University of São Paulo
"Luiz de Queiroz" College of Agriculture

Recovery of active compounds from avocado oil residues: sustainable alternatives for obtaining new ingredients

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Dissertation presented to obtain the degree of Master of Science. Area: Food Science and Technology

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Bachelor of Food Science

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versão revisada de acordo com a Resolução CoPGr 6018 de 2011

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DEDICATION

To all research lovers.

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“Science and everyday life cannot and should not be separated.” – Rosalind Franklin

SUMARY

RESUMO

Recuperação de compostos ativos de resíduos de óleo de abacate: alternativas sustentáveis para obtenção de novos ingredientes

Durante o processamento do óleo de frutos, como o de abacate, somente a polpa é utilizada, enquanto a casca, a semente e a fração sólida da polpa, geralmente, são descartadas como resíduos. Entretanto, esses resíduos são fontes promissoras de compostos bioativos, com potencial de aplicação como ingredientes funcionais na indústria alimentícia e cosmética, devido ao alto teor de compostos fenólicos e à alta capacidade antioxidante. Para tanto, a obtenção desses compostos de interesse deve ser estudada de forma holística, em que todas as etapas do processo de obtenção de ingredientes bioativos sejam eficientes e amigáveis ao meio ambiente. Com isso, essa dissertação teve como objetivo otimizar o processo para a recuperação de compostos bioativos, como os compostos fenólicos, a partir do resíduo da extração do óleo de abacate, agregando valor aos materiais descartados. Para tanto, no Capítulo 1, é apresentada uma revisão sobre o processamento do óleo de abacate, os principais resíduos gerados durante o processamento, o potencial de reutilização desses resíduos como fonte de compostos bioativos e as possíveis aplicações desses compostos nas indústrias alimentícias e cosméticas. No Capítulo 2, apresenta-se a otimização da obtenção dos compostos bioativos de resíduos da produção do óleo de abacate da variedade Breda, por meio da extração assistida por ultrassom. A metodologia de superfície de resposta com um arranjo experimental do tipo planejamento composto central rotacional foi utilizada para otimizar as variáveis experimentais (concentração de etanol (%), a temperatura (°C) e a razão entre amostra/solvente (g/mL)). A extração dos compostos fenólicos e dos taninos foi significativamente ($p < 0,05$) influenciada pela concentração do etanol e pela proporção entre amostra/solvente. A temperatura não influenciou significativamente no rendimento da extração, definindo-se, portanto, 30 °C como a temperatura usada, devido à sua proximidade com a temperatura ambiente, implicando em um menor gasto energético. Os extratos obtidos em condições otimizadas foram caracterizados quanto ao teor de compostos fenólicos totais (CFT), atividade antioxidante (DPPH, ABTS e FRAP), atividade antimicrobiana e compostos fenólicos (ácido 3,4-dihidroxibenzoico, 4-hidroxibenzoico, cafeico, siríngico e catequina). Para a casca, a concentração ótima de etanol foi de 40%, temperatura de 30 °C e a proporção de amostra/solvente de 0.225 g/mL. Para a semente, as condições ótimas foram 20% de etanol, 30 °C e proporção de amostra/solvente de 0.5 g/mL. Ambos os extratos apresentaram altas atividades antioxidantes, entretanto, não foram capazes de inibir os microrganismos estudados. Além disso, foi possível identificar altas concentrações de compostos fenólicos nos extratos otimizados. Com isso, os resultados indicam que os extratos obtidos podem ser considerados uma boa fonte bioativa sustentável para fins alimentícios e cosméticos.

Palavras-chave: Óleo de abacate, Resíduos, Capacidade antioxidante, Otimização, Superfície de resposta

ABSTRACT

Recovery of active compounds from avocado oil residues: sustainable alternatives for obtaining new ingredients

During the fruit oil processing, such as the avocado oil, only the pulp is used, while the peel, seed and the solid fraction of the pulp (pomace) are generally discarded as waste. However, these residues are promising sources of bioactive compounds, with potential application as functional ingredients in the food and cosmetics industry, due to the high content of phenolic compounds and the high antioxidant capacity. Therefore, the obtention of compounds of interest should be studied in a holistic way, in which all stages of the process of obtaining bioactive ingredients are efficient and friendly to the environment. Thus, this dissertation aimed to optimize the process for the recovery of bioactive compounds, such as phenolic compounds, from the residues of avocado oil extraction, adding value to discarded materials. Hence, in Chapter 1, a review is presented on the processing of avocado oil, the main residues generated during processing, the potential for reuse of these residues as a source of bioactive compounds and the possible applications of these compounds in the food and cosmetics industries. Chapter 2 presents the optimization of obtaining bioactive compounds from avocado oil production, of the Breda variety, through ultrasound-assisted extraction. The response surface methodology with a central composite rotatable design (CCRD) was used to optimize the experimental variables (ethanol concentration (%), temperature (°C) and solid/solvent ratio (g/mL)). The extraction of phenolic compounds and tannins was significantly ($p < 0.05$) influenced by the ethanol concentration and the solid/solvent ratio. Temperature did not significantly influence the extraction yield, so the temperature of 30 °C was chosen for energy-saving reasons. The extracts obtained under optimized conditions were characterized as the content of total phenolic compounds (TPC), antioxidant activity (DPPH, ABTS, and FRAP), antimicrobial activity and phenolic compounds (3,4-dihydroxybenzoic, 4-hydroxybenzoic, caffeic, syringic acid and catechin). For the peel, the optimal concentration of ethanol was 40%, temperature of 30 °C and the ratio of solid/solvent of 0.225 g/mL. For the seed, the optimal conditions were 20% ethanol, 30 °C and a ratio of solid/solvent of 0.5 g/mL. Both extracts showed high antioxidant activities, however, they were not effective in inhibiting the microorganisms studied. In addition, it was possible to identify high concentrations of phenolic compounds in the optimized extracts. Thus, the results indicate that the extracts obtained can be considered a good sustainable bioactive source for food and cosmetic purposes.

Keywords: Avocado oil, Waste, Antioxidant capacity, Optimization, Response surface

1. INTRODUCTION

The underutilization of waste streams or byproducts that have a high content of organic matter and bioactive compounds, produced by the development of new food products and the increase in agricultural activities, negatively affects the environment and the financial viability of the agricultural sector (AYALA-ZAVALA et al., 2011). This negative effect can be reduced by using better practices, such as upcycling, which is the conversion of food byproducts into new ingredients, animal feed or fuel, and circular strategies, e.g. the reuse of industry waste (GALANAKIS, 2018).

The processing of fruits with high nutritional and economic value, such as avocado, are examples of the generation of a significant volume of waste, both solid and liquid (ARAÚJO et al., 2018). However, they are also promising sources of bioactive compounds that can be recovered and used as valuable substances through the development of new processes (SAAVEDRA et al., 2017).

Avocado oil is extracted from the pulp of the fruit, in a similar process to olive oil extraction (COSTAGLI; BETTI, 2015). As only the pulp is used, the seed and the peel represent a large amount of waste (AVHAD; MARCHETTI, 2016). Due to the high content of phenolic compounds, functional proteins and antioxidant capacity present, recent studies have shown that these byproducts have wide application possibilities, both in the food and cosmetics industries (HÜRKUL et al., 2021; PERMAL et al., 2020; TESFAYE et al., 2022). In addition, consumers demand products that are safe and preferably free of synthetic additives (SANTOS-SÁNCHEZ et al., 2017). Synthetic antioxidants, for example, have demonstrated possible toxic effects during long-term intake. Due to this, common synthetic antioxidants such as BHA, BHT and TBHQ, are banned or have strict restrictions for food uses in some countries such as Japan and European countries (OUSJI; SLENO, 2020; SHARMA et al., 2019; SHASHA; MAGOGO; DZOMBA, 2014). This confirms the opportunity for studies to efficiently obtain antioxidants and bioactive compounds from natural sources.

However, the processing methods of these byproducts still need to be explored. Even though we live in an era of advanced food reuse and new technology development, without a doubt, we face one of the greatest challenges of human life, which is to develop a healthy and sustainable food system (GUERRA et al., 2015). Hence, the development of the food system requires the adoption of a holistic approach in which food production systems are competitive and environmentally friendly (PEIXOTO; PINTO, 2016; HULLOVA et al., 2019).

Therefore, although the sustainable food production chain includes a wide range of stages, such as agricultural food production, food processing, retail and consumption, this research focused on the development of strategies for the management and use of food waste,

by the conversion of these products into value-added materials. Thus, this project aimed to study the clean recovery of bioactive compounds, such as phenolic compounds, from agro-industrial waste, using process optimization techniques, to add value to discarded materials.

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2. CHAPTER 1. A REVIEW OF THE POTENTIAL USE OF RESIDUES FROM AVOCADO OIL PRODUCTION

Abstract

Background

Processing oil from different vegetable sources produces a significant amount of byproducts. The extraction of oil from vegetables and fruits, such as avocado, are examples of solid and liquid residues that generate direct environmental impacts in the regions where they are produced. In parallel, the demand for additives from sustainable and environmentally friendly sources has forced both the food industry and the academic community to find alternatives to synthetic antioxidants and preservatives.

Scope and Approach

In this review, the bioactive potential of residues from the production of avocado oil, especially peels and seeds, is described. This study emphasizes the importance of the reuse of residues generated during the production of avocado oil, as they are promising sources of bioactive compounds, which show high content of phenolic compounds, functional properties, and antioxidant capacity.

Key Findings and Conclusions

The studies analyzed identified a great nutritional and phytochemical potential of the peels and seeds, which can be used as food or cosmetics ingredients. However, an optimized process for the extraction of these compounds still needs to be better investigated, enabling the valorization of these materials in the productive sector.

Keywords: Bioactive compounds; Avocado oil byproducts; Extraction optimization

2.1 Introduction

Avocado is a fruit of high economic nutritional value that is rich in proteins, fibers and fat-soluble vitamins, including vitamins A and B and average levels of vitamins D and E. In addition, the lipid content is one of the most important factors in avocado, containing a large amount of oil compared to other fruits. It is widely used in pharmaceuticals and cosmetics industries, as well as in the food industry to obtain commercial oils similar to olive oil, due to its similar composition of fatty acids (Salgado et al., 2008; Duarte et al., 2016; Araújo et al., 2018).

Avocado oil is extracted from the pulp of the fruit, resembling many of the principles of olive oil extraction during its processing (Costagli & Betti, 2015). Since only the pulp is used, the seeds and the peels represent a large amount of waste (Avhad & Marchetti, 2016). Hence, the underutilization of these materials represents a serious threat to the environment and the financial viability of the agricultural sector and the food industry (Ayala-Zavala et al., 2011).

The conversion of avocado processing byproducts into food, fuel, application as ingredients or animal feed is therefore a fundamental step toward mitigating economic and environmental obstacles (Barba et al., 2015).

Recent studies have shown that these byproducts have wide application possibilities due to their high content of phenolic compounds, functional proteins, and antioxidant capacity (Jimenez et al., 2020). Thus, interest in techniques to efficiently extract these compounds has increased to add value to the discarded materials (Araujo et al., 2021; Ejiofor et al., 2018; Krumreich et al., 2018; Yepes-Betancur et al., 2021).

In this context, this work aimed to review available data on the functional properties and bioactive compounds of the byproducts obtained during the processing of avocado oil, especially peels and seeds. We also discussed possible alternatives to make agro-industrial waste recovery processes more attractive, clean, and efficient.

2.2 Avocado fruit

Avocado (*Persea americana*) originated in southern Mexico, where archaeological remains indicate that its cultivation began 6000 years ago (Popenoe, 1935; Costagli & Betti, 2015). The avocado varieties grown for agricultural purposes originate from three vegetable races, long recognized: the Mexican avocados (*Persea americana* var. Drymifolia), Guatemalan (*Persea americana* var. guatemalensis) and West Indian race (*Persea americana* var. americana) (Bergh & Ellstrand, 1986; Duarte et al., 2016).

The main cultivars planted in the world are hybrids obtained from natural crossings between different races, such as Breda, Fortuna, Geada, Margarida, Ouro Verde and Quintal (hybrids of the Antilhana and Guatemalense races) and Hass and Fuerte (hybrids of the Mexican and Guatemalan races) (Almeida et al., 2018).

In Brazil, the introduction of the fruit took place in the early 19th century in Pará and extended along the Atlantic coast to Rio Janeiro and São Paulo (Wiltbank, 1977). In the 1970s, avocado production greatly developed in the country due to the tax incentives granted by the Federal Government, which financed orchards with commercial characteristics from grafted seedlings (Campos, 1984 *apud* Santos Francisco & Baptistella, 2005). Today, the country stands out in this fruit's production, ranking seventh in the world production ranking, with a production of 266,784 t in 2020 (FAO, 2020).

Avocado is grown in almost all states in Brazil, with the state of São Paulo being the largest producer, with a production in 2018 of 130,202 t (49% of the national total). The second largest producing state, Minas Gerais, has a share of approximately 30%, followed by the states of Paraná with 10% and Espírito Santo and Ceará with 3% each (IBGE, 2020).

Among the main cultivars present in Brazil are Fucks (West Indian-race), Breda, Fortuna, Geada, Margarida, Ouro Verde, Quintal, Hass and Fuerte (Almeida et al., 2018). The fruits of these different cultivars can have variable chemical compositions, and they differ in size, color when mature and oil content (Duarte et al., 2016; Tango et al., 2004).

The proportion of peel, pulp and seed can vary from 8.6 to 22.9%, 52.9 to 81.3% and 10.1 to 25.1%, respectively, according to the species and geographic region in which the avocado tree was cultivated (Tango et al., 2004). The products of greatest commercial interest are derived from avocado pulp, such as oils, pulps, guacamoles, and pastes (Daiuto et al., 2014).

2.2.1 Avocado oil: chemical composition, bioactive compounds present and extraction process

The avocado oil is contained in a finely dispersed emulsion in the cells of the fruit pulp, requiring a rupture not only of the cell walls but also of the structure of the emulsion for its extraction (Costagli & Betti, 2015). Depending on the cultivation method and the variety of the fruit, the amount of oil present in the avocado pulp can vary from 8 to 30% (Gómez-López, 2002; Takenaga et al., 2008; Galvão, Narain, & Nigam, 2014; Peraza-Magallanes et al., 2017).

It is important to note that this product has stood out among other oils due to the presence of a great diversity of sterols, including β-sitosterol, and the high level of monounsaturated fat, such as oleic acid, which can be used as adjuvants in the treatment of hyperlipidemia (Salgado et al., 2008; Gupta et al., 2011; Santos et al., 2014). Other bioactive components, such as vitamin E (tocopherols and tocotrienols), phenolics, carotenoids, and chlorophylls, have also made this oil very attractive, since the presence of tocopherols increases the antioxidant activity and inhibits lipid oxidation (Tabee et al., 2008; Krumreich et al., 2018; Tan, 2019).

