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TocVit: Optimization of tocopherols extraction through conventional and emerging techniques, and bioactive characterization of the obtained extracts

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Abbreviations and Acronyms

°C: Degrees Celsius

µL: Microliter

µm: Micrometer

4HPP: 4-hydroxyphenylpyruvate

ATCC: American type culture collection

BHT: Butylated hydroxytoluene

CCCD: Central composite circumscribed design

CFU: Colony forming units

CSE: Conventional solvent extraction

CTT: Total concentration of tocopherol

DAHP: 3-deoxy-D-arabino-heptulosonate-7-phosphate

DMPB: Dimethyl-benzoquinols, namely 2,3-dimethyl-6-phytyl-1,4-benzoquinol

DPPH: 2.2-diphenyl 1-pycrilhydrazyle

Dw: Dry walnut

EAE: Enzyme-assisted extraction

EC_{50}: Half maximal effective concentration

FAD: Free fatty acid

FAOSTAT: Food and agriculture organization corporate statistical database

FFA: Free fatty acid

G: Grams

HMBPP: 4-hydroxy-3methylbutenyl diphosphate

HPLC: High performance liquid chromatography

LC: liquid chromatography

LSR: Solvent/Sample ratio

MAE: Microwave-assisted extraction

MBC: Minimal bactericidal concentration

MBC: Minimum bactericidal concentration

MEP: The methylerythritol phosphate

Mg: Milligram

MIC: Minimum inhibitory concentration

MIC: Minimum inhibitory concentration

Min: Minutes

mL: Milliliter

MPBQ: 2-methyl-6-phytylbenzoquinone

Na2So4: Anhydrous sodium sulfate

NaCl: Sodium chloride

OH: Hydroxide

PHWE: Pressurized hot water extraction

PLE: Pressurized liquid extraction

Rpm: Revolutions per minute

S: Samples

SC-CO2: Supercritical carbon dioxide

SDGs: The sustainable development goals

SE: Soxhlet extraction

SLE: Solid liquid extraction

SPE: Solid phase extraction

SPM: Secondary plant metabolites

TOC: Tocopherol

TSB: Tryptic soy broth

UAE: Ultrasound-assisted extraction

V/W: Volume-to-weight ratio

Vit-E: Vitamin E

W: Watts

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Abstract

Vitamin E, an essential fat-soluble vitamin, is well known for its antioxidant properties, ability to regulate cholesterol levels, among other benefits; Its consumption strengthens the immune system, prevents chronic diseases such as Alzheimer's, and reduces oxidative and inflammatory stress. It is composed of a group of molecules called tocopherols, classified into four isoforms: α-tocopherol, β-tocopherol, γ-tocopherol, and δ-tocopherol, responsible for its various biological activities. It can be found in several dry fruits such as almonds, hazelnuts, peanuts, and pistachios, with walnuts *(Juglans regia* L.*)* being one of them, produced in large quantities in Portugal. Vitamin E can be obtained from by-products of the nut industry, promoting the reuse of these matrices to obtain molecules with high added value. However, the extraction of this vitamin can be challenging due to its fragility, making it prone to degradation during the extraction process. Therefore, the present study aims to optimize tocopherol extraction from walnuts using conventional and emerging techniques, as well as to evaluate the obtained extracts from a bioactive perspective. Four different extraction methods, including conventional solvent extraction, soxhlet extraction, microwave-assisted extraction, and ultrasound-assisted extraction, were optimized demonstrating that γ-tocopherol is the most abundant isoform, followed by δ-tocopherol, β-tocopherol and α-tocopherol. The microwave-assisted extraction method stands out as a promising approach for tocopherol extraction from walnuts. It showed the highest efficiency in terms of both tocopherol content 1.060±0.007mg/100g (dry weight) and a quenching radical activity of 0.190±0.04mg/mL using the 2,2-diphenil-1-picrylhydrazil and 2.87±0.03mg/mL for the reducing power. Regarding the antimicrobial potential, UAE stands out in the inhibition of *A. fumigatus* in a concentration comparable with the one of the positive control ketoconazole. Furthermore, the microwave technique is more environmentally friendly and economical, requiring less solvent and shorter extraction times.

Keywords: vitamin E, tocopherols, antioxidant, antimicrobial, dry fruits, and walnuts

Resumo

A vitamina E, é uma vitamina essencial lipossolúvel, bem conhecida pelas propriedades antioxidantes, capacidade de regular os níveis de colesterol, entre outros benefícios; o seu consumo fortalece o sistema imunitário, previne doenças crónicas como o Alzheimer e reduz o estresse oxidativo e inflamação. É composta por um grupo de moléculas chamadas tocoferóis, classificadas em quatro isoformas: α-tocoferol, β-tocoferol, γ-tocoferol e δ-tocoferol, que lhe conferem as suas propriedades biológicas. Pode ser encontrada em vários frutos secos, como amêndoas, avelãs, amendoins e pistachos, sendo as nozes (Juglans regia L.) um deles, produzidas em grande quantidade em Portugal. A vitamina E pode também ser obtida a partir de subprodutos da indústria de frutos secos, promovendo a reutilização dessas matrizes para obter moléculas de valor acrescentado a partir de resíduos. No entanto, a extração pode ser desafiadora devido à sua fragilidade, tornando-a propensa à degradação durante o processo de extração. O presente estudo tem como objetivo otimizar a extração de tocoferol de nozes usando técnicas convencionais e emergentes, bem como avaliar os extratos obtidos do ponto de vista bioativo. Quatro métodos diferentes de extração, incluindo extração convencional por solvente, extração soxhlet, extração assistida por micro-ondas e extração assistida por ultrassom, foram otimizados, demonstrando que o γ-tocoferol é a isoforma mais abundante, seguido pelo δ-tocoferol, β-tocoferol e α-tocoferol. O método de extração assistido por micro-ondas destaca-se como o melhor para a obtenção de tocoferol a partir de nozes. Mostrou a maior eficiência em termos do conteúdo de tocoferol 1,060 ±0,007mg/100g (peso seco) bem como da atividade sequestradora de radicais de 0,190±0,04mg/mL usando o ensaio de 2,2-difenil-1-picril-hidrazilo e 2,87 ±0,03mg/mL para o poder redutor. Quanto ao potencial antimicrobiano, UAE destaca-se na inibição de A. fumigatus numa concentração comparável à do controlo positivo Ketoconazole. Além disso, a técnica de micro-ondas é mais amiga do ambiente e económica, exigindo menos solvente e tempos de extração mais curtos.

Palavras-chave: vitamina E, tocoferóis, antioxidante, antimicrobiano, frutos secos, nozes.

1. Introduction

1.1 Secondary metabolites

Carbohydrates, lipids, and amino acids are synthesized from primary metabolites and play a significant role in maintaining vital life elements in plants. Important physiological processes are produced through nutritional and reproductive functions. Secondary plant metabolites (SPMs) are compounds that play an important role in the ability of a plant to interact with its environment and be adaptive and protect itself (Cardoso et al., 2019; Jamloki et al., 2021).

Plant secondary metabolites can be divided into three main groups according to their origin of synthesis: terpenoids, alkali, acids, and phenolic compounds, the first group represent the major group of secondary metabolites with about 30.000 compounds, followed by alkaloids which have about 21.000 compounds and at third place, phenolic compounds constituting approximately 8.000 compounds (Jamloki et al., 2021).

1.2 Tocopherols

1.2.1 Homologues: nomenclature and structure

Among the plant secondary metabolites, we can mention the tocopherols, the name "tocopherol" is from the Greek words tokos and pherin, meaning in sum "to carry a pregnancy" with the ending -ol signifying its phenolic nature. In 1922, This name was given due to its initial identification as a nutritional fertility factor in rats (Evans and Bishop, 1922).

Tocopherol (Toc) is a chemical compound including four different forms that occur in alpha (α), beta (β), gamma (γ), and delta (δ) forms, the differences among them are only due to the position and number of the methyl substitutions on the aromatic chromanol ring as illustrated in **Table 1** (Niki and Traber, 2012).

Table 1. Chemical structure of tocopherol isoforms.

Tocopherol	R1	R2
Alpha (α)	CH_3	CH_3
Beta (β)	CH_3	H
Gamma (γ)	H	CH_3
Delta (δ)	H	H

1.2.2 Physicochemical properties

The physicochemical properties of α-tocopherol, the most abundant and active form of tocopherol in humans, the physicochemical properties are summarized in **Table 2** (Lucarini and Pedulli, 2010).

Table 2. Physicochemical properties of alpha-tocopherol (Lucarini and Pedulli, 2010).

Molecular formula	**$C_{29}H_{50}O_2$**
Molecular weight	430.7g mol^{-1}
Melting point	3°C
Boiling point	235°C
Solubility	Insoluble in water (1.9×10^{-6} mg. L^{-1} at 25°C), soluble in ethanol
Density	0.950g.cm^{-3} at 25°C
Partition coefficient	log P = 12.2
Stability	Uns to UV light, alkaline, and oxidation
Dissociation constant	pKa = 10.8
UV absorption maximum	292nm in ethanol
Fluorescence	Excitation 290–295nm, emission 320–335nm

Further properties of tocopherol are viscous oil at room temperature, insoluble in water, soluble in ethanol and some solvents, yellow, almost odorless, transparent, but oxidized and darkened when exposed to air or light.

1.2.3 Tocopherol biosynthesis and accumulation in plants

The biosynthetic pathway of tocopherol was revealed more than 30 years ago, and it is completely synthesized by photosynthetic organisms, plants, and algae (Hussain et al., 2013), being influenced by various factors such as plant age, organs, organelles, and plant hormones, factors such as abscisic acid, salicylic acid, and jasmonic acid (Falk et al., 2002; Sandorf et al., 2002; Munné-Bosch and Peñuelas, 2003).

The biosynthesis of tocopherols in plants involves two significant pathways the shikimate pathway and the methylerythritol phosphate (MEP) pathway. The shikimate pathway serves as the primary route for synthesizing aromatic compounds in plants and is also the precursor for tocopherol biosynthesis. This pathway initials with the conversion of erythrose 4-phosphate and phosphoenolpyruvate into 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) through the action of DAHP synthase. Subsequently, the formation of shikimate and its phosphorylation leads to the production of 4-hydroxyphenylpyruvate (4HPP). This key intermediate is further transformed into homogentisate, an essential precursor in tocopherol biosynthesis (Hasanuzzaman et al., 2014; Cela et al., 2009).

Conversely, the MEP pathway operates in the plastids and represents an alternative route for tocopherol synthesis. In this pathway, pyruvate and glyceraldehyde 3-phosphate undergo a series of enzymatic steps to form 2-C- methyl-D-erythritol 4-phosphate (MEP). MEP is then converted to 4-hydroxy-3methylbutenyl diphosphate (HMBPP), which enters the downstream pathway to produce homogenize and ultimately leads to tocopherol synthesis (Vidi et al., 2006).

