certificado de adição se refere a um sistema integrado de extração e hidrólise em água subcrítica e supercrítica com detecção *on-line* de compostos bioativos e açúcares fermentescíveis.

[002] A área de aplicação do presente certificado de adição pertence ao campo da química de produtos naturais, e pode ser aplicada em indústrias alimentícias, farmacêuticas e de biocombustíveis.

<u>4.2 FUNDAMENTOS DA INVENÇÃO</u>

[003] Compostos bioativos são intensamente procurados pela indústria alimentícia, farmacêutica e cosmética, devido a benefícios fisiológicos, nutricionais e medicinais que promovem a saúde humana. Esforços são feitos para obter esses compostos de fontes naturais, como plantas, animais e microrganismos, bem como substituir o uso dos produtos sintéticos. As principais classes de bioativos naturais são: compostos fenólicos, glicosinolatos, carotenoides, fitoesteróis, alcaloides e terpenos. A complexidade química e diversidade desses compostos não são prontamente alcançadas em compostos sintetizados em laboratório pelo homem.

[004] Entre os compostos que apresentam propriedades biológicas destacam-se os compostos fenólicos que estão presentes em diversos alimentos, como nas frutas, nos chás, nos grãos e em vários outros. Exemplos relevantes de compostos fenólicos são as isoflavonas presentes na soja, os ácidos fenólicos encontrados no café, os terpenóides comuns nas sementes e as antocianinas responsáveis por uma grande variedade de cores (vermelho, azul, roxo) de frutas, flores e folhas.

[005] Entretanto, como estes compostos são produtos do metabolismo secundário de plantas, sua distribuição e concentração são influenciadas por vários fatores, como a variedade, o local e ano de cultivo, práticas agrícolas, controle de pragas e doenças, etc. Adicionalmente, dependendo do tipo e da intensidade, o processamento pode provocar a
transformação e/ou a degradação destes compostos alterando suas propriedades e/ou diminuindo sua atividade. Todavia em muitos casos uma grande quantidade de compostos ainda permanece viável pós-processamento e são comumente subaproveitados ou até mesmo descartados como resíduos agroindustriais.

[006] Os resíduos agroindustriais de composição lignocelulósica podem passar por processos que promovem o aproveitamento energético para produção de biocombustíveis, esta aplicação tem sido o foco de diversas pesquisas em todas as partes do mundo, pois trata-se de um fonte renovável, abundante e não-alimentar. Capazes de conter as drásticas mudanças climáticas atribuídas à nossa dependência excessiva de combustíveis fósseis. Cada país normalmente foca em um resíduo de sua agroindústria local, entretanto o Brasil possui imensa e diversificada produção agroindustrial com diferentes resíduos que podem ser explorados em cada região.

[007] A água sub/supercrítica é uma tecnologia verde capaz de promover a conversão da biomassa por processos de extração e/ou hidrólise, podendo obter uma grande quantidade/variedade de produtos bioquímicos e açúcares fermentescíveis. Estes produtos são viáveis para aplicação nas indústrias alimentícias, farmacêuticas e de biocombustíveis.

[008] A tecnologia de fluidos supercríticos permite trabalhar com processos em diferentes faixas de temperatura, seu uso se difundiu inicialmente nos processos de extração, fracionamento e purificação. Entretanto novas aplicações vêm surgindo, como no caso da água subcrítica ou supercrítica comumente usadas para hidrólise de biomassa lignocelulósica. Água no estado subcrítico é definida como aquela que mantém o estado líquido por pressão a uma temperatura maior que seu ponto de ebulição natural de 100°C e menor que sua temperatura crítica de 374°C. A água acima do ponto crítico (Tc 374°C e Pc 22,1 MPa) é chamada de água supercrítica.

[009] A utilização da água sub/supercrítica é uma estratégia relevante para o desenvolvimento da economia circular, por ser vantajosa na valorização de resíduos agroindustriais. Além disso, é uma tecnologia atual, considerada mais ecológica que as convencionais e que também possibilita o acoplamento com sistemas de detecção *on-line*. Contudo os processos de extração, hidrólise, purificação e análise de compostos naturais envolvem uma série de etapas com diferentes técnicas e procedimentos.

[0010] A água sub/supercrítica apresenta baixa viscosidade e alta difusividade que facilita a penetração da água na estrutura complexa da matriz lignocelulósica, enquanto

sua baixa constante dielétrica, semelhante aos solventes orgânicos não polares, melhora a solubilidade dos compostos orgânicos. As propriedades físicas da água (como densidade, produto iônico e constante dielétrica) podem ser ajustadas variando a temperatura e a pressão, tornando um solvente rápido e seletivo tanto para processos de extração e hidrólise.

[0011] A água em ambas as condições promove uma rápida hidrólise de biomassa, embora a velocidade de reação seja uma vantagem para a intensificação do processo também é uma desvantagem significativa, principalmente para água supercrítica, no que diz respeito à seletividade em tempos de reação mais longos, podendo levar à degradação dos açucares e a formação de produtos da degradação que são inibitórios para processos biotecnológicos.

[0012] Portanto, é necessário entender os processos mecanismos de fracionamento hidrotérmico para melhorar a seletividade dos processos. A água subcrítica por utilizar condições mais brandas de temperatura e pressão surge como uma técnica promissora de extração, pois reduz consideravelmente o tempo de extração, evita o uso de solventes e enzimas com alto custo no mercado, desta forma podendo aumentar a lucratividade de uma biorrefinaria, visto que para recuperar os compostos bioativos de resíduos agroindustriais por vezes é necessário hidrolisar as ligações éster e/ou éter que as mantêm ligados.

[0013] A maioria dos métodos de extração e/ou hidrólise são operados de maneira *off-line* com o sistema de análise, ou seja, são realizadas separadamente. No entanto, em muitos casos estes processos poderiam ser integrados em um sistema *on-line*, onde todo o procedimento analítico ocorre em um sistema fechado, geralmente automatizado. Proporcionando dessa maneira benefícios adicionais, que incluem maior velocidade, sensibilidade e confiabilidade dos resultados. Além de evitar problemas associados às abordagens tradicionais, uma vez que o preparo da amostra em um sistema *on-line* tende a ser mais eficaz.

[0014] Muita ênfase foi colocada no desenvolvimento de técnicas de separação, particularmente, cromatografia líquida de alta eficiência (HPLC), que atualmente tem sido considerada uma ferramenta poderosa para separação e análise de componentes orgânicos de amostras complexas. Os compostos separáveis por HPLC são não voláteis ou de baixa volatilidade, o que abrange quase 75% dos compostos alvo em análises de rotina científica. No entanto, a determinação dos analitos presentes em matrizes complexas requer uma abordagem adequada de preparação de amostras para remover eficientemente os compostos de interesse de um meio contendo vários interferentes e, ao mesmo tempo, concentrá-los para melhorar a detecção do sinal de saída.

[0015] A preparação da amostra é uma etapa crucial para a maioria dos métodos analíticos. No caso de HPLC, mesmo considerando os avanços recentes na instrumentação, uma etapa preliminar ainda é necessária para aprimorar o procedimento analítico. As técnicas de preparação de amostra são consideradas como os procedimentos mais demorados, trabalhosos e propensos a erros de todo o processo analítico, que geralmente inclui etapas de: coleta de amostra, preparação de amostra, detecção e processamento de dados. Estatisticamente, a preparação da amostra ocupa cerca de dois terços do tempo de um procedimento analítico e é responsável por mais de um terço dos erros.

[0016] Apesar dos problemas que o preparo da amostra pode causar à análise, ela é extremamente necessária, dado que a amostra precisa de limpeza, purificação e concentração. Uma alternativa capaz de promover estas funções por meio de processos em linha é a técnica de extração em fase sólida (SPE). Na SPE em fase reversa, os analitos contidos numa matriz aquosa são extraídos, juntamente com os compostos interferentes, após passarem por um cartucho contendo adsorvente. Um solvente seletivo é geralmente utilizado para remover os interferentes e então, outro solvente orgânico é usado para lavar e eluir os analitos de interesse. Como os compostos são eluidos com um pequeno volume de solvente estes acabam concentrados na amostra. Assim pode-se obter uma amostra limpa e com alta concentração dos compostos de interesse.

[0017] Além da abordagem analítica, os processos e seu escalonamento no tratamento de resíduos agroindustriais têm ganhado notório destaque nos últimos anos. É sabido que um novo processo precisa ser concebido em escala laboratorial, quando em geral é realizada a otimização das variáveis que mais impactam nos resultados.

[0018] Diante do exposto, e de forma a solucionar os problemas técnicos relatados anteriormente, o desenvolvimento de sistemas e métodos de extração e hidrólise em escala laboratorial é uma etapa mandatória e crucial para um processo que vislumbra aplicações industriais. Fato este que é observado para os processos de extração e hidrólise de resíduos agroindustriais. Portanto, o desenvolvimento de sistemas e equipamentos capazes de integrar as etapas de extração, hidrólise e análise química dos produtos de forma automatizada, rápida e eficaz representa uma evolução para a concepção de métodos que, se em escala laboratorial mostrarem promissão, sejam levados às escalas maiores.

CHAPTER 4 – Patent (Extraction/Hydrolysis system with on-line detection)

[0019] O presente certificado de adição apresenta diversas vantagens e diferenciais em relação aos sistemas com a mesma finalidade presentes no estado da técnica, como o fato do sistema operar em condições subcrítica e supercríticas, tendo como aperfeiçoamentos o fato do sistema apresentar uma integração de técnicas como a extração em água subcrítica, hidrólise em água subcrítica e supercrítica, purificação em SPE, análise em HPLC e monitoramento de pH in-line, permitindo um controle preciso e automatizado da temperatura, pressão e vazão do solvente. O presente certificado de adição descreve um sistema que permite além da extração, a hidrólise, em um contexto de biorefinaria. Além disso, permite quebrar a matriz polimérica em diferentes graus, gerando moléculas menores, como por exemplo açúcares que podem ser usados para fermentação/geração de energia. O uso de dois reatores sequenciais, um operando em condições subcríticas e outro supercríticas, é mais eficiente do que os reatores únicos porque permitem um melhor controle da temperatura e do processo como um todo, além de minimizar reações indesejáveis de degradação.

4.3 ESTADO DA TÉCNICA

[0020] O documento BR1020190272562 protege um sistema e processo integrado sequencial de extração e hidrólise em água subcrítica de resíduos lignocelulósicos para obtenção de compostos bioativos e plataformas químicas. Comparado com o presente certificado de adição, ambas as invenções operam com água subcrítica. No entanto, no referido documento ocorre uma etapa de extração seguida de hidrólise no mesmo reator. E o presente certificado de adição se diferencia por apresentar um reator em linha capaz de operar em condições supercríticas. Além disso, o certificado de adição apresenta um sistema analítico acoplado capaz de detectar quase que instantaneamente os componentes presentes nos produtos.

[0021] A operação em condições supercríticas através do reator em linha permite obter produtos avançados em relação à quando somente o reator de hidrólise subcrítica está presente. A hidrólise supercrítica (segundo reator) quebra os oligossacarídeos produzidos na hidrólise subcrítica (primeiro reator), resultando em açúcares do tipo mono e dissacarídeos. Consequentemente, as duas etapas (hidrólise subcrítica seguida da hidrólise supercrítica) levam a produtos mais homogêneos e preferíveis para os processos de fermentação. A detecção *on-line* dos produtos do processo permite constante monitoramento em tempo real do processo que está sendo executado. Além do monitoramento instantâneo, a caracterização dos produtos é obtida juntamente com o processo de transformação, em único passo.

CHAPTER 4 – Patent (Extraction/Hydrolysis system with on-line detection)

[0022] O documento US10889795B2 propõe um sistema e método para hidrolisar biomassa, se resumindo em desenvolver um sistema e método para resfriamento de biomassa pré-tratada, através das técnicas convencionais, operando em batelada e não avaliando a extração de compostos bioativos. Enquanto o presente certificado de adição consiste em um sistema integrado de extração e hidrólise em água sub/supercrítica com monitoramento em tempo real de seus produtos gerados (compostos bioativos e açúcares).

[0023] O presente certificado de adição utiliza água sub/supercrítica, uma tecnologia verde e eficiente de hidrólise, capaz de promover também extração de compostos bioativos. Realiza monitoramento dos produtos formados em linha, proporcionando dessa maneira benefícios adicionais, que incluem maior velocidade, sensibilidade e confiabilidade dos resultados. Além de evitar problemas associados às abordagens tradicionais, uma vez que o preparo da amostra em um sistema on-line tende a ser mais eficaz.

[0024] O documento BR1020150314019 faz parte do estado geral da técnica e protege um sistema integrado para a produção de biocombustíveis a partir de resíduos e uso. Entretanto, o referido documento aborda o desenvolvimento de uma unidade experimental para a hidrólise da biomassa, sem a purificação e a análise dos compostos. A principal diferença do presente certificado de adição em relação ao referido documento está no monitoramento *on-line* dos produtos obtidos via hidrólise/extração em água sub/supercrítica. Isso se deve ao desenvolvimento de um novo sistema e processo de purificação e detecção *on-line*, que foi acoplado a um sistema de hidrólise e extração de compostos químicos da biomassa.

[0025] O documento de patente BR1020200172387 descreve um sistema bidimensional para a extração, purificação e análise de compostos bioativos, tendo como foco somente a extração com fins de análise de compostos. O referido sistema descrito no documento não opera em condições subcrítica e supercríticas e somente realiza processos de extração, além disso, não é capaz de realizar reações químicas e está projetado para analisar a amostra e estudar a composição. Como aperfeiçoamento apresentado pelo presente certificado de adição em relação ao referido documento é possível também citar que o sistema apresenta uma integração de técnicas como a extração em água subcrítica, hidrólise em água subcrítica e supercrítica, purificação em SPE, análise em HPLC e monitoramento de pH in-line, permitindo um controle preciso e automatizado da temperatura, pressão e vazão do solvente.

CHAPTER 4 – Patent (Extraction/Hydrolysis system with on-line detection)

[0026] A operação em condições de hidrólise subcrítica permite a obtenção de produtos diferentes após a extração. Sendo assim, o sistema do referido documento permite o processo de extração, enquanto o presente certificado de adição permite além da extração, a hidrólise, em um contexto de biorefinaria. Além disso, permite quebrar a matriz polimérica em diferentes graus, gerando moléculas menores, como por exemplo, açúcares que podem ser usados para fermentação/geração de energia. O uso de dois reatores sequenciais é mais eficiente do que os reatores únicos porque permitem um melhor controle da temperatura e do processo como um todo, além de minimizar reações de degradação. Enquanto o referido documento realiza a extração de todos os compostos para caracterizar e estudar a amostra.

[0027] E por fim, o documento CN108339496A protege um dispositivo e método de hidrólise subcrítica ou supercrítica para tratamento de biomassa. Já o presente certificado de adição realiza o aproveitamento de biomassa residual através da mesma tecnologia, entretanto promove uma etapa de extração de compostos bioativos antes do processo de hidrólise para aproximar as agroindústrias do conceito de biorrefinaria, obtendo assim o aproveitamento máximo dos recursos. E em adição realiza instantaneamente a caracterização química de seus produtos de modo *on-line*. O referido documento é aplicado somente para hidrólise de biomassa, não realiza extração dos compostos que possuem efeitos benéficos sobre o organismo humano, desperdiçando um material de alto valor econômico. Ademais não promove o monitoramento em linha dos produtos formados, acarretando maiores gastos com solvente e mão de obra para realização das análises.

4.4 BREVE DESCRIÇÃO DA INVENÇÃO

[0028] O presente certificado de adição se refere a um sistema integrado de extração e hidrólise em água subcrítica e supercrítica com detecção *on-line* para obtenção de compostos bioativos e açúcares fermentescíveis a partir de qualquer matéria-prima contendo esses compostos, mas preferencialmente resíduos agroindustriais. O objetivo da tecnologia é conceber um sistema de extração e hidrolise que realize instantaneamente a caracterização química de seus produtos de modo *on-line*. O sistema permite tanto a caracterização química dos resíduos industriais em termos dos compostos bioativos e açúcares, quanto o desenvolvimento de métodos integrados de extração, hidrolise e caracterização. Os compostos bioativos extraídos pelo sistema podem ser aplicados para aumentar a lucratividade da indústria, visto que possuem um alto valor de mercado e são extremamente requisitados no setor alimentício, farmacêutico e de cosméticos.

<u>4.5 BREVE DESCRIÇÃO DAS FIGURAS</u>

[0029] Na Figura 1 é apresentado um diagrama esquemático do equipamento de extração e hidrólise em água sub/supercrítica acoplada com análise cromatográfica em linha.

