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Olive by-products characterization and optimization of extraction techniques to recover bioactive compounds

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LIST OF ABBREVIATIONS

%: Percentage
°C: Celsius degree
µL: Microliter
µm: Micrometer
ABTS: 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
ANOVA: Analysis of variance
CCCD: Central Composite Circumscribed Design
CCD: Central composite Design
CV: Coefficient of variation

DE: Hydrodynamic maceration extraction
DNA: Deoxyribonucleic Acid
DP: Degree of polymerization
DPPH: 2,2-Diphenyl 1-picrylhydrazyl
EC_{50}: Half maximal effective concentration
EDA: Exploratory Data Analysis
ELISA: Enzyme-linked immunosorbent assay
Eq: Equation
FCCD: Face-Centered composite design
Fe III-TPTZ: Ferric tripyridylniazine
Fe II-TPTZ: Ferrous tripyridylniazine
FRAP: Ferric ion reducing antioxidant power
g: Gram
GHz: Gigahertz
HBA: Hydroxybenzoic acid
HCA: Hydroxycinnamic acid
HIV: Human immunodeficiency virus
HPLC-MS: high performance liquid chromatography - mass spectrometry
Hz: Hertz
KHz: kilo Hertz
M: Molar
MAE: Microwave-assisted extraction
mg: Milligram
min: Minutes
MLR: Multiple Linear Regression
mm: Millimeter
mM: Millimolar
mmol: Millimoles
nm: Nanometer
OMW: Olive mill-wastewater
pH: The negative log base 10 of the hydronium ion (H^+) concentration
RNS: Reactive Nitrogen Species
ROS: Reactive Oxygen Species
Rpm: Rotation per minute
RSM: Response Surface Methodology
SD: Standard deviation

SDGs: Sustainable development goals

SOD: Superoxide dismutase
SWOT analysis: Strengths, Weaknesses, opportunities, and Threats analysis
TCC: Total carotenoid content
TPC: Total phenolic compounds
UAE: Ultrasound-assisted extraction
UN: United Nations
V: Volume
VOCs: Volatile organic compounds
W: Watt
µg: Microgram

ABSTRACT

In the rapidly growing world, there's a substantial demand for food. The current challenge extends beyond the need for more food; it involves a concerning surge in food waste, posing interconnected risks to the environment, health, and the economy. Numerous studies have been addressing this issue and minimizing environmental harm. Plant waste, generated during cultivation, processing, and consumption, contains a wealth of bioactive compounds with various beneficial effects, for example antioxidant activities.

The *Olea europaea* leaves were extracted using dynamic maceration extraction (DE) for 1 h and 3 h, ultrasound-assisted extraction (UAE), and microwave-assisted extraction (MAE) to evaluate the antioxidant potential of the resultant extracts. Four antioxidant assays were performed, including 2,2-Diphenyl 1-picrylhydrazyl (DPPH), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), Ferric ion reducing antioxidant power (FRAP), and reducing power (RP). The total phenolic compounds (TPC) content was also determined. The results showed that DE-3h presents the highest TPC value compared to DE-1h, UAE and MAE which showed approximately similar amounts. DPPH, FRAP, and RP showed better results than ABTS test. Considering cost-effectiveness, DE comes up as the preferred extraction technique over UAE and MAE, particularly for evaluating various responses, with a specific emphasis on the DPPH test due to its specificity and simplicity compared to the other tests. DE extraction was performed using two solvents: methanol and acetone through a central composite design (CCD) to study the effect of three factors numerical (time of extraction, percentage of solvent, and pH level) and 1 categorical factor (Solvent type) on five responses (DPPH, TPC, yield of extraction, carotenoids, and chlorophylls determination). An exploratory data analysis (EDA) complemented this assessment. Carotenoids and chlorophylls demonstrated higher extractability when utilizing acetone, while the DPPH assay and extraction yields exhibited better results when methanol was used for extraction.

In terms of fitting models, response surface methodology (RSM) was applied for all independent and dependent variables, allowing us to suggested optimization with a desirability value of 65.6% including the following parameter values: Extraction time (A) = 120 minutes, Solvent percentage (B) = 78%, Solvent pH (C) = 5, and Solvent type = Acetone. Which yields 2.51 μg/mL of TCC, 6.05 μg/mL of Chlorophyll A content, 18.4 μg $_{\text{Trolox equivalent}}$/mL o for the DPPH test, 108.72 μg $_{\text{gallic acid equivalent}}$/mL for the TPC test, and an extraction yield of 50.7%.

Keywords: Antioxidant activity, CCD, EDA, hydrodynamic extraction, MAE, *Olea europaea* leaves, Phenolic content, RSM, UAE.

RESUMO

O rápido crescimento populacional leva a uma procura substancial por alimentos, que por consequência leva a um preocupante aumento de desperdício alimentar, que acarreta riscos ambientais, para saúde humana e a economia. Vários estudos têm vindo a ser realizados com intuito de diminuir os perigos ambientais causados por este problema. Os resíduos vegetais, gerados durante o cultivo, processamento e consumo, contém uma variedade de compostos bioativos com diferentes propriedades benéficas para a saúde humana, incluindo atividade antioxidante.

Foram realizados diferentes métodos de extração dos compostos bioativos presentes nas folhas de oliveira (*Olea europaea*): extração hidrodinâmica (DE) (1 h e 3 h), extração assistida por ultrassom (UAE) e extração assistida por micro-ondas (MAE) para avaliar o potencial antioxidante. Foram realizados quatro testes antioxidantes: 2,2-Difenil-1-picril-hidrazil (DPPH), ácido 2,2'-azinobis-(3-etilbenzotiazolina-6-sulfônico) (ABTS), poder antioxidante redutor de íons férricos (FRAP) e poder redutor (RP).; o conteúdo total de compostos fenólicos (TPC) também foi determinado. Os resultados mostraram que DE-3h apresenta o maior valor de TPC em comparação com DE-1h, UAE e MAE, que mostraram quantidades aproximadamente semelhantes. DPPH, FRAP e RP mostraram melhores resultados do que o teste ABTS. Considerando a relação custo-eficácia, a DE foi preferida em relação à UAE e MAE, com ênfase específica no teste DPPH devido à sua especificidade e simplicidade em comparação com os outros testes. A extração DE foi realizada com o uso de dois solventes: metanol e acetona, através de um design composto central (CCD) para estudar o efeito de três fatores numéricos (tempo de extração, percentagem de solvente e nível de pH) e um fator categórico (tipo de solvente) em cinco respostas (DPPH, TPC, rendimento da extração, carotenoides e clorofilas). Uma análise exploratória de dados (EDA) complementou esta avaliação. A extração com acetona demonstrou melhores resultados para os carotenoides e clorofilas, enquanto o metanol apresentou melhores rendimentos de extração e resultados no ensaio DPPH. A metodologia de superfície de resposta (RSM) foi aplicada a todas as variáveis independentes e dependentes, permitindo-nos sugerir a otimização com um valor de desejabilidade de 65.6%, incluindo os seguintes valores de parâmetros: tempo de extração (A) = 120 min, % de solvente (B) = 78%, pH (C) = 5 e Tipo de solvente = Acetona, que resulta em 2.51 μg/mL de TCC, 6.05 $\mu g_{\text{clorofila a}}$/mL, 18.4 $\mu g_{\text{eq. Trolox}}$/mL (DPPH), 108.72 $\mu g_{\text{eq. ácido gálico}}$/mL (TPC) e um rendimento de extração de 50.7%.