1.3 Sources of tocopherol

Tocopherols are present in many foods, including dried fruits, the different types of tocopherols, α-tocopherol, β-tocopherol, γ-tocopherol, and δ-tocopherol can be found in a variety of dried fruits, such as almonds, hazelnuts, and sunflower, but levels may vary depending on the type of dried fruit and growing, storage conditions and on the processing methods used. **Table 3** shows the contents of tocopherol in different types of nuts.

Table 3. The contents of individual tocopherol (mg kg^{-1}) in different types of nuts (Hejtmánková et al., 2018).

Nuts	Alpha (α)	Beta (β)	Gamma (γ)	Delta (δ)
Walnuts	42.2	< 3.33	442	52.4
Hazelnuts	803	8.39	31.5	2.39
Almond	1,132	4.04	21.4	< 1.67
Cashew	< 5.00	< 3.33	95.5	6.46
Pistachios	23.6	< 3.33	585	10.6
Brazil Nuts	162	< 3.33	427	2,298
Macadamia	435	< 3.33	95.1	4.31
Pecans	64.9	< 3.33	387	28.0
Peanuts	274	< 3.33	112	6.27

According to data from the food and agriculture organization corporate statistical database (FAOSTAT) global walnut production on a kernel basis reached approximately 870,000 metric tons during the 2017/2018 period, Portugal has also experienced growth in walnut production, with an output of 4,600 metric tons in 2017 (Nogales-Bueno et al., 2020).

Walnuts are an important member of the edible nut family and an excellent source of vitamin E (Rébufa, Artaud, and Le Dréau, 2022), indeed walnuts generally contain high levels of tocopherols essentially , α-tocopherol which is the most active form of vitamin E (Lu and Holmgren, 2014) and it's primarily located in the endosperm, the edible part of the nut (Amaral et al., 2005), there are many studies discussing the tocopherol content of walnut oil based on variety, maturity, year of harvest, time of harvest and storage conditions (Nguyen and Vu, 2023; Pycia et al., 2019; Özcan et al., 2020).

1.4 Role of tocopherols

1.4.1 In plant metabolism

Tocopherol possesses unique plant-specific properties that go beyond addressing nutritional requirements. In times of stress where natural factors such as high-intensity lighting or variations in temperatures cause oxidative damage on a cellular level within plants-tocopherols reveal potent antioxidant abilities by shielding cells from outside impacts while internally reinforcing processes simultaneously (DellaPenna and Pogson, 2006).

The primary mechanism for unveiling these antioxidant activities is through their intervention during lipid peroxidation events broken down into three essential phases initiation, propagation, and termination (Schneider, 2005). During the initiation phase, reactive oxygen species (ROS) are formed, usually by the interaction of molecular oxygen with polyunsaturated fatty acids (PUFAs) in cell membranes. This process generates lipid radicals, initiating the lipid peroxidation chain reaction (Muñoz and Munné-Bosch, 2019). In the propagation phase, lipid radicals react with molecular oxygen to form peroxyl radicals, these peroxyl radicals in turn trigger further lipid peroxidation reactions, leading to the formation of further lipid radicals, this perpetuating the chain reaction. The termination phase involves the termination of the chain reaction. Tocopherols play a crucial role in this phase by intercepting and neutralizing lipid radicals (Niki, 2014; Hasanuzzaman, Nahar, and Fujita, 2014).

1.4.2 In human health

Vitamin E plays an important role in the promotion of health and the prevention and treatment of certain diseases and disorders (Tucker and Townsend, 2005), the recommended daily intake is described in the following **Table 4** according to age and gender.

Table 4. Recommended Dietary for vitamin E (in mg) (Institute of Medicine (US) Panel on Dietary Antioxidants and Related Compounds 2000).

Age	Male	Female	Pregnancy	Lactation
0-6 months	4	4		
7-12 months	5	5		
1- years	6	6		
4-8 years	7	7		
9-13 years	11	11		
14 + years	15	15	15	19

Several functions of vitamin E have been demonstrated, including antioxidant by scavenging free radicals, especially peroxyl radicals and singlet oxygen (Niki, 2014). Vitamin E prevents disruption of the internal amphiphilic balance structure, a physiological regulator of enzyme activity, cell signaling, cell proliferation and gene expression, not directly related to antioxidant activity, inhibition of platelet coagulation, prevention of diseases, including neurological diseases, cardiovascular diseases, age-related eye and skin damage and infertility (Ramana et al., 2018).

Vitamin E is widely used as a dietary supplement, alone or in combination with other micronutrients such as vitamin C, to promote health and reduce or prevent the risk of diseases thought to be caused by harmful oxidative modifications of biomolecules (Zong et al., 2015).

1.5 Extraction methods

Extractions are an important step in studies to discover active compounds; ideally, an extraction procedure must be fast, simple, and inexpensive for routine analysis with high yield, and show high purity (Stévigny et al., 2007). Furthermore, parameters like pH, temperature, pressure, solvent, among many others obviously affect the outcome of the extraction (Ibañez et al., 2012).

The extraction methods can be classified into two main classes, conventional and emerging methods, usually, traditional or conventional methods such as hydrodistillation, maceration, and soxhlet extraction require long extraction time and have usually low efficiency

, often requiring large amounts of solvents, incurring in additional costs (Giergielewicz-Możajska, Dąbrowski, and Namieśnik, 2001; Sajfrtová et al., 2010; Bimakr et al., 2012), this has led to the development of novel extraction processes such microwave-assisted (MAE) and supercritical fluid extraction to improve product quality and quantity of active naturals (Menezes Maciel Bindes et al., 2019; Chemat and Strube, 2015).

1.5.1 Conventional

1.5.1.1 Direct solvent extraction method

Direct solvent extraction is the most used method to extract tocopherols from different plant species (Lim et al., 2007). In this method the extraction process typically involves three steps: preparation, extraction, and solvent dissolution. The solid sample is first prepared, which may involve grinding or homogenizing the plant material to increase its surface area and facilitate the extraction process. Next, the sample is immersed directly in a solvent for a shorter duration to extract the desired compounds such as vitamin E. The solvent dissolves the tocopherols from the solid matrix, effectively extracting them into the liquid phase. This technique has been widely standardized for extracting vitamin E from soybean seeds (Vinutha et al., 2015). A detailed procedure for the extraction and analysis of vitamin E from plant samples is given below in **Figure 1**.

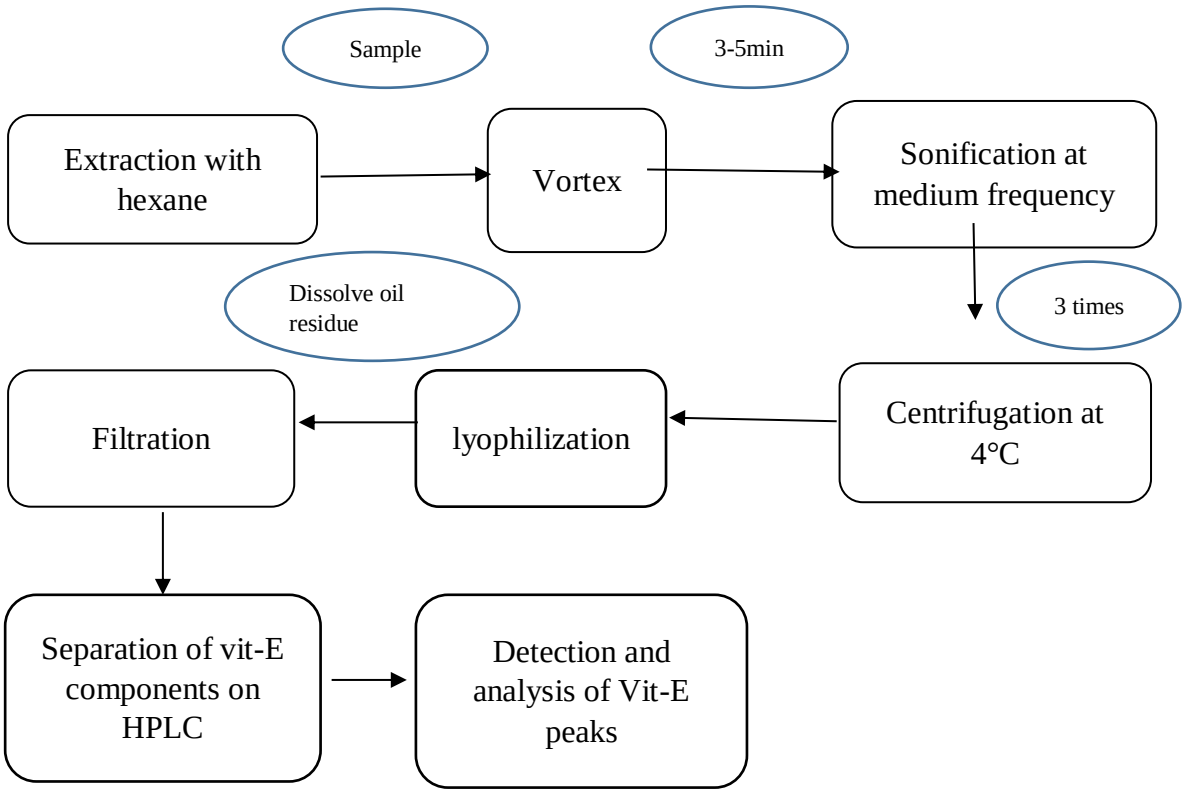

Figure 1. Process of solvent extraction method.

1.5.1.2 Saponification method

Saponification is widely used to extract vitamin E from many fortified foods, including nuts, meats, fruits (Lim et al., 2007), The process of saponification is commonly used in the extraction of vitamin E, involving the use of sodium hydroxide (NaOH) to hydrolyze fats or oils. Once the saponification reaction occurs, the resulting mixture is acidified, leading to the separation of two distinct phases: a tocopherol-enriched phase and a glycerin-containing phase (Brigelius-Flohé, 2019).

To further isolate and release vitamin E from its conjugates, the first fraction, which comprises the tocopherol-enriched phase, undergoes esterification using alkaline hydrolysis. This esterification process, often referred to as Fizet's method, helps convert the tocopherol conjugates into their free form, making them more accessible for analysis and utilization (Elst et al., 2018).

To recover the tocopherol-rich fraction, a rotary evaporator is commonly employed. The use of a rotary evaporator allows for the removal of the solvent under reduced pressure and at low temperatures, facilitating the concentration and recovery of the tocopherols (Ivanova et al., 2022). For a more detailed extraction procedure, including specific conditions and steps, refer to **Figure 2** in the publication by Brigelius-Flohé, (2019), which provides a comprehensive description of the extraction protocol.