[0030] Na Figura 2 é apresentado um perfil tridimensional da extração e dessorção da amostra de bagaço de uva obtido a 520 nm.

[0031] Na Figura 3 é apresentado um cromatograma do hidrolisado do bagaço de uva e do padrão de glicose.

<u>4.6 DESCRIÇÃO DETALHADA DA INVENÇÃO</u>

[0032] O presente certificado de adição se refere a um sistema integrado de extração e hidrólise em água sub/supercrítica com detecção *on-line* para obtenção de compostos bioativos e açúcares fermentescíveis a partir de qualquer matéria-prima contendo esses compostos, mas preferencialmente resíduos agroindustriais.

[0033] O sistema compreende dois módulos, um módulo de extração/hidrólise e outro módulo de análise, que engloba um sistema de purificação (SPE) e um sistema de cromatografia líquida (HPLC-PDA/RID). O módulo de extração/hidrólise compreende os seguintes componentes:

- Reservatório de água (1);
- Bomba HPLC (2);
- VB Válvula de bloqueio (VB-1, VB-2, VB-3 e VB-4)(3);
- Pré-aquecedor (4);
- Estufa (5);
- Reator subcrítico (6);
- Termopares (7);
- Manômetros (8);
- Reator supercrítico (9);
- Controlador de temperatura (10);
- Trocador de calor (11);
- Banho termostático (12);
- VM Válvula micrométrica (16);
- Coletor de amostra (18).

[0034] E o módulo de análise, com purificação (SPE) e cromatografia líquida (HPLC_PDA/RID) compreende os seguintes componentes:

- Filtro em linha (13);
- Coluna SPE (14);
- Válvula de interface (15);
- Sensor para monitoramento de pH in-line (17);
- Cromatógrafo líquido (19);
- Coletor de resíduo (20);
- Computador (21).

[0035] Como aperfeiçoamentos do presente sistema em relação aos encontrados no estado da técnica, é possível citar o fato do sistema operar em condições sub e supercríticas, apresentar dois reatores, um de extração/hidrólise e outro de hidrólise sequenciais e conectados em série, permitir um monitoramento *on-line* e permitir um monitoramento de pH in-line.

[0036] A estrutura base do sistema é construída com perfilados de alumínio 30 x 30, ligados com conectores de aço galvanizado de 90°. Chapas de inox de 2 mm de espessura são adicionadas para sustentação dos equipamentos e acessórios. Para possibilitar fácil movimentação da estrutura são adicionados seis rodízios de PVC 3", modelo de placa giratório com freio. As tubulações utilizadas no sistema de cromatografia líquida (HPLC_PDA/RID) possuem dimensões reduzidas (1/16" OD x 0,010" I.D).

[0037] Para o módulo de extração/hidrólise foram utilizadas tubulações com maiores diâmetros (1/8" OD x 0,500" ID). E para o sistema de purificação (SPE) foi utilizado tubulação com dimensões intermediárias (1/16" OD x 0,040" I.D). Os processos de extração e hidrólise de resíduos agroindústrias com composição lignocelulósica são realizados em um módulo de extração/hidrólise de fluxo semi-contínuo (batelada para o sólido e contínuo para o líquido) e um reator supercrítico de fluxo contínuo.

[0038] Na Figura 1 é apresentado o diagrama esquemático da invenção. O equipamento consiste em um módulo de extração/hidrólise construído em aço inoxidável 316, com volume útil de 20 mL, para evitar os vazamentos nas tampas existe sistema de vedação com gaxetas, além de filtros de 5 micrometros para retenção do material sólido não solúvel e assim evitar possíveis entupimentos na tubulação. A água antes de chegar ao módulo de extração/hidrólise é previamente aquecida por um pré-aquecedor (4) (sistema de aquecimento com camisa de resistência elétrica), o módulo de extração/hidrólise por sua vez é aquecido por uma estufa (5) com circulação forçada de ar. O reator supercrítico (9) conta com um

sistema de aquecimento do tipo camisa elétrica, onde a resistência está protegida por um material isolante de cerâmica para proteção do operador e para melhorar a distribuição do calor nas paredes do reator.

[0039] A bomba HPLC (2) de alta pressão foi usada para bombear água ao sistema. A pressão é controlada por uma válvula micrométrica (16) e medida por dois manômetros (8) conectados em pontos estratégicos da tubulação. As temperaturas dos processos de extração e hidrólise são monitoradas por um painel de controle com sistema de controle automatizado, as medidas são realizadas por termopares de tipo K devidamente calibrados para as faixas de temperatura utilizadas nestes procedimentos, o que garante um bom controle de temperatura.

[0040] O fluido quente após extração e hidrólise é resfriado a uma temperatura inferior a 30°C em um trocador de calor (serpentina) (11) acoplado a um banho termostático (12), operando a 10°C (utilizando água como fluido refrigerante), o resfriamento rápido assegura que as reações sejam cessadas evitando degradações. Após o resfriamento da amostra inicia-se os procedimentos analíticos. Primeiramente ocorre uma filtração em linha com filtro do tipo copo (13), com poros de dois micrômetros para retenção do material sólido que pode precipitar logo depois da amostra resfriada, em seguida é feito uma purificação utilizando as técnicas de adsorção e dessorção através da tecnologia de extração em fase sólida (SPE) (14).

[0041] A injeção da amostra ocorre por intermédio da válvula de interface (15), a válvula de interface (15) permanece na posição padrão (posição 1) para passagem da amostra pelo *loop* até o recipiente de coleta (18), no momento da injeção o sistema envia um sinal para válvula de interface (15) que muda para posição de injeção (posição 2), e o fluxo de solvente passa pelo *loop* descarregando a amostra no cromatógrafo líquido (HPLC_PDA/RID) (19), a válvula de interface (15) permanece na posição de injeção (posição 2) durante três segundos e depois retorna para posição padrão (posição 1).

[0042] Descrevendo o funcionamento do sistema de forma mais detalhada, o mesmo é iniciado com a etapa de empacotar o reator subcrítico (6) com a matéria-prima desejada. Primeiro adiciona-se uma camada de lã de vidro, em seguida a amostra, depois outra camada de lã de vidro e por fim completa-se o volume do reator subcrítico (6) com esferas de vidro. A etapa seguinte é empacotar a coluna SPE (14) com adsorvente. Acopla-se o reator subcrítico (6) e a coluna SPE (14) no equipamento e instala-se a coluna C18 no cromatógrafo

líquido (HPLC_PDA/RID) (19) para detecção de compostos bioativos. Em seguida a estufa (5) e o banho termostático (12) são ligados para que alcancem a temperatura de trabalho.

[0043] Antes de iniciar a análise, deve-se certificar de trocar e reabastecer os solventes aquosos e orgânicos. O canal A (água com 2,5% de ácido acético) e o canal B (acetonitrila com 2,5% de ácido acético).

[0044] Os nobreaks, o computador (21) e os demais equipamentos devem ser ligados. O *software* associado ao sistema é ativado. Abrem-se dois projetos, uma para o módulo extração/hidrólise e outro para o módulo de análise (HPLC_PDA/RID). Em seguida é ativado o adsorvente da coluna SPE (14), com o fechamento das válvulas da tubulação do processo extração/hidrólise (VB-1 e VB-3), a abertura da válvula do *by-pass* (VB-2) e com o bombeamento de 30 mL de etanol e depois 30 mL de água.

[0045] Em seguida, realiza-se a purga do cromatógrafo (19), (mín. 10 mL) para cada canal, ao final verifica-se se existem bolhas nas tubulações. Deve-se dar início nos métodos adequados de cada projeto e fazer uma verificação se a válvula de interface (15) está na configuração adequada para injeção automática. Em seguida é criado as sequências de injeções sincronizando o tempo para os módulos de extração/hidrólise, e módulo de análise (HPLC_PDA/RID). Em seguida, o pré-aquecedor (4) é ligado.

[0046] Após os procedimentos descritos anteriormente, a tubulação do processo é pressurizada com a válvula de bloqueio (VB-4) e a válvula micrométrica - VM (16) sendo fechadas, dando início ao processo de extração/hidrólise (iniciando o bombeamento da bomba HPLC (2) com a vazão desejada). Sendo que, quando atingir a pressão adequada, deve-se abrir a válvula de bloqueio (VB-4) e controlar a pressão com a válvula micrométrica - VM (16). Em seguida, deve-se iniciar as injeções do módulo HPLC_PDA/RID. Quando terminar a extração, inicia-se a dessorção com etanol 50%, parando as injeções do módulo extração/hidrólise e parando o bombeamento da bomba HPLC (2), fechando as válvulas (VB-1 e VB-3) da tubulação do processo de extração/hidrólise, abrindo a válvula do *by-pass* (VB-2) e iniciando o processo de dessorção (iniciando novamente o bombeamento da bomba HPLC (2) com a vazão desejada).

[0047] Deve-se trocar o coletor amostra (18) a cada 12 minutos. Quando observando os cromatogramas percebe-se que a dessorção terminou, inicia-se as mudanças no módulo HPLC_PDA/RID para análise de açúcares, instalando a coluna para detectar açúcares e iniciando o método de açúcares no sistema HPLC_PDA/RID. Em seguida, o procedimento

de hidrólise é iniciado, parando o processo de dessorção (parando o bombeamento da bomba HPLC (2)), fechando a válvula do *by-pass* (VB-2), abrindo as válvulas da tubulação do processo de extração/hidrólise (VB-1 e VB-3), aumentando a temperatura da estufa (5) e do pré-aquecedor (4) para condições de hidrólise subcrítica e ligando o aquecimento do reator supercrítico (9) na temperatura de 374°C.

[0048] É criado as sequências de injeções sincronizando o tempo para os dois módulos, módulo extração/hidrólise e módulo HPLC_PDA/RID. Quando as temperaturas do processo de hidrólise forem alcançadas, são iniciadas as injeções do módulo extração/hidrólise (iniciando o bombeamento da bomba HPLC (2) com a vazão desejada). As injeções no módulo HPLC_PDA/RID são iniciadas. Quando terminar a hidrólise, inicia-se a dessorção com etanol 50%, parando as injeções do módulo extração/hidrólise (parando o bombeamento da bomba HPLC (2)), fechando as válvulas (VB-1 e VB-3) da tubulação do processo de extração/hidrólise, e abrindo a válvula do *by-pass* (VB-2).

[0049] Em seguida, deve-se iniciar o processo de dessorção (iniciando novamente o bombeamento da bomba HPLC (2) com a vazão desejada). Quando observando os cromatogramas percebe-se que a dessorção terminou, inicia-se as etapas de desligamento do sistema, parando as injeções e parando o fluxo do módulo HPLC_PDA/RID, desligando o pré-aquecedor (4), a estufa (5) e o aquecimento do reator supercrítico (9). Quando as temperaturas estiverem abaixo de 100°C, deve-se parar o fluxo do módulo extração/hidrólise, (parando o bombeamento da bomba HPLC (2)), e abrindo a válvula micrométrica – VM (16) para despressurizar a tubulação do processo, e por fim, desligar os equipamentos.

<u>4.7 EXEMPLO DE CONCRETIZAÇÃO</u>

[0050] Os processos hidrotérmicos de extração e hidrólise sequencial de resíduos agroindústrias foram conduzidos utilizando como matéria-prima o bagaço de uva preta como forma de validação do sistema. O bagaço de uva é o principal resíduo sólido das vinícolas, atualmente é utilizado como fertilizante e ração animal. No entanto, apenas 30–40% das antocianinas contidas nas uvas são extraídas das cascas durante a produção do vinho. Portanto, o bagaço, que corresponde a 50% da massa total da uva, é rico em compostos bioativos com propriedades funcionais, sendo capaz de atuar no metabolismo humano, prevenindo doenças degenerativas, cardiovasculares e inflamatórias. Além das antocianinas e outros compostos fenólicos presentes no bagaço de uva preta, sua composição lignocelulósica

é atrativa para realização de hidrólise, visando obter açúcares fermentescíveis para produção de biocombustíveis.

[0051] Como amostra, foram utilizados 10g de bagaço de uva triturado. O adsorvente selecionado (PoraPakTM Rxn), polímero para limpeza foi empacotado em uma coluna de SPE (14) ativada com 30 mL de etanol e acondicionado com 30 mL de água a um fluxo de 3 mL/min, através do *by-pass* do equipamento. A temperatura de extração foi 40°C e o fluxo de solvente foi 2 mL/min, como solvente foi utilizado água pressurizada a 15 MPa.

[0052] Para dessorção foi utilizado novamente o *by-pass*, e como solvente de eluição etanol 50% com fluxo de 0,25 mL/min. Após o término da dessorção o bombeamento foi interrompido, o by-pass foi fechado e as temperaturas de hidrólise foram programadas (150°C na estufa e 250°C no reator supercrítico), no momento em que as temperaturas foram atingidas foi iniciado o bombeamento de água à 2 mL/min sob pressão de 15 MPa.

[0053] A coleta dos extratos fracionados foi feita a cada 12 minutos. A análise automática dos compostos presentes nos extratos foi realizada por meio de um método isocrático (60% de B), baseado no uso de uma coluna cromatográfica C18 à temperatura de 50°C, com um fluxo de 0,7 mL/min, utilizando como fase móvel água (solvente A) e acetonitrila (solvente B) acidificados com ácido acético (2,5%).

[0054] Os cromatogramas foram obtidos através do detector de arranjo de fotodiodo (PDA) no comprimento de ondas de 520 nm para produção do perfil bidimensional durante os processos de extração e dessorção. As injeções foram programadas para ocorrer no início e no meio das coletas das frações para representar a composição inicial e média dos extratos obtidos em cada intervalo, ou seja, foram realizadas a cada 6 minutos.

[0055] A coleta dos hidrolisados fracionados foi feita a cada 12 minutos. A análise automática dos açúcares presentes nos hidrolisados foi realizada por meio de um método isocrático (0,005N H₂SO₄), baseado no uso de uma coluna cromatográfica de troca iônica à temperatura de 50°C, com um fluxo de 1,0 mL/min, utilizando como fase móvel 0,005N H₂SO₄. Os cromatogramas foram obtidos através do detector de índice de refração (RID) para produção do perfil bidimensional durante o processo de hidrólise. As injeções foram programadas para ocorrer no meio das coletas de cada fração, para representar a composição média do hidrolisado obtido nos intervalos.

[0056] A Figura 2 mostra os cromatogramas tridimensionais dos processos de extração e dessorção obtidos para o bagaço de uva. Eles exemplificam como seriam os

cromatogramas de qualquer matéria-prima processada no equipamento que está sendo proposto. Eles são compostos por três eixos, o eixo do comprimento representa o tempo da análise cromatografia, o eixo da altura representa a intensidade do sinal emitido pelo detector do cromatógrafo, e o eixo de profundidade representa o tempo do processo de extração no qual a injeção de amostra é realizada.

[0057] Analisando a Figura 2, é possível observar que no início do processo (cromatograma 6 até 48 min) o detector sinaliza uma leitura de antocianinas muito baixa devido ao fato delas ficarem retidas no adsorvente da coluna SPE. A partir do cromatograma de 54 min, é realizada a eluição (dessorção) das antocianinas e podemos observar a curva cinética de extração, onde observamos um rápido crescimento na intensidade do sinal cromatográfico (pico) e depois ocorre uma diminuição gradativa conforme os compostos vão se esgotando no extrato.

[0058] Os cromatogramas das amostras podem ser expressos juntamente com um cromatograma de referência representando o padrão cromatográfico. Com a informação do padrão cromatográfico e as informações de cada tempo de residência é possível fazer a quantificação do composto obtido, bem como plotar as curvas cinéticas de extração e hidrólise de cada composto. Informação está de extrema relevância do ponto de vista da engenharia do processo, pois além de expressar a concentração das frações de extrato e hidrolisado, expressa o rendimento dos compostos alvo.

[0059] Desta forma, foi realizada uma análise quantitativa no hidrolisado do bagaço de uva, comparando o cromatograma da amostra com o de uma solução de padrão de glicose de concentração conhecida (10 g/L). A Figura 3 apresenta a comparação entre os cromatogramas do hidrolisado do bagaço de uva e do padrão de glicose. A partir da análise dos tempos de residência, identificou-se o pico da glicose no hidrolisado do bagaço de uva e através das áreas de cada pico pode-se calcular a concentração do composto no hidrolisado. Portanto pode-se inferir que na fração do hidrolisado analisado possui 0,5 g/L de glicose.