Hence, the composition of avocado oil is similar in many ways to olive oil, including oxidative instability during heating (Salgado et al., 2008; Berasategi et al., 2012).

Avocado oil can be extracted in different ways, aiming to rupture the structure of the emulsion. The main traditional methods commonly used in industry for the extraction of oil are the centrifugation/mechanical pressing, solvent extraction, and the cold-pressed mechanical method. For the first method, the oil is obtained by mixing the pulp in water, then decanting and centrifugating/pressing to separate the oil. In the second method, the whole ripe fruit is dried, mechanically pressed at a high temperature and then extracted with organic solvents (Reddy, Moodley, & Jonnalagadda, 2012; Costagli & Betti, 2015; Satriana et al., 2019). Despite presenting a high extraction yield, the use of organic solvents, such as hexane, heptane, and

methanol, has generated concerns about its environmental impacts. Additionally, edible oil may have its characteristics changed or even contain residues from the removal process of these solvents (Cheng et al., 2018; Ramezani, Saeedi & Hashemi, 2018; Tan et al., 2018a). Lastly, the cold-pressed method is a fast extraction technique that extracts less than the solvent extraction, however, the energy spent during the solvent evaporation and the oil refining can be avoided (Satriana et al., 2019).

Additionally, several alternative processes of pulp oil extraction have been studied, such as assisted by ultrasound or microwave and with supercritical CO_2 (Reddy, Moodley, & Jonnalagadda, 2012; Krumreich et al., 2018; Tan et al., 2018b; Flores et al., 2019; Tan, 2019). Figures 1 and 2 show the general flowcharts of these processes.

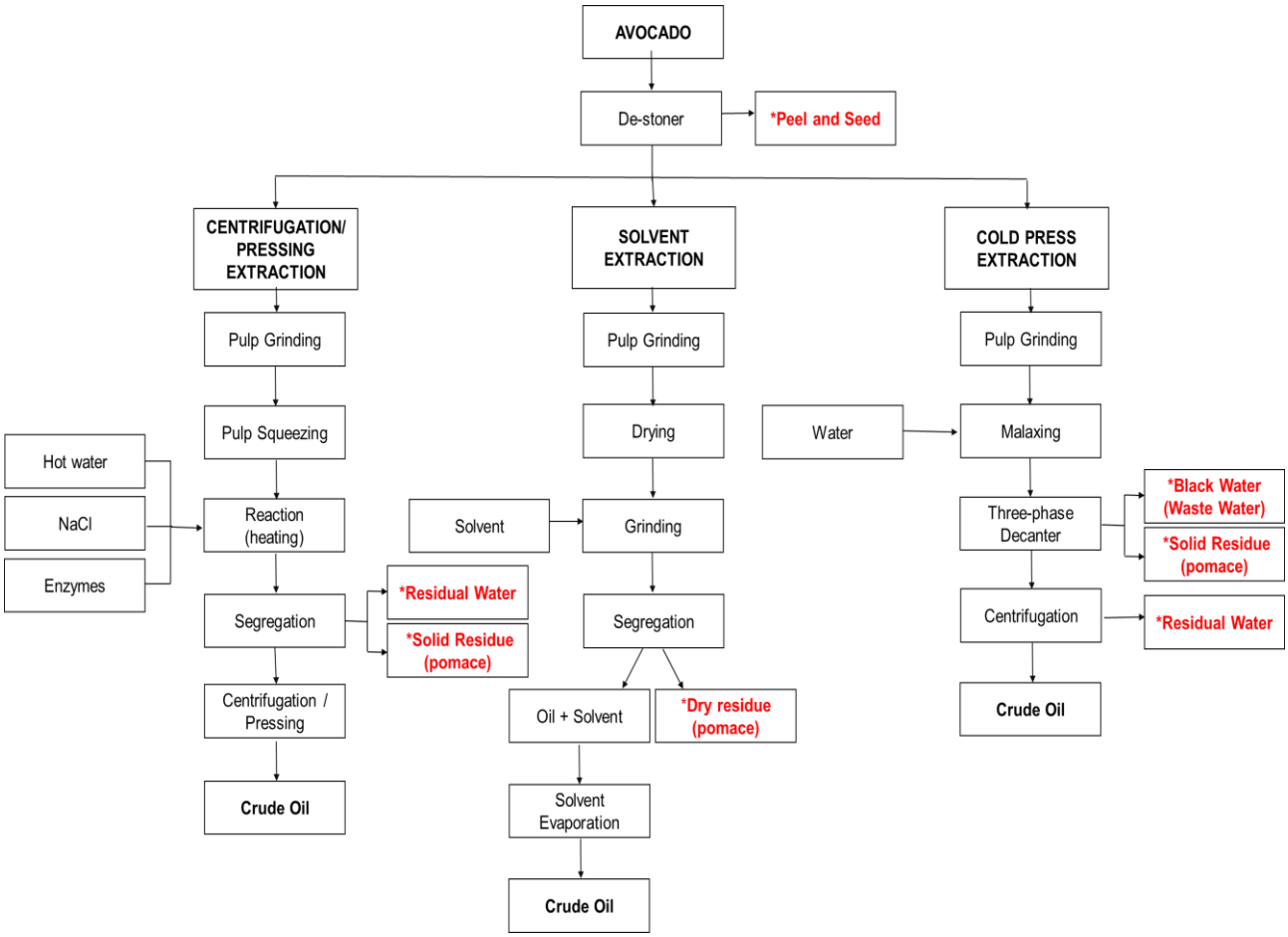

Figure 1. Flowchart of common extraction processes for avocado oil: centrifugation/pressing extraction (Satriana et al., 2019); solvent extraction (Salgado et al., 2008; Reddy, Moodley, & Jonnalagadda, 2012) and cold pressing extraction (adapted from Costagli & Betti, 2015 and Permal et al., 2020).

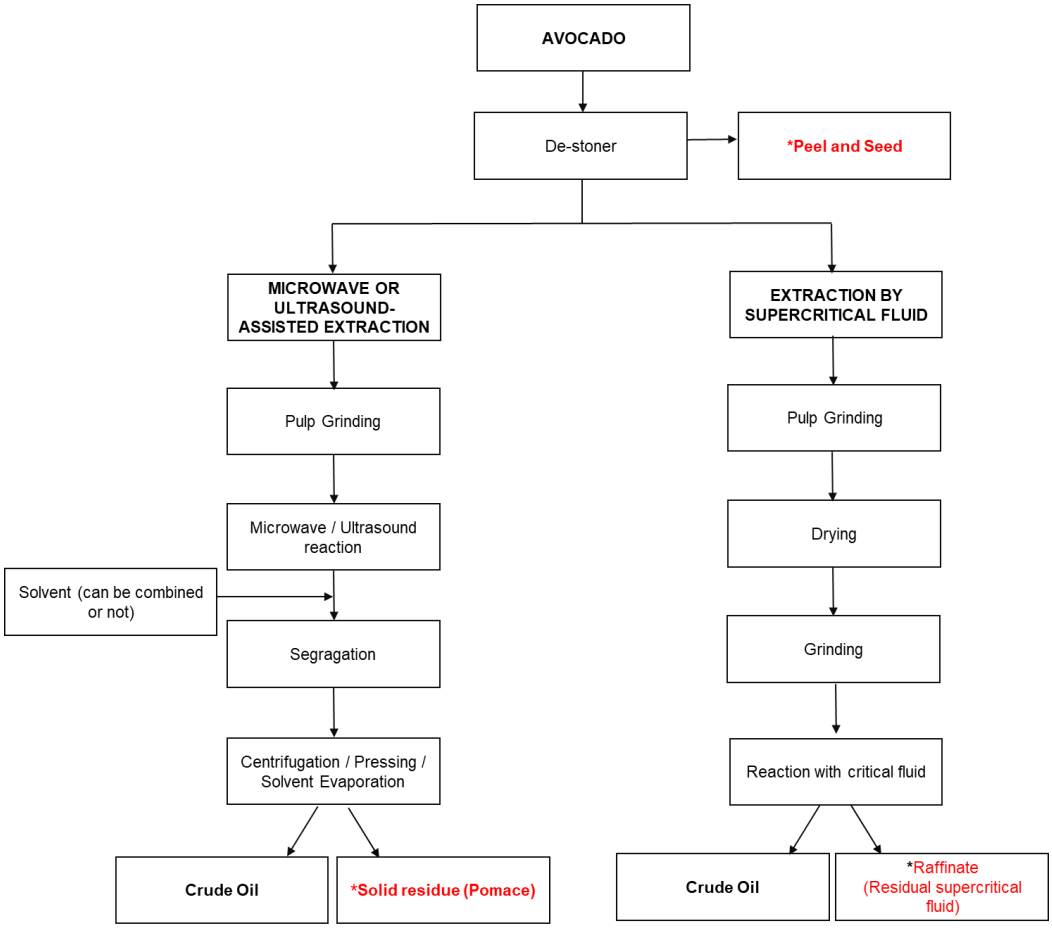

Figure 2. Flowchart of alternatives extraction processes for avocado oil: microwave-assisted extraction (Ortiz Moreno et al., 2003; Reddy et al., 2012); ultrasound-assisted extraction (Martínez-Padilla et al., 2018; Tan, Chong, et al., 2018) and extraction by supercritical fluid (Corzzini et al., 2017).

Even with several methods, in all the processes of extraction of avocado oil, only the pulp is used, generating large waste of peels, seeds and the solid residues of the pulp (pomace) (Araújo et al., 2018).

2.2.2 Lipid extraction residues

During the processing of avocado into oil and paste, approximately 20 to 50% of the weight of the fruit is discarded (Tango et al., 2004; Wang et al., 2010).

The main residues produced in the extraction of avocado oil are solid and liquid residues, such as:

- Solid waste: conventional pomace and vegetable remains, peels, seeds, earth and stones that were removed through the process of cleaning the fruits.

- Liquid waste: produced through the process of preparing the fruits for extraction (water for washing the fruits, water for washing the storage places), water used during the process (vegetation water), and the residual solvents associated with the avocado oil solvent extraction (Costagli & Betti, 2015; Qin & Zhong, 2016).

These large quantities of byproducts have aroused interest in the potential for use in the food, chemical and pharmaceutical industries due to the high content of phenolic compounds and antimicrobial, antifungal, and phytochemical compounds present. In addition, the significant growth in environmental concerns and sustainable development has driven the strategic reuse of these byproducts (Colombo & Papetti, 2019).

Avocado seed represents 10 to 25% of the fruit and is usually discarded during processing (Tango et al., 2004). This byproduct, as shown in Figure 3, is composed of a very thin shell (endocarp) that surrounds the nucleus (seed itself) (Araújo et al., 2018). Due to its high content of proteins, lipids, carbohydrates and fibers, it can provide the development of new food ingredients as an alternative source of starch and fibers (Barbosa-Martín et al., 2016; Chel-Guerrero et al., 2016). Additionally, it represents a potential material to the pharmaceutical industry, with uses in veterinary antibiotics, due to the presence of natural compounds related to antibacterial and fungicidal activities (Leite et al., 2009; Salinas-Salazar et al., 2017).

Similar to the seed, the peel represents 9 to 23% of the fruit and is discarded (Salazar-López et al., 2020). This residue corresponds to the outermost part of the fruit, the epicarp (Figure 3). In addition to the antioxidant and antimicrobial activity, some recent studies have also pointed to mosquito larvicidal activities due to the high presence of bioactive compounds in these byproducts (Louis et al., 2020).

Furthermore, studies have shown that seeds and peels are rich in phenolic compounds, presenting higher in vitro antioxidant activity than those found in edible portions (pulp) and common synthetic antioxidants, which may be of interest to the industry as sources of natural bioactive compounds (Melgar et al., 2018; Rodríguez-Carpena et al., 2011; Wang et al., 2010).

On the other hand, pomace, composed of solid residues from the pulp, represents the fruit's mesocarp. Despite limited information on the bioactivity of this residue, Permal et al. (2020) point out the presence of a significant amount of dietary fiber, a potential alternative ingredient for the food industry or animal nutrition.

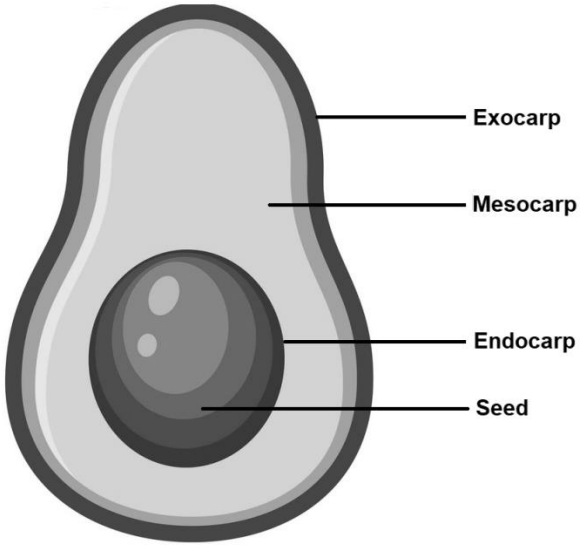

Figure 3. Composition of avocado fruit (Araújo et al., 2018).

Some studies that report these bioactive compounds with potential reuse from residues, both from avocado fruit and those obtained by extracting avocado oil, are summarized in Table 1.

Table 1. Bioactive compounds, antioxidant and antimicrobial activities reported for some residues from the use of avocado (seed, peel and pomace).