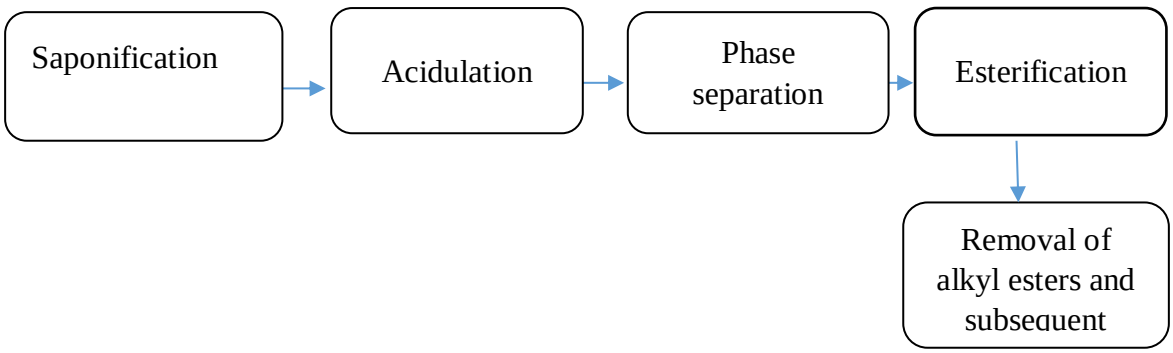

Figure 2. The steps of extraction by saponification.

The disadvantages of this approach are problematic emulsion formation when high fat samples are subjected to saponification, significant loss of vitamin E even in protected

conditions such as dark, high nitrogen conditions, and significant loss of α-tocopherol upon exposure, and prolonged exposure to alkaline conditions (Quek, Chu, and Baharin, 2007).

1.5.1.3 Soxhlet extraction

Soxhlet extraction of fats and oils from seed matrices is based primarily on solvent selection, and includes acetone, ethanol, methanol, and petroleum ether. According to literature reviews, petroleum ether is a common choice as a solvent for the extraction of tocopherol from nuts because it is safer to handle than hexane (Bourgou et al., 2016). The second solvent used in the extraction process is hexane. Hexane was chosen due to its low boiling point, which allows for easy recovery of the solvent, and its ability to dissolve most oils. It is effective in extracting vitamin E from nuts, yielding higher quantities, especially when temperature and agitation parameters are carefully controlled (Mamidipally and Liu, 2004). However, it is important to note that one of the drawbacks of using n-hexane is its potential for environmental pollution (Péres et al., 2006).

This method is one of the oldest extraction techniques. The principle of this method involves placing the sample in a thimble holder, which is then filled with fresh condensed solvent from a distillation bottle. As the liquid level in the thimble reaches the overflow point, a siphon is created, allowing the solution to be aspirated and expelled into the still. The solute extracted from the sample is collected in the solvent flask through distillation, while the residue remains in the flask. Fresh solvent continuously enters the fixed bed to maintain the extraction process (Danlami et al., 2014), then the extracts were then filtered through qualitative filter paper and then refiltered through a filter.

These extraction methods generally remove about 99% of the oil from the seeds, making them the preferred extraction method, while recovering about 60% of the solvent used, which is among its main advantages, it’s also a very simple and low-cost technique of extraction, however, it causes economic and environmental problems due to the high temperature, the target compound has a risk of thermal degradation, while also making selective extraction impossible and difficult to automate (Luque de Castro and Priego-Capote, 2010).

1.5.1.4 Cold press extraction

Cold-press extraction is a hydro-extraction method that offers several advantages compared to other extraction techniques. It requires less energy, making it more

environmentally friendly, and is cost-effective since it doesn't involve the use of heat, chemicals, or solvents. The cold-pressing process allows to produce high-quality oil at low temperatures, preserving the natural properties of the oil. It retains a higher concentration of lipophilic phytochemicals, including antioxidants, making the oil more nutritious than refined oils (Tańska et al., 2016).

One of the key benefits of cold-pressed oils is the retention of temperature-sensitive phenolic components, which can have significant health benefits. Since no heat is applied during the extraction process, the oxidation process is minimized, resulting in more oil with prolonged shelf life. The purification of cold-pressed oil typically involves washing with water, followed by filtering, and centrifuging to remove impurities (Masoodi et al., 2022).

However, it is worth noting that cold-press extraction may have limitations. The raw materials used may yield substandard oil, affecting the overall product quality. Achieving consistent and high-quality results can be challenging (İmer and Tasan, 2018). There are three main types of cold presses commonly used: expellers, expanders, and twin-cold systems.

1.5.2 Emerging techniques

1.5.2.1 Microwave-assisted solvent

The system of microwave assisted solvent extraction is specifically designed to operate at high temperatures, and pressure is controlled by a temperature sensor (Abuín, Carro, and Lorenzo, 2000), the parameters commonly studied for microwave-assisted optimization are pressure or temperature, extraction time, microwave power, solvent, solvent volume, and matrix properties, including water content (Camel, 2000), the principle of microwave-assisted extraction is that the high pressure exercised on cell walls and organelles causes physical changes in the matrix, causing the solvent to diffuse through the sample matrix, and thus a solute is released from the sample matrix into the solvent. The choice of solvent is a crucial step in achieving optimal extraction results, which ultimately leads to improved extraction efficiency, this extraction requires solvents that have a high selectivity for the analyte and good solvent-matrix interactions (Eskilsson and Björklund, 2000).

According to (Danlami et al., 2014), the microwave-assisted extraction instrument can be categorized into two types: open container and closed container systems. In the open vessel system, the extraction is carried out at atmospheric pressure, where the solvent is refluxed while controlled temperature is applied. This setup allows for efficient extraction of the target

compound. On the other hand, the closed container system employs high pressure and temperature conditions to facilitate the extraction process. The elevated pressure helps to enhance the solubility and diffusion rates, leading to improved extraction efficiency. The selection of the appropriate extraction system depends on the specific requirements and characteristics of the target compound.

1.5.2.2 Enzymatic-assisted extraction

Enzymes are biodegradable, non-hazardous, and have the ability to catalyze reactions with high selectivity (Alexandre et al., 2021), the principle of the enzymatic method is to employ particular enzymes to enhance the extraction of bioactive compounds from various sources (Fischer et al., 2016), For example, lipase enzymes can be used to convert Free Fatty Acid (FFA) to Fatty Acid (FAD) instead of using a chemical catalyst like methanol. When selecting the enzyme, factors such as the cell wall structure, and position of the oil droplets in the seed must be considered. Under mild conditions, multiple enzymes can effectively dissolve cell walls and membranes in aqueous solutions (Marić et al., 2018).

According to (Quek, Chu, and Baharin, 2007), under optimized conditions, it was possible to achieve a 96.5% Free Fatty Acid (FFA) yield using 2.7-4.3% lipase at 50 °C without the use of dehydrating agents. Additionally, this method resulted in a 1.7-fold increase in tocopherol concentration compared to the original attention. Enzyme-assisted extraction offers advantages, such as economical operating costs and fast extraction (Sowbhagya and Chitra, 2010).

Various factors should be considered when optimizing oil extraction, including pH, temperature, particle size, enzyme concentration, and the oil-to-water ratio. Moisture is essential for the proper functioning of enzymes, and inadequate moisture levels in oilseeds can result in the formation of thick suspensions that hinder enzyme activity. Enzymatic reactions are conducted under mild conditions, which is advantageous for extracting heat-sensitive compounds and saving energy (Sowbhagya and Chitra, 2010). However, despite numerous benefits, the utilization of enzyme-assisted extraction is constrained by the challenging drying process following enzyme treatment. The requirement for significant amounts of enzymes can also make these methods costly. Moreover, the lack of commercially available enzymes limits preventing oil emulsification, necessitating a demulsification stage after extraction to recover and enhance oil yield (Yousefi et al., 2019).

1.5.2.3 Ultrasound-assisted extraction

Ultrasound-assisted extraction is used for the extraction of analytes from solid samples, by applying ultrasonic high-frequency radiation in a water bath or with other devices, such as probes (Santos and Capelo, 2007), this method is based on the use of energy from ultrasonic waves to cause compression and expansion cycles (Pico, 2013), they lead to surface peeling, erosion, and particle breakdown to facilitate solvent access to the hydrophobic compounds contained within promoting the release of bioactive (Vilkhu et al., 2008).

Ultrasound possesses the capability to significantly enhance the extraction process by inducing agitation, thereby improving the contact between the solvent and the desired compounds. This method is relatively simple, cost-effective, and can reduce extraction times and temperatures. The effectiveness of this technique lies in the type of irradiation source employed (Lyytikäinen, Kukkonen, and Lydy, 2003). Recently, a more efficient system utilizing a cylindrical probe for sample sonication has been developed (Santos et Capelo, 2007). The selection between baths and probes depends on the specific requirements of the contaminant analysis being conducted (Li, Pordesimo, and Weiss, 2004; Pananun et al., 2012).

One significant advantage of ultrasound-assisted extraction is its simplicity, cost-effectiveness, and overall efficiency, making it a favorable alternative to conventional extraction methods. Ultrasound can enhance the extraction rate and expedite the kinetics of the process. Moreover, ultrasound is particularly useful for extracting heat-sensitive compounds, as it minimizes the potential for degradation during extraction (Chemat, Zill-e-Huma, and Khan, 2011).

1.5.3 Comparative of different extraction methods

This section aims to provide a comprehensive comparative analysis of the different extraction methods. This comparison is represented in **Table 5**, highlighting the advantages and limitations of each technique. This comparative analysis will enable selection of the most appropriate extraction methods based on factors such as extraction efficiency, selectivity, extraction time, energy consumption, cost-effectiveness, and scalability potential.

Table 5. Comparative of different extraction methods.

Extraction method	Advantages	Disadvantages	Refences
Saponification	Standard method Effective method	Loss of tocopherols due to the use of alkali In cases where saponification is followed by solvent extraction, the formation of lumpy soap mass makes the extraction difficult. May require powdering agent or chemicals for metathesis.	(Quek, Chu, and Baharin, 2007)
Solvent extraction method	Standard method Simple and faster Low-cost requires less apparatus and solvent Can extract more sample mass than other methods	One of the main limitations is that it may not be as efficient	(Vinutha et al., 2015; Luque de Castro and Priego-Capote, 2010)
Soxhlet extraction	Standard method Low costs Temperature during the extraction system could be maintained Quick and easy way to extract vitamin E	Slow method Large amount of solvent Compound sensitive to heat may be thermally decomposed	(Luque de Castro and Priego-Capote, 2010)
Enzyme-Assisted Extraction	Higher extraction yields Higher quality of extract	Require longer reaction time compared with chemical method.	(González-Gómez et al., 2019 ;

	Oxidation stability Eco-friendly. Milder reaction temperature at atmospheric pressure Lipases can be used repeatedly.	High cost non-availability of enzymes on a commercial scale	Nadar, Rao, and Rathod, 2018 ; Çakaloğlu, Özyurt, and Ötleş, 2018)
Cold-Press Extraction	Hydraulic extraction method Consumes less energy Eco-friendly High quality oils at low temperatures	Low Production Hard to extract uniform quality of oil	(Özcan et al., 2020 ; İmer and Tasan, 2018)
Ultrasonic-Assisted Extraction	High oil yield, high extract quality, and reduced solvent consumption Easy to handle with reduced working time	This method depends totally on ultrasound unit Oil extraction is weak Existence of a dispersed phase may contribute to an ultrasound wave attenuation	(Tan, Watson, and Preedy, 2012; Tiwari, 2015)
Microwave-Assisted Extraction	High returns on capital investment are expected Less time consuming than conventional methods	For heat sensitive compounds is not appropriate Difficult to operate and expensive equipment	(Hu et al., 2018; Danlami et al., 2014)

There are several other types of extraction, each extraction methods having its advantages and disadvantages for vitamin E extraction, it's important to choose the most appropriate method.