Palavras-chave: Atividade antioxidante, CCD, conteúdo fenólico, EDA, extração hidrodinâmica por maceração, folhas de *Olea europaea*, MAE, RSM, UAE.

1 INTRODUCTION

1.1 Olea europaea

1.1.1 Description of *Olea europaea*

The olive tree (*Olea europaea)* is an ancient tree that traces its origins back to the Mediterranean region, where it is believed to have been cultivated for over 6,000 years (Caporaso & Boskou, 2021; Vossen, 2007). Olive trees are predominantly found in countries bordering the Mediterranean Sea, such as Spain, Italy, Greece, Tunisia, Turkey and Portugal, which collectively produce most of the world's olive oil (Caballero et al., IOC, 2003, 2022; Vossen, 2007). The olive tree's adaptability has led to its proliferation across the globe, with plantations now flourishing the places as diverse as California, Chile, Brazil, and Australia (Vossen, 2007).

Olea europaea belongs to the Oleaceae family and comprises numerous subspecies distributed all over the world (Figure 1) that can include: *Olea europaea europaea* (commonly found in the Mediterranean region); *Olea europaea sylvestris* (considered the ancestor of cultivated olive trees), *Olea europaea* var. *oleaster* (broad category that include different wild and semi-wild olive varieties); *Olea europaea cuspidata* (can be found in India and Pakistan and is adapted to arid conditions); *Olea europaea maroccana* (native to Morocco, and presented the ability to thrive in desert-like conditions); *Olea europaea subsp. cerasiformis* (located in Madeira, Portugal) (Kassa et al., 2019). Moreover, *Olea europaea* is an attractive crop since it has been recognized in traditional medicine for its significant potential therapeutic utilities including anti-microbiological effects (Ahmed et al., 2014; Markin et al., 2003), anti-inflammatory activity (L. Wang et al., 2008), anti-hypertensive activity (Susalit et al., 2011), antioxidant activities (El & Karakaya, 2009) and, among others.

During olive oil production, different olive by-products are generated including olive leaves, olive stones, olive pomace, and olive mill-wastewater (OMW) as illustrated in Figure 2. Olive leaves, despite not being as prominent as olive pomace or OMW in terms of volume, present around 5 % of the total by-products yield and contain valuable bioactive compounds (Lama-Muñoz et al., 2020; Markhali et al., 2020). According to several studies, olive leaves are rich in different bioactive molecules including syringic acid, hydroxytyrosol, oleuropein, cinnamic acid, and others which represent interesting bioactivities (Acar-Tek & Ağagündüz, 2020; Servili et al., 2009).

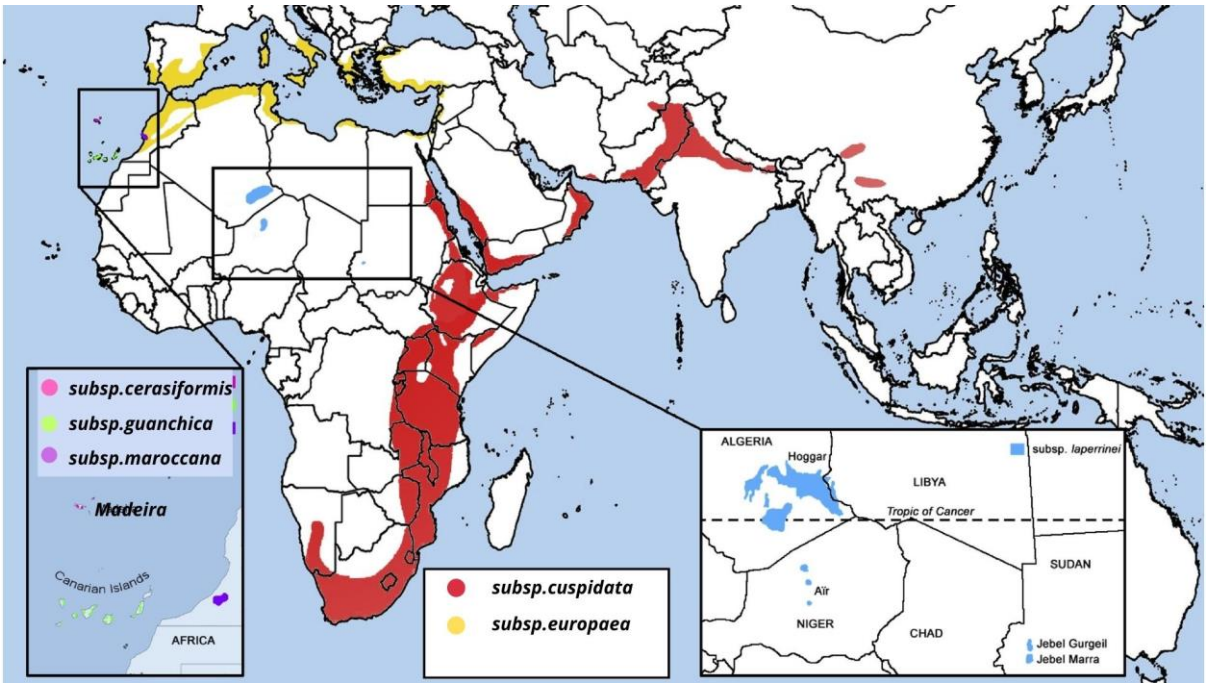


Figure 1: Distribution of some subspecies of Olea europaea (Kassa et al., 2019).

The pulp, skins, and stones of the olives during the milling process create a solid waste called olive pomace (Benincasa et al., 2021). Also, OMW represents the liquid by-product which is composed of the water used during the extraction process and the water mixed with discarded olives (Azzam & Hazaimeh, 2021). In addition, olive stones are olive by-products that account for about 10% of the weight of olive fruit (Rodrigues et al., 2015) and they present an interesting composition with 25.3 to 27.2% lignin, 28.1 to 40.4% cellulose, and 18.5 to 32.2% of hemicellulose (Rodríguez-Gutiérrez et al., 2014). Due to the high content of hazardous organic compounds and low pH (such as OMW), non-treated olive by-products represent a serious ecological problem, therefore, those should be controlled, treated, and then can be valorized (Rodrigues et al., 2015). However, solid waste which includes olive pits and dried pulp can be used as biomass for energy generation or as a component in animal feed (Molina-Alcaide & Yáñez-Ruiz, 2008).