References	Year	Compound studied	Byproduct	Results*	Avocado Variety	Origin of the Fruit	Extration type of bioactive compounds
Bioactive Compounds/Antioxidant Activity							
Rodríguez-Carpena et al.	2011	Total Phenolic Coumponds	Seeds	16.99 – 69.12 mg GAE.g^{-1} of dried sample	Hass and Fuerte	Spain	Solvent extractions: ethyl acetate; acetone/water (70:30 v/v) or methanol/water (70:30 v/v)
			Peels	32.93 – 172.18 mg GAE. g^{-1} of dried sample			
		Antioxidant Activity CUPRAC	Seeds	58.00-353.43 mmol TE.g^{-1} of sample			
			Peels	56.40-330.75 mmol TE.g^{-1} of sample			
		Antioxidant Activity ABTS	Seeds	21.57-194.80 mmol TE.g^{-1} of sample			
			Peels	16.12-242.26 mmol TE.g^{-1} of sample			
		Antioxidant Activity DPPH	Seeds	17.78-167.50 mmol TE.g^{-1} of sample			
			Peels	17.85-199.61 mmol TE.g^{-1} of sample			
Vinha; Moreira; Barreira	2013	Total Phenolic Coumponds	Seeds	7.04±13.0 mg GAE.g^{-1} of fresh sample	Hass	Portugal	Aqueous extraction at 40 °C
			Peels	6.79±11.70 mg GAE. g^{-1} of fresh sample			
		Flavonoids	Seeds	0.48±0.28 mg flavonoids. g^{-1} of fresh sample			
			Peels	0.44±0.31 mg flavonoids. g^{-1} of fresh sample			
		Carotenoids	Seeds	0.10±0.01 mg equivalentes de β-carotene. g^{-1} of fresh sample			
			Peels	0.03±0.00 mg equivalentes de β-carotene. g^{-1} of fresh sample			
		Vitamin C	Seeds	0.03±0.01 mg ascorbic acid. g^{-1} of fresh sample			
			Peels	0.04±0.03 mg ascorbic acid. g^{-1} of fresh sample			
		Vitamin E	Seeds	0.05±0.01 mg α-tocoferol. g^{-1} of fresh sample			
			Peels	0.02±0.01 mg α-tocoferol. g^{-1} of fresh sample			
Daiuto et al.	2014	Antioxidant Activity DPPH	Seeds	43% SRL	Hass	Bauru-SP, Brazil	Hydroethanolic extraction: ethanol:water (80:20 v/v) assisted by ultrasound for 15 minutes
			Peels	35% SRL			
		Total Phenolic Coumponds	Seeds	57.3 ± 2.7 mg GAE. g^{-1} of sample			
			Peels	63.5 ± 7.2 mg GAE.g^{-1} of sample			
		Antioxidant Activity DPPH	Seeds	410.7 ± 35.8 mg GAE. g^{-1} of sample			
			Peels	310.0 ± 36.9 mg GAE. g^{-1} of sample			
		Antioxidant Activity ABTS	Seeds	645.8 ± 17.9 mg GAE. g^{-1} of sample			

			Peels	791.5 ± 35.9 mg GAE. g^{-1} of sample			
Antasionas, Riyanto and Rohman	2017	Antioxidant Activity DPPH		9.47-78.33 EC_{50} µg.mL^{-1}	Bacon	Indonesia	Solvent extraction: petroleum ether, ethyl acetate and methanol
		Antioxidant Activity ABTS	Peels	1.12-156.70 EC_{50} µg.mL^{-1}			
		Reducing Power		63.98-742.87 EC_{50} µg.mL^{-1}			
Saavedra et al.	2017	Total Phenolic Coumponds	Seeds	12.52-33.23 mg GAE. g^{-1} of dried sample	Hass	Chile	Solvent extraction: acetone:water (70:30 v/v)
			Peels	12.42-31.10 mg GAE. g^{-1} of dried sample			
		Antioxidant Activity DPPH	Seeds	0.44- 1.11 mmol TE. g^{-1} of dried sample			
			Peels	0.60-1.53 mmol TE. g^{-1} of dried sample			
Melgar et al.	2018	Antioxidant Activity DPPH	Seeds	220 ± 3 EC_{50} µg.mL^{-1}	Hass	Portugal	Hydroethanolic extraction: ethanol:water (80:20 v/v)
			Peels	149 ± 5 EC_{50} µg.mL^{-1}			
		Reducing Power	Seeds	51 ± 1 EC_{50} µg.mL^{-1}			
			Peels	32 ± 1 EC_{50} µg.mL^{-1}			
Tremocoldi et al.	2018	Total Phenolic Coumponds	Seeds	57.3 - 59.2 mg GAE. g^{-1} of dried sample	Hass and Fuerte	Bauru-SP, Brazil	Hydroethanolic extraction: ethanol:water (80:20 v/v) assisted by ultrasound for 15 minutes
			Peels	63.5 – 59.2 mg GAE. g^{-1} of dried sample			
		Antioxidant Activity DPPH	Seeds	410.7 – 464.9 µmol TE.g^{-1} of dried sample			
			Peels	310 – 420.59 µmol TE.g^{-1} of dried sample			
		Antioxidant Activity ABTS	Seeds	580.8 – 791.5 µmol TE.g^{-1} of dried sample			
			Peels	791.5 – 1.004.5 µmol TE.g^{-1} of dried sample			
		Antioxidant Activity FRAP	Seeds	656.9 – 931.7 µmol Fe^{2+}.g^{-1} of dried sample			
			Peels	1.175.1 – 1.881.4 µmol Fe^{2+}.g^{-1} of dried sample			
		Total Phenolic Coumponds	Seeds	2.05 -11.36 mg GAE.g^{-1} of dried sample			
			Peels	5.45 - 38.82 mg GAE.g^{-1} of dried sample			
		Flavonoids	Seeds	0.19 – 0.65 mg. EQ.g^{-1} of dried sample			
			Peels	0.84 – 2.74 mg. EQ.g^{-1} of dried sample			
Amado et al.	2019	Antioxidant Activity DPPH	Seeds	42.75 – 379.63 µmol TE.g^{-1} of dried sample	Quintal, Hass, Margarida, and Fortuna	Londrina - PR, Brazil	Solvent extraction: ethanol 1:10 (m/v)
			Peels	156.47 – 482.65 µmol TE.g^{-1} of dried sample			
		Antioxidant Activity ABTS	Seeds	3.54 – 122.90 µmol TE.g^{-1} of dried sample			
			Peels	97.38 – 497.53 µmol TE.g^{-1} of dried sample			
		Antioxidant Activity FRAP	Seeds	5.85 – 160.00 µmol Fe II.g^{-1} of dried sample			
			Peels	3.62 – 8.58 µmol Fe II.g^{-1} of dried sample			
Permal et al.	2020	Total Phenolic Coumponds	Seeds	81 ± 10 mg GAE. g^{-1} of dried sample	Hass	New Zealand	Solvent extraction: methanol (50%) and acetone (70%)
			Peels	137 ± 4.0 mg GAE. g^{-1} of dried sample			
			Pomace	36 ± 1.3 mg GAE. g^{-1} of dried sample			
		Antioxidant Activity CUPRAC	Seeds	21 ± 0.9 mg TE. g^{-1} of dried sample			
			Peels	70 ± 1.20 mg TE. g^{-1} of dried sample			

		Antioxidant Activity FRAP	Pomace	71 ± 1.0 mg TE. g^{-1} of dried sample			
			Seeds	77 ± 3.5 mg TE. g^{-1} of dried sample			
			Peels	137 ± 7.6 mg TE. g^{-1} of dried sample			
		Antioxidant Activity fosfomolibdênio	Pomace	40 ± 0.1 mg TE. g^{-1} of dried sample			
			Seeds	81 ± 1.0 mg TE. g^{-1} of dried sample			
			Peels	120 ± 11.9 mg TE. g^{-1} of dried sample			
			Pomace	68 ± 19.0 mg TE. g^{-1} of dried sample			
		Antimicrobian activity					
Rodríguez-Carpena et al.	2011	Antimicrobial activity	Seeds	Inhibited growth of: *Bacillus cereus; Staphylococcus aureus; Listeria monocytogenes; Escherichia coli; Yarrowia lipolytica.*	Hass and Fuerte	Spain	Solvent extraction: ethyl acetate; acetone/water (70:30 v/v) or methanol/water (70:30 v/v)
			Peels	Inhibited growth of: *Bacillus cereus; Staphylococcus aureus; Listeria monocytogenes; Escherichia coli; Pseudomonas spp; Yarrowia lipolytica.*			
Nwaoguikpe; Braide; Ujowundu	2011	Antimicrobial activity	Seeds	Inhibited growth of: *Staphylococcus aureus; Listeria monocytogenes; Escherichia coli; Pseudomonas aeruginosa; Klebsiella pneumoniae.*	NA	Nigeria	Solvent extraction with experimental design: hot water; cold water; methanol and ethanol
Jiménez-Arellanes et al.	2013	Antimicrobial activity	Seeds	Chloroform extract inhibited the growth of: *Mycobacterium tuberculosis H37Rv*, isolado de *M. tuberculosis MDR SIN 4*, três cepas de referência resistentes a M*. tuberculosis H37Rv* e quatro micobactérias não tuberculosas (*M. fortuitum, M. avium, M. smegmatis e M. absessus*); Ethanol extract affected only the growth of two monoresistant strains of M*. tuberculosis H37Rv* e *M. smegmatis* (MIC ≤50 µg/ml).	NA	Mexico	Solvent extraction: chloroform and ethanol.
Salinas-Salazar et al.	2017	Antimicrobial activity	Seeds	Inhibited growth of: Listeria monocytogenes.	Hass	Mexico	Solvent extraction: Extract 1 - acetone (1:2 m/v); Extract 2 - Heptane:methanol (1:1 v/v)

*Legends= TE: Trolox equivalent; GAE: gallic acid equivalent; QR: Quercetin equivalent; EC50: sample concentration that achieves 50% of the antioxidant activity or 0.5 of absorbance in the reducing power assay; % SRL= percentage of free radical scavenging of 0.1 mg.mL^{-1} of extract; NA: Not available.

Through these studies, it is possible to see that each variety of avocado studied and each extraction method and solvent used has its particularities in the recovery of bioactive compounds. The amounts of bioactive compounds found by Tremocoldi et al. (2018) in the several varieties studied were different. Likewise, Amado et al. (2019) found different concentrations of phenolic compounds and antioxidant activity in residues of multiple varieties (Quintal, Hass, Margarida, and Fortuna). Thus, the obtainment of these compounds must be studied in detail for each variety and type of extraction, in addition to expanding the study to several other varieties not yet studied, to have a wide knowledge of the most efficient way to recover these bioactive compounds for each raw material. Furthermore, an optimized method for extracting these compounds is also essential for the efficiency of this recovery.

2.3 Bioactive compounds in avocado residues

Regardless of the particularities of each variety, tannins, phenolic acids, and flavonoids are the main groups of compounds found in avocado peels and seeds in previous studies.

Tannins can be defined as a group of natural polyphenols that have the ability to complex strongly with carbohydrates and proteins (Khanbabaee & Van Ree, 2001; Mateus et al., 2004; Sieniawska & Baj, 2017). These compounds are among the most studied as secondary metabolites of plants since they are considered an important defense factor, especially against herbivorous insects (Barbehenn & Peter Constabel, 2011; Salminen & Karonen, 2011). They can be found in leaf, seed, root and stem tissues, as well as in shoots (Helm, Ranatunga, & Chandra, 1997; Romani, Campo, & Pinelli, 2012). The condensed tannins derived were the most reported tannin group in avocado byproducts, with different polymerization degrees (Araujo et al., 2021; Figueroa et al., 2021).

In general, these polyphenols are highly bioactive due to their antioxidant, antimicrobial, antiviral and anticarcinogenic activities (Dong et al., 2018; Kaczmarek, 2020; Motta et al., 2020; Serrano et al., 2009). In addition, other physiological effects have been reported, highlighting anti-inflammatory and antidiabetic properties, as well as the protection of the cardiovascular system (Park et al., 2002; Kumari & Jain, 2012; Macáková et al., 2014). Furthermore, foods that contain low levels of tannins can be used as regulators of rumen microbiota. This occurs by the complexation of proteins and tannins, reducing the populations of proteolytic bacteria and, consequently, selecting the microorganisms present (Hoste et al., 2006).

Phenolic acids have a carboxyl group attached to a benzene ring and can be divided into compounds derived from benzoic acid (i.e., hydroxybenzoic acids) and cinnamic acid (i.e., hydroxycinnamic acids). The major hydroxybenzoic acids are gallic acid (3,4,5-trihydroxybenzoic acid), protocatechuic acid (3,4-dihydroxybenzoic acid) and p-hydroxybenzoic acid (4-hydroxybenzoic acid). Hydroxycinnamic acids are naturally synthetized in plants, producing the simplest hydroxycinnamic acid, p-coumaric acid, which can then be synthetized into caffeic, ferulic and sinapic acids (Bento-Silva et al., 2019; Khadem & Marles, 2010). Studies have shown that these compounds have antioxidant, antibacterial and antifungal activities, suggesting that they may significantly benefit the food, cosmetics and pharmaceutical industries (Gurbuzer, 2021; Hsu et al., 2021; Liu et al., 2020). Both groups of these compounds have been reported in avocado and its byproducts (Di Stefano et al., 2016; Weremfo et al., 2020).

Flavonoids are also included in the phenolic compound family and comprise a large group of different structures. In plants, the biosynthesis of flavonoids can proceed by two different pathways: through phenylpropanoids, which produce the phenylpropanoid skeleton (C6-C3), and the acetate pathway, which produces blocks for polymeric 2-carbon units (Dias et al., 2021; Nabavi et al., 2020). Flavonoids have many positive impacts on human health, including anti-allergenic, antimicrobial, anti-inflammatory and anti-carcinogenic activities (Ghani et al., 2020; Kobayashi et al., 2015; Omosimua et al., 2020). Among these bioactive qualities, the antioxidant effects are indubitably the most prominent. The antioxidant activity of flavonoids may be due to their direct scavenging of reactive oxygen species (ROS), inhibition of ROS formation through the chelation of trace elements, such as iron, or inhibition of the enzymes that generate free radicals (Dias et al., 2021; Nabavi et al., 2020; Nile et al., 2018). In avocado byproducts, some of the flavonoids previously reported were catechin, epicatechin, epicatechin gallate, rutin, naringenin and quercetin (Figueroa et al., 2021; Salazar-López et al., 2020; Weremfo et al., 2020).

2.3.1 Alternative processes for recovering the bioactive compounds

Despite numerous and important applications, most extraction processes for these compounds have been studied using chemical solvents, most of them petroleum derivatives, such as hexane, petroleum ether, methanol, acetone, among others (Rodríguez-Carpena, Morcuende, & Estévez, 2011; Gómez et al., 2014; López-Cobo et al., 2016; Saavedra et al., 2017). These, however, have disadvantages that go beyond the environmental impact, such as the permanence of solvent residues in the final products, which limits their use in food products (Qin & Zhong, 2016).

On the other hand, consumer demand for food and personal care products made from environmentally friendly materials is growing worldwide (Costa & Johnson, 2019; Quintin et al., 2019). Therefore, for greater efficiency in the recovery of compounds of interest present in avocado oil residues, alternatives to conventional extraction processes still need to be explored and optimized.

Some studies have shown that enzymatic extraction can be an alternative (Gómez-García et al., 2012; Nadar et al., 2018). Several enzymes, such as cellulases, pectinases and hemicellulose, are often used to disrupt the structural integrity of the plant cell wall, increasing the extraction of bioactive components, and can be used synergistically with other solvents or physical media (Puri, Sharma, & Barrow, 2012).

The use of ultrasound has also been shown to be efficient for extracting bioactive compounds, having several benefits and advantages over conventional extraction processes (Silva, Garcia, & Franciscato, 2016; Tan et al., 2018b; Tremocoldi et al., 2018). This method can offer greater penetration of the solvent into the cell material, shorter processing time and a low consumption of solvents, as well as high processing yield (Roselló-Soto et al., 2015).