1.6 Chemical characterization

1.6.1 Chromatographic method

Vitamin E quantification and extraction can be performed by high-performance liquid chromatography (HPLC), ion exchange chromatography and adsorption chromatography are the two most popular methods used, the commercially available ion-exchangers, such as Amberlite IRA 900, Dowex, XUS 40240, Amberlyst A 26 and Duolite A 161, are frequently utilized, the OH anion type of resin is the most used because of its large adsorption capacity for tocopherols, normal Phase Adsorption Chromatography continued polar hydrophilic and lipophilic base stationary and less polar mobile phase, conversely, it is sui for reversed-phase adsorption chromatography, extraction of vitamin E from a preconcentrated fraction containing alkyl esters using a normal phase adsorption column packed with alumina, Silica gel, magnesium oxide, calcium hydroxide or cellulose , pillar is first elution with medium-chain alkyl hydrocarbons, such as hexane to heptane, the fatty acid alkyl esters then desorb the vitamin E from the column sorbent by eluting column with lower alkyl alcohol such as isopropyl alcohol (Quek, Chu, and Baharin, 2007), ion exchange chromatography is better than adsorption chromatography, advantages of ion exchange chromatography over adsorption chromatography include larger adsorption capacity, higher reproducibility, and high comparing the selectivity or specificity of basic anion resins with inorganic resins such as alumina and magnesium oxides (Quek, Chu, and Baharin, 2007), Similar to other methods, chromatography also has its limitations. In the case of the normal adsorption method, there is a possibility of irreversible binding of components from raw materials to the stationary phase. This leads to a short lifespan of the normal phase media, making the process economically impractical due to frequent replacement requirements. An alternative approach is ion exchange chromatography, where the separation of vitamin E is based on the retention of charged molecules on a charged stationary phase, instead of their attraction to immobilized hydrophobic stages. However, a major drawback of this method is the unavailability of high resolution (Tsochatzis and Papageorgiou, 2013).

2. Materials and Methods

2.1 Chemicals, reagents, and samples

All chemicals and reagents used in this study were sourced from scientific suppliers with at least analytical-grade quality, except for those utilized in high-performance liquid chromatography (HPLC), which involved the use of HPLC-grade chemicals and reagents. The walnut *(Juglans regia L.)* plant material used in this study was collected from Vale da Porca (Portugal) at coordinates 41.54° N 4.887°S, and were produced by a local farmer. Upon arrival of the samples, they were weighed, and the shells were removed and stored.

2.2 Sample preparation

To preserve the integrity of the sample and facilitate further processing, they were frozen at a temperature of -30°C. After this initial step, a freeze-drying process was employed using the FreeZone 4.5 model (7750031, Labconco, KS, USA) lyophilizer. This freeze-drying process effectively removed the moisture content from the sample. The dried samples were further processed by reducing them to a fine powder through a grinding procedure. This transformation to a fine powder was crucial to ensure the homogeneity and suitability of the sample for subsequent analytical procedures. For a visual representation of this transformation, **Figure 3**, shown below, provides a clear contrast between the sample as it was received and its state after grinding.

Figure 3. Samples before and after grinding.

2.3 Tocopherol extraction

2.3.1 Conventional solvent extraction method (CSE)

For the extraction procedure, butylated hydroxytoluene (BHT) solution (100µl) and tocol solution (400µL) were pre-added to 0.5 g of each sample (S1=0.554g, S2=0.523g, S3=0.527g), which were homogenized with methanol (4mL) using vortex mixing for 1 minute. Then, hexane (4mL) was added, and the mixture was vortexed for another minute. Concentrated sodium chloride (NaCl) solution (2mL) was then added, and the resulting mixture was stirred for 1 min. The mixture was centrifuged (5minutes, 4000rpm), and the supernatant was carefully transferred to a separate vial (25mL). Two additional extractions of the sample were performed with hexane and NaCl, followed by dehydration with anhydrous sodium sulfate (Na_2SO_4). The combined solution was dried under a stream of nitrogen, redissolved in 2mL of hexane, filtered through a 0.22µm disposable filter, transferred into a dark injection vial, and analyzed by HPLC.

2.3.2 Soxhlet extraction method (SE)

For this extraction method, 5g (S1= 5.042g, S2 = 5.056g, S3 =5.087g) of sample was placed in a paper filter was extracted with petroleum ether for 7h at 120°C. The solvent was recycled in a closed loop, allowing for repeated extraction of tocopherols from the samples. The combined solution was dried under a stream of nitrogen, redissolved in 2mL of hexane, filtered through a 0.22µm disposable filter, transferred into a dark injection vial, and analyzed by HPLC. This technique is depicted in **Figure 4**.

Figure 4. Soxhlet used for tocopherol extraction from walnut.

2.3.3 Microwave-assisted extraction (MAE)

The MAE procedure was conducted using (Microwave Synthesis System, NuWay-uno, Nutech Analytical Technologies Pvt. Ltd, Kolkata, India) inside sealed high precision glass containers (**Figure 5**). In this emerging extraction technique, an optimization was performed to understand which were the conditions that yielded the highest amounts of tocopherols. Thus, the samples (1g) were extracted according to different conditions, namely time (t), temperature (T), and solvent to sample ratio (SRL), defined as the independent variables by the response surface methodology design (RSM). During processing, samples were stirred at 600rpm using a magnetic stirring bar. After extraction, the combined solution was dried under a stream of nitrogen, redissolved in 2mL of hexane, filtered through a 0.22μm disposable filter, transferred into a dark injection vial, and analyzed by HPLC-fluorescence.

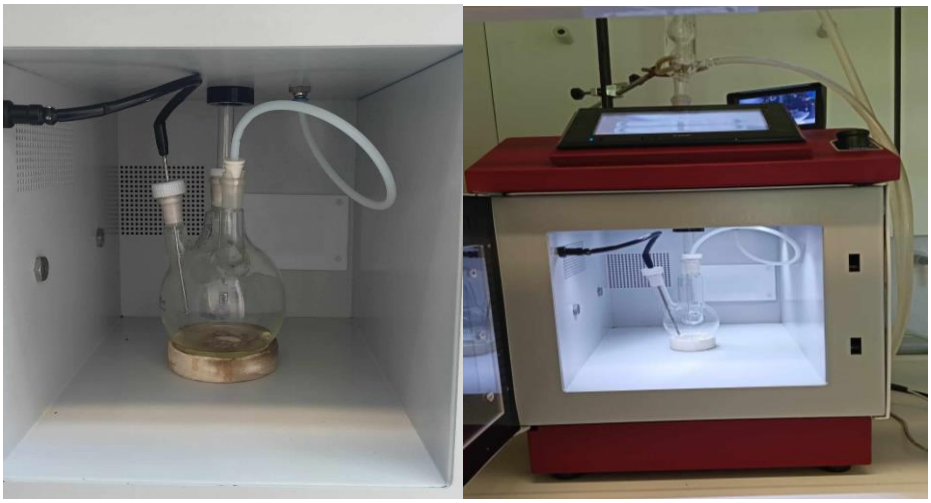

Figure 5. Microwave oven used for tocopherol extraction from walnut.

2.3.4 Ultrasound-assisted extraction (UAE)

The UAE was also subject to an optimization procedure. The extraction was performed with (Ultrasonic homogenizer, model CY-500, Optic Ivymen Systen, Barcelona, Spain), which was equipped with a tapered microtip (dimeter: 6mm) and an integrated temperature controller (**Figure 6**). The wight of the samples and the volume of the solvent used according to the optimization plan in 2.4.3 experimental design part. The samples were combined with a solvent, namely acetone: water, and subjected to extraction while being maintained under an ice bath to prevent any temperature rise. Subsequently, the combined solution was dried under a stream of nitrogen, redissolved in 2mL of hexane, filtered through a 0.22μm disposable filter, transferred into a dark injection vial, and analyzed by HPLC. A second methodology was also performed after some vials showed 2-phases, which did not allow them to be injected in the HPLC. Thus,

the changes in this new methodology included a removal of water through rotary evaporator (in substitution of nitrogen current drying), redissolution in 2 mL of hexane and subsequent filtration. Unfortunately, due to lack of sample and time, only 8 extractions could be performed with this new method.

Figure 6. Ultrasound used for tocopherol extraction from walnut.

2.4 Optimization extraction

2.4.1 Methodology of optimization - RSM polynomial model

As stated above, the MAE and the UAE were subject to optimization processes to understand which conditions favored higher tocopherol yields. For this, a three-level, three-factor Box-Behnken design (Box and Behnken, 1960) was implemented. The design consisted of 13 experiments, with two additional center points replicated to measure experimental error. The maximum and minimum levels were selected by preliminary screening tests and instrumental aspects. Considering the response variables, a second-degree polynomial model was used for data fitting (**Eq. 1**) using Least Squares method (Björck, 1990), where *Y* represents the predicted response for dependent variables; b_1, b_2, and b_3 are the linear terms; b_{11}, b_{22}, and b_{33} are the quadratic terms; b_{12}, b_{13}, and b_{23} are the interaction terms; and *t*, *T*, and *S* represent the Box-Benken design encoded values for the three independent variables.

$$Y = b_0 + b_1 t + b_2 T + b_3 S + b_{11} t^2 + b_{22} T^2 + b_{33} S^2 + b_{12} tT + b_{13} tS + b_{23} TS \quad (2)$$

An analysis of variance was performed to verify the robustness and statistical significance of the models and their respective terms (b). For the construction of the model, only the statistically significant terms were kept. Values of R2, adjusted R2, p-value of the model and mean square error were used to measure the adequacy of the models to the response variables. The determination of the experimental points and the construction of the polynomial model were performed using Matlab R2021b (MathWorks Inc.).

2.4.2 Optimization

Optimized conditions were estimated considering the response variables individually, based on a RSM combined with a Genetic Algorithm (RSM-GA), using the same software. The genetic algorithm supports non-differentiable objective functions and can estimate the optimal parameters (Lima et al., 2021). This way, it is possible to achieve more accurate and reliable results compared to a RSM alone.

2.4.2.1 Experimental design

In this work, the points are generated based on a central composite circumscribed design (CCCD) represented by a circumscribed sphere in which each factor has five levels of midpoint points (-1.68 to 1.68). The points of the headquarters should correspond to the best combination of responses. Therefore, multiple iterations are performed to maximize the predictions (Box, Hunter, and Hunter, 2005). The trial runs are performed to minimize the impact of an unexpected change in the observed response. In **Table 6** the range for each variable is shown for the UAE (Lee et al., 2012; Elouafy et al., 2022; Pop et al., 2021; Masoodi et al., 2022) and MAE (Hu et al., 2018; Shabanian, Sari, and Daraei Garmakhany, 2021; Singh et al., 2023), these variables include the solvent: sample ratio, time, ultrasonic power and temperature. **Table 7** presents a comprehensive overview of the independent variables, including their coding and nature.