An increased awareness implies that, when properly harnessed, these wasted by-products might be viewed as valuable assets that not only improve health but also wield significant impact across numerous sectors, such as the food industry. There is a growing understanding that, if correctly utilized, these underutilized by-products might be seen as a valuable resource that promotes health and has a significant impact in different industries like the food industry

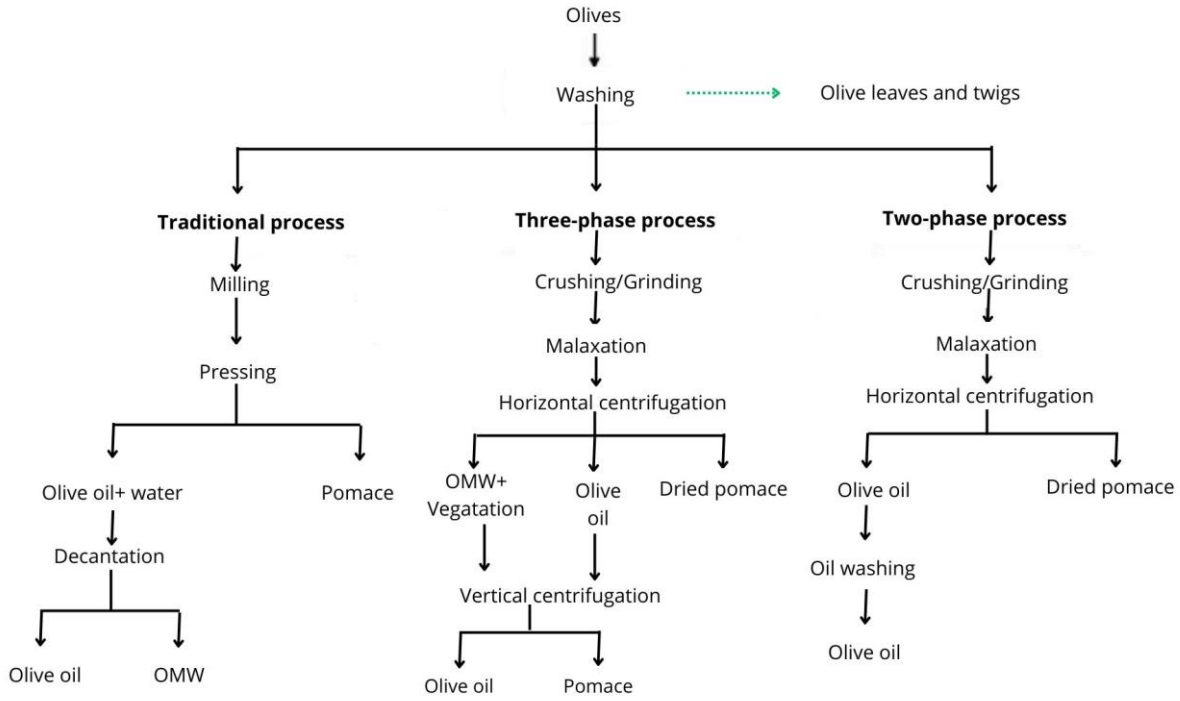


Figure 2: Olive oil production process (Adapted from (Morillo et al., 2009)).

1.1.2 Relevance of *Olea europaea*

Traditionally, the infusion of *Olea europaea* leaves was used to prevent and treat different illnesses, including hypoglycemia (Gullón et al., 2020; Tahraoui et al., 2007) Olive oil shows high and statistically significant nutritional value renders it a predominant choice, notably in gastronomy, where it is often used. Furthermore, it is imperative to note that olive oil is a good source of fatty acids, predominantly featuring oleic acid as the primary fatty acid (constituting 55-83% of the lipid fraction). Additionally, it contains minor compounds such as vitamins E and K, tocopherols, and polyphenols which assume a crucial role as intrinsic natural antioxidants (Negro et al., 2019). Besides, due to its high content of antioxidant compounds and other factors (for example barrier to oxygen and moisture retention), olive oil can function as a food preservative (Serra et al., 2008).

1.1.3 *Olea europaea* markets side streams

Olive trees and their products, such as oil, are commonly applied in a variety of industries including agriculture and pharmaceuticals, among others. *Olea europaea* has the potential to generate different side streams from these industrial activities that are known to generate huge amounts of waste, as well as gas emissions, which could be toxic to the environment (Espeso et al., 2021). Olive leaves are widely studied to be used in different

applications and sectors due to their significant chemical composition. The whole intact leaves have the potential to undergo treatments for dual purposes: energy generation owing to their lignin, cellulose, and hemicellulose and or extraction of phenolic compounds and other interesting molecules from their constituents (Espeso et al., 2021). In other studies, *Olea europaea* by-products were also used for their important potential to produce energy and biogas, as described above (Tekin & Dalgıç, 2000). Different *Olea europaea* applications are summarized in Table 1.

Table 1: Olea europaea side streams

Substrate	Processes	Products	References
Olive leaves	Pretreatment using high water temperatures at high pressures: Hydrothermal treatment or solvents such as sulphuric acid.	Ethanol	(Espeso et al., 2021)
Olive pomace	Anaerobic digestion using microorganisms growing in wastewater of a landfill area, in a batch mode.	Methane Biogas	(Tekin & Dalgıç, 2000)
OMW	Electrocoagulation alone Electrocoagulation+ Centrifugation Electrocoagulation+ Precipitation	Bioethanol Methane Hydrogen	(Ntaikou et al., 2020)
Olive stones	Processing and Refining of bio-oil	Biomass Biofuel	(Pattara et al., 2010)

1.1.4 Sustainable development goals

Inaugurated for the first time within the United Nations (UN) in 1972, the concept of sustainable development aims to address environmental challenges, health concerns, and poverty issues, as well as socio-economic circumstances. It presents the potential to ensure the fulfillment of current societal needs while safeguarding the imperatives of forthcoming generations. Sustainable development goals (SDGs) are a set of 17 interlinked and ambitious objectives (Figure 3) established by the UN in 2015 as part of the 2030 Agenda for Sustainable Development.

Figure 3: Sustainable development goals (United nations site https://sdgs.un.org/goals, 21-09-23).

These SGDs were adopted by all UN member states and provide a shared framework for countries, organizations, and individuals to work towards a more sustainable and equitable future by addressing pressing global challenges, including poverty, inequality, environmental degradation, and climate change. In this sense, *Olea europaea* is a magnificent source of natural compounds that could be related to the SDGs program. In regards of this, the SGDs analysis for an Andalucian variety was explored, and four main key hypotheses were given (Antonio Parrilla González & Ortega Alonso, 2022):

1) Olive oil can contribute to solving the problem of poverty and hunger (SDG 2). The production of olive oil and the olive table is important, about 82.2% of olive oil is produced in the country —Spain—, and 79.2% of olive for table consumption;

2) It proposes gender equality (SDG 5), so both women and men can participate in the innovation in this sector so they can influence success;

3) Achieving sustainable economic growth (SDG8) entails establishing an environment that enables individuals to access high-quality employment opportunities within olive oil cooperatives;

4) Good Health and Well-being (SDG 3): Can contribute to keeping humans in good health. This is explained by the fact that *Olea europaea* is rich in powerful antioxidants and keeps well-being.

Additionally, various studies have concentrated on the utilization of by-products from olive oil production, such as olive pomace and OMW, to generate clean and sustainable energy sources as described in Table 1. This effort may contribute to the realization of the goal outlined in SDG 7 – Affordable and Clean Energy.