REIVINDICAÇÕES

- Sistema integrado de extração e hidrólise em água subcrítica e supercrítica que compreende um módulo de extração/hidrólise e um módulo HPLC_PDA/RID, sendo <u>caracterizado</u> por compreender detecção *on-line* para obtenção de compostos bioativos e açúcares fermentescíveis, e adicionalmente consistir em:
 - um reservatório de água (1),
 - uma bomba HPLC (2) para bombear água,
 - uma válvula de bloqueio VB (3),
 - um pré-aquecedor (4) para aquecer a água antes de chegar ao módulo de extração,
 - uma estufa (5) com circulação forçada de ar,
 - um reator subcrítico (6) de extração,
 - um termopar (7),
 - pelo menos um manômetro (8) para medição da pressão,
 - um reator supercrítico (9) de hidrólise operado em série com o reator subcrítico (6) de extração,
 - um controlador de temperatura (10),
 - um trocador de calor (11) para resfriamento do fluido após a extração e hidrólise a uma temperatura inferior a 30°C,
 - um banho termostático (12) operando a 10°C e utilizando água como fluido refrigerante,
 - um filtro em linha (13),
 - uma coluna SPE (14),
 - uma válvula de interface (15) que possibilita a injeção da amostra a ser analisada,
 - uma válvula micrométrica VM (16) para controle da pressão,
 - um sensor de monitoramento de pH in-line (17),
 - um coletor de amostra, (18)
 - um cromatógrafo líquido, (19)
 - um coletor de resíduo (20); e
 - um computador (21).
 - Sistema, de acordo com a reivindicação 1, <u>caracterizado</u> pelo fato de a amostra ser empacotada no reator subcrítico (6) com uma camada de lã de vidro, em seguida a amostra, depois outra camada de lã de vidro e por fim esferas de vidro.

- Sistema, de acordo com a reivindicação 1, <u>caracterizado</u> pelo fato de a coluna de SPE (14) ser empacotada com adsorvente e acoplando-se entre o módulo de extração/hidrólise e módulo HPLC_PAD/RID permitir a análise on-line.
- 4) Sistema, de acordo com a reivindicação 1, <u>caracterizado</u> pelo fato de o adsorvente da coluna SPE ser ativado com o fechamento das válvulas da tubulação do processo extração/hidrólise (VB-1 e VB-3), a abertura da válvula do *by-pass* (VB-2) e com o bombeamento de 30 mL de etanol e 30 mL de água.
- 5) Sistema, de acordo com a reivindicação 1, <u>caracterizado</u> pelo fato de o reator subcrítico
 (6) de extração e o reator supercrítico (9) de hidrólise serem operados em série.

Sistema, de acordo com a reivindicação 1, <u>caracterizado</u> pelo fato de o coletor de amostra (18) ser trocado a cada 12 minutos.



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FIGURA 1



CHAPTER 4 – Patent (Extraction/Hydrolysis system with on-line detection)





FIGURA 3

CHAPTER 5 – Extraction of anthocyanins from açaí with on-line detection

PRESSURIZED LIQUID EXTRACTION COUPLED IN-LINE WITH SPE AND ON-LINE WITH HPLC (PLE-SPEXHPLC) FOR THE RECOVERY AND PURIFICATION OF ANTHOCYANINS FROM SC-CO₂ SEMI-DEFATTED AÇAÍ (EUTERPE OLERACEA)

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ABSTRACT

This study aimed to extract anthocyanins from dried and semi-defatted acaí pulp using green technologies based on the coupling of pressurized liquid extraction (PLE) with inline purification through solid-phase extraction (SPE) and on-line analysis by highperformance liquid chromatography (HPLC). Critical parameters that affect the extraction efficiency and purification were investigated and optimized by response surface methodology (RSM). PLE was performed with acidified water at different pH (2.0, 4.5, and 7.0) and temperatures (40, 80, and 120 °C) at 15 MPa, 2 mL/min, and solvent-to-feed mass ratio equal to 40. SPE was optimized in a column packed with the adsorbent PoraPakTM Rxn. Different ethanol concentrations (50, 75, and 100%) and temperatures (30, 40, and 50 °C) were evaluated for the anthocyanin's elution. The optimal conditions of the two experimental designs were determined by the RSM, firstly for PLE: 71 °C and pH 2; then using this PLE condition, the optimization of the SPE was obtained: 30°C and 50% ethanol. The developed PLE method provided similar anthocyanin yield to other techniques, and the coupling with SPE in-line produced an extract 5-fold more concentrated than PLE alone. Therefore, the system (PLE-SPE×HPLC-PDA) proved to be a powerful tool for monitoring the extraction process in real-time.

Keywords: Anthocyanins; Açaí; Green extraction; Solid-phase extraction; On-line Analysis

5.1 INTRODUCTION

Euterpe oleracea is a native palm tree of the Amazon region whose fruits are popularly known as açaí. Recently, due to açaí's high energy and nutritional value, the fruit has received attention from the food, pharmaceutical, and cosmetic industries (de Jesus et al., 2020; P. S. Melo et al., 2021). The açaí pulp is rich in bioactive compounds related to antioxidant, anti-inflammatory, antiproliferative, antimicrobial, analgesic, and cardioprotective properties (Costa et al., 2018). Anthocyanins are one of the main bioactive compounds in the açaí pulp and are classified as polyphenols belonging to the flavonoid group. They are water-soluble pigments responsible for colors in shades of red, blue, and purple, whose intensity depends on the concentration of the compound in the raw material (Dantas et al., 2021).

Interest in developing anthocyanins-based products has grown mainly due to their nutritional attributes and coloring power (Yousuf et al., 2016). The consumption of anthocyanins-rich products has been shown to exert preventive effects against many diseases (Bakuradze et al., 2019; Jamar et al., 2017). In addition, due to its intense purple color and functional properties, anthocyanins have also been used to produce natural dyes to replace synthetic ones (Sigurdson et al., 2017).

Obtaining anthocyanins from açaí requires extraction processes employing polar solvents. Nonetheless, dried açaí pulp presents a high lipid content (about 50 wt.%) (Buratto et al., 2021; Lucas et al., 2018; M. O. Souza et al., 2010), which also claims a first extraction process to obtain the lipid fraction preferentially without extracting anthocyanins. Supercritical fluid extraction (SFE) using carbon dioxide (CO₂) as the solvent has been successfully used for this purpose (Carolina et al., 2022; de Cássia Rodrigues Batista et al., 2016; M. P. Silva et al., 2019a). Since the supercritical CO₂ extracts the nonpolar compounds and leaves the raw material free of solvent without damaging the remaining compounds, it can selectively remove different types of compounds (Martínez et al., 2021). Açaí's oil has a lipid profile rich in mono and polyunsaturated fatty acids, respectively, 68-71% and 7.7-10.6% (Nascimento et al., 2008; Rogez, 2000), which makes the oil a valuable product for industrial application, given its unique sensory properties and potential health benefits (Silva & Rogez, 2013).

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Therefore, the dried açaí pulp can be submitted to sequential extraction processes to produce extract fractions with different chemical compositions and high concentrations of the desired compounds.

Several techniques have been applied to recover anthocyanins from a wide range of fruits and vegetables (de Sousa Sabino et al., 2021; Ferreira et al., 2020; Gadioli Tarone et al., 2021; Kurambhatti et al., 2020; Loarce et al., 2021; Teixeira et al., 2021). The selection of extraction methods and operational conditions are essential to produce extracts with high yield and well-defined composition and functionality (M. C. Souza et al., 2021). In this context, pressurized liquid extraction (PLE) is an effective method to recover polar compounds (such as anthocyanins) by applying polar and green solvents, such as ethanol and water (de Sousa Sabino et al., 2021; Papagiannopoulos et al., 2002; Teixeira et al., 2021). PLE uses solvents at high temperatures and pressure to allow the solvent to remain in the liquid state even at temperatures above the normal boiling point (Wianowska & Gil, 2019). Another important effect of PLE is the decreasing polarity of water at increasing temperatures (Haar et al., 1984; Miller & Hawthorne, 1998). Higher temperatures, except for thermosensitive compounds, provide higher extraction yields since temperature increases mass transfer and extraction rate, mainly due to the improved ability to solubilize the extract, break solute-matrix bonds, decrease solvent viscosity, and reduce the surface tension. The effect of PLE temperature on anthocyanins extraction has already been studied and reported for different raw materials. Nonetheless, strong divergences between optimal temperatures are commonly found in the literature, e.g., it has been reported optimal temperatures range from 40 to 100 °C (Aliaño-González et al., 2020; Machado et al., 2015; Pereira et al., 2019; Zou et al., 2011), which can be associated with the required energy to desorb and solubilize the anthocyanins in a shorter time able to avoid their thermal degradation. In addition to the temperature, the higher pressure improves the direction of solvent to the raw materials' pores and increases the target compound's solubility (Viganó et al., 2022).

The choice of solvents recognized as safe, such as ethanol and water, meets the environmental requirements of the green chemistry and makes PLE an eco-friendly process for an integrated biorefinery (Pagano et al., 2021; Raut et al., 2015). Indeed, PLE has been successfully integrated with SFE to recover different extract fractions (Viganó

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et al., 2016). However, PLE is generally not selective among the polar compounds (Viganó et al., 2021). Depending on the raw material target compound concentration, a large solvent volume is required to achieve a high extraction yield. To overcome this challenge, PLE has been coupled in-line with solid-phase extraction (SPE) (L. C. da Silva et al., 2020; M. C. Souza et al., 2021). SPE is widely used to remove unwanted compounds and concentrate complex samples, allowing a high level of automation and eliminating post-extraction steps such as centrifugation, filtration, and heated concentration (Hoff & Pizzolato, 2018; Viganó et al., 2021).

Moreover, PLE allows coupling analytical tools such as chromatography, making feasible the on-line detection of target compounds simultaneously with extraction in a closed system, usually automated (Clavijo et al., 2015; Liang & Zhou, 2019). From an analytical chemistry perspective, the coupling of these techniques may provide additional benefits, including higher speed to obtain results, sensitivity, reliability (Frenzel & Markeviciute, 2017; Mejía-Carmona et al., 2019), and the possibility of avoiding disadvantages associated with traditional analytical approaches, like sample loss and contamination at each preparation stage (Rutkowska et al., 2019; Sammani et al., 2019). On the other hand, the coupling may reduce the time to obtain product characterization and allow fast decision-making in industrial processes, which avoids product losses as reprocesses.

Considering what was introduced, the present study aimed to evaluate the anthocyanins extraction and concentration from dried and semi-defatted açaí pulp pretreated by SFE. Green technologies based on coupling PLE with in-line purification through SPE were used. Additionally, on-line analysis of extracts was performed by high-performance liquid chromatography (HPLC). The presented work is the first to deal with simultaneous extraction, purification, and analysis of anthocyanins in a single step to the best of our knowledge.

5.2 MATERIAL AND METHODS

5.2.1 Materials

The standard used in the HPLC were cyanidin-3-glucoside and cyanidin-3rutinoside (Sigma Aldrich, St. Louis, MO, USA). HPLC-grade acetonitrile was supplied by Dinâmica[®] (Indaiatuba, SP, Brazil). Ultrapure water was obtained from Millipore Milli-Q Simplicity[®] system (Burlington, MA, USA). Acetic acid (99.7%), Ethanol (99.5%), and phosphoric acid (85%) were purchased from Dinâmica[®] (Indaiatuba, SP, Brazil). The adsorbent was PoraPak[™] Rxn, purchased from Waters Corporation (Milford, MA, EUA).

The fresh açaí fruits, employed as raw material, were collected from cultivation in the Amazon region - 1° 21′ 46″ S, 47° 18′ 25″ W (Bonito, PA, Brazil). Managed/Cultivated açai is the predominant form of production in the Amazon as of 2015 (Maciel-Silva et al., 2021). Açaí pulp was manually obtained and immediately frozen, transported to the research laboratory, and freeze-dried. Dried pulp was semi-defatted by supercritical fluid extraction (SFE) using carbon dioxide (CO₂) as the solvent (99.9% purity, White Martins Gases Industriais Ltda, Sumaré, SP, Brasil). SFE was carried out in an apparatus described by (Viganó et al., 2016) at 40 °C, 35 MPa, and solvent-to-feed mass ratio (S/F) equal to 80. About 70 g were loaded in each extraction batch, and the solvent flow rate was 18 g/min. The SFE yield was 31,20 \pm 1,49 wt.%. The SFE açaí extract was recovered and stored for another application, and the semi-defatted pulp was recovered and stored at -20°C for further use in this work. The semi-defatted raw material presented moisture of 2.22 \pm 0.15 wt % and lipid content of 14.13 \pm 0.82 wt.%.

5.2.2 PLE-SPE×HPLC-PDA apparatus

The experimental trials were carried out in a new system coupling PLE with inline SPE and on-line HPLC (patent-pending, BR 1320210187220). The system was conceptually divided into two modules, one based on PLE and SPE and the other dedicated to the HPLC. The modules can be operated independently or simultaneously. The main components of the system are HPLC pumps, degasser, PDA detector, six-port valve, and XBridge C18 Column, both from Waters Corporation (Milford, MA, USA), in addition to a PLE Cell (15 mm x 100 mm I.D.) and an SPE column (4.6 mm x 50 mm I.D.). The simplified system configuration is shown in **Figure 1**.



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Figure 1. Schematic diagram of the integrated system (PLE-SPExHPLC-PDA). Gray line: Module one (PLE-SPE); Green line: Module two (HPLC-PDA). On the 6-port valve, dotted line: Inject position; continuous line: Load position.

5.2.3 PLE-SPE experimental design

Two experimental designs were carried out to optimize the PLE and SPE processes. At first, the experiments were off-line performed (without coupling SPE and HPLC to the PLE) to evaluate the PLE variables. The planning applied to optimize the PLE was the face-centered central composite design (FCCCD) of two variables and three levels, using the response surface methodology (RSM) to characterize the advantages, efficiency, simplicity and also to provide the effects and interaction on the responses (Jankovic et al., 2021; Wang et al., 2018). The variables and input levels used to optimize the extraction process were: X1, extraction temperature (40, 80, and 120 °C); and X2, water pH (2.0, 4.5, and 7.0) acidified with phosphoric acid. The pressure was set to 15 MPa, the flow rate to 2 mL/min, and the S/F equal to 40 kg H2O/kg semi-defatted açaí pulp during extraction. The response variable (Y) was the total anthocyanins yield, e.g., the sum of cyanidin-3-glucoside and cyanidin-3-rutinoside, the two main anthocyanins present in açaí. All extractions were performed in duplicate, totalizing 18 experiments. The recovered extracts were filtered through a 25 mm x 0.22 µm nylon syringe filter from Analítica (Campinas, SP, Brazil) for further off-line chromatographic analysis.

After optimizing the total anthocyanin yield from PLE, the global extraction yield (X_0) in the optimal condition was evaluated, calculated by the mass ratio between the dry extract (mext) and the dried and semi-defatted raw material (*F*), as shown in **Equation 1**. X_0 was determined by drying a 10 mL aliquot of sample extract at 105 °C in a laboratory oven (Fanem, 315-SE, São Paulo, Brazil) until constant weight. mext was calculated by multiplying the solids content obtained (g/mL) by the volume (mL) of extract recovered from the extraction.

$$X_0$$
 (%) = $\frac{M_{ext}}{B}$.100 Eq. (1)

The planning applied to optimize the in-line PLE-SPE process was a 2^2 factorial design with a central point. For this, the optimal PLE conditions previously determined were used. Thus, the SPE process input variables and levels were: X₁, SPE column temperature (30, 40, and 50 °C); and X₂, ethanol concentration for desorption (50, 75, and 100%). The response variables were the total anthocyanin yield that the adsorbent

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failed to retain in the SPE column during the PLE process (Y_1) and the total anthocyanin yield obtained after desorption (Y_2) . The central point SPE experiments were done in triplicate and the other conditions in duplicate, totalizing 11 experiments. The experimental designs for PLE and SPE are shown in **Table 1**.

| D | PLE Conditions | | |
|-----|------------------|----------------|--|
| Kun | Temperature (°C) | pH* | |
| 1 | 40 | 2.0 | |
| 2 | 40 | 4.5 | |
| 3 | 40 | 7.0 | |
| 4 | 80 | 2.0 | |
| 5 | 80 | 4.5 | |
| 6 | 80 | 7.0 | |
| 7 | 120 | 2.0 | |
| 8 | 120 | 4.5 | |
| 9 | 120 | 7.0 | |
| | SPE Conditions | | |
| Run | Temperature (°C) | Ethanol (%) | |
| 1 | 30 | 50 | |
| 2 | 30 | 100 | |
| 3 | 40 | 75 | |
| 4 | 50 | 50 | |
| 5 | 50 | 100 | |

Table 1. Experimental design for determination the best PLE and SPE conditions.