Table 6. The range for each UAE and MAE variables.

	Solvent: Sample ratio	Time	Ultrasound Power	Temperature
UAE	20:1 to 20:5 (v/w)	5 – 30 min	10-80%	
MAE	5:1 to 20:1 (v/w)	1–15 min		50–85 °C

The fixed variables for those methods were solvent acetone: water (80:20 v/v), the UAE solvent volume was fixed to 20mL and the MAE weight of the sample (1g).

Table 7. Experimental domain and codification of independent variables of both extraction techniques.

Coded values	Natural values					
	Ultrasound-assisted extraction			Microwave-assisted extraction		
	LSR	Time	Ultra-sound Power	LSR	Time	Temperature
-1.68				5	1	50
-1	1	5	10	8	4	57
0	3	17.5	45	13	8	68
+1	5	30	80	17	12	78
+1.68				20	15	85

For each type of extraction, 17 different runs with varying conditions were proposed, however, in both cases only sum runs reached the final step of quantification of tocopherols due to experimental errors.

Table 8 and **Table 9** represent the experimental runs performed in the study. A controlled experimental procedure in which the materials were systematically designed was used and the corresponding biological responses were accurately measured. The experiment was designed for reliability.

Table 8. The experimental plan for MAE.

Experimental Run	Coded Factors			MAE		
	X_1	X_2	X_3	LSR (v/w)	Time (min)	Temperature (°C)
1	-1	-1	-1	8	4	57
2	-1	-1	1	8	4	78
3	-1	1	-1	8	12	57
4	-1	1	1	8	12	78
5	1	-1	-1	17	4	57
6	1	-1	1	17	4	78
7	1	1	-1	17	12	57
8	1	1	1	17	12	78
9	-1.68	0	0	5	8	68
10	1.68	0	0	20	8	68
11	0	-1.68	0	13	1	68
12	0	1.68	0	13	15	68
13	0	0	-1.68	13	8	50
14	0	0	1.68	13	8	85
15	0	0	0	13	8	68
16	0	0	0	13	8	68
17	0	0	0	13	8	68

Table 9. The experimental plan for UAE.

Experimental Run	Coded Factors			UAE		
	X_1	X_2	X_3	LSR (v/w)	Time (min)	Ultrasonic power (%)
1	-1	0	0	1	17.5	45
2	1	0	0	5	17.5	45
3	0	-1	0	3	5	45
4	0	1	0	3	30	45
5	0	0	-1	3	17.5	10
6	0	0	1	3	17.5	80

7	0	0	0	3	17.5	45
8	0	0	0	3	17.5	45

2.5 Yield of walnut extract

Yield is a critical indicator of extraction method efficiency and was calculated in accordance with the following equation:

$$yield\ (\%) = 100\%\ x\ \frac{weight\ of\ dry\ extract}{weight\ of\ initial\ sample}$$

The weight of the dried extract refers to the weight of the extract obtained after evaporation and lyophilization, ensuring that all water content has been removed. The weight of the walnut seed represents the initial weight of the walnut seeds used as the raw material for extraction.

2.6 Statistical analysis

The confidence intervals were set at 0.95, employing a student distribution, as a means of identifying factors with statistical significance. To assess the overall significance of the regression models in describing the observed data, an analysis of variance (ANOVA) was employed. This ANOVA used the fisher's F- test to ascertain whether the constructed models adequately captured the underlying data patterns. Additionally, the coefficient of determination (R2) and its adjusted counterpart (R2A), which considers the number of coefficients in the model, were employed to gauge the extent to which the regression model explained the variability in the response variable (Mather, Moran, et Smith 1967).

2.7 Chromatographic identification and quantification

The HPLC equipment consisted of an integrated system, composed of a Smartline Pump 1000 (Knauer, Germany) as showed in **Figure 7**, degassing system Smartline Manager 5000, AS-2057 and 2500 Autosamplers UV detector at 295nm (Knauer, Germany) with FP-2020 fluorescence detector (Jasco, Japan) programmed for excitation at a wavelength of 290nm and the emission of 330nm. Data was analyzed using Clarity 2.4 software (DataApex). Chromatography Separation was performed at 30°C using a normal phase polyamide II column (250 x 4.6 mm) from YMC Waters (Japan) (7971 R Grace Furnace). The mobile phase used

was n-Hexane and ethyl acetate (70:30, v/v) at a flow rate of 1mL/minute with an injection volume of 20μL. The samples were identified by chromatographic comparison with authentic standards, using the external standard method. The content of tocopherol in walnut samples is expressed as nanograms per gram of dry walnut.

Figure 7. HPLC-fluorescence equipment.

2.8 Antioxidant activity evaluation

Two different methods were used for the antioxidant activity of *Juglans regia L*: the quenching of 2,2- diphenyl-1-picrylhydrazyl radical (DPPH) (Škrovánková, Mišurcová, and Machů, 2012), and the reducing power of ferric ions (FRAP).

2.8.1 DPPH radical-scavenging activity

For the DPPH analysis a microplate reader was used, namely an ELX800 (Bio-Tek. Instruments, Inc; Winooski, USA). The procedure involved the following steps: In each well of the microplate, 30μL of the extract solution at different concentrations was added, along with 270μL of a methanolic solution containing DPPH radicals ($6x10^{-5}$mol/l). A blank solution was prepared by adding 30μL of the extraction solvent instead of the sample. The mixture was allowed to stand for 60minutes in a dark environment. The reduction of the DPPH radical was measured by determining the absorption at 515nm using the microplate reader.

The percentage of DPPH discoloration was calculated using the following equation:

$$\%\, RSA \;=\; [(ADPPH \;-\; AS) \,/\, ADPPH] \;\times\; 100$$

(AS: absorbance of the solution when the sample extract was added at a specific level, ADPPH: absorbance of the DPPH solution). The absorbance of the control reaction, which included all reagents except for the tested compound, was labeled as Ablank, while the absorbance of the tested compound was designated as Asample. All experiments were performed in triplicate to ensure reliable results.

2.8.2 Reducing power assay

To assess antioxidant potential (Barros, Baptista, and Ferreira 2007), the tocopherols extracts were mixed with sodium phosphate buffer, potassium ferricyanide (0.5mL of each solution), incubated at 50°C for 20 minutes, followed by the addition of trichloroacetic acid, deionized water, and ferric chloride (0.5mL, 0.8mL, 0.16mL respectively), with absorbance being measured at 690nm using the same microplate reader.

2.9 Antimicrobial activity evaluation

Two different methods were used for the antimicrobial activity of *Juglans regia L*: antibacetrial activity and antifungal activity.

2.9.1 Antibacterial activity

The assessment of antibacterial effectiveness was conducted using the microdilution technique. 100 mg samples of the extract were prepared and subjected to analysis to ascertain the minimum inhibitory concentration (MIC), which signifies the lowest concentration preventing observable bacterial growth, and the minimum bactericidal concentration (MBC), representing the minimum concentration at which bacteria are effectively eradicated. The extracts were tested against a group of foodborne pathogens, comprising five Gram-negative bacteria: *Enteribacter clocae* (ATCC 49741), *Escherichia coli* (ATCC 25922), *Salmonella enterica* (ATCC 13076), *Yersinia enterocolitica* (ATCC 8610), and three Gram-positive bacteria: *Bacillus cereus* (ATCC 11778), *Listeria monocytogens* (ATCC 19111), and *Staphylococcus aureus* (ATCC 25923). Prior to the analysis, the bacterial strains were cultured at 37°C in an appropriate fresh medium for 24hours to ensure they were in the exponential growth phase. The MIC assessments for all bacterial species were carried out using a colorimetric assay, as described by Pires et al. (2018).

The extract samples were initially dissolved in a solution of 5% (V/V) dimethyl sulfoxide and 95% autoclaved distilled water, resulting in a stock solution with a final concentration of 20mg/mL. subsequently, 90µL of this solution was added to the first well of 96-well microplate in duplicate, along with 100µL of Tryptic soy broth (TSB). In the remaining wells, 90µL of TSB medium were added. Serial dilutions of the extract samples were performed to establish a concentration rang of 10 to 0.03125mg/mL. Following this, 10µL of a standardized inoculum (containing 1.5 x 10^6 colony forming units (CFU) /mL) was introduced to all wells, resulting in a concentration of 1.5 x 10^5 CFU/mL. To ensure consistency, two negative controls (one with TSB and another with the extract) and two positive controls (one with TSB and the inoculum, and another with medium, antibiotics, and bacteria) were included. Ampicillin and streptomycin were employed against all bacterial strains, while methicillin was exclusively used for *S. aureus*. The microplates were then incubated at 37°C for 24hours.

MIC determination involved the addition of 40µL of 0.2mg/mLp-iodonitrotetrazolium chloride to each well, followed by an incubation at 37°C for 30minutes. The MIC was identified as the lowest concentration that hindered visible bacterial growth, causing a color change from yellow to pink if the microorganisms remained viable. To ascertain the MBC, 10µL of liquid, from wells displaying no color change, was plated on solid blood agar medium (7% sheep blood) and incubated at 37°C for 24hours. The MBC was the lowest concentration at which no bacterial growth occurred, indicating the minimum concentration required for bacterial elimination.

2.9.2 Antifungal activity

The evaluation of antifungal efficacy was carried out using a methodology previously described by Heleno et al. (2013). For this study, *Aspergillus fumigatus* (ATCC 204305) and *Aspergillus brasiliensis* (ATCC1604) were used. The micromycetes were maintained on malt agar, stored at temperature of 4°C, and subsequently transfered to a fresh medium and incubated at 25°C for 72hours. To explore the antifungal properties, fungal spores were gently removed from the surface of agar plates using sterile 0.85% saline solution containing 0.1% tween 80 (V/V). The spore suspension was adjusted using sterile saline to achieve a concentration of around 1.0 x 10^5 spores per 100µL per well. The test samples were dissolved using a combination of % (V/V) dimethyl sulfoxide and 95% autoclaved distilled water, resulting in a stock solution with a final concentration of 20mg/mL. Subsequently, 90µL of this solution was

added to the first well of a 96-well microplate in duplicate, along with 100µL malt extract broth (MEB). In the remaining wells, 90µL of MEB medium was added. The determination of the minimum inhibitory concentration (MIC) was conducted using a serial dilution technique in a 96-well microplate. The lowest concentrations at which visible fungal growth was inhibited under microscopic observation were identified as MIC values. To ascertain the minimum fungicidal concentration (MFC) serial sub cultivation of 2µL of the tested compounds dissolved in a medium was performed. This subculture was then inoculated into microplates containing 100µL of MEB per well and further incubated for 72hours at 26°C. the lowest concentration that resulted in no visible fungal growth was defined as the MFC, indicating a 99.5% reduction in the original inoculum. For comparative purposes, the commercial fungicide ketoconazole was used as a positive control.