1.2 Bioactive compounds

Plants exhibit two fundamental processes comprising their intriguing metabolic apparatus: catabolism, which involves the catalysis of molecules, and anabolism, which is based on the synthesis of molecules. Phytochemical metabolites are broadly classified into two different categories, primary and secondary; primary metabolites include amino acids, organic acids, fatty acids, and carbohydrates that play vital functions for plants (Zaynab et al., 2019); while secondary metabolites encompass different organic compounds such as alkaloids, flavonoids, terpenoids, phenolics, among others (Erb & Kliebenstein, 2020; Zaynab et al., 2019).

1.2.1 Secondary metabolites

The secondary metabolites play an important role in plant defense against predators, attracting pollinators, and ecological interactions with other organisms (Erb & Kliebenstein, 2020; Zaynab et al., 2019). The following subsections provide details on a relevant example of secondary metabolites.

1.2.1.1 Alkaloids

Alkaloids were discovered and exploited as early as 4000 years ago. They are synthesized by a wide range of organisms, with plants being a prominent source (Heinrich et al., 2021). Alkaloids are mainly biosynthetically derived from amino acids, they serve as precursors to the true alkaloids and protoalkaloids, contrary to pseudo-alkaloids (Dey et al., 2020). Despite their differences, all alkaloids can be characterized by the presence of one or more nitrogen atoms in their structure, the majority within a heterocyclic ring structure. They show remarkable diversity in terms of both their structure and biological effects, adding their botanical and biological sources. It can be defined through three different types of alkaloids based on their biosynthetic pathways: true alkaloids (morphine, nicotine, cocaine), protoalkaloids (ephedrine), and pseudo-alkaloids (caffeine), which are represented in Figure 4 (Dey et al., 2020). Furthermore, other alkaloids are found with less abundance such as polyamine alkaloids and cyclopeptide alkaloids. Alkaloids exhibit diverse pharmacological

and medicinal properties and can have a profound effect on the physiology of living organisms (Debnath et al., 2018; Heinrich et al., 2021). Morphine (Figure 4) which is a potent narcotic is employed to alleviate pain with a precisely determined dose to avoid addiction (Dewey, 2007). Furthermore, they are applied to treat vomiting, constipation, and eye irritations (Amirkia & Heinrich, 2014). Alkaloids present also different biological activities including antioxidant power (Czapski et al., 2014), anticancer effect (Johnson-Ajinwo et al., 2019; Och et al., 2019), anti-inflammatory activity (Osmakov et al., 2019; Ribeiro-Filho et al., 2019), antimicrobial activity (Casciaro et al., 2019; Zielińska et al., 2019), antiviral activity (Thawabteh et al., 2019) and among others.

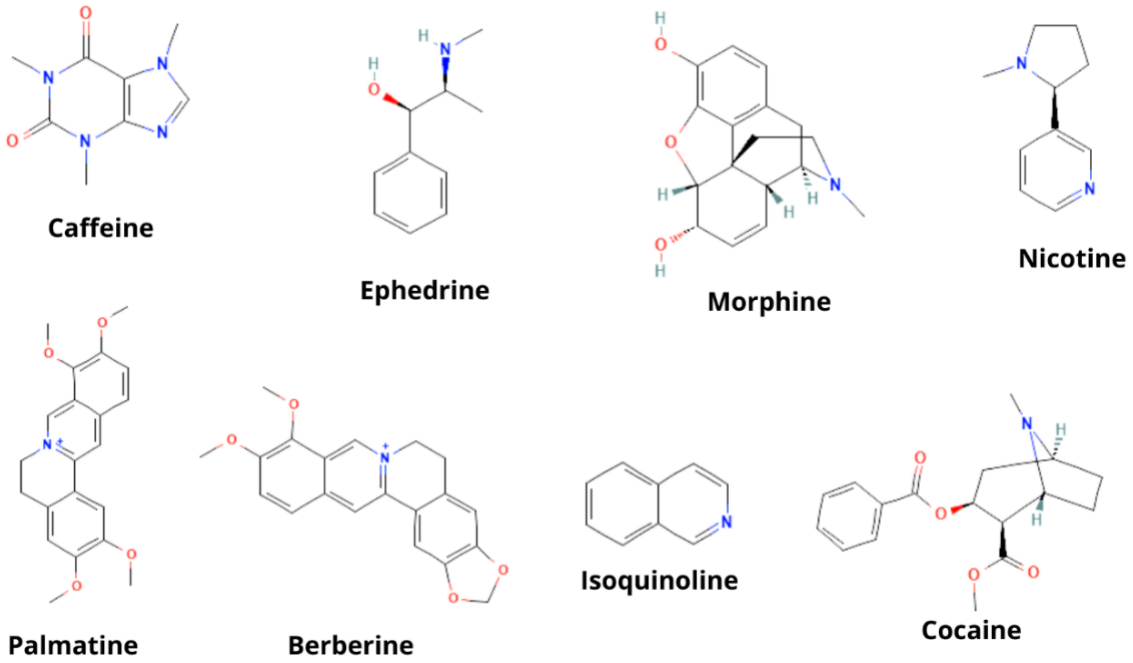


Figure 4: Some alkaloids structures (PubChem Database, with modifications).

1.2.1.2 Terpenes and terpenoids

Terpenes and terpenoids represent the most diversified natural compounds (Abdallah & Quax, 2017). They are mostly used for their aromatic properties as fragrances. In addition, they show interesting functions including therapeutic effects such as anti-inflammatory (Del Prado-Audelo et al., 2021), and antibacterial properties (Guimarães et al., 2019). Terpenes and terpenoids are characterized by their structure, which consists of repeating isoprene units. Their complexity and diversity arise from the various arrangements and modifications of the isoprene units. However, terpenoids which are hydrocarbons containing oxygen, can be described as a subclass of terpenes characterized by the relocation or elimination of oxidized methyl groups at different positions and the presence of various function groups such as hydroxyl group,

carbonyl group, carboxylic acid and esters (Perveen & Al-Taweel, 2018). Some examples of terpenoids are shown in Figure 5.