* Water acidified with phosphoric acid.

5.2.4 PLE-SPE methods

PLE is performed in a stainless-steel extraction cell with a semi-continuous flow (batch for solid and continuous for liquid). The cell is packed with a layer of glass wool, sample, another layer of glass wool, and the cell volume is filled with glass pearls. The SPE column was packed with adsorbent and connected to the system. Adsorbent activation was performed through the by-pass with 30 mL ethanol and conditioned with 30 mL water. The PLE process was carried out in a semi-continuous mode. For this, 1.5 g of semi-defatted açaí pulp was loaded in the extraction cell, and water continuously flowed at 2 mL/min for 30 min at the desired temperature and pH. For each PLE

experiment, 60 mL of extracts were collected in a container. After the extraction, desorption from the adsorbent was started using the by-pass and applying ethanol as eluent at 0.25 mL/min and the different concentrations described in Section 2.3. At this stage, the volume collected varied according to the SPE conditions adopted, considering its interference with the extract concentration and the elution rate.

After optimizing the off-line PLE and in-line PLE-SPE, the experiments were carried out with the new fully coupled system (PLE-SPE×HPLC-PDA). PLE-SPE and on-line HPLC analysis were connected through the six-port valve responsible for making the interface between the extraction and analysis module. The valve has been programmed to change from "LOAD" to "INJECT" position every 6 minutes, and 5 μ l from the PLE-SPE module were injected into the HPLC module. The ethanol flow rate during the desorption was decreased to 0.25 mL/min aiming to allow more injections and consequently a more detailed desorption kinetic profile.

5.2.5 Comparison with other extraction techniques

The comparison with other extraction techniques was made with the optimized PLE-SPE method. The S/F equal to 40 and extraction time of 30 min were maintained in the relative extraction techniques. The solvents used were ethanol, ultrapure water at pH 7.0, and ultrapure water acidified with phosphoric acid at pH 2.0. The extraction techniques evaluated were heating under orbital agitation and ultrasound-assisted extraction (UAE). The first took place in a Tecnal® TE-421 orbital incubator (Piracicaba, SP, Brazil) at 71 °C and 100 rpm. The UAE was performed in a SolidSteel® SSBu-6L ultrasound bath (Piracicaba, SP, Brazil), maintained at 35 °C and operating at 40 kHz and 100% power (100 W). After extraction, the samples were centrifuged at 4,000 rpm for 10 min; the supernatant was collected and filtered through a 25 mm x 0.22 µm nylon syringe filter from Analítica® (Campinas, SP, Brazil) for further chromatographic analysis. All extractions were performed in duplicate.

5.2.6 HPLC-PAD analysis

The samples from the off-line and on-line processes were analyzed in the same equipment by a Waters HPLC, equipped with a binary pump with column heater (1525),

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in-line degasser (AF 186001273 DG2), and photodiode array detector (2998). The analytical column was an XBridge BEH C18 (130Å, 3.5 µm, 4.6 mm x 50 mm). An isocratic separation method was applied for the anthocyanins analysis in which the mobile phase was composed of water (solvent A) and acetonitrile (solvent B), both containing 2.5% acetic acid (v/v). The elution conditions applied were: 5% B for 6 min, flow rate 1.0 mL/min, and column temperature of 50 °C. The injection volume was five µL, and Chromatograms were obtained at 520 nm. The identification of the compounds was carried out by comparing the retention times of the compounds separated from the samples with the retention times of the standard compounds. Quantification was performed using calibration curves, cvanidin-3-glucoside (y = 14725x - 1226.2; $R^2 =$ 0.9994) and cyanidin-3-rutinoside (y = 11290x + 8566.8; $R^2 = 0.9997$), both with six points ranging the concentration from 3.125 to 100 mg/L. All off-line analyses were performed in duplicate. The total anthocyanin yield was calculated by summing the cyanidin-3-glucoside (C3G) and cyanidin-3-rutinoside (C3R). The result was expressed in mg total anthocyanin yield / g of dried and semi-defatted raw material (mgTAY/gDDRM).

5.2.7 Statistical analysis

The results were expressed as mean \pm standard deviation. In the experimental designs, statistical analysis was performed to assess the effect of factors under the response variables. The interactions were tested statically by ANOVA at a significance level of 95% (p \leq 0.05). The Tukey test was run for mean comparisons. Additionally, the response variables were optimized using the SIMPLEX method. All the analyses were run in STATISTICA 7 software by StatSoft Inc. (Round Rock, TX, United States).

5.3 RESULTS AND DISCUSSION

5.3.1 PLE optimization

The first steps were to freeze-dry the açaí pulp to obtain a solid material suitable for the PLE process, resulting in a dry material with 2.2% moisture. Then, 31.2% of lipids were removed using SFE with CO₂, approximately 70% of the total lipids in the sample. The total lipids were 45.3% (dry basis), determined by Soxhlet extraction, a value that corroborates the literature reports (Buratto et al., 2021; Lucas et al., 2018; M. O. Souza et al., 2010). Soxhlet consists of an intermittent method that uses heated organic solvent reflux, and it is the classic laboratory method used to extract oils. However, the solvents used are toxic, flammable, expensive, and environmental pollutants. In addition, high temperatures degrade thermosensitive bioactive compounds and leave chemical residues after the extraction process (Danlami et al., 2014). SFE using CO₂ was chosen for its non-toxic, non-flammable, and non-polluting properties. Additionally, the use of pressurized CO₂ results in the absence of chemical residues, for being a gas at room temperature and atmospheric pressure and, hence easily removed after the extraction (M. M. R. de Melo et al., 2014; Týskiewicz et al., 2018).

PLE was firstly off-line performed to understand the effect of the variables on the anthocyanins' extraction yield and the kinetic extraction behavior and to optimize the process without coupling the SPE and the HPLC. The extraction yield of the two and the total anthocyanins are shown in **Figure 2**.

The information from the analysis of variance (ANOVA) for PLE are listed in **Table 1 of Supplementary Material**. According to the statistical results, the regression model was highly significant (P < 0.05), and the coefficient of determination ($R^2 = 0.9929$) and the coefficient of determination adjusted ($R^2 = 0.9812$) suggest that the model is highly accurate and reliable. Furthermore, the non-significant lack of fit showed that the selected model better fits the predicted response. Additionally, the value of the coefficient of variance (CV = 6.01) was less than 10%, so the data have low dispersion and high homogeneity, reinforcing the statistical strength of the model and its adequacy to the experimental data. The second-order empirical polynomial model for açaí total anthocyanin yield by PLE is presented in **Equation 2**.

$$Z = 2.524 - 0.814X + 0.0456X^{2} + 0.057Y - 0.00048Y^{2} + 0.00252XY$$
 (Eq. 2)

Where, Z represents the total anthocyanin yield responses; X and Y are the independent variables, pH and temperature, respectively.



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Figure 2. Anthocyanin extraction yield obtained by PLE at S/F equal to 40. Results are the replicate means with standard deviation estimates. Same lower-case letters do not differ between each anthocyanin yield, and the same upper-case letters do not differ between total anthocyanin yield ($p \le 0.05$) [ANOVA and Tukey test].

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The modeling of the PLE process and optimization of operational parameters for maximum yield of total anthocyanins from açaí were performed using RSM, which is a collection of statistical methods for designing experiments, building models, evaluating the impact of processing parameters on responses, and optimizing processes (Shafi et al., 2018). **Figure 3** illustrates the response surfaces graphs, where **Figure 3** (**A**) is threedimensional – 3D and **Figure 3** (**B**) is two-dimensional – 2D. On the 3D response surface, the slope of the surface indicated the degree of influence of the variables on the response value; the more accentuated the slope, the greater the interaction between them. Furthermore, the color of the response surfaces is a preliminary determination of the optimal conditions and can be better observed on the 2D surface. The color tended to increase in yield with the change from green to red. The trends generated by the variation of the independent variables (Temperature and pH) were different. The increase in temperature provided a greater yield of anthocyanins to some extent, followed by a steady decline.

On the other hand, the pH generated an increasing trend in the dependent variable as the pH values decreased. **Table 2** presents the optimized conditions for PLE. According to the results of the optimization study, the optimal conditions were pH 2.0 and extraction temperature 71 °C. The maximum value predicted by the model was 3.33 mgTAY/gDDRM.

| Fable 2. | Optimized | conditions | for PLE |
|----------|-----------|------------|---------|
|----------|-----------|------------|---------|

| PLE Conditions | Total Anthocyanin Yield | |
|------------------|-------------------------|---------------------|
| Temperature (°C) | pН | (mgTAY/gDDRM) |
| 71 | 2.0 | $3,\!01 \pm 0,\!15$ |

The extraction kinetics was performed in duplicate; the results are shown in **Figure 4** to validate the proposed conditions. The individual concentration of the major anthocyanins of açaí during the 2 h kinetics is presented in **Figure 4** (**A**). The duration of the PLE process occurred until the extract's concentration started to fall below the limit of quantification. In **Figure 4** (**B**), it is observed that the chromatograms show peaks with large areas in the first points of the kinetics that rapidly decay and further exhibit a smoother decay.



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Figure 3. Response surface plots of total anthocyanin yield showing interaction effects of PLE parameters. (A) three-dimensional -3D and (B) two-dimensional -2D.



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Figure 4. Extraction kinetics of PLE at 71 °C and pH of 2.0. (A) Accumulated yield of anthocyanins; (B) Chromatograms at 520nm; and (C) Visual apparency of fractions collected.

Corroborating this fact, **Figure 4** (**C**) shows this behavior through the extracts coloring throughout the kinetics. At the end of the kinetics, the yield was 4.37 ± 0.13 mgTAY/gDDRM, being 1.47 ± 0.03 mgC3G/gDDRM and 2.90 ± 0.09 mgC3R/gDDRM. When considering the extraction over 30 min (S/F equal to 40), the total anthocyanin yield was 3.01 ± 0.15 mgTAY/gDDRM, 91% close to the value predicted by the model. On this occasion, the S/F of 40 and yields approximately 70% of the maximum anthocyanins from açaí extracted by the PLE process in 2h. However, the spent time is 4-fold lower, which justifies the choice of the S/F used.

After the validation of the extraction method, an extraction test was performed to assess the importance of performing SFE in the sample. The total anthocyanins yield for the only freeze-dried sample was $1.48 \pm 0.02 \text{ mgTAY/gDDRM}$, indicating that a high concentration of lipids in the sample significantly affects the extraction of anthocyanins from açaí as well as could make purification difficult by the SPE process. According to (Alcázar-Alay et al., 2017; Moraes et al., 2015) the initial removal of oil via SFE favors the sequential separation of bioactive compounds by modifying the sample structure and increasing the proportion of analytes. Therefore, removing lipids by SFE is justified to improve the yield of anthocyanin extraction and to take advantage of açaí oil, a valuable by-product of excellent composition with good sensory properties and rich in health-beneficial properties (Silva et al., 2019b).

The overall yield of the PLE Extraction was 11.18 ± 1.42 % (g/gDDRM) in the optimal condition of 71°C and acidified water at pH 2.0 with S/F equal to 40. Usually, the X₀ is higher at temperatures extraction rates, due to increased desorption of compounds from the raw material matrix, weakening van der Waals forces, hydrogen bonding and dipolar attraction. Furthermore, high temperatures decrease the surface tension of the solvent, solute and matrix, decreasing the solvent viscosity, increasing diffusion within the matrix particle, and finally increasing the mass transfer of molecules in the solvent (Mustafa & Turner, 2011; Wijngaard et al., 2012). However, the highest overall yield is not always the most attractive answer, as co-extraction of undesirable groups of molecules increases the yield but decreases the concentration of target molecules.

5.3.2 SPE optimization

The results obtained from the experimental design in which PLE was coupled with SPE are shown in **Figure 5**. The objective of the performed planning was to reduce total anthocyanins loss during the adsorption; in other words, the amount of anthocyanins trapped by the adsorbent would be maximized. After extraction/adsorption the elution (desorption) was performed, and in this case, a maximum yield was desired.

The statistical results presented in **Table 2 of the Supplementary Material** demonstrated that a highly significant regression model was obtained for temperature (P < 0.05). The model aimed at greater anthocyanin yield after the desorption step portrayed the coefficient of determination ($R^2 = 0.9797$) and the adjusted coefficient of determination ($R^2 = 0.9392$). Hence, the model presented a coefficient that suggests high precision and reliability. Furthermore, the lack of fit of the model was not significant, showing that the selected model predicted good responses. Additionally, the value of the coefficient of variance (CV = 5.64) was less than 10%, so the data have low dispersion and high homogeneity, reinforcing the statistical strength of the model and its adequacy to the experimental data. The second-order empirical polynomial model for the desorption project in SPE is shown in **Equations 3**.

$$Z = 1.69 + 0.0258X - 0.000243X^2 - 0.038Y + 0.00014XY$$
 (Eq. 3)

Where, Z represents the total anthocyanin yield responses; X and Y are the independent variables, temperature and ethanol concentration, respectively.

Through the RSM, the optimization of the operational parameters for the two stages of the SPE process was carried out. **Figure 6** illustrates the response surface graphs; subfigures (**A**) and (**B**) are from the desorption stage. During the SPE adsorption step, the only significant variable is temperature. At this moment, what is happening is the purification of the extract, and the retention of anthocyanins, the solvent that is passing through the SPE column is water at pH 2.

The ethanol concentration did not reach significance (p < 0.05) in the desorption step model, mainly because the loss of anthocyanins in the adsorption was very high at the highest temperature (50°C), which strongly impacted the anthocyanins yield in


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Figure 5. Results to determine the best conditions for SPE of anthocyanins from açaí. Results are the replicate means with standard deviation estimates. Same lower-case letters do not differ between adsorption tests, and the same upper-case letters do not differ between desorption tests ($p \le 0.05$) [ANOVA and Tukey test].



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Figure 6. Response surface plots of total anthocyanin yield showing interaction effects of SPE parameters. (A) Three-dimensional – 3D of yield in desorption and (B) two-dimensional – 2D of yield in desorption.

desorption. Temperature is highly influential in the adsorption process (Pivokonsky et al., 2021). At first, where the PLE process is being carried out together with in-line adsorption, the SPE column temperature is the only significant variable. Thus, 30 °C is the condition that promotes less anthocyanins loss and consequently greater retention of the compound on the SPE column. According to Park et al. (2017), adsorption is affected by the temperature of the solution and favored at its lowest values.

Considering the desorption process, the ethanol concentration affects the response variable (**Figure 5**). In this aspect, the ethanol concentration was the most significant for the desorption regression model, with p close to 0.05 (P = 0.06). **Figures 6** (**A**) and (**B**) show circular level curves, confirming a more significant interaction between the independent variables. The desorption model and the graphic indication through the more intense red color show that 30°C and 50% ethanol are the optimal conditions for desorption.

The SPE optimization study was essential to determine the best process conditions. However, a considerable loss of anthocyanins was observed (**Figure 5**). As the SPE temperature increased, the adsorption losses increased. Only at a temperature of 30 °C the anthocyanin yield in desorption be more significant than the loss in adsorption. In the optimal condition (30 °C and 50% ethanol), approximately 50% of the total anthocyanins obtained in the PLE process were recovered. This low yield was probably due to an insufficient amount of adsorbent used. The SPE process contained 0.25 g of the PoraPakTM Rxn. Therefore, a new SPE column was made (4.6 mm x 100 mm), with the same diameter and twice as long as the previous one, which now fits 0.5 g. **Table 3** presents the optimized conditions for SPE.

| Table 3. | Optimized | conditions | for | SPE. |
|----------|-----------|------------|-----|------|
|----------|-----------|------------|-----|------|

| SPE Cond | itions | Total Anthocyanin Yield (mgTAY/gDDRM) | |
|------------------|-------------|--|--|
| Temperature (°C) | Ethanol (%) | | |
| 30 | 50 | $2.54\pm0,\!08$ | |

5.3.3 PLE-SPExHPLC-PDA

After optimizing the PLE and the PLE-SPE, the system was configured to perform the PLE coupled in-line with SPE and on-line with HPLC. Therefore, allowing the simultaneous extraction, concentration, and analysis of the produced extract. The results are shown in **Figure 7**. The extraction yield of the major anthocyanins in açaí along the kinetics is shown in **Figure 7** (**A**). In the first 30 min of the process, both anthocyanins extraction yields were very low (under the limit of quantification), showing the retention in the adsorbent. After when the desorption begins, a rapid increase in anthocyanin content is noted (**Figure 7** (**D**)), followed by fast stabilization, which demonstrates the efficiency of the developed process in concentrating the extract. The PoraPakTM Rxn adsorbent used in the SPE, in addition to purifying the solution, was responsible for a 5fold higher concentration, as the anthocyanins were eluted in a smaller volume than the adsorbed volume (60 mL), with only the first 6 points of desorption being collected (12 mL). The remaining subfigures present other ways of looking at the complete extraction, purification/concentration, and on-line detection system.