Husen et al. (2014) evaluated the extraction of phenolic compounds from hydroalcoholic extracts from avocado pulp and observed that ultrasound-assisted extraction allowed for a greater obtainment of phenolic compounds in the extracts. Likewise, Che-Galicia et al. (2020) studied the incorporation of ultrasound in the extraction of bioactive compounds from avocado leaves and realized that the method significantly increased the extraction yield, demonstrating that it is a promising technology to extract phenolic substances from these residues.

Thus, although possible, extraction processes using environmentally friendly methodologies still need to be optimized so that they are carried out efficiently and without significant loss of the compounds of interest from the waste (Qin & Zhong, 2016). Therefore, the goal is to intensify studies related to the improvement of existing methods, combining different extraction and pretreatment processes (mechanical, ultrasonic, enzymatic, or thermal). As a result, a method that is effective in recovering the compounds and sustainable and viable on an industrial scale is expected.

2.4 Conclusion

It is possible to recover bioactive compounds from avocado oil agro-industrial residues using clean processing technologies, adding value to currently discarded solid materials and enabling a holistic approach in which all stages of the food production system are competitive and friendly to the environment. The type and amount of the bioactive compounds vary according to the avocado variety, solvents used for extraction, and the

extraction parameters. Then, optimized processing methods can improve the extraction efficiency, adding value to a product that can be reused in the food and cosmetic industries.

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3. CHAPTER 2: INDUSTRIAL BYPRODUCTS FROM AVOCADO OIL PRODUCTION: RECOVERY OF BIOACTIVE COMPOUNDS BY OPTIMIZED ULTRASOUND-ASSISTED EXTRACTION

Abstract

Brazil is one of the largest avocado producers in the world, and, in, addition to fruit consumption, avocado oil has become popular. To find an alternative to pressed oil production, which generates a large amount of byproducts, the ultrasound-assisted extraction of bioactive compounds was optimized. A central composite rotatable design (CCRD) was used, and extraction parameters were studied. The optimal conditions for the extraction of phenolic compounds and tannins from the peels were an ethanol concentration of 40%, a temperature of 30 °C, and a solid-solvent ratio of 0.225 g/mL. For seeds, the optimal conditions were an ethanol concentration of 20%, a temperature of 30 °C, and a solid-solvent ratio of 0.5 g/mL. Under these conditions, the experimental results agreed with the predicted values. The extracts presented high antioxidant capacity. High-performance liquid chromatography (HPLC) analysis of the optimized extract revealed the presence of phenolic compounds in the peel and seed extracts, with 3,4-dihydroxybenzoic acid being the major compound in both extracts. Therefore, this optimized ultrasound-assisted extraction method has been demonstrated to be a potential technique for the efficient recovery of polyphenolic antioxidants from avocado oil byproducts, transforming these materials into value-added products with high antioxidant properties that may have different food and cosmetic uses.

Keywords: Breda Avocado, Surface response, Optimization, Avocado waste, Phenolic compounds

3.1 Introduction

The avocado fruit (*Persea americana Mill)* is an evergreen crop extensively cultivated in tropical and subtropical areas. Brazil is the seventh largest producer of avocado in the world. The Brazilian avocado harvested area occupied, in 2020, an area of 16,211 ha situated mainly in the southeast of the country, comprising mostly hybrids of the West Indian and Guatemalan races, such as the Breda and Margarida varieties.[1–3]

Breda avocado, the most consumed variety in Brazil, is constituted primarily by the fresh pulp (72%), followed by the seed (21%), and peel (7%).[4] In addition to fruit consumption, due to its high content of lipids and its high nutritional value, the pulp has been used for extracting avocado oil, which has generated growing interest among consumers.[5] The oil has a very good nutritional value, since it is rich in oleic acid, tocopherols, and total phenols, which allows for a multitude of applications in foods, cosmetics, pharmaceuticals, and

nutraceuticals.[5,6] However, similar to oils extracted from other fruits and vegetables, avocado oil processing generates a large amount of byproducts that are mainly composed of peels, seeds, residual water, and residual pulp (pomace).[7,8] These solid residuals are commonly disposed of, improperly discarded, or incinerated, generating serious environmental and public health concerns. Instead, these large amounts of agricultural waste require other solutions to convert them into value-added materials, which should be incorporated into the company's strategy, to shift from a linear to a circular economy.[9]

Avocado oil byproducts are sources of dietary fiber (peel 20.1%; seeds 8.4%; wastewater 2.6%; pomace 12.4%), carbohydrates (peel 0.3%; seeds 29.7%; wastewater 0.1%; pomace 0.1%), protein (peel 2%; seeds 2.1%; wastewater 2.6%; pomace 1.2%), lipids (peel 1.7%; seeds 1.6%; wastewater 6.3%; pomace 1.6%).[8] In addition, seeds and peels are rich sources of bioactive compounds, such as polyphenols with antioxidant and antimicrobial proprieties.[10–14] These byproducts require processing methods that allow for the least amount of unusable residues and highest yield while also preserving the integrity of bioactive compounds of interest. Nonetheless, due to the unstable nature of phenolic compounds, each vegetable phenolic source demands an individual approach for extraction.[15]

The extraction process depends on several factors, such as the amount of sample, time, solvent composition, temperature, and solvent-to-sample ratio.[16] The optimization of these factors is essential to achieve maximum yield of the compounds of interest, avoiding the environmental and economic impacts using fewer toxic solvents, in lesser volumes, and increasing the commercial value of the raw material and its profitability. Beyond the obvious requirements for developing economical, safe, effective, and environmentally friendly extraction techniques, it is important that such techniques not only allow a clean label product but also ensure higher yields with minimal impact on the quality of the final product. The Central Composite Rotatable Design (CCRD) has been effectively used in determining ideal conditions for the extraction of antioxidant compounds from different vegetable extracts, such as green propolis, acerola residues, and coffee leaves.[17–19] In addition to exploring several factors, this experimental design requires fewer tests and is less time-consuming compared to the full factorial design experimentation, reducing the experimental error.[20,21] Additionally, in association with this methodology, the application of the ultrasonic approach is a potential alternative to the extraction of bioactive compounds, since the mechanical effects of ultrasound provide greater solvent penetration into cellular materials and improve mass transfer, allowing for the reduction of toxic solvents and the use of lower temperatures and periods.[16,22]

Some studies on avocado byproducts have been published, which report on antioxidant compounds and the different extraction processes, such as extraction solvent (ethanol:water) [8,23], microwave-assisted extraction (MAE) [24,25,] Soxhlet (SE), supercritical carbon dioxide ($scCO_2$) extraction [26] and ultrasound-assisted extraction (UAE) [27]. However, to

our knowledge, there is a lack of studies involving UAE in Breda's avocado oil byproducts, evaluating the optimization of the solvent composition, temperature, and solvent-to-sample ratio at the same time. Thus, the objective of this study was to optimize the extraction of bioactive compounds from the waste of industrial Breda's oil using solvents from a sustainable source at minimal processing temperature and assisted by ultrasound extraction.

3.2 Materials and Methods

3.2.1 Chemicals

Gallic acid, ABTS (2.2'-azinobis-(3-ethylbenzothiazoline-6-acid)), DPPH (2,2-diphenyl-1-picrylhydrazyl), TPTZ ((2,4,6-Tris(2-piridil)-s-triazina), TBA and Trolox (6-hydroxy2,5,7,8-tetramethylchroman-2-carboxylic acid) were obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Folin–Ciocalteau reagent was purchased from Êxodo Científica (Sumaré, SP, Brazil). Other reagents were analytical grade.

3.2.2 Samples

Avocado seeds, peel and pomace from the Breda variety were collected from the Paraíso Verde avocado oil processing plant, located in São Tomás de Aquino, Minas Gerais, Brazil. Approximately 4 kg of seeds and 4 kg of peels were collected directly from the destoner, washed, dried for 4 h in the sun, and placed in plastic bags. The residual pulp (pomace) (4 kg) was obtained from the decanter immediately after avocado oil extraction. Samples were immediately stored at -18 °C and transported in an ice bath to the laboratory at ESALQ/USP - Brazil. A random portion of the fresh samples was used for physical-chemical analysis, while the remaining portion was freeze-dried using a Liotop Laboratory Freeze Dryer (model L101) for 96 h at −52 °C and 500 μHg, ground in a common blender, homogenized, sift through a 1 mm mesh, and then stored at −18 °C until further analysis. In this study, Breda's avocado oil waste samples were collected during the beginning of the season (June 2020).

3.2.3 Proximate analysis of avocado byproducts

Standard methods were used to determine pH, water activity (aw), moisture, ash, protein, fat, nitrogen-free extract, crude fiber, and dietary fiber.[28,29] The pH was determined using a digital bench-top pH meter (Tecnal model Tec-3MP) calibrated with buffer solutions of pH 4.0 and 7.0. The water activity was measured by a bench-top water activity analyzer

(TESTO 650, Lenzkirch, Germany). Moisture content was determined based on the mass difference after oven drying at 105 °C until constant weight.[28] Ash content was calculated from the difference between the initial dried and final sample weight after combustion at 550 °C for 5 h using a muffle furnace (Quimis). Fat content was determined using hexane extraction according to the Soxhlet principle. Protein content was measured using the Micro Kjeldahl method. Approximately 50 mg of dried and degreased samples were mixed with potassium sulfate, copper, and concentrated sulfuric acid. This mixture was digested on a heating block at 360 °C for 4 hours. After cooling to room temperature, the samples were then distilled in a Microkejeldhal distillation apparatus using sodium hydroxide and saturated boric acid solution (containing 1% indicator solution: bromocresol green/methyl red). The distillate was titrated using 0.02 N sulfuric acid. A nitrogen conversion factor of 6.25 was used. The nitrogen-free extract (NFE) was calculated according to the AOAC standard:

$$\%NFE = 100 - (\%water + \%\ crude\ protein + \%\ crude\ fiber + \%\ ash + \%\ crude\ fat)$$

3.2.4 Microbiological analysis of avocado byproducts

Microbiology analyses were performed following the recommendations of Brazilian Health Regulatory Agency (Anvisa) issues regulation No. 60, which establishes the list of microbiological standards for food. According to this resolution, the frozen fruit derivatives must not contain *Salmonella ssp*., present up to 10^2 UFC/g *Escherichia coli*, and fruit pulps must not contain *Salmonella ssp*., present up to 10^2 UFC/g *Escherichia coli*, and up to 10^4 UFC/g Molds and Yeasts. The enumeration of E. coli in avocado byproducts was performed by the rapid method of plate counting Petrifilm™ EC (3 M Company). The presence of *Salmonella ssp*. was analyzed according to ISO 6579:2002.[30] Finally, potato dextrose agar (PDA) was used to enumerate yeast and mold counts. The plates were incubated at 25 °C for 5 days.

3.2.5 Exploratory analyses of bioactive activity of avocado byproducts

As a first exploratory analysis, the bioactive compounds of peel, seeds and pomace were extracted according to Tremocoldi et al. (2018)[13]. Each lyophilized material was weighed (1g) and mixed with 10 mL of solvent (ethanol/water, 80/20 v/v). These extracts were sonicated in ultrasound bath, 40 kHz (LS Logen Scientific, LSUC2-120-3.0, Diadema, Brazil), for 15 minutes at room temperature (25 °C), centrifuged at 5000 g (Hitachi Koki Co, Himac CF 16 RN) for 15 minutes, and filtered and analyzed for total phenolic compounds and tannins content.

3.2.6 Design of experiments (DOE)

A Central Composite Rotatable Design (CCRD) was used to optimize three independent extraction parameters: ethanol concentration (%, X1), temperature (°C, X2), and solid-solvent ratio (g/mL, X3) of two dependent variables: total phenolic content (Y_{TPC}) and tannin content (Y_{Tannin}). These parameters were selected due to their significant influence on extraction efficiency.[31] The extraction of bioactive compounds was performed by dispersing the sample in the selected solvent system, with the temperature and the solid/solvent ratio adjusted according to the experimental design, using an ultrasound bath, 40 kHz (LS Logen Scientific, LSUC2-120-3.0, Diadema, Brazil), for 15 minutes. After the extraction period, the extracts were centrifuged at 5000 g at 4 °C (Hitachi Koki Co, Himac CF 16 RN) for 15 minutes, filtered with qualitative filter paper, and stored refrigerated at 7 °C until the time of analysis.

Due to its low toxicity when compared to other organic solvents, ethanol was chosen as the solvent, given the interest in applying the extracts in foods. The independent variables were coded at three levels, and their real values were selected based on literature data and preliminary experimental tests. The independent variables and their related codes and levels are displayed in Table 2. A total of 18 experimental runs were performed randomly, which included four replicates at the central point (Table 3). The results were evaluated by the Response Surface Methodology and submitted to multiple regression analysis, analysis of variance and F test using the Statistica 13 program (Statsoft, Inc. 2015) to adjust the mathematical models.

Table 2. Three levels of three variables of the extraction process

		Coded Levels				
Independent variables	Symbols	-1.68	-1	0	1	1.68
Ethanol concentration (%)	X_1	0	20	50	79	99
Temperature (°C)	X_2	30	36	45	54	60
Solid/solvent ratio (g/mL)	X_3	0.01	0.05	0.11	0.16	0.20

Table 3. Central composite rotatable design (CCRD) with observed responses of the dependent variables from the extraction of avocado byproducts

Run	**Independent Variables**			**TPC (mg GAE/mL of extract)**		**Tannins (mg GAE/mL of extract)**	
	X_1 (%)	X_2(°C)	X_3 (g/mL)	Peel	Seed	Peel	Seed
1	-1	-1	-1	2.78	3.27	2.34	2.93
2	1	-1	-1	2.63	3.17	2.03	2.66
3	-1	1	-1	3.23	2.44	2.76	2.08
4	1	1	-1	2.81	3.32	2.21	2.84
5	-1	-1	1	7.58	10.90	6.70	9.74
6	1	-1	1	6.82	7.97	5.41	6.79
7	-1	1	1	8.35	9.62	7.32	8.65
8	1	1	1	6.94	8.98	5.42	7.33
9	-1.68	0	0	3.50	4.77	3.09	4.35
10	1.68	0	0	2.22	2.56	1.40	1.87
11	0	-1.68	0	7.11	7.08	5.71	6.01
12	0	1.68	0	7.71	7.35	6.24	6.48
13	0	0	-1.68	0.79	0.79	0.69	0.70
14	0	0	1.68	8.00	13.27	6.70	11.60
15	0	0	0	6.02	6.80	4.95	6.06
16	0	0	0	7.30	7.63	5.85	6.78
17	0	0	0	5.61	7.07	4.29	6.20
18	0	0	0	7.39	7.19	6.05	6.37

3.2.7 Chemical analysis

3.2.7.1 Total Phenolic Compounds (TPC)

The total phenolic content (TPC) was determined according to Singleton, Orthofer, Lamuela-Raventós (1999), with some modifications.[32] After the extraction (presented in section 2.4), 0.25 mL of sample or standard was mixed with 1.25 mL of Folin-Ciocalteau reagent (diluted 1:10 v/v), and after 5 min of reaction, 1.0 mL of 7.5% Na_2CO_3 was added. The mixture was vortexed and kept in the dark at room temperature (23 - 25 °C) for 2 h. The solution absorbance was measured at 760 nm against a water blank. The TPC results were expressed as gallic acid equivalents (mg GAE/mL of extract), calculated through a standard curve of gallic acid solutions at different concentrations (5–95 ug/mL).