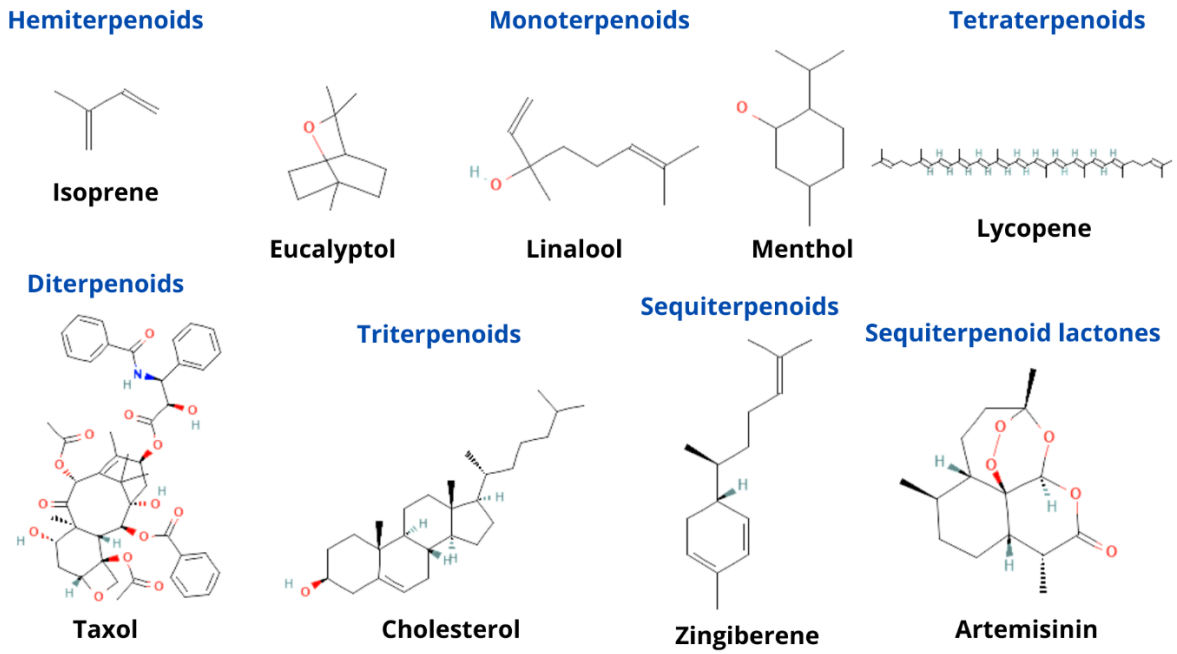


Figure 5: Some structures of terpenoids (PubChem Database, with modifications.

1.2.1.3 Phenolic compounds

Phenolic compounds are, probably, the most studied secondary metabolites for their interesting properties, diversification, and functions. They perform crucial functions in numerous physiological roles, including shaping plant structure and function, contributing to the rigidity of cell-walls, acting as antioxidants, conferring resistance against stress, as well as participating in pigmentation and interactions with other organisms, and conferring flavor, (Y. Zhang et al., 2022). The number of confirmed molecules in the family of phenolic compounds is over 8000, and it keeps expanding. Phenolic compounds can be divided into distinct classes including phenolic acids, flavonoids, tannins, lignans, and stilbenes (Alu'datt et al., 2017).

1.2.1.3.1 Phenolic acids

Phenolic acids represent a sub-group of phenolic compounds within the broader class of plant-derived phenolic compounds. They are found in different plant-based foods; the highest concentration is found in seeds, fruit skins, and leaves. These organic molecules are characterized by the presence of a phenol ring with at least one hydroxyl (-OH) group attached to a carboxylic acid group (COOH) (Kumar & Goel, 2019). They can be classified into two different groups: hydroxycinnamic acid (HCA) and hydroxybenzoic acid (HBA). Some

examples are illustrated in Figure 6. These molecules show several biological activities including antimicrobial activity (Campos et al., 2009), antidiabetic activity (Jung et al., 2006; Prabhakar & Doble, 2011), anticancer activity (Badhani et al., 2015; Gupta et al., 2022) and antioxidant activity (Fonseca et al., 2010).

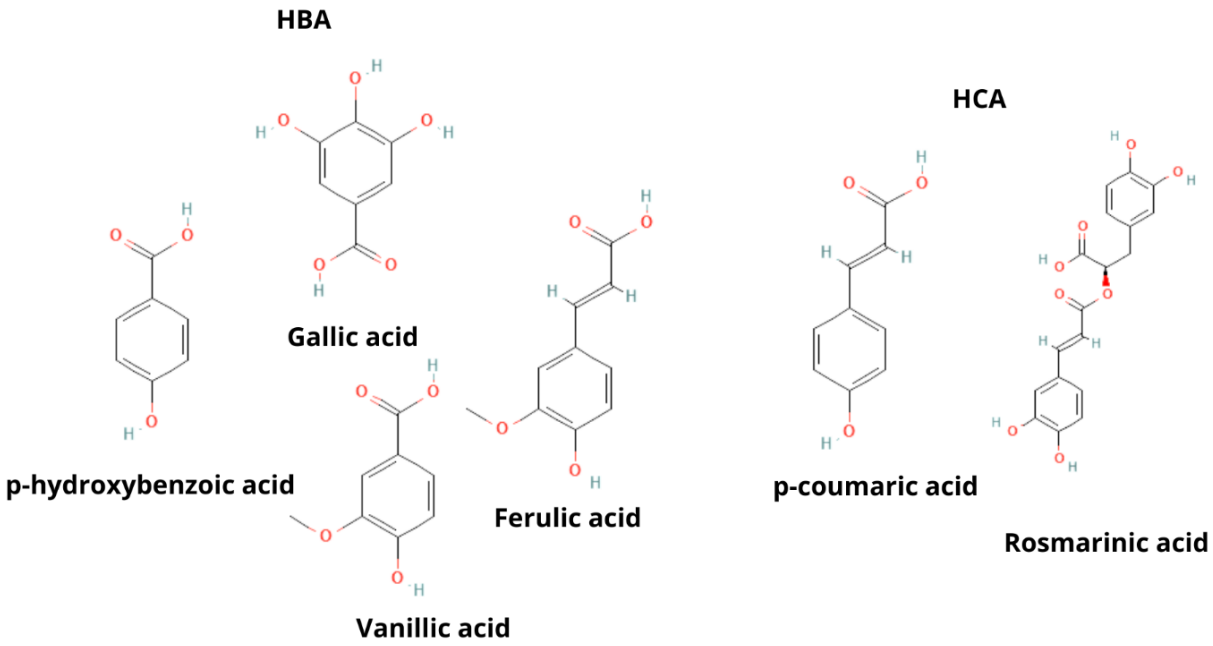


Figure 6: Examples of phenolic acids (PubChem Databse, with modifications).

1.2.1.3.2 Flavonoids

Flavonoids play several biological functions that reduce and/or inhibit harmful effects on the human body. They are used in different applications such as pharmaceuticals (Guven et al., 2019), and cosmetics (Malinowska, 2013). The main bioactivity of flavonoids extensively examined is their role as antioxidants (Fonseca et al., 2010; Shen et al., 2022). They present also anti-inflammatory activity (L. Yang et al., 2021), and cardiovascular effects (Sánchez et al., 2019). Furthermore, flavonoids are essentially produced by plants to fight against insects, predators, and microorganisms and participate in coloring fruits and flowers (Shen et al., 2022). They are generally located in different parts of the plant such as roots, leaves, and flowers (Santos et al., 2017). It can be distinguished numerous subclasses of flavonoids including flavones, flavanones, flavonols, flavanols, isoflavones, anthocyanidins, and chalcones (Figure 7) (Shen et al., 2022).