3. Results and discussion

Walnuts are recognized in scientific literature as rich sources of tocopherols, offering a valuable natural alternative in combating oxidative stress and as a source of antioxidant molecules. This activity has significant applications in the food, chemical, and pharmaceutical industries, where walnut tocopherols can be used in food preservation, cosmetic product formulation, and in the development of antioxidant supplements.

3.1 Tocopherol analysis

In this work, the conventional tocopherol extraction method (solvent extraction), and the soxhlet technique were compared to emerging green extraction methods (UAE and MAE), with a view to exploiting tocopherols for industrial applications, increasing the yields in these compounds and implementing sustainable practices. The data corresponding to conventional and soxhlet extraction is shown in the **Figure 8**.

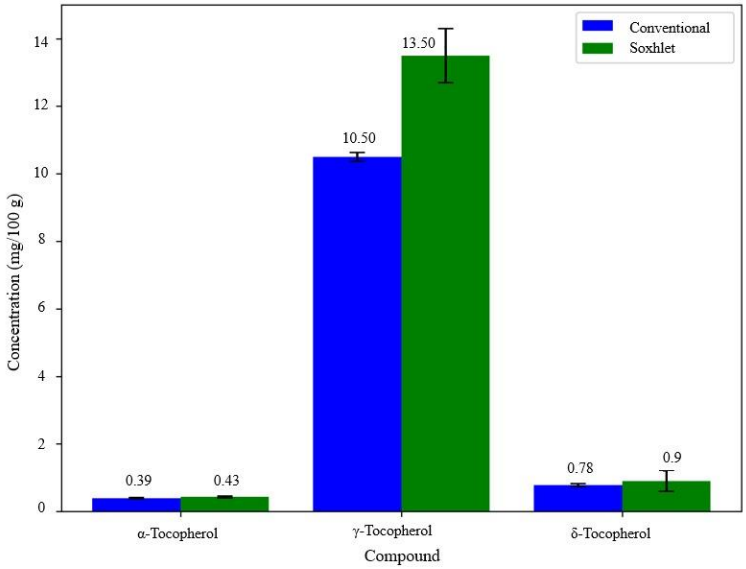

Figure 8. Profile of tocopherols quantified by HPLC in solvent extraction and soxhlet extracted samples of walnuts.

In **Figure 8**, the yield of each tocopherol is shown for the conventional and soxhlet extractions, showing the differences between these two extraction techniques. The comparison was performed using a Student's T-test with a 95% confidence level. In the conventional extraction, using hexane, the following tocopherol contents were obtained: 0.39mg/100g of α-tocopherol, 10.50mg/100g of γ-tocopherol, and 0.78mg/100g of δ-tocopherol. Regarding the

soxhlet extraction, with petroleum ether, the obtained yields were 0.43mg/100g of α-tocopherol, 13.50mg/100g of γ-tocopherol, and 0.9mg/100g of δ-tocopherol. β-Tocopherol was present in a significantly smaller percentage in the conventional and soxhlet extraction which has been deemed as insignificant. Rabrenovic et al. (2011) made the same observation for cold pressing and soxhlet extraction. Mitsikaris et al. (2022) and Elouafy et al. (2022), wrote that the amounts of α-, γ-, δ-tocopherols ranged respectively from 1.08 to 4.05, 10.62 to 26.46, and 2.51 to 4.23 (mg/100 g), which are in accordance with the results found in this work. For α- and δ-tocopherol, there were no significant differences between the yields, showing that statistical differences were only sought for γ-tocopherol, in which the soxhlet extraction showed a statistically higher amount of tocopherols. This is an interesting finding, as soxhlet extraction uses high temperatures for the extraction, while the conventional extraction uses low temperatures. This is particularly important due to tocopherols being quite sensible to high temperature. Still, the soxhlet extraction takes considerably longer to extract and could also be a reason for the higher amount of tocopherols found. However, it is essential to understand that the use of petroleum ether in this method can be potentially hazardous if the tocopherols are to be used for food incorporation, as this solvent should not be present in foods.

Table 10. Total tocopherol extracted using CSE, SE, MAE and UAE in (mg/100g dw) from walnut sample.

Run MAE	α-Tocopherol	β-Tocopherol	γ-Tocopherol	δ -Tocopherol	Total tocopherol content
Conventional solvent extraction	0.39±0.02	n.a	10.523±0.134	0.777±0.043	11.689±0.119
Soxhlet extraction	0.428±0.0033	n.a	13.478±0.796	0.9±0.028	14.806±0.801
Microwave-assisted extraction					
MAE-1	0.221±0.001	n.a	0.363±0.000046	0.476±0.005	1.060±0.007
MAE-2	0.208±0.0002	n.a	0.358±0.0005	n.a	0.566±0.001
MAE-3	0.218±0.0001	0.301±0.001	0.359±0.0002	n.a	0.879±0.001
MAE-4	0.218±0.002	n.a	0.354±0.007	0.462±0.002	1.035±0.011
MAE-5	n.a	0.324±0.008	n.a	n.a	0.324±0.008
MAE-6	0.226±0.0001	n.a	0.366±0.008	n.a	0.592±0.007
MAE-7	0.23±0.0001	n.a	0.365±0.008	n.a	0.590±0.007
MAE-8	n.a	0.306±0.001	n.a	n.a	0.306±0.001
MAE-9	0.221±0.0002	n.a	0.363±0.0001	n.a	0.583±0.0001
MAE-10	0.222±0.00004	n.a	0.365±0.00005	n.a	1.061±0.00004
MAE-12	0.221±0.001	0.304±0.0002	0.368±0.008	n.a	0.893±0.007
MAE-14	n.a	0.315±0.004	0.374±0.002	n.a	0.688±0.004
MAE-15	0.22±0.001	0.325±0.0001	0.353±0.00002	n.a	0.9±0.001
MAE-16	0.218±0.0001		0.359±0.001	n.a	0.577±0.001
Ultrasound-assisted extraction					
UAE-1	n.a	n.a	0.622±0,015	0.093±0.008	0.730±0.024

UAE-2	n.a	n.a	0.043±0.002	0.006±0.002	0.05±0.0001
UAE-3	n.a	n.a	0.142±0.006	0.02±0.002	0.166±0.009
UAE-4	n.a	n.a	0.101±0.001	0.014±0.002	0.118±0.001
UAE-5	n.a	n.a	0.174±0.003	0.019±0.001	0.198±0.005
UAE-6	n.a	n.a	0.136±0.002	0.0016±0.001	0.155±0.001
UAE-7	n.a	n.a	0.129±0.01	0.016±0.001	0.148±0.011
UAE-8	n.a	n.a	0.07±0.005	0.009±0.001	0.081±0.004

n.a. – not available

Table 10 shows the different amounts of the 4 isoforms in the conventional extractions (CSE and SE) and the different runs for the optimization in the emerging extraction types (UAE and MAE). As stated in the methodology, to understand which are the best conditions for extraction of tocopherols, the two emerging techniques were subject to 16 extraction runs each, with varying conditions. In the case of UAE, after carrying out the extractions, prior to HPLC injection, a problem was found with the vials, in which they were composed of two phases and thus could not be injected. Due to restrictions in time and amount of sample available, only 8 extractions could be performed, and thus, the strategy relied in compare all runs against the other extractions, prior to the optimization for MAE which can be found in the next sections of the thesis. Considering tocopherol isoforms, γ-tocopherol is the most abundant isoform for all extraction types, followed by δ-tocopherol and β-tocopherol. As for α-tocopherol, it was found in the lowest quantities of all isoforms, being these results similar to the ones found by Kafkas et al., (2017, and Ada et al. (2021) who observed a comparable ranking in tocopherol isoforms. In the context of MAE, β-tocopherol was detected at 0.325±0.001mg/100g dw, this stands in contrast with the amounts found in the conventional extraction, which was deemed insignificant. Elouafy et al. (2022) found the same results. None of the emerging extraction techniques could rival the conventional or soxhlet extraction, with none of the extraction runs reaching a threshold of 2mg/100g, only showing similar amounts for α- and δ-tocopherol. This proves that soxhlet and conventional extraction are still the best in extracting these biomolecules, and that the emerging techniques destroyed most of the γ-tocopherol, which were found 10-fold in the conventional and soxhlet techniques. Still, there are advantages of using MAE and UAE over traditional methods, namely a reduced extraction time and solvent amount, leading to cost savings and a smaller environmental footprint (Hu et al., 2018; Danlami et al., 2014).

3.2 Statistical analysis results

The statistical results presented in this section of the study are essential to evaluate the relationship between extraction parameters and tocopherol content and for determining the optimal extraction conditions. **Tables 11**, **12** and **13** show, for MAE, the experimental conditions and their respective results, the estimated regression coefficients and their standard error, along with the p-value, the results of the analysis of variance (ANOVA), respectively. These were only analyzed for MAE due to the problems with the extraction of UAE detailed above. Furthermore, due to β- and δ- tocopherols only occurring in some runs of MAE, they were excluded from the optimization analysis (outliers).

Table 11. Experimental dataset applied to the response surface analysis (outliers excluded) for MAE.

LSR (v/w)	Time (min)	Temperature (°C)	α-Tocopherol	γ-Tocopherol	Total
8	4	57	0.221	0.363	0.584
8	4	78	0.208	0.358	0.566
8	12	57	0.218	0.359	0.577
8	12	78	0.218	0.354	0.572
17	4	78	0.226	0.366	0.592
17	12	57	0.23	0.365	0.595
5	8	68	0.221	0.363	0.584
20	8	68	0.222	0.365	0.587
13	15	68	0.221	0.368	0.589
13	8	85	0	0.374	0.374
13	8	68	0.22	0.353	0.573
13	8	68	0.218	0.359	0.577
13	8	68	0.219	0.356	0.575

Excluding outliers helped improve the accuracy and reliability of the model by eliminating atypical data points, indeed, the number of experiments has been reduced from 16 to 13, which can be explained by the fact that tocopherol is highly volatile, making the extraction and measurement more complex, and the challenging to quantify it accurately.

Table 12. Estimation of the regression coefficients of the second-order polynomial model, individual *p*-value and coefficient of variation considering the total tocopherol content for MAE.

	Estimate	**Standard error**	***p*-value**
Intercept	0.575	0.005	>0.001
x1	0.005	0.003	0.180
x2	-0.057	0.006	0.002
x3	-0.061	0.006	0.002
$x1^2$	0.004	0.003	0.295
x1x2	-0.057	0.007	0.004
$x2^2$	0.039	0.004	0.002
x1x3	-0.055	0.007	0.004
x2x3	0.000	0.004	0.949
$x3^2$	-0.035	0.004	0.003

Table 13. Results of the analysis of variance (ANOVA) for MAE.