Figure 7 (B) shows the three-dimension chromatogram. The detector signal is presented on the y-axis, the x-axis shows the chromatographic separation, and the z-axis gives the separation achieved by the PLE-SPE. Such a figure illustrates qualitative data that corroborates **Figure 7 (A)**. At first, the detection showed no signal of anthocyanins, showing the period of PLE with adsorption in SPE. Once this period ended, the desorption in SPE started, and there were intense peaks with high absorbance followed by a decrease until no signal observation, indicating the complete desorption. In **Figure 7 (C)**, the results obtained from the SPE present the losses in the adsorption step and the yield in the desorption step. The efficiency of the SPE process proved to be satisfactory with the new SPE column. 2.54 \pm 0,08 mgTAY/gDDRM of total anthocyanins were recovered, representing 84% of the total obtained in the PLE. **Figure 7 (D)** shows the visual apparency of the extract fraction. Although the color reflects what was observed in **Figures 7 (A)** and **7 (B)**, the anthocyanins are retained in the SPE column when starting the PLE process.



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Figure 7. Extraction kinetics under optimal PLE (71 °C and pH of 2.0) and SPE (30 °C and 50% ethanol) conditions with the complete integrated system (PLE-SPExHPLC-PDA). (A) Accumulated yield of anthocyanins; (B) Chromatograms of the extract fraction at 520nm; (C) SPE total anthocyanin yield; and (D) Visual apparency of fractions collected.

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Over time, the adsorbent presents a decrease in its absorptive capacity. It can be seen from the change in the color, from the initial transparent to reddish color with a slight increase in intensity during the kinetics to the point where desorption starts, with a change in the extraction solvent (water pH 2) to the solvent of elution (50% Ethanol), at that moment a solution with a dark red was obtained. This one, during desorption, exhibits a faster decay in color intensity compared to PLE (**Figure 4 (C)**), considering that the compounds come out in a much smaller volume.

Through the different ways used to analyze the results, it was possible to evaluate the efficiency of the developed process. The HPLC method conditions provided a short analysis time, allowing the collection of enough chromatograms to plot individual kinetic extraction curves and to produce a comprehensive chromatogram. Through the visual analysis of the collected extract fractions, it is possible to easily follow the changes, even if the concentration of the analytes is below the quantification limit by the HPLC. The greater amount of adsorbent used in the new SPE column was essential for the excellent performance of the complete process. Hence, the necessary purification for on-line detection and concentration of anthocyanins enabled a satisfactory yield without excessive losses.

Unfortunately, it was impossible to determine the extract yield for every condition. The amount of extract produced is small when using a few grams of sample. This small extract mass is also spread over several fractions collected during the process, resulting in only a few milligrams of extract in each fraction. Evaporating the solvent with a few milligrams of the solid extract is difficult and prone to losses, leading to errors. However, it is essential to highlight that when using an adsorbent, "contaminants" (such as sugars and other more polar compounds) are not retained and are removed from the extract. Therefore, due to the increased selectivity of the adsorbent for the anthocyanins and the removal of more polar compounds, it is expected that their relative concentration in the extract (mg of anthocyanin/g of extract) will be higher than those obtained with conventional techniques.

5.3.4 Comparison of PLE with other extraction techniques

PLE with in-line SPE and on-line HPLC was investigated as green and rapid technology for extracting and purifying anthocyanins from açaí. The conditions of PLE, extraction, temperature of 71 °C, and water as solvent at pH 2.0, recovered the highest concentration of anthocyanins (3.01 mgTAY/gDDRM). **Figure 8** shows the results obtained from the other evaluated extraction techniques, orbital stirring and UAE, using three green solvents (ethanol, water, and acidified water at pH 2.0), maintaining the extraction time parameters (30 min) and S/F ratio (40) similar to those in PLE.

Figure 8 (A) illustrates the anthocyanin yield obtained using the agitation technique at 71 °C, the extraction temperature optimized in the PLE technique. Water at neutral pH was the solvent with the lowest total anthocyanin yield $(0.79 \pm 0.05 \text{ mgTAY/gDDRM}, approximately 25\% of PLE yield). Although the optimum temperature for PLE was 71 °C, the process was semi-continuous with low residence time, while the extraction agitation was carried out in batch mode; that is, the residence time of the solvent was the time of the entire process (30 min.). Although raising the extraction temperature to some extent improves the anthocyanins yield ($ **Figure 2**), long residence times at a constant temperature result in logarithmic degradation of anthocyanins (Alvarez-Suarez et al., 2021). Mainly from cyanidin-3-glucoside, which proved to be more thermosensitive to water (pH 7.0).

The total of anthocyanins in açaí is given by the sum of the two significant anthocyanins. The proportion between them varied a lot between the different solvents. For water (pH 7.0), the cyanidin-3-glucoside concentration was 5-fold lower than the cyanidin-3-rutinoside concentration in the agitation technique. While for ethanol, there was no significant difference between the cyanidin content. For water (pH 2.0), the cyanidin-3-rutinoside value was twice of cyanidin-3-glucoside, a proportion similar to that observed in other techniques (PLE and UAE) for this solvent. It is important to point out that the aim was to compare the developed method with other extraction methods in similar conditions of temperature and S/F.



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Figure 8. Comparison between different extraction techniques and solvents. (A) Agitation Extration; (B) Ultrasound-Assisted Extration; (C) Extract collected Agitation Extration; and (D) Extract collected Ultrasound-Assisted Extration.

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Regarding the solvent, besides the water, ethanol was used for comparison due to greener issues. It is also worth mentioning that organic mixtures, including methanol, are known to produce higher anthocyanins yield; however, only ethanol and water were selected due to the toxic character of methanol. On the other hand, mixtures of ethanol and water also have reported higher anthocyanins yield (Raut et al., 2015; Viganó et al., 2022; Wianowska & Gil, 2019); however, such solvent is not suitable for SPE. In that case, a previous step to SPE is needed to evaporate the organic fraction.

There was no significant difference between the three extraction techniques in total anthocyanins using water at pH 2.0 as solvent. The stability of anthocyanin pigments is affected by several factors: pH, temperature, enzymes, light, and oxygen (Chen & Stephen Inbaraj, 2019). The pH is highly relevant for the stability of anthocyanins, which are more stable in acidic media (Echegaray et al., 2020). For this reason, acidified water was the best solvent.

The UAE results are shown in **Figure 8** (**B**). The temperature applied was 35°C due to the limitations of the available ultrasound bath. Temperature plays an essential role in the totality of anthocyanins (Zhang & Jing, 2020). Thus, the solvent that showed the most significant difference concerning the total anthocyanin yield between the studied techniques was water (pH 7.0). The UAE presented a good yield ($2.22 \pm 0.09 \text{ mgTAY/gDDRM}$), even better than ethanol ($1.53 \pm 0.11 \text{ mgTAY/gDDRM}$). Ethanol showed a greater yield in the agitation technique ($1.88 \pm 0.10 \text{ mgTAY/gDDRM}$).

Figure 8 (**C**) and (**D**) show the color of the extracts collected for agitation and UAE extraction, respectively. The color variation of the solutions results from the extraction techniques and the different configurations of anthocyanins obtained mainly by solution pH change (Freitas et al., 2020). The chemical structure of anthocyanins affects their stability and color (Echegaray et al., 2020). At pH values below 3.0, the flavylium cation responsible for the red color is the predominant form of anthocyanins (Ghareaghajlou et al., 2021). When increasing the pH to close to 7.0, the structure of anthocyanins changes from a flavylium cation to a neutral quinoidal base, resulting in red color (Moloney et al., 2018). The green coloration in the ethanolic solution is due to the extraction of traces of more fat-soluble compounds such as chlorophyll that may not have been completely removed in the SFE. And also, due to the 8.5

pH of the ethanol used, the color of the anthocyanin solution changes to green at pH above 7.0 due to the formation of natural and anionic chalcones (Fenger et al., 2019).

5.4 CONCLUSION

Coupling of PLE with in-line SPE and on-line HPLC was successful for extraction, purification, concentration, and simultaneous detection of anthocyanins from açaí. The preliminary process of SFE using CO₂ removed approximately 70% of the total lipids in the sample. The SFE was performed to enable an excellent on-line detection to improve the SPE purification step, increase the PLE yield, and take advantage of açaí oil, a by-product of excellent composition and high value-added. The best conditions to maximize anthocyanins yield by PLE were extraction temperature of 71 °C and water at pH 2.0 as solvent. The best conditions to obtain lower losses in the adsorption step and greater yield in the desorption step of the SPE were 30°C and 50% ethanol. The developed PLE method provided an anthocyanin yield similar to other extraction techniques, and water acidified to pH 2.0 was the best green extraction solvent.

Additionally, PLE with in-line SPE produced an extract 5-fold more concentrated than PLE alone. Coupling the on-line HPLC to the extraction process proved simple, as it was possible to obtain a short HPLC method with good separation of the different anthocyanins from açaí. The developed system (PLE-SPExHPLC-PDA) proved to be a powerful tool for monitoring the extraction process in real-time. The evidence presented in this study shows that the application of the developed method can be used for a wide range of samples and analytes.

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CHAPTER 6 – An automated system to integrate sequential extraction and hydrolysis

DESIGN, ASSEMBLY AND VALIDATION OF A NEW AUTOMATED SYSTEM TO INTEGRATE SEQUENTIAL EXTRACTION AND HYDROLYSIS COUPLED WITH INLINE SPE PURIFICATION AND ONLINE HPLC ANALYSIS

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ABSTRACT

The growing demand for healthy products that generate less impact on natural resources has driven the development of new ingredients and processes. Therefore, this study aimed to design, assemble and validate a new green processing system coupled with inline purification and online analysis to obtain high value-added products from natural raw materials. The automated system (PHWE/SubCWH \times SPE \times HPLC-PDA/RI) consists of coupling of pressurized hot water extraction (PHWE) and sequential subcritical water hydrolysis (SubCWH) with inline purification by solid phase extraction (SPE) and online analysis by high-performance liquid chromatography (HPLC) using photodiode array (PDA) and refractive index (RI) detectors. For validation, the recovery of anthocyanins, sugars and organic acids from black grape pomace (BGP) was evaluated. Twelve peaks of anthocyanin compounds were identified, and the extraction yield obtained by HPWE was 47.60 ± 0.69 milligrams of cyanidin-3-glycoside equivalent per gram of dried black grape pomace (mgC3G.gDBGP⁻¹). Seven organic acids and five sugars were identified and quantified after SubCWH. Tartaric acid represented 53% of the total organic acids and mannose 52% of the total sugars. Therefore, the newly developed system proved viable for performing HPWE and SubCWH of BGP with inline SPE purification and online HPLC analysis of different classes of natural compounds. The real-time monitoring of the products allowed for quick and efficient responses, shorter processing time, low consumption of solvents, less labor, using less energy and generating less impact on the environment.

Keywords: Black grape pomace, Recovery of natural compounds, Green processes, Solidphase extraction, High-performance liquid chromatography, on-line coupling.



CHAPTER 6 – An automated system to integrate sequential extraction and hydrolysis

GRAPHICAL ABSTRACT

6.1 INTRODUCTION

Due to the constant generation of solid by-products from the agri-food sector, sustainable technologies for waste management are urgently needed. The fractionation of biomass by-products is a challenge for effectively implementing a biorefinery. It requires a more eco-friendly, intensive, and faster process to manage large volumes of biomass at a decentralized location without the generation of effluents (Cocero et al., 2018; Maciel-Silva, Lachos-Perez, et al., 2022). From the perspective of an integrated biorefinery, the feedstocks could be exploited in an integrated close-to-zero waste system to recover bio-based products for the circular economy transition (Ferreira et al., 2021; Maciel-Silva et al., 2021). Beyond, one approach that could reduce the environmental impacts of extraction processes is the application of non-conventional green solvents. A green solvent should be environmentally friendly throughout its entire life cycle. The use of green solvents entirely fits the biorefinery concept offering low cost, simple preparation, negligible volatility, and a low toxicity profile (Panić et al., 2021).

Pressurized water under different temperatures has been studied as an alternative to conventional chemicals (Sarker et al., 2021). Subcritical water (SubCW) is defined as liquid water below its critical point maintained at a temperature of between 100 and 374 °C and pressure between 0.1 and 22.1 MPa (Ruiz et al., 2021). Supercritical water (SupCW) is defined as a state in which both the temperature and pressure of water are higher than the critical point, 374°C and 22.1 MPa (Cocero et al., 2018). The solubilization of macromolecules at SubCW and SupCW occurs at increased pressure and temperature without using hazardous chemicals (Gan & Baroutian, 2022). This characteristic is primarily used for hydrolysis biomass (hemicellulose, cellulose, and lignin) into oligomers and monosaccharides (Rostagno et al., 2015; Ruiz et al., 2023). The application of SubCW can be enclosed in both hydrolysis and extraction processes since it is possible to use high or moderate temperature and pressure to recover sugars and bioactive compounds. However, SubCW is still conducted at relatively high temperatures (>100°C), and depending on the target compound, the use of pressurized hot water (PHW) is an advantage, such as in the extraction of anthocyanins (T. Barroso et al., 2022; Maciel-Silva). Therefore, a critical step for recovering value-added bioproducts in a biorefinery

concept is the development of sustainable processes that can sequentially extract and hydrolyze biomass under different operational conditions (Lachos-Perez et al., 2022).

Nevertheless, normally these techniques require large volume of solvent to obtain high yield of extraction and hydrolysis, depending on the concentration of the target compound of the raw material, a significantly diluted product is obtained. To overcome this challenge, inline coupling with solid phase extraction (SPE) can be performed (Batchu et al., 2013; Campone et al., 2015). SPE is widely used to remove unwanted compounds, separate and concentrate complex samples, allowing a high level of automation and eliminating later sample preparation steps such as centrifugation, filtration and concentration (Hoff & Pizzolato, 2018).

In this way, to obtain close to full use of the resources, it is possible to carry out processes that sequentially integrate pressurized hot water extraction (PHWE) to recover bioactive compounds and hydrolysis in subcritical water (SubCWH) to obtain sugars and organic acids from the breakdown of biomass lignocellulosic (Lachos-Perez et al., 2020). Then perform coupling to analytical tools such as high performance liquid chromatography (HPLC), enabling online analysis of various biocompounds simultaneously with extraction and hydrolysis in a closed system (Borisova et al., 2016; Clavijo et al., 2015; Liang & Zhou, 2019). From the point of view of analytical chemistry, the coupling of these techniques can bring additional benefits, including greater sensitivity, reliability and speed to obtain results (Frenzel & Markeviciute, 2017; Mejía-Carmona et al., 2019). In addition to avoiding some problems associated with traditional analytical techniques, such as sample loss and contamination at each stage of preparation (Sammani et al., 2019). Additionally, application of time monitoring can reduce product characterization time and allow quick decision-making in industrial processes, which avoids product losses such as reprocessing (Viganó et al., 2021).

Technological advance is still necessary to increase the recovery of value-added bioproducts from lignocellulosic biomass by-products and to accelerate the application in the Industry 4.0. Based on mentioned above, this study developed a novel automated and sustainable system based on PHW and SubCW to integrate sequential extraction and hydrolysis of biomass with real-time monitoring of value-added bio-based products. The advantages of the process are enclosed in the optimization of sample preparation, real-time monitoring of the bioproducts generated, and minimizing experimental errors. This process will contribute to implementing an Industry 4.0, towards digitalization and automation of extraction and hydrolysis processes.

Black grape pomace (BGP) is the primary solid residue of wineries; only 30–40% of the anthocyanins contained in black grapes are extracted from the peel during wine production. Therefore, the BGP, which corresponds to 50% of the total mass of the black grape, is rich in bioactive compounds with functional properties, able to act on human metabolism, preventing degenerative, cardiovascular, and inflammatory diseases (Pereira et al., 2019). In addition to anthocyanins and other phenolic compounds, BGP has an attractive lignocellulosic composition for hydrolysis, making it possible to obtain fermentable sugars for the production of food bioproducts and biofuels. Thus, BGP ('BRS Vitória') was selected as a sample model to validate the automated system (PHWE/SubCWH × SPE × HPLC-PDA/RI) based on the coupling of PHWE and SubCWH with inline purification by SPE and online analysis by HPLC, using photodiode array (PDA) and refractive index (RI) detectors.