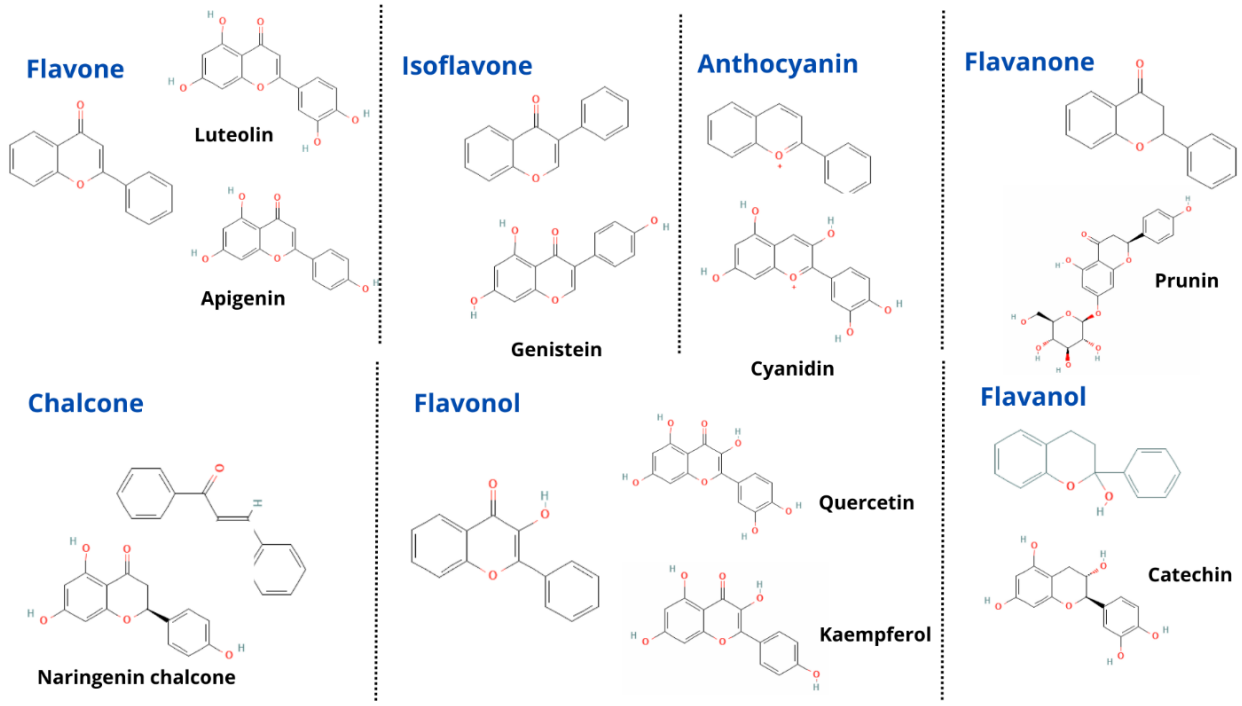


Figure 7: Flavonoids subclasses structures and some examples for each subclass (PubChem Database with, modifications).

1.2.1.3.3 Tannins

Tannins are widely distributed in the plant kingdom, especially in fruits, leaves, and bark. They can bind proteins and carbohydrates, which reduces the digestibility of tannin-rich food for herbivores (Shadkami et al., 2009). Tannins include two essential classes, condensed tannins, and hydrolysable tannins. The difference is that condensed tannins are derived from the oxidative condensation of flavonoids, whereas hydrolyzable tannins are formed by the bonding of gallic acid, hexahydroxydipheric and meta-digallic acid residues with sugars (Khanbabaee & van Ree, 2001).

1.2.1.3.4 Other subclasses

Stilbenes, lignans, lignin, and coumarins make parts of the phenolic compounds family (Saltveit, 2009). These types of secondary metabolites are of relevant interest mostly for their biological effects. For example, coumarins exhibit an antibacterial effect (Nagamallu et al., 2016; Souza et al., 2005) and anti-inflammatory activity (Dawood et al., 2015). Stilbenes are known for cancer prevention and treatment and anti-diabetic effects (Akinwumi et al., 2018; Signorelli & Ghidoni, 2005; Soufi et al., 2012). Lignan and lignin show in turn interesting biological activities such as antioxidative and anti-inflammation activities (Shu et al., 2021; Zálešák et al., 2019).

1.2.1.4 Pigments

Pigments in plants are a complex and diverse group of organic compounds, responsible for the vibrant colors in different plant structures (Mortensen, 2006), and present various functions in the growth, survival, and development of plants (Pavlovic et al., 2014; Schwartz et al., 1997; Sun et al., 2022). Two essential types of pigments exist, carotenoids and chlorophylls. Carotenoids are liposoluble compounds derived from tetraterpenoids (Zaghdoudi et al., 2015). They present different functions including the photosynthesis process and pigmentation or the coloration of fruits and leaves providing red, orange, and yellow hues (Sun et al., 2022). They play a crucial role in photosynthesis since they capture and absorb light energy within the 450-550 nm spectrum, a range in which chlorophylls do not absorb light (Hashimoto et al., 2016). Moreover, carotenoids present antioxidant effects by protecting plants from reactive oxygen species (ROS) damage. They also contribute to preventing chronic diseases (Agarwal & Rao, 2000) such as cataracts, HIV, and cancer as described by (Rao & Rao, 2007). Furthermore, they are applied in gap junction communication and immune response (Bertram, 2009). Lutein and zeaxanthin are two carotenoids that were studied to show potent disease prevention (Ribaya-Mercado & Blumberg, 2004). Some examples of carotenoids are shown in Figure 8.

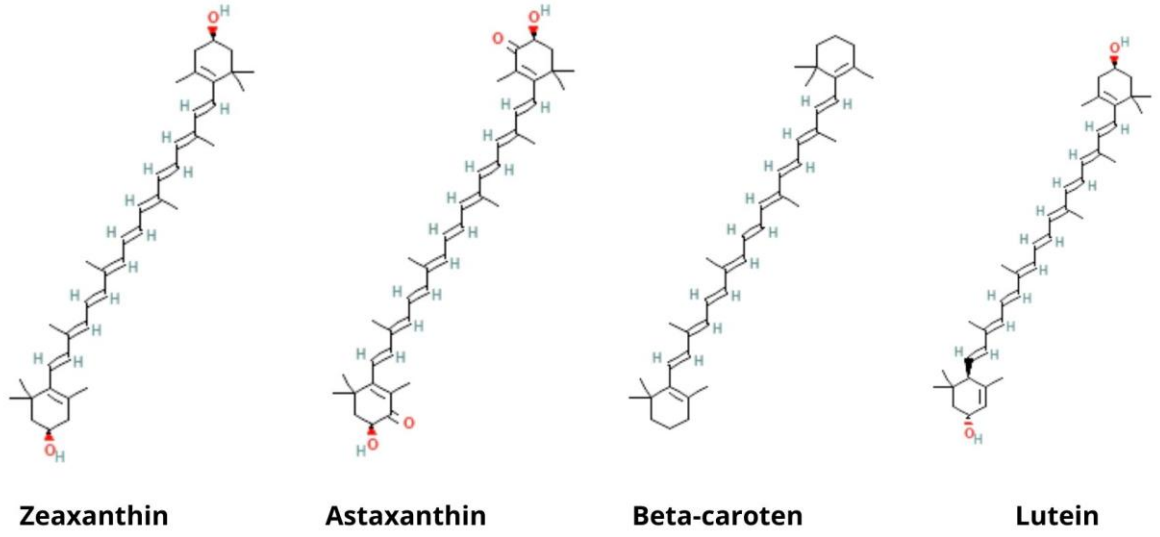


Figure 8: Structures of some examples of carotenoids (PubChem Database with, modifications).