3.2.7.2 Tannin content

Tannins contents were determined according to Makkar et al. (2016) and Calderón-Oliver et al. (2016) using insoluble polyvinyl-polypyrrolidone (PVPP), which binds tannins[33,34]. The byproduct extracts (1.5 mL), in which the total phenolic content was determined, were mixed with 150 mg of insoluble PVPP, vortexed, kept for 15 min at 4 °C and then centrifuged for 10 min at 15,000 g. Non-tannin phenolics were determined in the clear supernatant, using the same method as that of total phenolics. Tannin content was calculated as a difference between total and non-tannin phenolic content and expressed as gallic acid equivalents (mg GAE/mL of extract).

3.2.7.3 Antioxidant activity

Antioxidant activity by the DPPH method (2,2-diphenyl-1-picryl-hydrazyl) was carried out according to Brand-Williams, Cuvelier and Berset (1995).[35] Diluted avocado byproduct extracts with volumes of 500 µL were mixed with 3.0 mL of ethanol and 300 µL of a 60 mM DPPH ethanolic solution. The mixture was vortexed and left to react in the dark at room temperature for 45 min. The reduction in the DPPH radical in the samples was spectrophotometrically measured against an ethanol blank at 515 nm. The exact concentration of DPPH was calculated using a standard curve. The radical scavenging activity of the extracts was calculated as the amount of DPPH inhibited by the sample when compared to the control. A Trolox solution (Trolox equivalent antioxidant capacity—TEAC) with concentrations varying from 20 to 200 µM was used to obtain a standard curve, and the results were expressed in µmol TEAC/mL of extract.

Antioxidant capacity was determined by the ABTS method [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] as described by Re et al. (1999).[36] A radical solution (7 mM ABTS and 2.45 mM potassium persulfate in equal proportions) was prepared and left to react in the dark at room temperature (25 °C) for 16 hours until ABTS radical formation. After the reaction, the solution absorption was stable at 734 nm. This solution was diluted with ethanol P.A. till reaching an absorbance value of 0.70 ± 0.02 at 734 nm. The diluted avocado byproduct extracts (20 µL) were mixed with 2 mL of diluted ABTS solution and kept in the dark for 15 min at room temperature. The reduction of the blue–green ABTS radical by hydrogen-donating antioxidants was measured by a spectrophotometer (Shimadzu, model UV 1240, Japan) at 734 nm. The concentration of ABTS was calculated using a standard curve with different Trolox concentrations (800-2000 µmol). The radical scavenging activity of the extracts

was measured as the amount of ABTS inhibited by the sample against the blank (diluted ABATS solution). The results were expressed in µmol TEAC/mL of extract.

The ferric reducing antioxidant power (FRAP) assay was performed as outlined by Thaipong et al. (2006).[37] FRAP reagent was prepared with 20 mM ferric chloride solution, 0.3 M sodium acetate buffer and a solution of 10 mM TPTZ, previously prepared with 40 mM HCl (10:1:1, v/v/v). This FRAP solution was mixed with 90 µL of the sample extracts or standard solution and 270 µL of distilled water. The mixtures and the blanks (only FRAP reagent) were vortexed and incubated for 30 min at 37 ± 2 °C. They were then measured against a blank at 595 nm. Ferrous sulphate solutions with concentrations varying from 200 to 2000 µM were used to generate a standard curve. The results were expressed as µmol ferrous sulphate/mL of extract.

3.2.8 Individual phenolic compounds analyzed by HPLC

Phenolic compounds present in the seed and peel extracts after optimization was analyzed using an HPLC system (AGILENT, 1100 Series model, Waldbronn, Germany) equipped with a UV detector. Chromatographic separation was performed by employing a reversed-phase C18 column (4.6 × 250 mm–5 µm). The mobile phase consisted of 5% aqueous acetic acid (Solvent A) and acetonitrile (Solvent B). The run time was set at 55 min with 95% mobile phase A and 5% mobile phase B. The flow rate was 0.8 $mL.min^{-1}$, and the injection volume was 30 µL.

The phenolic compounds were identified by comparing their retention times with corresponding standards. Each standard solution and sample were analyzed in triplicate. The peaks were detected by UV at wavelengths of 280 and 320 nm. All the identified compounds were quantified using external standard calibration curves, and their concentration was expressed as mg/L of extract.

3.2.9 Antibacterial activity

The antibacterial activity against *Staphylococcus aureus*, *Escherichia coli*, *Salmonella enterica*, and *Listeria monocytogenes* strains was evaluated using the microtiter broth dilution method to determine the minimum inhibitory concentration (MIC) according to Mann and Markhan (1998).[38] A stock solution of the avocado byproduct extracts was prepared to a final concentration of 10% and 4% (m/v - g/mL) for seeds and peels, respectively. A serial dilution from the stock solution was tested with 12 different concentrations (0.1%, 0.25%, 0.5%, 0.75%, 1%, 5%, 15%, 5%, 15%, 50%, 75%, 100% (v/v)).

The bacterial suspension, containing approximately 10^6 colony-forming units/mL, was prepared from a 24 h culture growth. From this suspension, 75 µL was inoculated with 75 µL of each of the extract concentrations in a sterile 96-well microplate (Kasvi, Brazil). Positive control with no extract, negative control with no inoculum (only extract), and one blank (only culture medium) were also studied for each strain. The microplates were incubated for 24 h at 37 °C under static conditions. After this time, 10 µl of 0.01% (w/v) resazurin sodium salt (Sigma–Aldrich #R7017) was added to each well as an indicator of microbial growth. The plates were incubated at 37 °C for an additional 2 h. Bacterial growth was visually determined as color changes from blue to pink, which indicated the presence of viable cells in cultures. Thus, the last blue colored well indicated the MIC of the extracts. Lastly, 100 µL aliquots of all blue wells were plated onto Tryptone Soy Agar (TSA) medium and incubated for 24 h for the further confirmation of the bacterial inhibition.

3.2.10 Statistical analysis

The optimization statistics and the response surface plots were performed using the Protimiza Experimental Design and Statistica software (version 13, Statsoft, Inc. 2015). The effectiveness of the models was measured by evaluating the p value, the coefficient of determination (R^2), and the Fisher test value (F value) obtained from the analysis of variance (ANOVA) at a 95% confidence level. Validation of optimized conditions was performed with at least four samples. ANOVA, followed by Tukey´s test, was performed for analysis with multiple mean comparisons using GraphPad Prism 8 (GraphPad Software, Inc., La Jolla, CA).

3.3 Results and Discussion

3.3.1 Avocado oil byproducts characterization

The avocado oil extraction byproducts were obtained as shown in Figure 4. The first step consisted of the selection and washing of the avocado fruits. The fruit was then pulped, separating the avocado pulp from the seed and skin. Seeds and peels represented between 27 and 32% of the whole fruit. Subsequently, the oil was extracted from the flesh mixed with water in a malaxing stage. In this step, the avocado paste was continuously stirred at a controlled temperature of 30 °C for 1 h 30 min. After that, the paste was separated in a 3-phase decanter centrifuge at 40 °C into three products: oil, wastewater, and pomace. This process is similar to that reported by Permal et al. (2020)[8] for Hass avocado oil extraction.

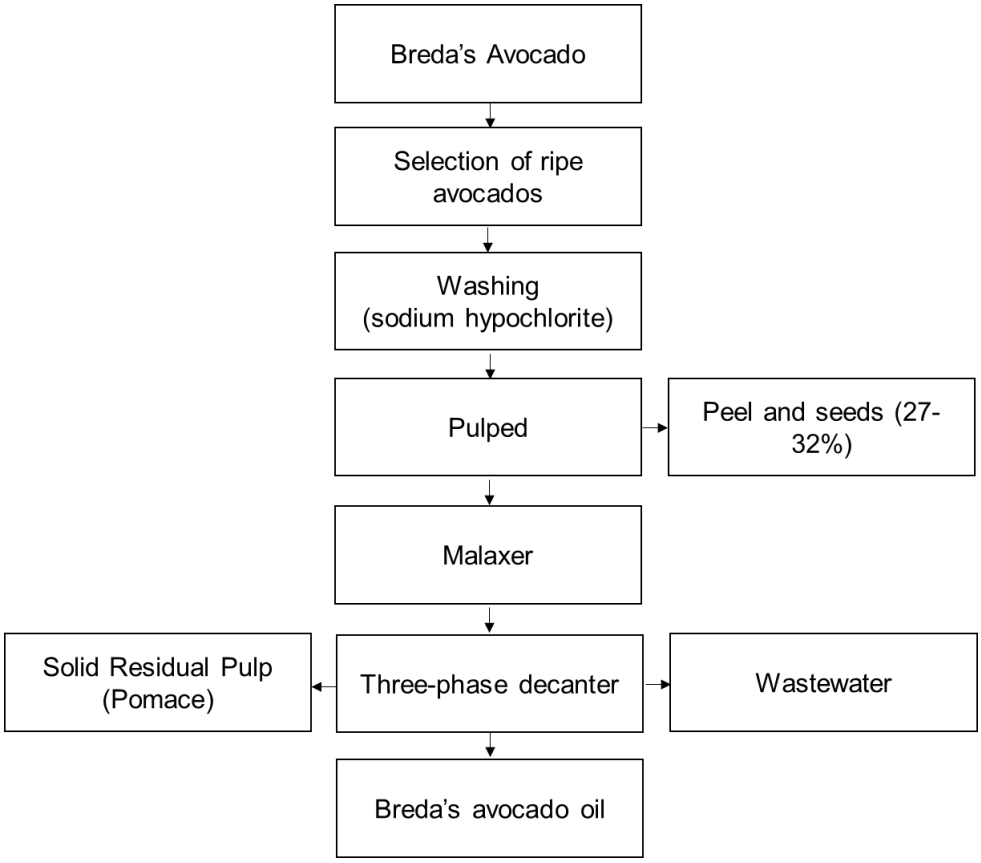

Figure 4. Breda´s avocado oil extraction process flows in the Paraíso Verde company production line with a three-phase decanter system during the early harvest season.

The approximate composition of Breda avocados' byproducts is shown in Table 4. The pomace presented the highest moisture content (83.92%), followed by the peel (67.74%) and seed (59.88%). The lipid content was also significantly higher in the pomace than in the peels and seeds. Otherwise, the peel showed more ash, crude fiber, and total dietary fiber compared to the pomace and seeds (wet basis). The moisture content is aligned with the results found by Permal et al. (2020). However, there are some differences when comparing other parameters, such as the lipid content found by these authors, for all byproducts (6.9% peel, 3.7% seed, and 9.3% pomace – wet basis).[8] These differences can be explained by the different avocado varieties, different environmental conditions, ripeness, and oil extraction in the case of the pulp pomace. This is presented by Mardigan et al. (2019), showing that the composition of avocados of five different varieties is distinct.[39]

Table 4. Proximate analysis of avocado byproducts.

Physical-chemical composition of avocado byproducts (dry basis % w/w)			
	Peel	Seed	Pomace
Protein (%)	3.82 ± 0.1 b	3.13 ± 0.09 c	5.13 ± 0.05 a
Lipid (%)	9.39 ± 0.07 b	2.9 ± 0.04 c	35.91 ± 0.05 a
Ash (%)	2.71 ± 0.01 a	2.08 ± 0.04 b	1.34 ± 0 c
Crude Fiber (%)	22.95 ± 0.35 b	3.85 ± 0.47 c	27.76 ± 0.37 a
Nitrogen-Free Extract (NFE) (%)	61.14 ± 0.27 b	88.04 ± 0.33 a	29.86 ± 0.46 c
Total Dietary Fiber (%)	63.66 ± 12.78 a	29.32 ± 0.45b	63.41 ± 1 a
Insoluble Fiber (%)	58.75 ± 12.75 a	16.39 ± 0.85 c	38.05 ± 0.25 b
Soluble Fiber (%)	4.91 ± 0.26 b	12.08 ± 1.29 a	2.59 ± 0.69 c
Physical-chemical composition of avocado byproducts (wet basis % w/w)			
	Peel	Seed	Pomace
Protein (%)	1.23 ± 0.03 a	1.26 ± 0.04 a	0.82 ± 0.01 b
Lipid (%)	3.03 ± 0.02 b	1.16 ± 0.02 c	5.77 ± 0.01 a
Ash (%)	0.87 ± 0.00 a	0.83 ± 0.02 b	0.22 ± 0.00c
Crude Fiber (%)	7.40 ± 0.11 a	1.54 ± 0.19 c	4.46 ± 0.06 b
Nitrogen-Free Extract (NFE) (%)	19.73 ± 0.58 b	35.32 ± 0.77 a	4.8 ± 0.12 c
Total Dietary Fiber (%)	20.54 ± 4.12 a	11.42 ± 0.18 b	6.53 ± 0.1 b
Insoluble Fiber (%)	18.95 ± 4.11 a	6.57 ± 0.34 b	6.12 ± 0.04 b
Soluble Fiber (%)	1.58 ± 0.08 b	4.85 ± 0.52 a	0.42 ± 0.11 c
Moisture Content (%)	67.74 ± 0.51 b	59.88 ± 0.78 c	83.92 ± 0.07 a
pH	6.00 ± 0.02 a	6.04 ± 0.11 a	4.68 ± 4.68 b
aW	0.99 ± 0.002 b	0.98 ± 0.003 b	1.00 ± 0.001 a

Data are expressed as the mean ± standard deviation. Different letters within a row indicate significant differences ($p < 0.05$). ANOVA followed by Tukey´s test was performed for multiple mean comparisons.