6.2 MATERIAL AND METHODS

6.2.1 Chemicals and sample

The standard used in the HPLC was cyanidin-3-glucoside (Sigma Aldrich, St. Louis, MO, USA). HPLC-grade acetonitrile was supplied by J.T.Baker[®] (São Paulo, SP, Brazil). Ultrapure water was obtained from the Millipore Milli-Q Simplicity[®] system (Burlington, MA, USA). Acetic acid (99.7%), ethanol (99.5%), and phosphoric acid (85%) were purchased from Dinâmica[®] (Indaiatuba, SP, Brazil). The adsorbent was PoraPakTM Rxn, purchased from Waters Corporation (Milford, MA, EUA).

The BGP used as sample in this work presented moisture of 79.67 ± 0.92 wt%. To obtain the BGP, the black grape ('BRS Vitória') was purchased in a supermarket in the metropolitan region of Campinas, SP, Brazil. The grapes were macerated, the BGP was separated from the pulp, crushed in a domestic blender and stored at -20 °C protected from light until the sequential extraction and hydrolysis experiments were carried out.

6.2.2 System components

The components used in the PHWE/SubCWH × SPE × HPLC-PDA/RI system were purchased individually. From Waters Corporation (Milford, MA, USA): 1515 Isocratic HPLC Pump; 1525 binary HPLC pump with column heater; 2998 Photodiode Array Detector; 2414 Refractive Index Detector; In-Line Degasser AF 186001273 DG2; Rheodyne EV100-106-WA Interface Valve. From Novus Produtos Eletrônicos Ltda (Canoas, RS, Brazil): Fieldlogger. From Marconi Equipamentos Para Laboratórios Ltda (Piracicaba SP, Brazil): Ultrathermostated bath. From RSA Comércio de Equipamentos para Instrumentação Ltda (Paulínia, SP, Brazil): Laboratory oven and electric heating system jacket type insulated by ceramic fiber. From Parker Autoclave Engineers (Erie, PA, USA): Micrometer valve. From High-Pressure Equipment Company - HIP (Erie, PA, USA): Check valve.

The base structure was built with 30x30 aluminum profiles, connected with 90° galvanized steel connectors. 2 mm thick stainless-steel plates were added to support equipment and accessories. Furthermore, 6 PVC 3" casters were added to allow easy movement of the structure, a model of a rotating plate with a brake. The structural materials were acquired from Forseti Soluções (Curitiba, PR, Brazil). In addition, several custom-made parts were designed and manufactured. As the pH electrode support and other components, including the extraction / hydrolysis (E/H) cell (15 mm × 100 mm I.D.), SPE column (4.6 mm × 50 mm I.D.), heating system for the E/H cell, and cooling system for the E/H cell output line. Finally, a computer controls the system through the Waters EmpowerTM 3 software.

6.2.3 Extraction and hydrolysis conditions

To validate the PHWE/SubCWH × SPE × HPLC-PDA/RI system. A sample of 5 g of BGP was loaded into the E/H cell. The cell was connected to the system piping inside the oven. The SPE column was packed with PoraPakTM Rxn adsorbent and connected to the system tubing inside the ultra-thermostated bath. The adsorbent was activated through the by-pass with 30 mL of ethanol and conditioning with 30 mL of water at 2.0 mL.min⁻¹. Afterwards, PHWE was performed at 60 °C, 10 MPa, and 2.0 mL.min⁻¹ of ultrapure water at pH 2.0 acidified with phosphoric acid for 30 min. After the extraction, adsorbent desorption was started using the by-pass and applying 0.25 mL.min⁻¹ of 50% ethanol (v.v⁻¹) as eluent for 60 min. After desorption, SubCWH was performed at 180 °C, 15 MPa, and 5.0 mL.min⁻¹ of ultrapure water at pH 7.0 for 60 min (T. Barroso et al., 2022; Maciel-Silva, Viganó, et al., 2022). The selected operational conditions were applied to validate the developed PHWE/SubCWH × SPE × HPLC-PDA/RI system.

6.2.4 Chromatographic analysis

The samples were analyzed in the same equipment by a Waters HPLC, equipped with a binary pump with column heater (1525), inline degasser (AF 186001273 DG2), photodiode array detector (2998), and refractive index detector (2414). The analytical columns were Phenomenex Kinetex C18 Core-Shell (100Å, 2.6 μ m, 4.6 mm x 100 mm) for anthocyanins and Phenomenex RezexTM ROA-Organic Acid H+ (8%, 300 x 7.8 mm) for sugars and organic acids.

Initial conditions were based on a previously developed method for phenolic compounds (Rostagno et al., 2011). A gradient separation method was applied for the anthocyanins analysis in which the mobile phase was composed of water (solvent A) and acetonitrile (solvent B), both containing 2.5% acetic acid (v.v⁻¹). The elution conditions applied were: 0-1 min (8% B), 1-3 min (8-15% B), 3-5 min (15-30% B), 5-6 min (30-50% B), 6-7 min (50% B), 7-8 min (50-8 % B), and 8-10 min (8% B). Flow rate 1.0 mL.min⁻¹, column temperature of 50 °C, and Chromatograms were obtained at 520 nm. The injections (5 μ L) were programmed to occur at half the time of each extract fraction collection to represent the average fraction composition (every 10 min). Quantification was performed using the calibration curve, cyanidin-3-glucoside (y = 12216x – 17934; R² = 0.9969), with 6 points ranging from 3.0 to 150.0 mg.L⁻¹. The result was expressed as a milligram of cyanidin-3-glycoside equivalent per gram of dried black grape pomace (mgC3G.gDBGP⁻¹). The total anthocyanin yield was calculated by summing the concentration of each anthocyanin peak quantified as a function of cyanidin-3-glycoside.

Two isocratic separation methods were applied for sugars and organic acids. The first was a qualitative method for online analysis, where a flow rate of 1.5 mL.min⁻¹ of H2SO4 (5 mmol.L⁻¹) was adopted, column temperature at 60 °C, IR detector temperature at 40 °C, and injection volume of 5 μ L scheduled to occur halfway through the collection time of each hydrolysate fraction to represent the average composition of the fraction (every 10 min). The quantitative method adopted the same parameters, except for the mobile phase flow rate, which was 0.6 mL.min⁻¹. The quantification of sugars was performed using calibration curves, cellobiose (y = 116773x – 5082.3; R² = 0.9991); glucose (y = 113884x + 729.4; R² = 0.9989); mannose (y = 126599x + 607.95; R² = 0.9997); fructose (y = 124560x – 110.65; R² = 0.9996); arabinose (y = 102944x – 117.1; R² = 0.9976), with 5 points varying the concentration from 0.2 to 1.0 g.L⁻¹. The quantitation of organic acids was performed using calibration curves, tartaric

acid (y = 22935 x - 5133.8, $R^2 = 0.999$), citric acid (y = 94738x - 57.333; $R^2 = 0.9998$); succinic acid (y = 88336x - 872.2; $R^2 = 0.9996$); lactic acid (y = 87713x - 415.33; $R^2 = 0.9999$), acetic acid (y = 50955x - 379.26; $R^2 = 0.9998$); propionic acid (y = 69250x - 710.98; $R^2 = 0.9994$); isobutyric acid (y = 87385x - 465.69; $R^2 = 0.9995$), with 5 points varying the concentration from 0.25 to 2.0 g.L⁻¹.

6.3 RESULTS AND DISCUSSION

6.3.1 System concept

An integrated sequential extraction and hydrolysis system with on-line detection and inline pH monitoring to obtain bioactive compounds, organic acids, and fermentable sugars requires combined strategies at each step. The extraction was performed using water as a solvent, and the pH can be varied to obtain a wide range of compounds. Hydrolysis occurs after extraction is complete through changes in process operating conditions. The in-line purification system performs the cleaning and allows the fractionation and concentration of biocompounds. The HPLC analysis occurs through sequential injections of samples, synchronizing the time for the extraction/hydrolysis and analysis modules. Short times were selected for the HPLC analysis to provide sufficient chromatograms to characterize the samples. In this context, the system comprises three modules: i) Extraction/hydrolysis (PHWE/SubCWH), ii) Purification (SPE), and iii) Analysis (HPLC-PDA/RI).

i) Extraction/hydrolysis module: The module extracts the bioactive compounds from the sample using PHWE. Sequentially, rapid hydrolysis of residual lignocellulosic biomass in the extraction/hydrolysis cell is carried out using SubCWH. Both processes are carried out at high pressure with controlled temperature. For extraction, lower temperatures are used to avoid degradation and decrease the functionality of the extract. For hydrolysis, higher temperatures are used to promote the rupture of the structural walls of the sample and release the sugars and organic acids.

ii) Purification module: First, there is an in-line filtration to retain the solid material that can precipitate after the fluid has cooled. Then, purification is performed using the techniques of adsorption and desorption through SPE technology. Compounds interact differently with the SPE column adsorbent depending on their chemical characteristics. Compounds that do not interact with the adsorbent pass through the column more quickly, while compounds that

interact weakly are slightly retained and pass through the column more slowly. Compounds that strongly interact with the adsorbent are retained. These differences in interactions provide fractionation, purification, and sample concentration. The compounds that pass through the SPE column arrive at the valve that connects the purification modules with the analysis module. This interface valve allows injection alternately into the HPLC and collection of extract/hydrolysate fractions.

iii) Analysis module: Module responsible for on-line analysis in HPLC using a PDA detector for bioactive compounds and an RI detector for sugars and organic acids. Through the development of rapid HPLC analytical methods, it is possible to collect multiple chromatograms during extraction and hydrolysis to create a more defined process profile, resulting in detailed information on every compound present in any fraction. Furthermore, as the number of injections during processes increases, the resolution of the sample profile increases. All sample compounds can be monitored in real-time, which allows for identifying the ideal moment to end the extraction and hydrolysis, consequently obtaining more concentrated fractions and avoiding unnecessary dilutions. Additionally, in-line monitoring of the pH of the fluid is carried out before collecting the sample fractions. The pH is an operational parameter for process control, mainly for hydrolysis. At this stage, there is a release of sugars and the production of organic acids.

The three modules of the system can operate either simultaneously or in individual modes. When the modules are synchronized, the system allows real-time monitoring of the extraction/hydrolysis processes, making it possible to obtain a chromatographic profile that chemically characterizes the sample and its fractions throughout the processes. The possibility of operating the modules individually is crucial as it allows for various system configurations. The HPLC can be used for multi-sample analytical purposes, regardless of the system. The extraction/hydrolysis module can be used individually to obtain crude extracts or hydrolysates. It can also be coupled in-line with the purification module to obtain treated products, mainly after optimization of the operational parameters that can be quickly determined with the operating system with all modules synchronized. Finally, the purification module can be used alone to prepare external samples.

6.3.2 Assembly details of PHWE/SubCWH × SPE × HPLC-PDA/RI system

Figure 1 shows the schematic diagram of the sequential extraction and hydrolysis equipment coupled with in-line purification and on-line analysis. The system design sought to position the equipment in such a way as to minimize the piping length and dead volume, resulting in lower costs and high chromatographic efficiency.

The extraction/hydrolysis module starts in the water reservoir, connected to a highpressure isocratic pump. Following the path of the piping, there is the Check valve (CV-1), the first valve of the by-pass system together with the CV-2 and CV-3 used to allow the flow to the purification module without passing through the cell of extraction/hydrolysis. In this way, it is possible to activate and condition the adsorbent contained in the SPE column before starting the sample extraction or to elute the compounds extracted from the sample retained in the adsorbent without letting the solvent pass through the extraction/hydrolysis cell, which could remove other compounds from the sample matrix and contaminate the obtained extract.



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Figure 1. Schematic diagram of the PHWE/SubCWH-SPE × HPLC-PDA/RI system.

(1) Water reservoir, (2) HPLC pump, (3) Check valve, (4) Manometer, (5) Extraction / hydrolysis cell, (6) Heat exchanger - electric jacket, (7) Temperature controller, (8) Thermocouple, (9) Oven, (10) Heat exchanger - serpentine, (11) Ultra-thermostatized bath, (12) In-line filter, (13) SPE column, (14) Interface valve, (15) Micrometer valve, (16) in-line pH sensor, (17) Sample collector, (18) Liquid chromatograph, (19) Computer, and (20) Waste collector.

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The CV-4 valve is used to pressurize the system, a micrometric valve is used to control the pressure, and 2 manometers are installed in strategic positions to monitor the pressure throughout the system to easily identify any possible overpressure points. To control the temperature, an electric jacket-type heating system and an oven are used to obtain a controlled environment and serve as a security system. The temperature inside and at the exit of the extraction/hydrolysis cell is monitored using thermocouples. After exiting, the fluid is cooled through a serpentine-type heat exchanger with an ultra-thermostated bath.

The purification module has a cup-type in-line filter with 2 μ m pores to retain solid material. After passing through the filter, the fluid follows the SPE column, whose temperature is controlled by an ultra-thermostated bath. Then there is the interface valve (**Figure 2**), which separates an aliquot of extract that flows through the valve to be instantly injected into the HPLC system. The automatic valve has six ports and operates in two positions. Position 1 (LOAD position) allows in-line pH monitoring and collection of sample fractions. In position 2 (INJECT position), the sample is injected into the HPLC.

The analysis module consists of an HPLC from Waters company, which contains a binary solvent pump, an inline solvent degasser, a manual injection valve, a chromatographic oven, a PDA detector, a RI detector, and a waste collector. A computer controls this entire system through Waters EmpowerTM 3 software. This module also has an inline pH probe connected through a Fieldlogger to the control computer for monitoring the pH of the sample throughout the process.

6.3.3 Process description

The procedure to perform sequential extraction and hydrolysis with in-line purification and on-line analysis, in addition to in-line sample pH monitoring, was as follows:

i) Initially, the E/H cell is packaged with the desired raw material. Adding a layer of glass wool, then the sample, then another layer of glass wool, and finally completing the volume with glass spheres. With the packaging finished, connect the E/H cell to the heating system. Then, the selected adsorbent is packed in the SPE column, and the column is connected to the ultra-thermostated bath.



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Figure 2. Schematic diagram of interface valve operation.

ii) The extraction and hydrolysis solvents are checked, in addition to the level of the HPLC mobile phases. If necessary, the solutions are exchanged before starting the experiment. All equipment in the system is turned on, the Waters EmpowerTM 3 software and the Fieldlogger software are opened, and the equipment responsible for monitoring the temperature and pH values. Then, the isocratic pump of the extraction/hydrolysis module and the binary pump of the analysis module are purged. After the purge, the chromatographic method is set up to stabilize the column temperature, system pressure, and detector signal. The HPLC system is ready for online analysis when starting the experiment. Then, the activation of the adsorbent with organic solvent and conditioning with water begins. In this study, 30 mL of ethanol and 30 mL of ultrapure water were used, both steps at 3 mL.min⁻¹. The valves were configured to pass the flow through the by-pass (CV-1 and CV-3 closed and CV-2 open).

iii) Once the preparation steps are finished, the extraction process starts with system pressurization. For this purpose, the CV-4 valve is closed, and the other Check valves are configured to pass the flow through the E/H cell (CV-1 and CV-3 open and CV-2 closed), then the solvent pumping is started. With the system pressurized, the pumping is stopped, the heating of the E/H cell starts and the ultra-thermostated baths are turned on to control the temperature of the heat exchanger for cooling the fluid and the SPE column. When the temperatures are reached, the extraction method is synchronized with the automatic injection sequence of the sample in the HPLC system, which is performed through the interface valve, as shown in Figure 2. The "PULSE" command is performed, activating the valve to position (INJECT) in a programmed time. The valve returns to the initial position (LOAD), where the sample loads the loop and proceeds to in-line pH monitoring and collection of sample fractions. Thus, injections are performed at each interval, defined by the analytical method. Simultaneously, sample fractions are collected at each interval. In the current work, isocratic methods were used for HPLC analysis of the compounds from the extraction and hydrolysis, both with 10 min, a short time to allow a high number of analyses during the experiments, thereby providing a detailed kinetic profile. Using a PDA detector and reverse phase column with C18 packing for HPWE, RI detector with an Ion Exclusion column packed with Sulfonated Styrene Divinyl Benzene for SubCWH. Then, the extraction process begins with sequential hydrolysis with inline purification and on-line analysis. The micrometric valve maintains the desired pressure while the extraction and hydrolysis processes pump solvent.