Chlorophylls are green pigments involved directly in the photosynthesis process, capturing light energy, and converting it into chemical energy, found in chloroplasts, a small organelle in plants. Several types of chlorophyll exist however chlorophyll-a and chlorophyll-b are the most abundant and studied as shown in Figure 9 (Pareek et al., 2017). The biological activity of chlorophylls is described as their capacity to function as antioxidants, anticarcinogens, and antimutagens (Martins et al., 2023). Their distinctive chemical structure

enables them to effectively neutralize detrimental free radicals, regulate cellular mechanisms, and reduce DNA damage associated with disease development (Perez-Galvez et al., 2018; Queiroz Zepka et al., 2019). Furthermore, they present anti-inflammatory effects (Hannan et al., 2020), anti-obesity activity (Galgani & Ravussin, 2008), and neuroprotective activity (De Vogel, 2004).

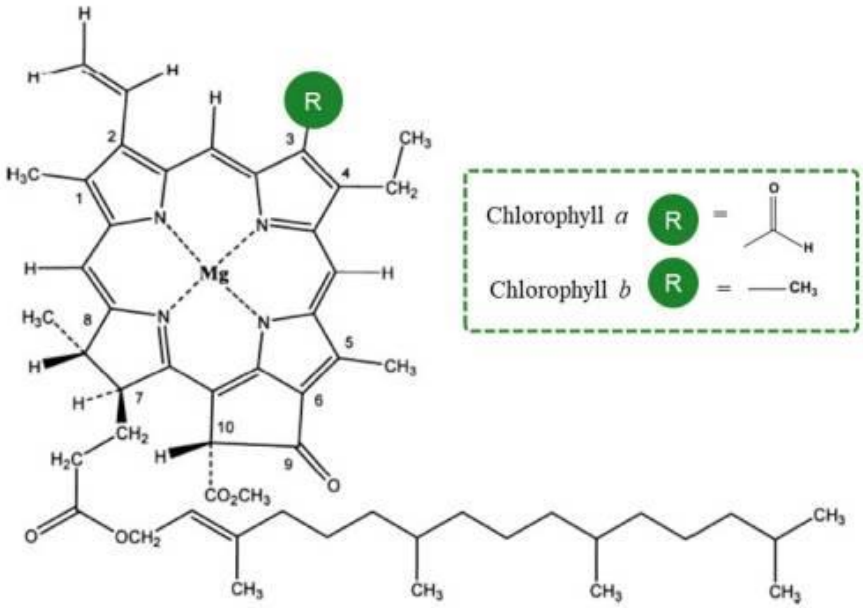


Figure 9: Chemical structures of chlorophyll a and b (Martins et al., 2023).

1.2.2 Free radicals and antioxidants

A free radical is a highly reactive and unstable molecule with unpaired electrons in its structure. This unpaired electron makes them highly chemically reactive and can either remove an electron from nearby molecules or donate an electron to other molecules thus acting as a reductant or an oxidant, which can lead to a chain reaction of electron theft (Zaric et al., 2023). The adenosine triphosphate (ATP) production generates also free radicals. Two types of these elements can be found; ROS and reactive nitrogen species (RNS) which are produced during the cellular redox process (Mandal & Biswas, 2020).

Free radicals have two antagonist effects on cells. They can be harmful and beneficial substances at the same time depending on their concentrations (Pham-Huy et al., 2007). They contribute to the detoxification process, to chemical signaling, and to other functions. They are continually generated and regulated by natural compounds including natural enzymes such as catalase (El & Karakaya, 2009). However, they have a very toxic effect on cells when they are produced in excess due to the unpaired electron (Zaric et al., 2023). Free radicals are a real danger to human health, causing many serious and chronic diseases, including eye diseases such

as cataracts (Fletcher, 2010), cancer (Young, 2001), cardiovascular problems (Ceriello, 2008; Pacher et al., 2007), inflammatory diseases (Zaric et al., 2023), among others. The different diseases caused by free radicals are summarized in Figure 10. It can damage essential molecules such as proteins, lipids, and nucleic acids (Zaric et al., 2023). One of the most serious health problems is cancer, which is essentially generated due to oxidative stress that damages the deoxyribonucleic acid (DNA). It starts by producing hydroxylated bases of DNA and affects the normal development of cells by inducing genetic mutations (Dizdaroglu et al., 2002).

The scientific community has demonstrated that plants present an excellent source of antioxidant compounds that can inhibit free radicals. Phytochemicals such as beta-carotene, flavonoids, fatty acids, phenolic compounds among others, are powerful molecules to inhibit free radicals (Pham-Huy et al., 2008). Understanding the intricate balance between free radicals and antioxidants has important implications not only in botany and agriculture, but also in the potential for utilizing these natural antioxidants in human health and nutrition.

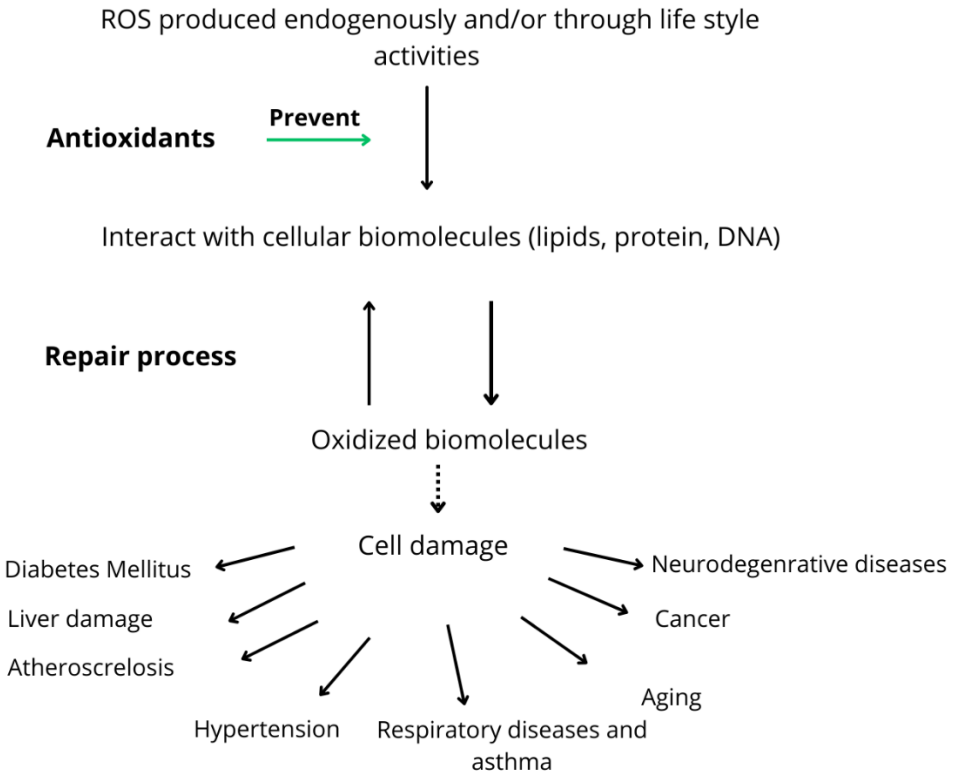


Figure 10: Scheme of harmful consequences of free radicals in Human health (Rao & Rao, 2007; Zaric et al., 2023).