	Sum of Squares	**Degrees of Freedom**	**Mean Square**	**F-value**	***p*-value**
x1	>0.0001	1	>0.001	0.02	0.906
x2	0.0004	1	>0.002	4.69	0.119
x3	0.0129	1	0.013	156.09	0.001

$x1^2$	0.0001	1	0.000	1.61	0.295
x1x2	0.0058	1	0.006	70.55	0.004
$x2^2$	0.0081	1	0.008	98.25	0.002
x1x3	0.0054	1	0.005	65.69	0.004
x2x3	>0.0001	1	>0.002	0.00	0.949
$x3^2$	0.0065	1	0.006	78.85	0.003
Error	0.0002	3	>0.002	1.00	0.500

The results were promising and testified the effectiveness in explaining and predicting total tocopherol content. The coefficient of determination (R^2) explained around 99.4% of the variation observed in tocopherol content. The adjusted R^2 had a value of 0.976, confirming that the model can explain the behavior of the experiment considering the number of predictors. The F-statistic with a calculated value of 54.1 and a low *p*-value of 0.0037 underlined the overall significance of the model. A low *p*-value for the model reinforced the idea that there is a statistically significant relationship between the predictors and tocopherol content. The low root-mean-square error (RMSE) of 0.0091 confirmed that the model's predictions were very close to the actual values, revealing its accurate predictive power. In **Table 13**, the predictor variables (x2, x3) - time and temperature respectively - play a crucial role in the model. The coefficients associated with these variables indicate how they influenced tocopherol content, with low *p*-values confirming their significance. The coefficients x1 (LSR), $x1^2$ (quadratic effect of LSR) and x2x3(interaction between time and temperature) presented high p-values, therefore they are not considered significant for the model. In conclusion, all these results suggested that the model was a strong fit for the data, with a high explanatory and predictive capacity. This model enabled a prediction of the optimal extraction conditions being set at 20.12v/w, 1.07 min and 50.06 °C, with a maximum tocopherol content of 1.1214 mg/100 dw, higher than any of the carried out individual runs, shown in **Table 11**.

3.3 Response surface analysis

The three-dimensional (3D) contour plots are the graphic representations of regression models, providing a method to visualize the relationship between responses and experimental levels of each variable and the type of interactions between them. The relationship between independent and dependent variables was illustrated in 3D representation of the response surfaces generated by the model for total tocopherols, in the MAE (mg/100g dw).

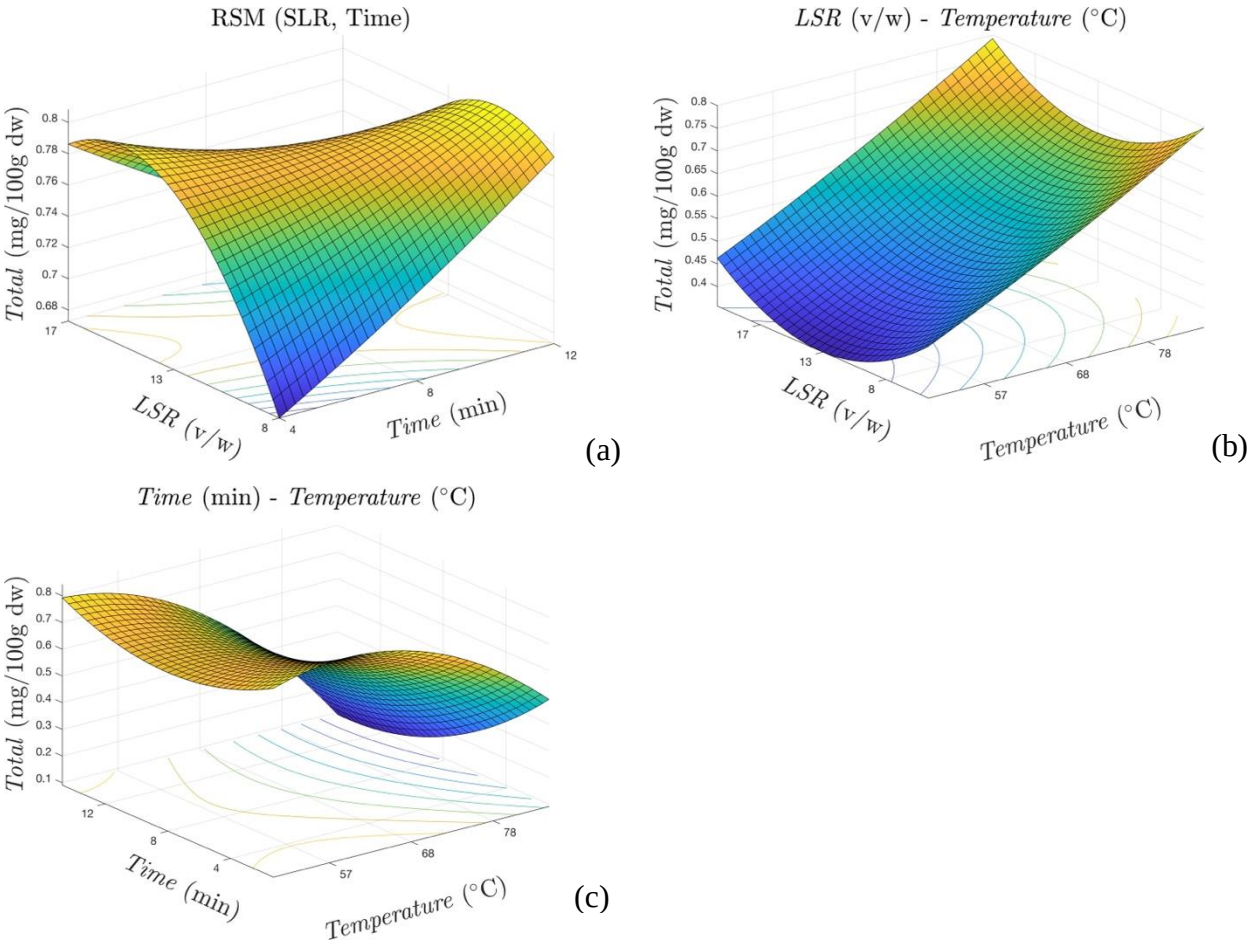

Figure 9. Response surface plots illustrating the binary effects of independent variables on total tocopherol content. In each plot, the excluded variable was fixed at its optimal response value. For (a) there are two interactions LSR (v/w) and time (min), (b) LSR (v/w) and temperature (°C) and for (c) time (min) and temperature (°C) for MAE.

The 3D plots for the total as a function of extraction temperature, time and LSR are shown in **Figure 10**. a, b, and c. As it can be seen in **Figure 10**. a, the use of 8:1 LRS, an increase of time from 4 to 12min, promoted a total increase from 0 to 0.79mg/100g dw, highlighting the significant effect of the linear term of time. Moreover, when around 13:1 LRS

with time variation from 4 to 8 min and from 8 to 12 min, the tocopherol total is high 0.79 to 0.74mg/100g dw and 0.74 to 0. 8mg/100g dw respectively, which may mean that the optimum point 13:1 LRS may be an optimal condition, this can be confirmed by the study made by Karami et al. (2015) which found optimal condition in microwave assisted method liquid/solid ratio of 12.7/1. In the same figure **(Figure 10**. a), when the maximum of CTT reaches 0.79mg/100g, and this occurs when the extraction time is close to 12 min, it suggests that 12min is the optimal extraction time to achieve the highest concentration of tocopherol. As it can be seen in **Figure 10.** b, the use of 5:1 LRS, an increase of temperature from 50 to 85°C, promoted a total increase from 0.48 to 0.8mg/100g, highlighting the significant effect of the linear term of temperature. In **Figure 10**, c when temperature is equal to 50°C, the total tocopherol is high, equaling 0.8mg/100g, while when temperature increases, total tocopherol decreases, which is in line with Hu et al. (2018). The obtained results suggest that optimal tocopherol extraction conditions can be achieved with an LSR of around 13:1 (v/w), an extraction temperature of around 50°C and 1 min of extraction time. This finding is interesting, specifically the fact that the extraction time is so low, as it seems that longer extraction times tend to reduce the amount of total tocopherols, probably due to destruction of their structure by the microwaves.

3.4 Bioactivities

3.4.1 Antioxidant activity

In terms of antioxidant activity tests, two were used, namely the DPPH scavenging activity and reducing power (RP). Each extract obtained from the four techniques was tested, being the UAE and MAE tested at the optimal conditions. A one-way ANOVA was used to evaluate the variance of the samples with an interval of confidence of 95%, with prior evaluation of the distribution of the data. A Tukey's test was used as a post-hoc to further classify the samples using the SPSS program, version 18. (SPSS, IBM - Armonk, USA).

Table 14. Antioxidant activity of MAE, UAE, soxhlet and conventional extraction from walnut sample expressed in EC_{50} (concentration that inhibits 50% of radicals), mg/mL).

	Extraction Methods			
	Conventional	**Soxhlet**	**UAE**	**MAE**
DDPH	2.47±0.29d	0.119±0.004a	0.32±0.09c	0.19±0.04b
RP	53.26±0.46d	0.73±0.12a	14.4±0.15c	2.87±0.03b

Table 14 shows the EC_{50} values of the two tested antioxidant activity assays. A lower EC_{50} indicates stronger antioxidant activity, as a smaller concentration of the extract is needed to neutralize 50% of radicals or reduce 50% of the targeted species. In essence, the lower the EC_{50}, the more effective the antioxidant, as it can combat oxidative stress and protect against damage from free radicals or other reactive species with a smaller amount of the extract. The results of the DDPH analysis demonstrate the superior antioxidant activity of soxhlet and MAE extractions, as reflected in their lower EC_{50} values (0.119±0.004mg/mL and 0.19±0.04 mg/mL, respectively). In contrast, UAE showed an EC_{50} of 0.32±0.09mg/mL, while the conventional method performed significantly worse with an EC_{50} of 2.47±0.29mg/mL. Overall, soxhlet, MAE and UAE were in average 10 time lower than the conventional extraction, even though in the emerging techniques the amount of total tocopherols was lower. Kantar et al., (2022) found that MAE's EC_{50} value was between 2.4 to 2.6mg/mL, but Rahimipanah et al. (2010) found similar EC_{50} results to this work, specifically 0.143±0.007mg/mL. Those results can confirm that our results are in the average of this types of extraction.

For the reducing power, the same pattern of EC_{50} was found, with all extraction types showing statistically different values, although the magnitudes were quite different. In this assay, only soxhlet showed an antioxidant value below 1mg/mL, standing out as the best extraction type for reducing power. Bourgou et al. (2016), also reported values between 0.95±0.02 and 2.16±0.06mg/mLwith different solvents, namely ethanol and water, respectively.

3.5 Antimicrobial Activity

3.5.1 Antibacterial activity

Table 15 shows the outcomes of the microbiological assays carried out to evaluate the inhibitory/bactericidal/fungicidal capacity of the extracts against foodborne contaminants. These tests bear immense significance in upholding the integrity and wholesomeness of food items, as the intrusion of bacteria can culminate in food spoilage, precipitate foodborne illnesses in consumers, and incur substantial economic repercussions within the food sector. **Table 15** presents the results obtained against clinical isolates, given the diverse application that the obtain extracts can have, that can include the pharmaceutical sector.