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iv) In purification by SPE during the sample matrix extraction process, the analytes contained in the extract can be fractionated or retained after passing through the column containing the adsorbent, depending on their chemical characteristics. Compounds that do not interact with the adsorbent move through the column more quickly, while compounds that interact poorly are held back slightly and move through the column more slowly. Compounds that strongly interact with the adsorbent are retained. For the particular case evaluated in this study, the PHWE of the grape marc lasted for 30 min at a temperature of 60 °C with a flow rate of 2 mL.min⁻¹ at a pressure of 10 MPa, the extraction solvent used was acidified water at pH 2.0 with phosphoric acid. Thus, the anthocyanins, the main compounds extracted from the sample, were retained in the SPE column by the adsorption process due to their high affinity with the PoraPakTM Rxn adsorbent.

v) A selective solvent is used to wash/elute the analytes of interest retained in the SPE column, a process called desorption. As the compounds are eluted with a small volume of solvent, they end up concentrated in the collection. Thus, a clean sample with a high concentration of the compounds of interest can be obtained. Therefore, in this study, at the end of the PHWE, the extraction solvent was replaced by the desorption solvent, 50% ethanol, responsible for eluting the anthocyanins retained in the SPE column. For this, the isocratic pump was purged with the desorption solvent, and the flow was directed to pass through the by-pass instead of passing through the E/H cell. The flow rate performed in this step was 0.25 mL.min⁻¹ to observe a more detailed chromatographic profile in the on-line analysis and to facilitate the definition of the desorption endpoint.

vi) After the end of the Desorption, the preparation for hydrolysis is started, stopping the pumping of the isocratic pump and the HPLC binary pump. Specifically for this work, the HPLC mobile phases were changed, the binary pump was again purged, the reserving phase C18 column was also changed by the ion exchange column, and then a setup was given in the chromatographic method of analysis of sugars and organic acids. While the HPLC system stabilizes, the desorption solvent is exchanged for the hydrolysis solvent, ultrapure water, and the isocratic pump is purged with the new solvent. Increase the temperature of the E/H cell to hydrolysis condition. In this case, SubCWH was performed at 180°C, 15 MPa, with a flow rate of 5mL.min⁻¹. The hydrolysis flow rate was greater than the extraction flow rate to reduce the residence time of the solvent with the sample matrix, thereby avoiding the large formation of

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unwanted compounds and inhibitors of biotechnological processes, such as fermentation. Upon reaching the desired temperature and stabilization, the chromatographic system synchronizes the programming of the injection sequence with the pumping of the solvent, initiating the hydrolysis. At that moment, the SPE column works as a purification system, retaining impurities and solid particles that may precipitate after cooling. The in-line pH monitoring of the sample throughout the hydrolysis works as an operational parameter for process control because, in this step, it occurs together with the release of sugars and the production of organic acids.

6.3.4 Extraction results

Figure 3 shows the result of PHWE kinetics from DBGP. From the chromatographic profile it was possible to observe that a good separation of the anthocyanin compounds was achieved in less than 6 min, the remaining time was used to clean the column and stabilize the system for the next injection, completing the run in 10 min. The main objective of the analytical method in this work was to reasonably separate the compounds present in the sample in the shortest possible time to obtain a representative profile of the processes, resulting in a greater number of injections. Therefore, 3 injections were performed during PHWE and 6 injections during desorption, where the flow was lowered from 2.0 to 0.25 mL.min⁻¹ in order to obtain an expressive chromatographic profile, useful for determining the end point of desorption and that after the optimized process could be done in a much shorter time with a flow similar to the extraction. The decrease in process time is interesting to reduce the cost of operation and materials.

During the first 30 min of operation, no peak was observed in the chromatograms because, at first, the SPE cell filled with the adsorbent manages to retain all the compounds in the extract, allowing only the extraction solvent to (acidified water) reach the PDA detector. However, when exchanging the extraction solvent for the desorption solvent (50% ethanol), the compounds began to be detected after 30 min and continued to be detected until the end of the process.



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Figure 3. On-line HPLC-PDA anthocyanins chromatograms (520 nm) of PHWE kinetics from DBGP.
At the beginning of desorption, the concentration of anthocyanins is maximum (40 min chromatogram). At this point, the presence of 12 anthocyanin compounds was qualitatively detected, with peak number 3 at 2.90 min being cyanidin-3-glucoside, which was used as a standard for the quantitation of other anthocyanins. Throughout the desorption, which lasted 60 min, a decrease in the intensity and number of compounds detected was observed. In the last 3 chromatograms, only 5 compounds were detected.

Table 1 shows the individual and total yield of anthocyanins by PHWE process from DBGP. Twelve anthocyanins were found, the majority represented by peaks 2, 3, 5, 6, 8, and 12 with concentrations greater than 3 mgEC3G.gDBGP⁻¹. By summing the concentration of each anthocyanin, a total yield of 47.6 mgEC3G.gDBGP⁻¹ was obtained. This value is similar to that observed by Ribeiro et al., (2015), who worked with Terci grape pomace and obtained an anthocyanin recovery of nearly 43 mg.g⁻¹. On the other hand, other authors obtained much lower yields. Pereira et al., (2019) obtained an anthocyanin value of approximately 11 mg.g⁻¹ when working with grape pomace and Panić et al., (2019) obtained an anthocyanin yield of 1.2 mg.g⁻¹.

| Peaks | Retention time (min) | mgEC3G.gDBGP ⁻¹ |
|-------------------------|-----------------------------|----------------------------|
| 1 | 1.43 | 0.51 ± 0.02 |
| 2 | 2.25 | 8.57 ± 0.09 |
| 3: C3G | 2.90 | 3.00 ± 0.06 |
| 4 | 3.20 | 2.23 ± 0.04 |
| 5 | 3.82 | 5.42 ± 0.03 |
| 6 | 3.93 | 3.74 ± 0.11 |
| 7 | 5.02 | 1.97 ± 0.10 |
| 8 | 5.35 | 8.78 ± 0.09 |
| 9 | 5.62 | 2.28 ± 0.03 |
| 10 | 5.68 | 2.76 ± 0.01 |
| 11 | 5.82 | 0.58 ± 0.01 |
| 12 | 5.96 | 7.76 ± 0.10 |
| Total Anthocyanin Yield | | 47.60 ± 0.69 |

Table 1. Anthocyanin extraction yield obtained by HPWE from DBGP.

Results are replicate means and their standard deviation.

The high total anthocyanin yield of this work is due to the efficiency of the PHWE system using acidified water as the solvent and suitable process conditions. The anthocyanin

extraction was accompanied by inline SPE using a reverse phase adsorbent, which made the obtained extract much superior to those found in the literature. Loarce et al., (2021) evaluated PHWE aided by natural deep eutectic solvents (NADESs) for the extraction of anthocyanins from grape pomace, obtained extracts with an anthocyanin concentration of 0.02 g.L⁻¹ only using PHWE and 0.17 g.L⁻¹ for PHWE assisted by 20% of NADESs as modifiers, values significantly lower than those found in this study. This shows that the combination of PHWE with inline SPE for recovery of anthocyanins is highly efficient and should be evaluated for large-scale application.

The polar character of the anthocyanins caused their complete retention in the SPE column during the PHWE. When removed in the desorption in a smaller volume, a concentration four times higher was obtained, because PHWE at 2 mL.min⁻¹ for 30 min used 60 mL and desorption at 0.25 mL.min⁻¹ for 60 min resulted in 15 mL. The concentration of the final extract was 1.6 gEC3G.L⁻¹; without desorption it would be 0.4 gEC3G.L⁻¹. Although considerable concentration was achieved with inline SPE, it could have been even greater. Since it can be observed through the last chromatograms (**Figure 3**) that the desorption was no longer eluting a significant anthocyanins recovery. With 40 min of desorption, 91% of the total anthocyanins were recovered, if the process were stopped at that moment, the concentration would be approximately 2.2 gEC3G.L⁻¹.

6.3.5 Hydrolysis results

Figure 4 shows the SubCWH kinetics from DBGP results obtained in real-time. The time needed to obtain a good separation of the compounds from DBGP hydrolysate with the chromatographic system used takes 48 min due to the sensitivity and the time needed to stabilize the baseline of the RI detector. However, the objective of the online analysis is to obtain a control parameter and a representative profile of the process, thereby determining the best point to end the operation. An HPLC-RI short method of 10 min was developed, where it was possible to obtain a stable system to perform the injections, even without a perfect separation of the compounds; through this method, it was possible to follow the evolution of the hydrolysis by the reduction of the areas of the peaks of the compounds co-eluted, as can be seen in **Figure 4** (A).



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Figure 4. Real-time results of SubCWH kinetics from DBGP. (A) HPLC-RI chromatograms and (B) pH profile.

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All kinetics chromatograms have an inverted solvent peak. The first chromatogram has a prominent peak with a residence time of approximately 5 min. It can be observed in this and the other peaks that there is co-elution of the compounds, as they present valleys (the lowest point of the peak) that do not reach the baseline, unlike the cases of chromatograms with good separation. In the following chromatograms, it is observed that the first peak had a slight displacement in the residence time, appearing around 5 min. This variation may be due to a probable overload of the chromatographic column, since the overall concentration of compounds passing through the column is higher in the first fraction of the hydrolysate. However, this can be avoided by using less sample mass in the E/H cell or by making changes to the system structure, so that sample dilutions are made before automatic injection. In the last chromatogram analyzed, practically no peak appears, indicating that the production of sugars and organic acids is no longer occurring effectively.

Additionally, there is inline pH monitoring to monitor the hydrolysis kinetics, as shown in **Figure 4** (**B**). For SubCWH, ultrapure water with neutral pH was used. However, due to the release and formation of organic acids right at the beginning of the experiment, it is observed that the pH of the solution is approximately 4.5, which rapidly decays to close to 3.5 in 5 min of kinetics and returns to 4.5 with 10 me. The pH continues to increase until it stabilizes at about 5.25 after 25-30 min.

Figure 5 shows the results of kinetic SubCWH from DBGP obtained off-line, performed from the collected fractions. The characterization of the organic acids during the hydrolysis is shown in **Figure 5** (**A**), of the sugars in **Figure 5** (**B**), and the representative chromatogram of the hydrolysis kinetics obtained from the first fraction collected is shown in **Figure 5** (**C**).

Several studies that performed hydrolysis in semi-continuous flow-through subcritical water with different raw materials obtained good yields in the recovery of sugars, such as for grape pomace (Pedras et al., 2020), brewers' spent grains (Sganzerla et al., 2022), jabuticaba peel (T. L. C. T. Barroso et al., 2022), coffee waste (Mayanga-Torres et al., 2017), sugarcane straw (Lachos-Perez et al., 2017). However, in all these works, inhibitors of biotechnological processes were formed. That is, degradation compounds commonly found in lignocellulosic hydrolysates, called 5-(hydroxymethyl)furfural, resulting from the degradation of hexoses and furfural produced by the degradation of pentoses (Yu et al., 2008).



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Figure 5. Off-line accumulated results of SubCWH kinetics from DBGP. (A) Organic acids profile, (B) Sugars profile, and (C) Representative HPLC-RI chromatogram from the sample. Peaks: 1 – Tartaric acid; 2 – Cellobiose; 3 – Citric acid; 4 – Glucose; 5 – Mannose; 6 – Fructose; 7 – Arabinose 8 – Succinic Acid; 9 – Lactic acid; 10 – Acetic acid; 11 – Propionic acid; and 12 – Isobutyric acid.

The formation of these compounds occurs due to the severe conditions used to obtain biomass hydrolysis (Prado et al., 2016). However, using inline SPE, the hydrolysate obtained in this work was purified, leaving the inhibitors retained in the adsorbent. Therefore, it was possible to reduce the running time from 48 to 22 min, as shown in **Figure 5** (**C**). Remember that in the chromatographic method used, 5-hydroxymethylfurfural comes out after 30 minutes of running and furfural after 44 minutes. The sugars and organic acids already come out in less than 22 minutes. Although this time is relatively short, it would not be suitable for online detection, as a representative hydrolysis profile would not be obtained. Therefore, a qualitative method was necessary for online analysis, and another quantitative one was conducted offline.

Through SubCWH, seven organic acids (tartaric acid, citric acid, succinic acid, lactic acid, acetic acid, propionic acid and isobutyric acid) and five sugars (cellobiose, glucose, mannose, fructose and arabinose) were identified and quantified. The most significant compounds were tartaric acid representing 19.5 mg.gDBGP⁻¹, 53% of the total organic acids, and mannose, 21.8 mg.gDBGP⁻¹, 52% of the total sugars. Although tartaric acid has the highest percentage compared to other organic acids, only lactic acid showed significant production until the end of hydrolysis, and the other acids practically ceased after 30 min. This fact corroborates the pH profile illustrated in **Figure 4 (B)**, which remained constant after that moment. Mannose behaved similarly to lactic acid, obtaining production until the end of hydrolysis. Cellobiose was released up to half of the hydrolysis, while the remaining sugars were produced only at the beginning of the process.

6.3.6 Potential applications

The sustainable process developed can be used for a range of applications. The product from extraction is purified in the process, allowing the further application to produce pure chemicals, avoiding downstream processing for purification. Additional advantages of the developed process are the fast extraction time (30 min), concentration of the target compound after desorption, and low volume of solvent for elution. The application of SPE allows the concentration by adsorption and desorption process, allowing the fractionation of different compounds depending on the solvent applied in the process. These advantages allow the practical application of green chemistry principles.

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Nevertheless, the product obtained from the hydrolysis reactor can be applied for fermentative processes. Depending on the adsorbent used, the hydrolysate can be produced with a high concentration of monosaccharides and a low concentration of organic acids, furfural, and 5-HMF, which are considered inhibitors for fermentative processes. The hydrolysis of biomass followed by purification is a sustainable approach for recovering a detoxified stream rich in sugars. This aspect allows the application of PHWE/SubCWH \times SPE \times HPLC-PDA/RI to produce several biofuel and value-added bio-based products.

6.4 CONCLUSION

The new automated system (PHWE/SubCWH \times SPE \times HPLC-PDA/RI) was designed, developed, and validated to serve as an alternative method of valorization of agro-industrial waste capable of integrating three modules into a single system, which can be operated individually or together. The 1st module promotes sequential extraction and hydrolysis, allowing almost full use of the material. The 2nd module performs sample preparation through the inline SPE, and can act as a purification, fractionation, and concentration agent. Finally, the 3rd module is responsible for real-time analysis, allowing a greater understanding of processes and enabling immediate decision-making. The system's versatility is very innovative, and the validation results with DBGP were promising, demonstrating the possibility of transitioning from linear production to a circular economy.

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CHAPTER 7 – General Discussion

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Given the need to develop circular economy processes and biorefineries, this thesis sought to produce an integrated sequential extraction and hydrolysis system with sample preparation and real-time analysis. The açaí industry was chosen because it is an emerging market. Data from IBGE (2020), Brazil produced in 2018 the amount of 1.7 million tons of açaí fruits, generating a revenue of 3.9 billion reais. Additionally, the processing of açaí has a low yield of pulp, on average only 10%. The rest is the residual biomass (açai seed) which currently does not exist in the large-scale use of this residue. The other agroindustry evaluated was the grape industry, whose production, according to IBGE (2021), is around 1.5 million tons/year. Of this volume, about 25% is destined for the production of wines and after processing, 50 % of the total mass of the grapes becomes waste, called grape pomace.

The açaí fruit review summarizes research knowledge from the last 27 years (1993 to 2019), applying a longitudinal bibliometric study. The results showed that few documents addressed the recovery of energy from açaí seeds, and residues from fruit processing are not always disposed of in an environmentally sound way. Furthermore, the results show that the replacement of electricity contributes to the mitigation of greenhouse gases. In addition, technological advances can stimulate sustainable agro-industrial intensification in areas such as the Legal Amazon, considered a hotspot for mitigating climate change.

The review on extraction processes coupled to online detection systems highlighted the importance of developing this integration. Detecting analytes in complex matrices requires a series of preparation steps to obtain an analytical result. Sample preparation can be time-consuming and error-prone, reducing investigation reliability. The bibliometric analysis evaluated publications over the last 31 years (1990 to 2020) on on-line detection through a longitudinal bibliometric analysis, applying statistical and mathematical methods to analyze the evolution and trends in this area of research. In addition, the recent state of the art of extraction techniques most suitable for coupling with chromatographic systems is presented.