Microbiological analyses showed that the byproducts showed satisfactory results regarding the analysis of *E. coli, Salmonella*, yeasts, and mold. *E. coli* was absent in all byproducts, and *Salmonell*a was also absent in 25 g of each sample. Yeast and mold were absent in the pomace as well. This information on the microbial quality and safety of avocado byproducts could be useful for developing control measures to reduce the risk of further application of these products. Additionally, counts of indicator microorganisms on the avocados' byproducts may be useful for evaluating their handling and storage conditions.[40]

As a first exploratory experiment, total phenolic (TCP) and tannin analyses were performed. The avocado peel and seeds contained higher phenolic and tannin contents than the pomace. The peel extracts presented the highest TPC concentration (4.08 mg GAE/mL of extract); however, they did not show a significant difference in relation to the seed extracts (3.69 mg GAE/mL of extract). Tannins represent a large fraction of the phenolic compounds present in these byproducts, as they made up approximately 88% of the peels and 80% of the seed extract (Figure 5). Due to the lowest phenolic content, the pomace was disregarded for further optimization of the extraction of the bioactive compounds. Permal et al. (2020) found higher phenolic content in the peels (13.7 g/100 g), followed by the seeds (8.1 g/100 g), and, last, the pomace (3.6 g/100 g).[8] However, Vinha et al. (2013) reported a slightly higher TPC

content in avocado seeds than in peels (0.70 g GAE/100 g in fresh seeds and 0.68 g GAE/100 g in fresh peels).[41] The differences between the avocado phenolic contents could be explained by the solvent used during the extraction - Permal et al. (2020) extracted the bioactive compounds with methanol (50%) and acetone and Vinha et al. (2013) with water – the production region, variety and the harvesting time of the avocados since these factors can interfere with the characterization of the fruit.[8,13,41,42]

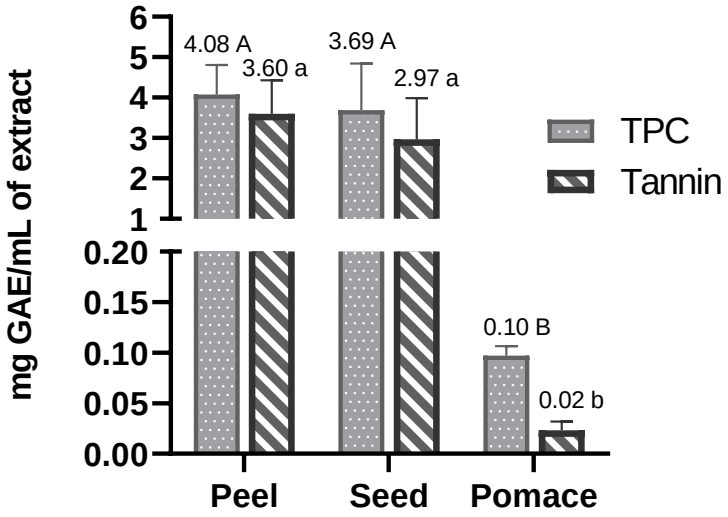

Figure 5. Total phenolic compounds (TPC) and tannins from avocado byproducts. Different uppercase letters indicate significant differences ($p<0.05$) in the TPC content. Different lowercase letters indicate significant differences ($p<0.05$) in the tannin content.

3.3.2 Model Fitting

A central composite rotatable design (CCRD) was used to evaluate the effects of the extraction parameters (ethanol concentration, temperature, and solid-solvent ratio) on the total phenolic and tannin contents. The experimental design with the corresponding responses is shown in Table 3.

The TPC experimental values ranged from 0.79 to 8.35 mg GAE/mL and 0.79 to 13.27 mg GAE/mL of peel and seed extracts, respectively. Tannins ranged from 0.69 to 7.32 mg GAE/mL and 0.70 to 11.60 mg GAE/mL in peel and seed extracts, respectively. These results indicate the noticeable influence of the extraction parameters, which suggests the importance of the optimization process. In addition, the central points showed small variation ($CV_{TPC\ peels}$ = 14%; $CV_{TPC\ seeds}$ = 5%; $CV_{Tannins\ peels}$ = 15%; $CV_{Tannins\ seeds}$ = 5%), indicating a good experimental repeatability. Thus, a second order polynomial model was obtained, and ANOVA was used to verify the adequacy and fitness (Table 5). ANOVA results revealed that the model was

statistically significant since the R^2 values for TPC (0.9624 and 0.9867, peels and seeds, respectively) and for tannins (0.9500 and 0.9868, peels and seeds, respectively) were close to 1, and the lack of fit values was not significant ($P > 0.05$), indicating the adequacy of the model.[43]

Table 5. Analysis of Variance (ANOVA) on the adjusted models for total phenolic content (TPC) and Tannins from avocado by-products.

Parameters	R^2	Sum of Squares	D.F	Mean Square	Fcalc.	F tab	*p Value*	Second-order polynomial equation
Peel TPC	0.96							TPC = 6.56 - 0.36 x_1 - 1.24 x_1^2 + 0.37 x_2^2 + 2.22 x_3 - 0.70 x_3^2
Regression		98.0	5	19.6	61,5	3.11	<0.0001 *	
Residuals		3.8	12	0.3				
Total		101.8	17					
Seed TPC	0.99	182.8	5	36.6	178.1	3.11	<0.0001 *	TPC = 7.25 - 0.48 x_1 - 1.19 x_1^2 + 3.39 x_3 + 0.41 x_1 x_2 - 0.54 x_1 x_3
Regression		2.5	12	0.2				
Residuals		185.3	17					
Total								
Peel Tannins	0.95	69.6	5	13.9	45.6	3.11	<0.0001 *	Tannins = 5.27 - 0.50 x_1 - 0.99 x_1^2 + 0.33 x_2^2 + 1.88 x_3 - 0.48 x_3^2
Regression		3.7	12	0.3				
Residuals		73.2	17					
Total								
Seed Tannins	0.99	143.6	5	28.7	178.9	3.11	<0.0001 *	Tannins = 6.35 - 0.58 x_1 - 1.09 x_1^2 + 2.95 x_3 + 0.33 x_1 x_2 - 0.59 x_1 x_3
Regression		1.9	12	0.2				
Residuals		145.5	17					
Total								

PC = total phenolic content; * *p* value significant ($p \leq 0.05$).

3.3.3 Influence of the Extraction Parameters on Total Phenolic Content (TPC)

The highest TPC recovery in avocado peel was achieved mainly with 40% ethanol and the highest solid-solvent concentration. However, the temperature did not influence the TPC results. Hence, only ethanol concentration (X1) and solid-solvent concentration (X3) had significant ($P < 0.05$) effects on TPC. The quadratic effect of all parameters ($X1^2$, $X2^2$, and $X3^2$) also had a significant ($P < 0.05$) influence on TPC. There were no significant interaction effects between the parameters.

The effect of temperature and ethanol concentration (%) in avocado peel extract is presented in Figure 6a. This shows that only ethanol concentration had effects on TPC. The highest TPC contents were observed in the range between 20 and 70%, with the highest TPC content observed using 40% ethanol. In contrast, increasing the temperature did not have a

significant effect on the TPC content. In addition, the effect of the solid-solvent ratio (g/mL) and ethanol concentration is shown in Figure 6b.

The results indicate that the solid-solvent ratio had a significant enhancing effect on the TPC. As the solid-solvent concentration increased, the TPC concentration increased. In addition, Figure 6c presents the effects of temperature and sample-solvent ratio, in which only the solid-solvent ratio influenced the TPC.

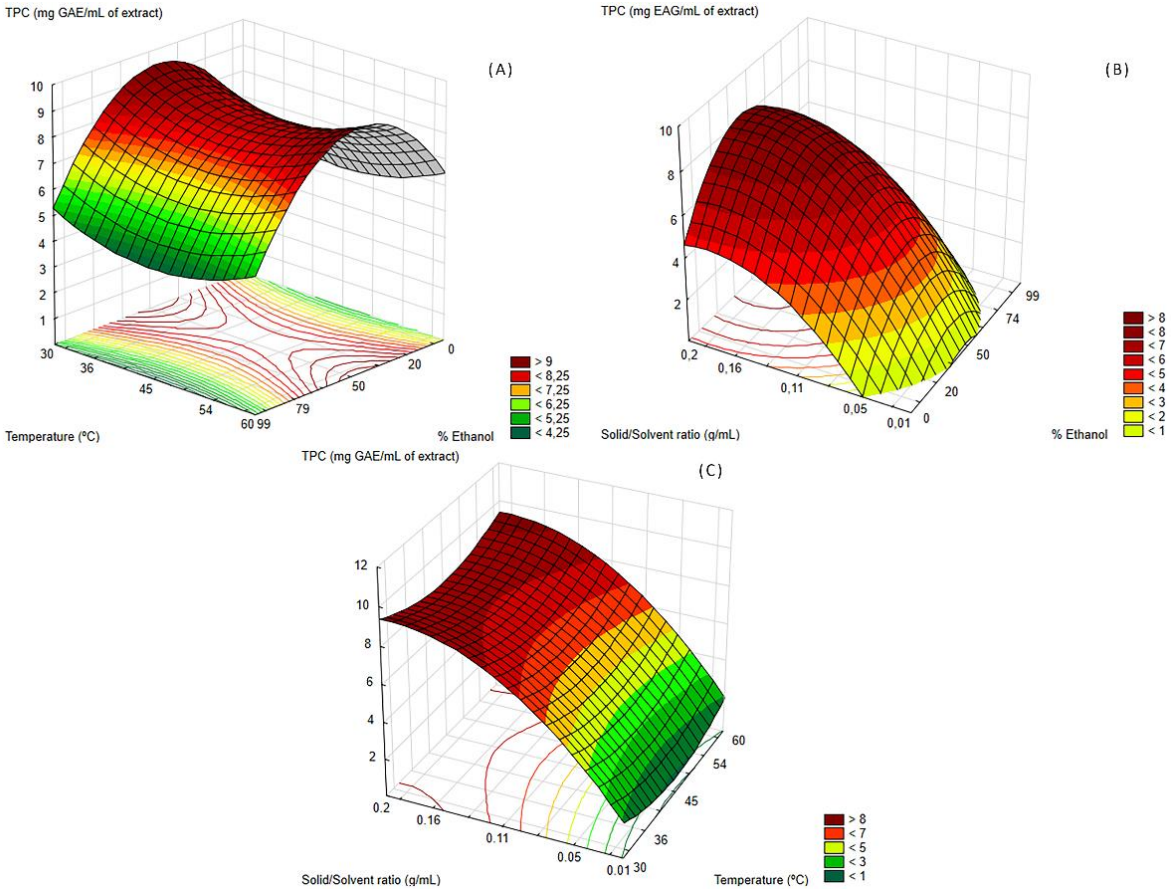

Figure 6. Response surface plot showing the effect of extraction variables on TPC of avocado peel (mg GAE/mL of extract). (A) Effect of ethanol concentration and temperature; (B) effect of solid/solvent ratio and ethanol concentration and (C) effect of solid/solvent ratio and temperature.

In the avocado seed extracts, the highest TPC content was obtained in the range of 20-40% ethanol concentration and the highest solid-solvent concentration. Temperature also did not influence the TPC of avocado seed extracts. In addition, only ethanol concentration had a significant quadratic effect ($X1^2$) ($p<0.05$) on TPC. Moreover, there was a significant ($p<0.05$) interaction between the ethanol concentration and temperature (X1X2), as well as ethanol concentration and solid-solvent ratio (X1X3). The second-order polynomial equations for TPC in avocado peel and seed extracts are shown in Table 5.

The effects of the extraction parameters on the TPC of avocado seed are shown in Figure 7. Figure 7a shows that the concentration of ethanol affected the TPC content, but the temperature did not. The concentration between 20 and 40% was the most effective in the TPC extraction. Figure 7b shows that the highest concentrations of TPC were reached with the maximum concentration of sample-solvent. Ethanol concentration ranges were also better between 20 and 40%. Finally, Figure 7c clearly shows that the temperature did not interfere with the TPC content. This non-influence of temperature can be a positive factor since it may vary throughout the extraction on a scale-up process.

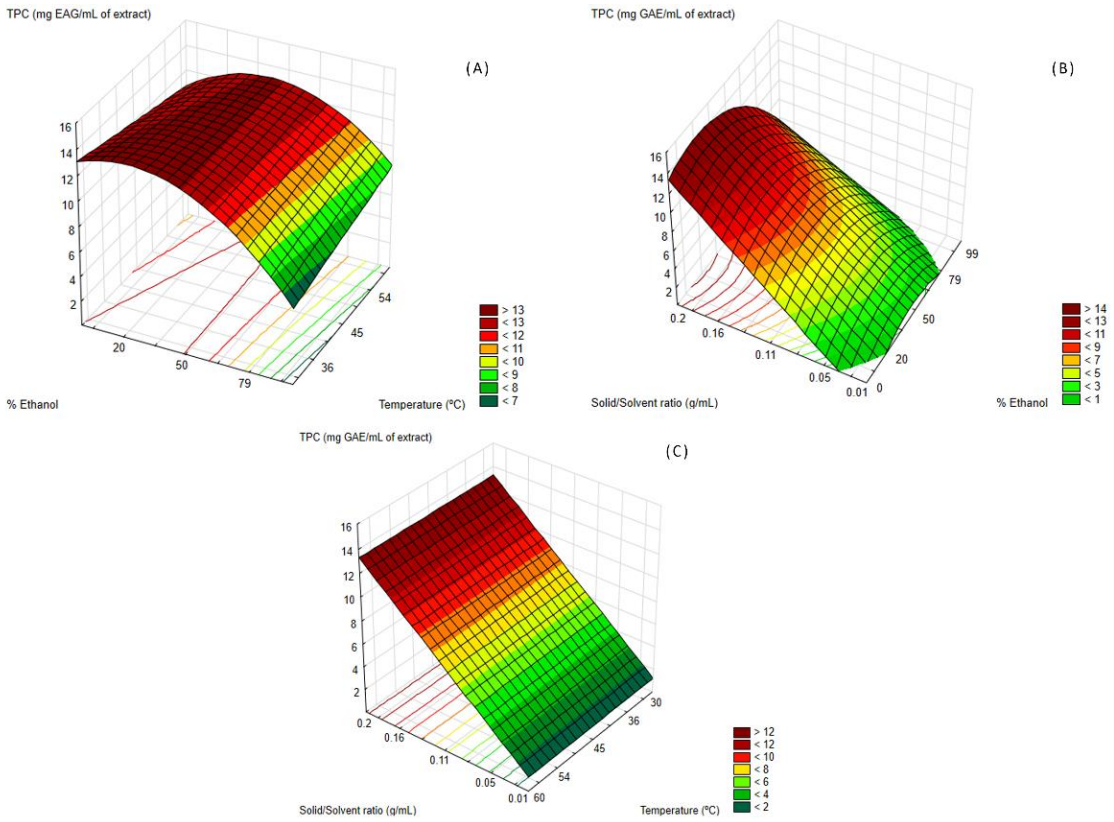

Figure 7. Response surface plot showing the effect of extraction variables on TPC of avocado seed (mg GAE/mL of extract). (A) Effect of ethanol concentration and temperature; (B) effect of solid/solvent ratio and ethanol concentration and (C) effect of solid/solvent ratio and temperature.