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Table 15. Minimum inhibitory concentrations (MIC, mg/mL) and minimal bactericidal concentration (MBC, mg/mL) of MAE, UAE, soxhlet, and conventional extracts from walnut. Maximum extract concentration tested of 10mg/mL.

									Positive Control					
	SE		CSE		MAE		UAE		Streptomycin 1mg/mL		Methicillin 1mg/mL		Ampicillin 10mg/mL	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MBC	MBC	MIC	MBC	MIC	MBC
Bacteria tested: food bacteria														
Gram-negative bacteria														
Enterobacter cloacae	>10	>10	>10	>10	10	>10	2.5	>10	>10	0.007	n.a	n.a	0.15	0.15
Escherichia coli	>10	>10	>10	>10	>10	>10	2.5	>10	>10	0.01	n.a	n.a	0.15	0.15
Pseudomonas aeruginosa	>10	>10	>10	>10	5	>10	2.5	>10	>10	0.06	n.a	n.a	0.63	0.63
Salmonella enterica	>10	>10	>10	>10	10	>10	2.5	>10	>10	0.007	n.a	n.a	0.15	0.15
Yersinia enterocolitica	10	>10	10	>10	0.6	>10	1.25	>10	>10	0.007	n.a	n.a	0.15	0.15
Gram-positive bacteria														
Bacillus cereus	>10	>10	>10	>10	5	>10	0.6	>10	>10	0.007	n.a	n.a	n.a	n.a
Listeria monocytogenes	10	>10	>10	>10	5	>10	1.25	>10	>10	0.007	n.a	n.a	0.15	0.15

Bacteria tested: clinical bacteria														
Gram-negative bacteria														
Escherichia coli	>10	>10	>10	>10	5	>10	5	>10	<0.15	<0.15	<0.0078	<0.0078	n.a	n.a
Klebsiella pneumoniae	>10	>10	>10	>10	10	>10	5	>10	10	>10	<0.0078	<0.0078	n.a	n.a
Morganella morganii	>10	>10	>10	>10	10	>10	1.25	>10	>10	>10	<0.0078	<0.0078	n.a	n.a
Proteus mirabilis	>10	>10	>10	>10	5	>10	1.25	>10	<015	<0.15	<0.0078	<0.0078	n.a	n.a
Pseudomonas aeruginosa	>10	>10	>10	>10	10	>10	2.5	>10	>10	>10	0.5	1	n.a	n.a
Gram-positive bacteria														
Enterococcus faecalis	>10	>10	>10	>10	>10	>10	2.5	>10	<0.15	<0.15	n.a	n.a	<0.0078	<0.0078
Listeria monocytogenes	>10	>10	>10	>10	10	>10	5	>10	<0.15	<0.15	<0.0078	<0.0078	n.a	n.a
MRSA	>10	>10	>10	>10	2.5	>10	5	>10	<0.15	<0.15	n.a	n.a	0.25	0.5
Staphylococcus aureus	>10	>10	>10	>10	2.5	>10	1.25	>10	>10	0.007	0.007	0.007	0.15	0.15

n.a: not applicable; MIC- minimum inhibitory concentration; MBC – minimum bactericidal concentration; MFC – minimum fungicidal concentration.

The results obtained show that tocopherol extracts exhibit moderate to high antibacterial activity, but their efficacy varies according to the applied extraction method and bacterial strain. Conventional solvent extraction and the soxhlet method revealed minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) equal to or greater than 10mg/mL for most food and clinical bacterial strains, suggesting that this extraction method did not show significant antibacterial activity.

However, the microwave-assisted extraction (MAE) method showed more promising antibacterial activity against certain foodborne bacteria, in particular against *Pseudomonas aeruginosa*, *Yersinia enterocolitica*, *Bacillus cereus*, *Listeria monocytogenes* and *Staphylococcus aureus*, with MICs ranging from 0.6 to 5mg/mL. Gram-negative bacteria exhibited higher resistance to the extracts tested than Gram-positive bacteria, which may be due to the presence of lipopolysaccharides in the membrane of Gram-negative cells, limiting access to the membrane; as also observed by (Żurek et al., 2022) that reported MIC from 0.3125 to 20mg/mL.

These results suggest that the MAE method may be more effective against specific bacterial strains compared to conventional and soxhlet methods. These observations are in line with previous studies by other researchers (Kanatt, Chander, and Sharma, 2010), who also demonstrated improved antibacterial activity with MAE.

Regarding the Ultrasound-Assisted Extraction (UAE), the obtained extract was able to inhibit certain Gram-negative bacterial strains, especially *P. aeruginosa*, with a MIC of 2.5mg/mL. However, for *E. coli* and *S. enterica*, the MIC is higher than 10mg/mL.

Concerning Gram-positive bacteria, the UAE extract was able to inhibit *S. aureus*, with a MIC of 1.25mg/mL. However, it did not inhibit *B. cereus* and *L. monocytogenes*, where the MIC scales beyond 10 mg/mL.

These insights emphasize that UAE might wield its antibacterial influence more potently against certain strains, while others remain considerably more resistant, being in line with prior research (Dolatabadi, Moghadam, and Mahdavi-Ourtakand, 2018). For clinical bacteria, the MAE method continued to exert a strong inhibitory effect, particularly against Gram-negative strains such as *E. coli* and *P. mirabilis*, with a MIC of 5mg/mL, as well as Gram-positive strains, with a MIC of 2.5mg/mL. These results are in line with previous research by other investigators (Vieira et al., 2019). The results from UAE show considerable variation depending on the specific bacterial strain under examination. For clinical Gram-negative

bacteria, UAE's effectiveness is quite diverse. While it has been successful in inhibiting the growth of certain strains, such as *K. pneumoniae* and *M. morganii*, with MICs ranging from 1.25 to 10mg/mL, it faces notable resistance from other strains like *E. coli* and *P. mirabilis*. In the latter case, the MIC exceeds 10mg/mL, indicating limited antibacterial activity against these strains, in agreement also with previous research (Rather et al., 2012). UAE demonstrates its capacity in suppressing the growth of some strains, especially *S. aureus* and *L.monocytogenes*, with MIC values ranging between 5 and 10mg/mL. However, it´s activity was not so effective against some Gram-positive bacteria such as *E. faecalis* and MRSA, with MIC values exceeding 10mg/mL.

3.5.2 Antifungal activity

Table 16 exhibits the antifungal capacity of the extracts.

Table 16. Minimum inhibitory concentrations (MIC, mg/mL) and minimal fungicidal concentration (MFC, mg/mL) of MAE, UAE, soxhlet, and conventional extracts from walnut.

	Aspergillus brasiliensis		*Aspergillus fumigatus*	
	MIC	**MFC**	**MIC**	**MFC**
Soxhlet	10	>10	10	>10
Conventional	10	>10	10	>10
MAE	>10	>10	10	>10
UAE	5	>10	0.6	>10
Ketoconazole	0.06	0.125	0.5	1

The results indicate that the tested tocopherol extracts did not show significant antifungal activity against *A. brasiliensis* and *A. fumigatus*, with MICs and MFCs equal to or greater than 10 mg/mL for the soxhlet, conventional and MAE extract.

The UAE method displayed higher potential with a MIC of 5mg/mL and 0.6mg/mL against *A. brasiliensis* and *A. fumigatus*, respectively, highlighting its capacity to suppress the growth of these fungi when compared to the other extraction techniques. This implies that UAE significantly outperforms the other methods in inhibiting the growth of those fungi, that is in line with previous researchers (Noumi et al., 2010). It is also important to highlight the UAE MIC obtained against A. *fumigatus* (0.6mg/mL), comparable to that exhibited by the positive

control Ketoconazole, a synthetic fungicidal compound commonly applied. This is important because this extract could be exploited as a potential candidate to apply as a natural alternative to ketoconazole.

4. Conclusion

The results of this study compared different extraction methods of tocopherols from walnut, including conventional solvent extraction (CES), soxhlet extraction, Microwave-Assisted Extraction (MAE), and Ultrasound-Assisted Extraction (UAE), shedding light on their efficiency. The findings demonstrate that γ-tocopherol is the most abundant isoform in this nut, as in most extractions it was found in higher amounts. The MAE stands out as a promising approach for tocopherol extraction from walnuts, exhibiting high efficiency in terms of both tocopherol content 1.060±0.007mg/100g dw and antioxidant activity 0.19±0.004mg/mL for the DPPH and 2.87±0.03mg/mL for the reducing power. Furthermore, MAE is more environmentally friendly, requiring less solvent and shorter extraction time when compared to the other investigated techniques. The extraction conditions yielded 3.028% of tocopherol content at 13:1v/w, 12 min and 50°C according to the response surface methodology plots. MAE also displayed moderate antibacterial activity against specific bacterial strains. These findings indicate that MAE stands out as a great choice for extracting tocopherols from walnuts. It strikes a balance between high extraction efficiency and reduced extraction time. MAE is particularly valuable in industries where time and precision are critical, such as food, chemical, and pharmaceutical sectors. Furthermore, UAE also holds promise as a greener and more environmentally friendly alternative to traditional methods. Indeed, with a CTT equal to 0.730±0.024mg/100g dw and yield equal 32.517%, higher than MAE yield, and also antibacterial activity against specific bacterial and fungal, but higher EC_{50} for DPPH and PR assays comparing with MAE, (0.32±0.09mg/mL and 14.4±0.015mg/mL), means that it is less effective at neutralizing free radicals (DPPH assay) and inhibiting radical peroxidation (PR as say). However, UAE extract exhibited excellent performance in inhibiting A. fumigatus in concentration comparable to the one of ketoconazole, an important finding that could be exploited as a natural alternative to this synthetic fungicidal agent. Still, comparing the emerging techniques with the conventional and soxhlet extractions, the latter show much higher content of tocopherols, especially the most abundant and active form in dried nuts, γ-tocopherol. Thus, even though the emerging techniques seem to be safer due to not using hazardous solvents and less time consuming, the long-lasting soxhlet and the conventional maceration techniques yield more of these bioactive molecules, although this does not translate directly to a better antioxidant or antimicrobial activity, which was better for MAE and UAE.

5. Future perspectives

Regarding future work, due to the problems found in the UAE, further analysis is needed to allow for a 17-run response surface assay, to better understand the optimal conditions for extraction. While the soxhlet and conventional extraction are widely used, further optimizations of these technique could be carried out. Beyond this, other extraction techniques can also be studied, namely cold-press, supercritical CO_2-assisted, and enzymatic extractions, as well as a combination of techniques to further extract the maximum yield in tocopherols.

Also, considering the great activity of the UAE extract against A. fumigatus, this extract could also be exploited a potential inhibitor of this fungus in real environment.

In the realm of potential applications, extracting tocopherols from walnuts holds immense potential for various industries, namely food, cosmetics, and pharmaceutical sectors.

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