A new sub/supercritical water extraction and hydrolysis coupling system with in-line SPE and online HPLC was assembled, validated and patented. The validation of the system used black grape skin as a raw material, a by-product rich in bioactive compounds and lignocellulose, an attractive substrate for extraction and hydrolysis. During extraction and hydrolysis, the system automatically injected a sample fraction (5 μ L) into the HPLC, from which anthocyanin and sugar concentrations were recovered. Obtaining bioactive compounds

CHAPTER 7 – General Discussion

can increase the profitability of the industry, as they have a high market value and are in great demand in the food, pharmaceutical and cosmetic sectors. Furthermore, lignocellulosic hydrolyzate, rich in fermentable sugars, can be applied in the production of biofuels.

A study of the extraction of anthocyanins from freeze-dried and semi-defatted açaí pulp was carried out using the newly developed system. The removal of lipids using SFE (approximately 70% of the total lipids in the sample) was performed to improve the SPE purification step, provide excellent on-line detection without harming the analytical column of the HPLC, and harness the açaí oil, a product of excellent composition and high added value. Thus, coupling of PLE with in-line SPE and on-line HPLC succeeded in extraction, purification, concentration, and simultaneous detection of anthocyanins from açaí.

Additionally, a study was carried out on the sequential extraction and hydrolysis from grape pomace, the main solid residue generated during the industrial processing of grapes. The yield of extraction and hydrolysis of this raw material was attractive, and can be applied in the manufacture of various products with high added value. In addition, the use of green technologies and solvents to obtain natural compounds from agro-industrial waste is an alternative for a circular economy capable of slowing down the advance of environmental changes. **CHAPTER 8 – General Conclusion**

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Two reviews have been produced, addressing advances in scientific research on the açaí fruit and the area of extraction processes coupled with on-line detection systems. Afterward, the mount and validation of a new extraction and hydrolysis unit in sub / supercritical water coupled with an on-line detection system were carried out. The new unit was used to evaluate the extraction of anthocyanins from açaí. In addition, to be used to evaluate the sequential extraction and hydrolysis from grape pomace. Based on the activities developed, the following conclusions were reached.

Chapter 2 presented a review of the research carried out on the açaí fruit through a bibliometric analysis and an assessment of the energy potential of açaí seeds, allowing the following conclusions to be reached:

- The growing açaí market has become of great interest for future investments. Furthermore, extractive production is seen by many researchers as a sustainable alternative to preventing deforestation in the Legal Amazon;
- The evaluation of scientific knowledge related to this approach revealed that research on the recovery of açaí residues is still in its infancy. In addition, a large part of the investigations on the energy recovery of açaí occurs in a regionalized way, mainly in Brazilian universities and research institutions;
- The integration of hydrolysis technology in subcritical water followed by anaerobic digestion may be an innovative way to rethink the açaí production chain towards a circular economy;
- The anaerobic digestion of all the açaí seeds generated in Brazil indicated that, theoretically, the electrical energy from burning biogas could supply all the needs of the frozen pulp industry with an additional surplus for sales to the network.

Chapter 3 develops a review of the different entry techniques, sample preparation techniques, automation, and coupling with online detection, in addition to evaluating publications on the subject through longitudinal bibliometric analysis.

- Advanced extraction techniques for solid matrices PLE, SubCWE, SFE, UAE, and MAE are best suited for coupling with on-line chromatography systems (LC, GC, and SFC);
- On-line coupling systems employ intermediate trapping, multiport valves, and one or more pumps for the dynamic extraction or transfer of the extract to the chromatographic system;

- Research on automation and coupling extraction processes with on-line detection started in 1990. The total number of publications has reached the mark of 2255 articles, and since 1995 more than 60 publications have been made annually;
- Spain, China, and the USA are the countries that contributed most to the development of this research front. The USA leads the ranking of international collaborations, making publications in partnership with 30 countries.

Chapter 4 refers to the patent for an integrated extraction and hydrolysis system in sub/supercritical water with on-line detection to obtain bioactive compounds and fermentable sugars from any raw material containing these compounds, but preferably agro-industrial residues.

- The hydrothermal processes of extraction and sequential hydrolysis of agro-industrial residues were carried out using black grape bagasse as raw material to validate the system;
- The real-time monitoring of the products enabled quick and efficient responses, less process time, low solvent consumption, fewer analytical errors, and less labor;
- The system allows both the chemical characterization of industrial waste in terms of bioactive compounds and sugars, as well as the development of integrated methods of extraction, hydrolysis, and characterization.

Chapter 5 refers to the experimental article that aimed to extract anthocyanins from dehydrated and defatted açaí pulp using the new patented extraction system with in-line purification by solid-phase extraction and on-line analysis by high-performance liquid chromatography.

- Critical parameters affecting extraction efficiency and purification were investigated and optimized by response surface methodology (RSM). The optimal conditions were: 71 °C and pH 2 for PLE; and 30 °C and 50% ethanol for SPE;
- PLE was compared to conventional extraction methods, presenting the highest anthocyanins yield (3.01 mg/g), and PLE+SPE produced an extract 5-fold more concentrated than PLE alone;
- The newly developed method can prepare samples and perform on-line HPLC of different anthocyanin classes from different raw materials.

Chapter 6 presents an experimental article whose objective was to detail the design, assembly and validation of the new developed system, in addition to promoting the recovery of anthocyanins, sugars and organic acids from black grape pomace.

- The newly developed system proved to be feasible to perform sequential extraction and hydrolysis of grape residue with inline SPE purification and online HPLC analysis. It can be used as an alternative method of valorization of agro-industrial residues;
- The real-time monitoring of the products allowed for quick and efficient responses, shorter processing time, low consumption of solvents, less labor, using less energy and generating less impact on the environment.

Taking into account the advances developed in this doctoral thesis and aiming to continue the work, the following suggestions are proposed for future work:

- Evaluate the sequential extraction and hydrolysis process using the new system developed with other promising agro-industrial residues for the recovery of bioactive compounds, sugars, amino acids and organic acids;
- Evaluate the stability, antioxidant activity and toxicity of bioactive compounds obtained from the extraction step;
- Develop foods enriched with extracts of bioactive compounds;
- Carry out fermentation tests for the production of 2G ethanol using hydrolysates as substrate;
- Carry out a technical-economic and environmental analysis of the sequential extraction and hydrolysis processes developed with a new system using the SuperPro Designer® software.

CHAPTER 9 – Doctorate Memorial

Francisco Weshley Maciel Silva holds a Bachelor's degree in Food Engineering from the Federal University of Pará - UFPA (2012-2016) and a Master's degree in Food Engineering from the State University of Campinas - UNICAMP (2017-2019). In 2019, he joined the Doctorate course in Food Engineering at UNICAMP, with a grant from CNPq (Process number. 140479/2019-8), developing research activities at the Laboratory of Bioengineering and Treatment of Water and Waste - BIOTAR, under the supervision of Prof. Dr. Tânia Forster-Carneiro and Co-supervision of Prof. Dr. Mauricio Ariel Rostagno.

18 credits were taken through the disciplines of Biochemical Engineering (TP319), Special Topics in Energy Systems Planning I (PE180), Life Cycle Assessment Applied to Energy (PE172), Topics in Food Engineering (TP121), Seminars (TP199), and two participations in the Teacher Training Internship - PED C (CD003) in the disciplines Laboratory of Unitary Operations (TA035) and Unitary Operations III (TA831).

During the doctorate period, 18 abstracts were published in the annals of national and international events, 9 articles in international scientific journals, and 3 patent deposits. Highlight for:

• Abstract awarded in event:

Third best abstract presented in "2nd International Congress on Bioactive Compounds and 3rd International Workshop on Bioactive Compounds: Personal Nutrition, Ageing and Food Science", UNICAMP.

SILVA, F.W.M.; CASTRO, L.E.N.; SGANZERLA, W.G.; COSTA, J.M.; ROSTAGNO, M.A.; FORSTER-CARNEIRO, T. Sequential extraction and hydrolysis process coupled in-line purification and on-line analysis applied to grape pomace (*Vitis vinifera L.*) for recovery of anthocyanins, sugars and organic acids. In: 2nd ICBC - International Congress on Bioactive Compounds and 3rd International Workshop on Bioactive Compounds, 2022, Campinas - SP.

• Articles published in international journals:

1. FERREIRA, S.F.; BULLER, L.S.; MACIEL-SILVA, F.W.; SGANZERLA, W.G.; BERNI, M.D.; FORSTER'CARNEIRO, T. Waste management and bioenergy recovery from açaí processing in the Brazilian Amazonian region: a perspective for a circular economy. Biofuels Bioproducts & Biorefining-Biofpr, v. 1, p. 37-46, 2020. 2. **MACIEL-SILVA, F.W.**; BULLER, L.S.; GONÇALVES, M.B.B.M.L.; ROSTAGNO, M.A.; FORSTER-CARNEIRO, T. Sustainable development in the Legal Amazon: energy recovery from açaí seeds. Biofuels Bioproducts & Biorefining-Biofpr, v. 1, p. bbb.2222, 2021.

3. SGANZERLA, W.G.; VIGANÓ, J.; CASTRO, L.E.N.; **MACIEL-SILVA, F.W.**; ROSTAGNO, M.A.; MUSSATTO, S.I.; FORSTER-CARNEIRO, T. Recovery of sugars and amino acids from brewers' spent grains using subcritical water hydrolysis in a single and two sequential semi-continuous flow-through reactors. Food Research International, v. 157, p. 111470, 2022.

4. BARROSO, T. L.C.T.; SGANZERLA, W.G.; ROSA, R.G.; CASTRO, L.E.N.; **MACIEL-SILVA, F.W.**; ROSTAGNO, M.A.; FORSTER-CARNEIRO, T. Semi-continuous flow-through hydrothermal pretreatment for the recovery of bioproducts from jabuticaba (*Myrciaria cauliflora*) agro-industrial by-product. Food Research International, v. 158, p. 111547, 2022.

5. **MACIEL-SILVA, F.W.**; VIGANÓ, J.; CASTRO, L.E.N.; SGANZERLA, W.G.; BULLER, Z.S.; MARTÍNEZ, J.; ROSTAGNO, M.A.; FORSTER-CARNEIRO, T. Pressurized liquid extraction coupled in-line with SPE and on-line with HPLC (PLE-SPExHPLC) for the recovery and purification of anthocyanins from SC-CO2 semi-defatted Açaí (*Euterpe oleracea*). Food Research International, v. 160, p. 111711, 2022.

6. **MACIEL-SILVA, F.W.**; LACHOS-PEREZ, D.; BULLER, L.S.; SGANZERLA, W.G.; PÉREZ, M.; ROSTAGNO, M.A.; FORSTER-CARNEIRO, T. Green Extraction Processes for Complex Samples from Vegetable Matrices Coupled with On-Line Detection System: A Critical Review. Molecules, v. 27, p. 6272, 2022.

7. BARROSO, T.L.C.T.; DA ROSA, R.G.; SGANZERLA, W.G.; CASTRO, L.E.N.; **MACIEL-SILVA, F.W.**; ROSTAGNO, M.A.; FORSTER-CARNEIRO, T. Hydrothermal pretreatment based on semi-continuous flow-through sequential reactors for the recovery of bioproducts from jabuticaba (*Myrciaria cauliflora*) peel. Journal Of Supercritical Fluids, v. 1, p. 105766, 2022.

8. SGANZERLA, W.G.; CASTRO, L.E.N.; ROSA, C.G.; ALMEIDA, A.R.; MACIEL-SILVA, F.W.; KEMPE, P.R.G.; OLIVEIRA, A.L.R.; FORSTER-CARNEIRO, T.; BERTOLDI, F.C.; BARRETO, P.LM.; VEECK, A.P.L.; NUNES, M.R. Production of nanocomposite films functionalized with silver nanoparticles bioreduced with rosemary (*Rosmarinus officinalis L.*) essential oil. Journal of Agriculture and Food Research, v. 11, p.100479, 2023.

9. CASTRO, L.E.N.; SGANZERLA, W.G.; BARROSO, T.L.C.T.; **MACIEL-SILVA**, **F.W.**; COLPINI, L.M.S.; BITTENCOURT, P.R.S.; ROSTAGNO, M.A.; FORSTER-CARNEIRO, T.; Improving the semi-continuous flow-through subcritical water hydrolysis of grape pomace (*Vitis vinifera L.*) by pH and temperature control. The Journal of Supercritical Fluids, v. 196, p. 105894, 2023.

• Patents filed:

1. ROSTAGNO, M.A.; FORSTER-CARNEIRO, T.; SOUZA, M.C.; SILVA, L.C.; CHAVES, J.O.; SANCHES, V.L.; **MACIEL-SILVA, F.W.** Register number: BR1020200172387. Title: Sistema bidimensional para a extração, purificação e análise de compostos bioativos. Registration institution: INPI - Instituto Nacional da Propriedade Industrial. Deposit date: 08/24/2020. National Publication Date: 03/03/2022. Funding institution: FAPESP - Fundação de Amparo à Pesquisa do Estado de São Paulo.

2. FORSTER-CARNEIRO, T.; ROSTAGNO, M.A.; SGANZERLA, W.G.; MACIEL-SILVA, F.W.; BULLER, L.S.; BARROSO, T.L.C.T. Register number: BR1320210174020. Title: sistema com reatores carregados em série de hidrólise em água subcrítica para produção de percursores de biocombustíveis e bioprodutos. Registration institution: INPI - Instituto Nacional da Propriedade Industrial. Deposit date: 09/01/2021. National Publication Date: 11/01/2022. Funding institution: FAPESP - Fundação de Amparo à Pesquisa do Estado de São Paulo.

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CHAPTER 11 – Appendices

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APPENDIX A - Supplementary material from chapter 3.

"GREEN EXTRACTION PROCESSES FOR COMPLEX SAMPLES FROM VEGETABLE MATRICES COUPLED WITH ON-LINE DETECTION SYSTEM: A CRITICAL REVIEW"

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Figure S1. Methodological synthesis applied to the bibliometric analysis.

APPENDIX B - Supplementary material from chapter 5.

"PRESSURIZED LIQUID EXTRACTION COUPLED IN-LINE WITH SPE AND ON-LINE WITH HPLC (PLE-SPEXHPLC) FOR THE RECOVERY AND PURIFICATION OF ANTHOCYANINS FROM SC-CO₂ SEMI-DEFATTED AÇAÍ (EUTERPE OLERACEA)"

| Sourco | DF | Total anthocyanin yield (mgTAY/gDDRM) | | | | | |
|-------------------------------|----|---------------------------------------|----------------------|---------|---------|--|--|
| Source | DF | Sum of squares | Mean square | F value | p-value | | |
| Temperature (°C) | 1 | 0.5828 | 0.5828 | 66.46 | 0.0039 | | |
| Temperature (°C) ² | 1 | 1.1704 | 1.1704 | 133.47 | 0.0014 | | |
| pH | 1 | 1.5301 | 1.5301 | 174.49 | 0.0009 | | |
| pH ² | 1 | 0.1624 | 0.1624 | 18.52 | 0.0231 | | |
| Temperature (°C) vs pH | 1 | 0.2550 | 0.2550 | 29.08 | 0.0125 | | |
| Residual | 3 | 0.0263 | 0.0088 | | | | |
| Lack-of-fit | 3 | 0.0029 | 0.0009 | 0.03 | 0.0993 | | |
| Total | 8 | 3.7272 | 0.4659 | | | | |
| | | R ² =0.9929 | $R^{2}_{adj}=0.9812$ | CV=6.01 | | | |

Table SM1. ANOVA results for the quadratic model of response surface for PLE.

Table SM2. ANOVA results for the quadratic model of response surface for SPE.

| Source | DF | Total anthocyanin yield (mgTAY/gDDRM) | | | | | |
|---------------------------------|----|---------------------------------------|---------------------------------------|---------|---------|--|--|
| Source | Dr | Sum of squares | Mean square | F value | p-value | | |
| Temperature (°C) | 1 | 0.2970 | 0.2970 | 60.21 | 0.01620 | | |
| Temperature (°C) ² | 1 | 0.0112 | 0.0112 | 2.27 | 0.2708 | | |
| Ethanol (%) | 1 | 0.0600 | 0.0600 | 12.17 | 0.0733 | | |
| Temperature (°C) vs Ethanol (%) | 1 | 0.0042 | 0.0042 | 0.86 | 0.4524 | | |
| Residual | 2 | 0.0099 | 0.0049 | | | | |
| Lack-of-fit | 2 | 0.0003 | 0.0008 | 0.01 | 0.0893 | | |
| Total | 6 | 0.3823 | 0.0637 | | | | |
| | | R ² =0.9797 | R ² _{adj} =0.9392 | CV=5.64 | | | |

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