3.3.4 Influence of the Extraction Parameters on Tannins

The tannins followed a very similar pattern to the total phenolic content. For avocado peel extracts, the highest tannin content was also observed at approximately 40% ethanol, with the highest solid-solvent concentration and with no influence from the temperature. The

quadratic effect of ethanol concentration, temperature, and solid solvent ($X1^2$; $X2^2$; $X3^2$) had a significant ($P < 0.05$) influence on tannins. Additionally, it did not show a significant ($p<0.05$) interaction between the parameters.

For avocado seed extract, ethanol concentration and the solid-solvent ratio influenced the tannin recovery. Moreover, the temperature did not influence the tannin content. In addition, only ethanol concentration had a significant quadratic effect ($X1^2$) ($p<0.05$) on TPC. Additionally, there was a significant ($p<0.05$) interaction between the ethanol concentration and temperature (X1X2), as well as ethanol concentration and solid-solvent ratio (X1X3).

These results agree with Weremfo, Adulley and Adarkwah-Yiadom (2020), who found that TPC was strongly influenced by the concentration of ethanol used in avocado seed bioactive compounds microwave-assisted extraction. For these authors, the optimal ethanol concentration was 58% (v/v).[25] The significant ($p < 0.05$) quadratic effect of ethanol concentration can be explained by the improvement in the solubility of phenolic compounds with the increase in solvent concentration. However, this increase has a limit since the polarity of the solvent can change with modifications in the concentration, which may lead to an increase in the extraction of impurities, reducing the phenolic compounds extracted.

The second-order polynomial equations for tannins in avocado peel and seed extracts are shown in Table 5, and the surface plots showing the effects of the extraction parameter on tannins from the two extracts are presented in Figures 8 and 9. Figure 8 shows the influence of these parameters in tannins extracted from avocado peels. Figure 8(a) shows that the tannins were better extracted in the range of 20-70% ethanol concentration, while Figure 8(b) illustrates the effect of the ethanol concentration and solid solvent on tannins, in which the highest concentration of sample-solvent showed the highest recovery of tannins. Figure 8(c) shows the influence of the temperature and solid solvent on tannins.

Figure 9 presents the effects of these parameters in tannins extracted from avocado seeds. As shown in Figure 9(a), the extraction of tannins from avocado seeds increased with ethanol concentrations ranging from 20 to 40%. Figure 9(b) illustrates the effect of ethanol concentration and solid solvent on tannins, both of which influenced the tannin recovery. Moreover, Figure 9(c) shows the influence of the effect of temperature and solid solvent on tannins, with temperature having no effect on recovery.

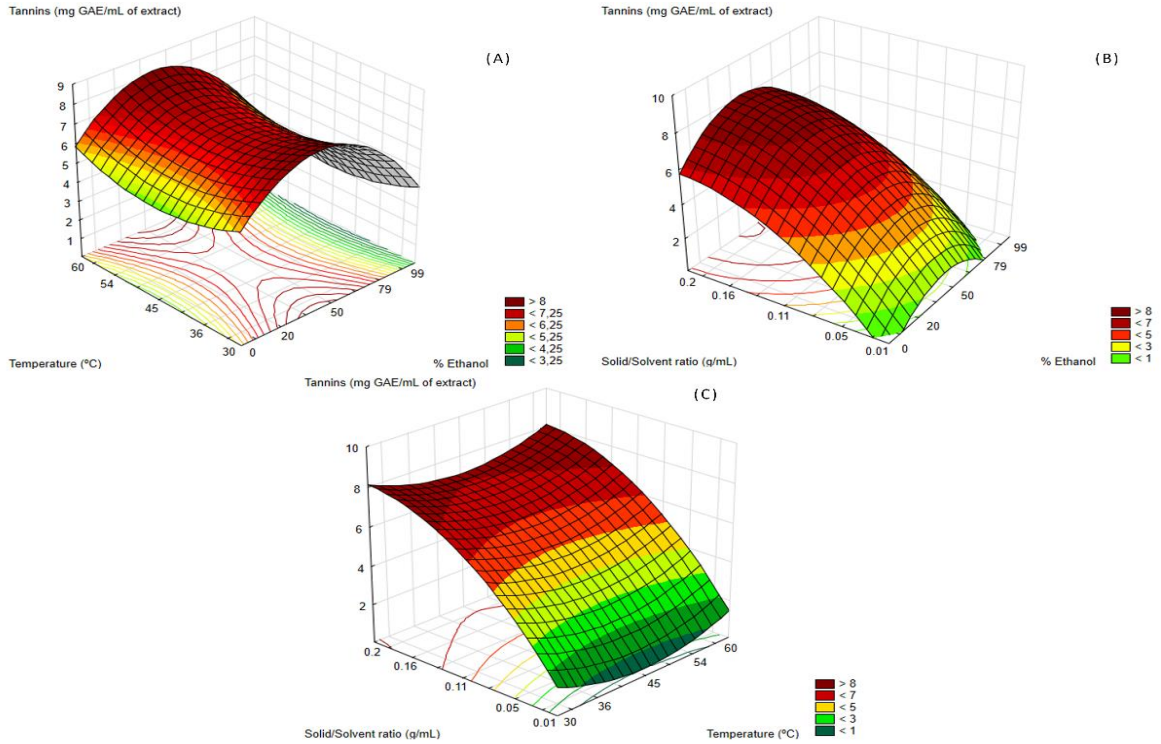

Figure 8. Response surface plot showing the effect of extraction variables on tannins of avocado peel (mg GAE/mL of extract). (A) Effect of ethanol concentration and temperature; (B) effect of solid/solvent ratio and ethanol concentration and (C) effect of solid/solvent ratio and temperature.

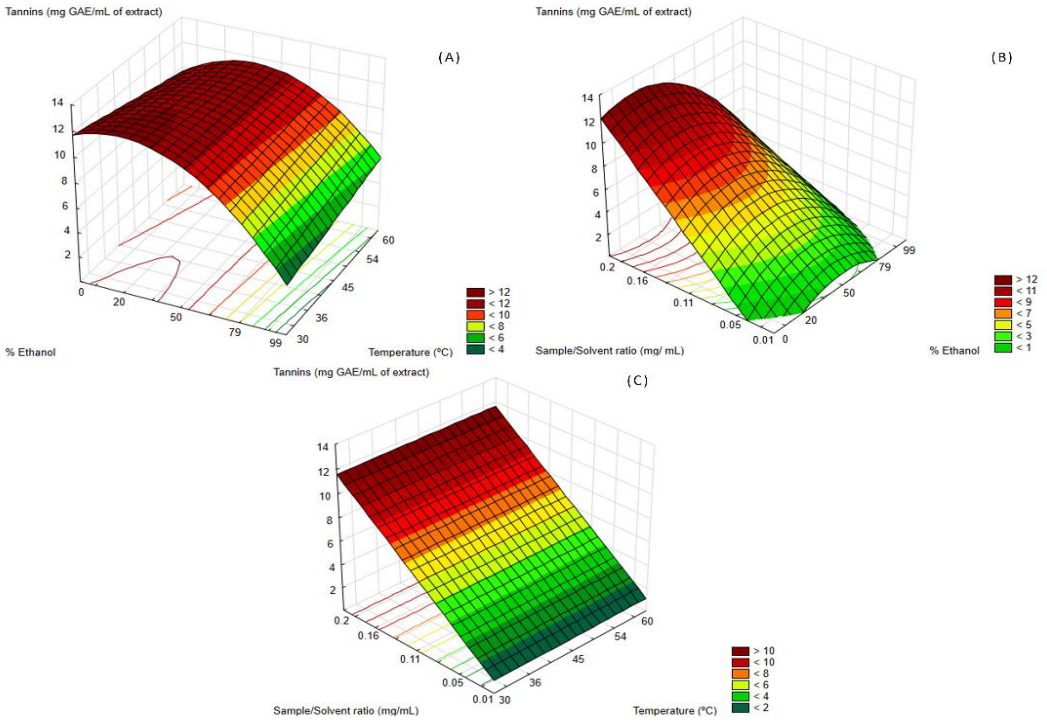

Figure 9. Response surface plot showing the effect of extraction variables on tannins of avocado seeds (mg GAE/mL of extract). (A) Effect of ethanol concentration and temperature; (B) effect of solid/solvent ratio and ethanol concentration and effect of solid/solvent ratio and temperature.

3.3.5 Optimal Extraction Conditions and Verification of the Predictive Model

Within the experimental conditions, the recovery of TPC and tannins from peel extracts were highest at the optimum ethanol concentration of 40%, at the maximum solid-solvent ratio of 0.2 g/mL, and at any temperature. In this case, for energy-saving reasons, the lowest temperature of the experimental design was defined as optimal. For avocado seed extracts, both the TPC and tannins had the highest recovery using 20 or 40% ethanol, 0.2 g of solid/mL solvent, and 30 °C.

The optimization provided the maximum predicted values of 9.34 mg·GAE/mL and 8.06 mg·GAE/mL of extract for TPC and tannins of peels, respectively, 13.83 and 12.34 mg·GAE/mL of extract for TPC and tannins of seeds extracted with ethanol 20%, and 14.57 and 11.89 mg·GAE/mL of extract for TPC and tannins of seeds extracted with ethanol 40%. Quadruplicate experiments were performed at the optimum conditions to evaluate the validity of the predicted results, and the results are presented in Table 6. The experimental values agreed with the predicted values, confirming the reliability of the model obtained in predicting the contents of phenolic compounds and tannin activity using ultrasound extraction. In addition, the TPC and tannin contents recovered with 20 and 40% ethanol in the seed extracts did not differ significantly ($p<0.05$). However, considering the use of a minimum concentration of solvent (ethanol), for further analyses, 20% ethanol was chosen as the optimal concentration. Furthermore, these experimental values were much higher than the first exploratory experiments (shown in section 3.3.1). The TPC values increased approximately 3 times for peels and 4 times for seeds, indicating the importance of the optimization process.

Table 6. Predicted and experimental values of response variables at optimum extraction conditions.

*Responses	Optimum extraction conditions			Predicted value	Experimental value
	X_1 (%)	X_2 (°C)	X_3 (g/mL)		
Peel					
TPC	**40**	**30**	**0.2**	9.34	13.54 ± 0.55
Tannins				8.06	12.16 ± 0.73
Seed					
TPC	**20**	**30**	**0.2**	13.83	13.28 ± 1.05^{A}
	40			14.57	13.67 ± 0.36^{A}
Tannins	**20**	**30**	**0.2**	12.34	12.64 ± 1.21^{a}
	40			11.89	13.07 ± 0.44^{a}

Note: The two different conditions on the seed extraction optimum point did not have a statistically significant difference. Different uppercase letters in the seed experimental results indicate significant differences ($p<0.05$) in the TPC content. Different lowercase letters in the seed experimental results indicate significant differences ($p<0.05$) in the tannin content. *TPC and tannin concentrations were measured as mg GAE/mL of extract. An unpaired t test was performed for two mean comparisons.

3.3.6 Solid-Solvent optimization

Despite high phenolic contents, the solid-solvent ratio parameter did not reach a peak, showing continuous growth with increasing concentration, indicating the possibility for further studies on the optimization of this parameter. Thus, other concentrations were studied considering the solubility of the raw materials. Therefore, for avocado peel, the concentrations studied were 0.2 to 0.3 g/mL and, for seeds, from 0.2 to 0.6 g/mL. Above these concentrations, it was not possible to separate enough volume after the extraction. The TPC results for these extracts at different concentrations are shown in Table 7. From the total sample obtained after extraction, it was also possible to obtain the yield of TPC recovery at these different concentrations. These results are shown in Figure 10.

The results show that when the peel was extracted using 0.225 g/mL (solid-solvent ratio), there was no significant increase in TPC recovery per mL of extract, but on the other hand, the yield of the amount of TPC/total extract obtained significantly reduced (Table 7 and Figure 10). For the seed extracts, the highest levels of TPC per mL of extract were obtained using 0.6 g/mL, however no statistically significant differences between 0.5 - 0.6 g/mL (Table 7) were found. In addition, this concentration had a good yield, also not differing statistically from the yield of the 0.5 g/ml concentration (Figure 10). For this reason, and since a smaller concentration implies in less quantities of raw material, the concentration of 0.5 g/mL was chosen. For the tannins, the solid-solvent ratio optimization followed the same tendency, and the results are shown in Appendix A (Table A_A). Thus, for further analyses, the following optimal extraction conditions were used: 40% ethanol at 30 °C and a ratio of 0.225 g/mL of solid/solvent for peels and 20% ethanol at 30 °C and a ratio of 0.5 g/mL of solid/solvent for seeds.

Table 7. Sequential optimization of the solid/solvent ratio of avocado byproduct bioactive compound extraction.

	Concentration g/mL	mg GAE/mL of extract	mL of extract after filtration	mg GAE/total extract
TPC Peel				
	0.200	13.53 ± 0.39 b	3.14 ± 0.16 a	42.55 ± 2.68 a
	0.225	14.25 ± 0.67 ab	3.15 ± 0.37 a	44.71 ± 3.80 a
	0.250	15.10 ± 0.51 ab	2.63 ± 0.27 ab	39.73 ± 4.87 ab
	0.275	15.59 ± 0.81 ab	2.13 ± 0.30 bc	33.24 ± 5.04 bc
	0.300	16.10 ± 1.23 a	1.47 ± 0.14 c	23.72 ± 2.87 c
TPC seed				
	0.200	11.94 ± 0.31 d	6.38 ± 0.36 a	76.18 ± 4.86 b
	0.275	15.67 ± 1.46 c	5.78 ± 0.22 ab	90.73 ± 11.98 ab
	0.350	19.83 ± 0.58 b	5.15 ± 0.44 bc	102.04 ± 8.99 ab
	0.425	22.17 ± 1.10 b	4.59 ± 0.21 cd	101.73 ± 3.17 a
	0.500	32.27 ± 1.05 a	3.64 ± 0.20 de	117.38 ± 7.99 a
	0.575	32.30 ± 0.80 a	3.44 ± 0.45 e	111.18 ± 15.43 a
	0.600	34.42 ± 1.14 a	3.48 ± 0.52 e	120.17 ± 21.45 a

Data are expressed as the mean ± standard deviation. Different letters within a column indicate significant differences ($p < 0.05$). ANOVA followed by Tukey´s test was performed for multiple mean comparisons.

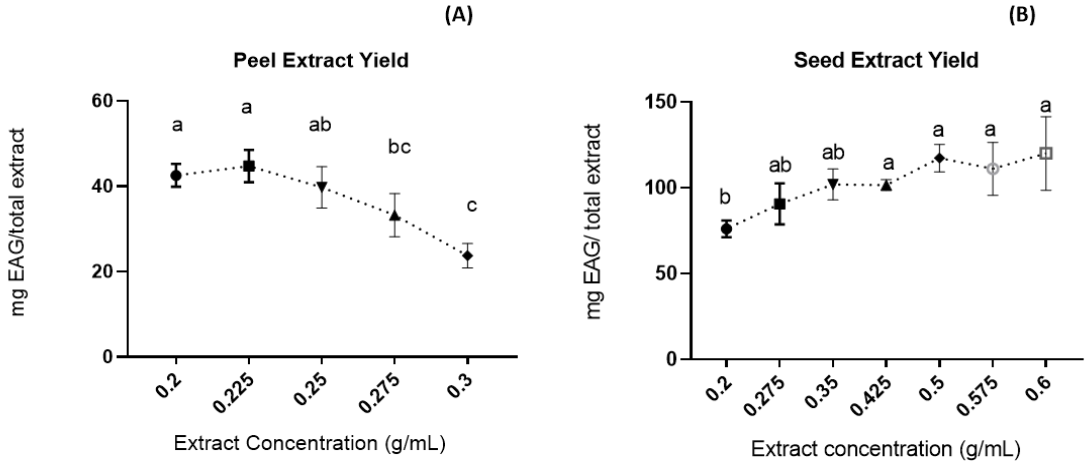

Figure 10. Total extract yield of TPC. (A) Yield in peel extracts and (B) yield in seed extracts.

3.3.7 Phenolic compounds, antioxidant, and antibacterial capacity of the extracts

Antioxidant (ABTS, DPPH, and FRAP) and antimicrobial analyses were performed on the optimized extracts. ABTS, DPPH, and FRAP tests are widely used to investigate the antioxidant activity of various food samples due to their high-quality reproducibility and simple and convenient control.[44] The results in Table 8 show that the extracts possess a good antioxidant capacity. However, the optimized avocado seed extract presented higher TPC, tannins, and antioxidant activities than peel extracts. This may be explained by the possibility of the use of a higher ratio of solid/solvent for avocado seed extracts in comparison to avocado peels.

Some natural plant extracts have gained attention in food research as possible growth inhibitors of foodborne pathogenic bacteria, such as *Bacillus cereus*, *Staphylococcus aureus*,

Escherichia coli, *Salmonella enterica*, and *Listeria monocytogenes*.[45] However, despite the high antioxidant capacity, neither extract was effective in inhibiting any of the studied microorganisms (*Staphylococcus aureus, Escherichia coli, Salmonella enterica,* and *Listeria monocytogenes* strains). Fernández-Castañeda et al. (2018) also did not observe any inhibiting effect of avocado seed extracts on Staphylococcus aureus.[46] Nevertheless, Salinas-Salazar et al. (2017) showed that isolated fatty acid derivatives (*acetogenins*) extracted from avocado seeds effectively inhibited *Listeria monocytogenes* strains.[12]

Table 8. Results of TPC, tannins, and antioxidant activity using different methods for optimized extracts of avocado peel and seed

	Ethanol (%)	Temperature (°C)	Solid Solvent Ratio (g/mL)	TPC[1]	TANNINS[1]	ABTS[2]	DPPH[2]	FRAP[3]
Peel	40	30	0.225	14.25 ± 0.67	12.75 ± 0.67	134.24 ± 8.74	175.36 ± 14.52	267.2 ± 23.89
Seed	20	30	0.500	32.27 ± 1.05	29.7 ± 0.93	262.01 ± 13.7	176.06 ± 14.58	624.06 ± 18.08

[1]TPC and tannin results are expressed as mg GAE/mL of extract.
[2]ABTS and DPPH results are expressed in µmol Trolox/mL of extract.
[3]FRAP results are expressed in µmol $FeSO_4$/mL of extract.

3.3.8 Phenolic composition of extracts produced under optimized conditions

The phenolic compounds present in the extracts, produced under optimized conditions, were identified by HPLC at wavelengths of 280 and 320 nm (Table 9). The identified compounds were 3,4-dihydroxybenzoic acid, caffeic acid, and syringic acid for peels and 3,4-dihydroxybenzoic acid, catechin, 4-hydroxybenzoic acid, caffeic acid, and syringic acid for seeds. The retention times of these phenolic compounds, the range used to construct their standard curves and their respective R^2 are shown in Table 9. It was also possible to see the peaks from gallic acid, ferulic acid, 3-hydroxybenzoic acid, and quercetin, but the elution was not clear for the quantification. The HPLC chromatograms are shown in Appendix B (Figure A_B).

The main compound in both extracts was 3,4-dihydroxybenzoic acid, with 460.94 mg/L in peel extract and 5793.53 mg/L in seed extract (Table 9). These phenolic compounds have been previously identified in avocado peels and seeds. Weremfo, Adulley, and Adarkwah-Yiadom (2020) did not identify 3,4-dihydroxybenzoic acid, but they identified rutin as the most abundant compound in Hass avocado seed extract. These authors also identified gallic acid, vanillic acid, p-coumaric acid, and quercetin, which we could not quantify in the seed extract.[25] Tremocoldi et al. (2018) identified procyanidin B2 as the main phenolic compound from Hass avocado peels and epicatechin for Fuerte avocado peel, while for Hass and Fuerte seeds, the major compound present was epicatechin.[13] In addition, Hurtado-

Fernández et al. (2013) only found gallic acid in the Sir Prize avocado variety, and 4-hydroxybenzoic acid was only detected at the second degree of ripeness.[47] Thus, the difference between the identified elements could be due to the extraction technique employed, avocado varieties studied, and the ripening degree, among other factors.[13,25,48] However, condensed tannins, phenolic acids, and flavonoids were generally the most representative groups of compounds found in avocado peels and seeds in previous studies.[10,13]

Table 9. Phenolic compounds in avocado peel and seed extract produced under optimal conditions

Compounds	Retention time (min)	Standard Curve range (mg/L)	Wavelength (nm)	R^2	Content (mg/L of extract)
Peel					
3,4-dihydroxybenzoic acid	7.12	2000 - 8000	320	0.9992	460.94 ± 5.39
Caffeic acid	18.45	0.025 - 2.5	320	0.9997	0.08 ± 0.00
Syringic acid	23.83	20 - 400	280	0.9996	41.76 ± 1.17
Seed					
3,4-dihydroxybenzoic acid	7.04	2000 - 8000	320	0.9992	5793.53 ± 267.71
Catechin	10.74	100 - 700	280	0.9988	499.73 ± 13.32
4-Hydroxybenzoic acid	12.79	5 - 200	280	0.9992	8.50 ± 0.32
Caffeic acid	18.70	0.025 - 2.5	320	0.9997	0.05 ± 0.00
Syringic acid	23.78	20 - 400	280	0.9996	130.30 ± 2.94

3.4 Conclusions

Using CCRD for optimization and ultrasound-assisted extraction proved very effective in defining the maximum yield of polyphenolic compounds and the antioxidant activity of avocado peels. The results of modeling by the response surface showed that the most important variables were ethanol concentration and sample-solvent ratio, whereas temperature did not affect the extraction. The optimal conditions for the extraction of maximum phenolic compounds (TPC) and tannins from avocado peels were an ethanol concentration of 40%, a solid-solvent ratio of 0.225 g/mL, and a temperature of 30 °C. For avocado seeds, the conditions were an ethanol concentration of 20%, a solid-solvent ratio of 0.5 g/mL, and a temperature of 30 °C. In addition, despite not showing antimicrobial effects, the extracts presented high contents of different phenolic compounds, making them suitable sources of natural ingredients with antioxidant capacity targeted for food or cosmetic purposes.

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4. GENERAL CONCLUSIONS

This dissertation demonstrated that the avocado oil production residues have a high content of bioactive compounds with potential use in food, cosmetic and pharmaceutical sectors as natural ingredients.

The literature research allowed us to understand the waste generated by avocado oil extraction, as well as their potential source of nutritional food ingredients due to the high content of compounds with high biological activity. In addition, it was possible to identify the recovery and extraction methods already existing and what is needed to implement more adequate and efficient techniques.

The outlined design and optimization method, assisted by ultrasound, allowed us to find the best conditions of ethanol concentration, temperature, and solid/solvent ratio to extract bioactive compounds with high antioxidant capacities using a safe and green method of extraction. The 3,4-dihydroxybenzoic acid was the major bioactive compound found in both avocado peel and seed extracts.

Therefore, the results obtained encourage the use of avocado oil byproducts as a source of bioactive compounds by the optimized ultrasound extraction, since the method is simple and easy to scale up. However, future investigations on their direct or non-direct use as a food ingredient in different formulations, as well as the safety doses to be applied are still needed.

APPENDICES

APPENDIX A

Table A_A. Sequential optimization of the solid/solvent ratio of avocado byproduct tannin extraction

	Concentration g/mL	mg GAE/mL of extract		mL of extract after filtration		mg GAE/total extract	
Tannin Peel							
	0.200	11.93 ± 0.46	b	3.14 ± 0.16	a	37.49 ± 2.52	ab
	0.225	12.75 ± 0.67	ab	3.15 ± 0.37	a	40.00 ± 3.24	a
	0.250	13.34 ± 0.43	ab	2.63 ± 0.27	ab	35.09 ± 4.25	ab
	0.275	13.82 ± 0.75	ab	2.13 ± 0.30	bc	29.48 ± 4.64	bc
	0.300	14.67 ± 1.28	a	1.47 ± 0.14	c	21.62 ± 2.90	c
Tannin seed							
	0.200	10.96 ± 0.11	e	6.38 ± 0.36	a	69.91 ± 4.21	b
	0.275	14.35 ± 1.50	d	5.78 ± 0.22	ab	83.11 ± 11.93	ab
	0.350	17.95 ± 0.82	c	5.15 ± 0.44	bc	92.34 ± 7.94	ab
	0.425	20.44 ± 1.06	c	4.59 ± 0.21	cd	93.79±3.63	ab
	0.500	29.70 ± 0.93	ab	3.64 ± 0.20	de	108.01 ± 7.15	a
	0.575	29.39 ± 1.03	b	3.44 ± 0.45	e	101.20 ± 14.98	ab
	0.600	32.24 ± 1.13	a	3.48 ± 0.52	e	112.60 ± 20.58	a

Data are expressed as the mean ± standard deviation. Different letters within a column indicate significant differences ($p < 0.05$). ANOVA followed by Tukey´s test was performed for multiple mean comparisons.

APPENDIX B

Figure A_B.

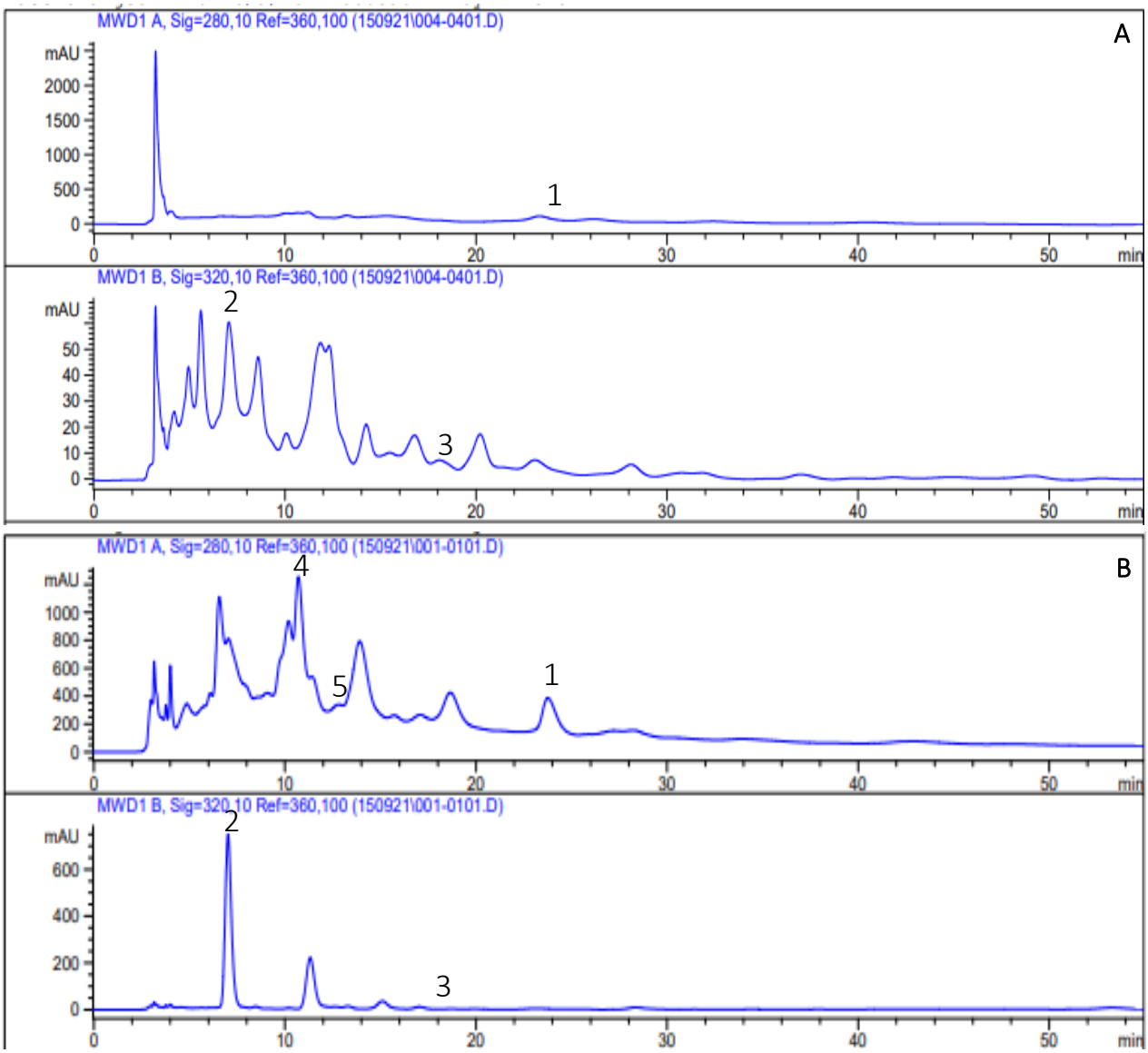

Figure A_B. High-performance liquid chromatography (HPLC) of peel (a) and seed avocado extracts (b) of avocado detected at 280 nm and 320 nm. Analyte legend: (1) Syringic acid; (2) 3,4-dihydroxybenzoic acid; (3) Caffeic acid; (4) Catechin; (5) 4-Hydroxybenzoic acid.