1.2.3 Synergetic and antagonistic effects of *Olea europaea* compounds

Bioactive molecules extracted from *Olea europaea* can show interesting reactions against many diseases and health problems (Benavente-García et al., 2000; Lee & Lee, 2010;

Y. Zhang et al., 2023). Benavente-Garcia and co-workers (2000) suggest that when polyphenols are mixed, as seen in olive leave extract containing a significant amount of oleuropein and other active polyphenols, they demonstrate a synergetic effect in their capacity to scavenge radicals which was greater than what individual phenolic compounds can achieve in their own (Benavente-García et al., 2000). Another study was conducted by Lee and Lee (2010) to test the antimicrobial and antioxidant effects of both the individual and combined phenolics in olive leaf extract. The findings indicate that both individual and combined phenolic compounds demonstrated effective radical scavenging capabilities, along with a notable resemblance to the activity of superoxide dismutase (SOD). The studies demonstrated that the synergistic effect of the combined phenolic compounds yielded significant antimicrobial activity against the different strains tested, Gram-positive *Bacillus cereus* and *Staphylococcus aureus* and Gram-negative *Escherichia coli* and *Salmonella enteritidis*, compared to the individual compounds (Lee & Lee, 2010).

1.3 Exploitation of olive by-products

1.3.1 Composition of *Olea europaea* by-products

Over the years, demand for natural products with high nutritional value has risen steadily. Plants and their waste products in turn provide materials rich in organic compounds beneficial for health. Not only are *Olea europaea* products incredibly wealthy in bioactive compounds, but also its by-products including olive pomace, OMW, and olive leaves. According to Nunes and co-workers (2018), olive pomace is rich in phenolic compounds, being hydroxytyrosol the most representative at 53.78% of the total phenolic content, followed by tyrosol, oleuropein derivates (Antónia Nunes et al., 2018). Additionally, in its composition, it can be found, tocopherols, sterols, squalene, and carotenoids (Gallardo-Guerrero et al., 2002; Ibáñez et al., 2000). OMW is considered a highly polluting waste due to its high organic composition, as well as its acidic and phenolic nature. OMW, depending on different factors, could contain oleuropein, hydroxytyrosol, caffeic acid, tyrosol, lutein-7-O-glucoside, luteolin, and cinnamic acid derivates (Mulinacci et al., 2001). For olive leaves, they present a smooth and leathery texture with a glossy to dark green coloration. For olive leaves, they present a smooth and leathery texture with a glossy to dark green coloration. They are considered the most important part of *Olea europaea* since they have an interesting biochemical composition. They present a high amount of fiber 32.83g/100g $_{\text{dried leaves}}$, protein 28.5 g/100g $_{\text{dried leaves}}$, and ash with 32.62 g/100g $_{\text{dried leaves}}$ which present important levels due to the presence of calcium,

potassium, and other minerals (Ismail et al., 2016). Relatively to the minor compounds, olive leaves contain significant amounts of polyphenols. Phenolic compounds such as oleuropein (the most abundant), gallic, ellagic, chlorogenic and, caffeic acids, rutin, epicatechin, quercetin, quercitrin and, kaempferol can be found in olive leaves extracts (Khaliq et al., 2015; Otero et al., 2020). Furthermore, olive leaf extract may contain quinic acid, verbascoside, and luteolin-7-O glucoside (Nicolì et al., 2019). This rich composition may explain the interesting antioxidant power of *Olea europaea*.

1.3.2 Benefits of antioxidants from *Olea europaea*

Olea europaea is a great source of diversified compounds that represent bioactive functions and therapeutic properties. These molecules contribute to scavenging oxidants and treating and/or preventing serious diseases such as cardiovascular problems, hypertension, inflammatory problems, and others (Esmaeili-Mahani et al., 2010; Somova et al., 2004). Various studies were carried out to prove the antioxidant effect of *Olea europaea* (De Leonardis et al., 2008; Fares et al., 2011; Lins et al., 2018; Somova et al., 2004; B. Wang et al., 2018). *Olea europaea* is well-known for its high concentration of phytochemicals which are mostly studied for their exceptional antioxidant abilities. Oleuropein is one of the most important and abundant molecules (Figure 11), identified in *Olea europaea* with significant concentrations and showed an important antioxidant effect (Hassen et al., 2015). Oleuropein is distributed in distinct parts of the tree including stems, roots, leaves, stones, fruits, and olive oil.

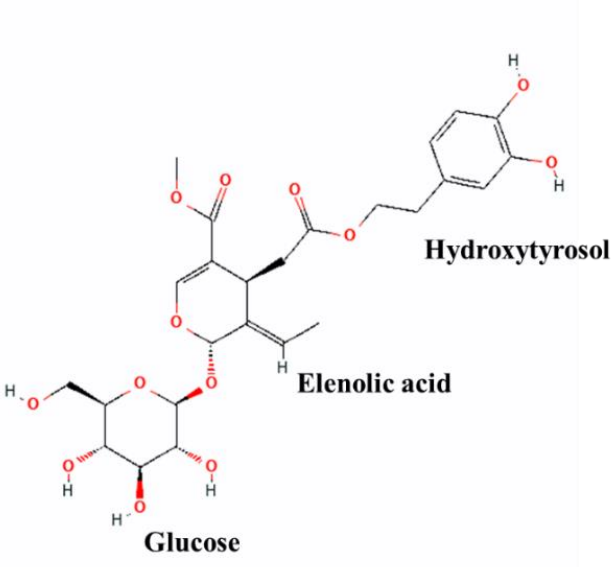


Figure 11: Oleuropein chemical structure (PubChem Database, with modifications).

Several studies were conducted to test the antioxidant capacity of extracts of Olea europaea and other biological activities (Burò et al., 2022; Papageorgiou et al., 2022; Šimat et al., 2022). For example, Mansour and co-workers (2023) compared the phenolic content and antioxidant activity of leaf extracts from three *Olea europaea* cultivars: picual, tofahi, shemlali, and *cv.* picual exhibited notably high antioxidant activity compared to the other two cultivars (Mansour et al., 2023). The potential anticarcinogenic, antirheumatic, and antidiabetic properties of oleuropein have been studied by several research groups (Al-Azzawie & Alhamdani, 2006; Juan et al., 2010; Milanizadeh et al., 2014; Visioli & Galli, 1998). Phenolic compositions and antioxidant activities of olive leaves and olive fruits were tested by Xie and colleagues (2015). Results demonstrated the potent antioxidant capacity of both olive leaf extracts and olive fruit extracts with the strongest antioxidation for the extracts from leaves (Xie et al., 2015).

1.4 Green extraction

The principles of green extraction represent a set of environmentally conscious guidelines aimed at minimizing the environmental impact of extraction processes while maximizing the efficiency of obtaining valuable compounds from natural sources. These principles align closely with the 17 SDGs set by the UN, reflecting a shared commitment to the environment with sustainable practices. The green extraction encourages the minimization of waste generation and advocates for the reuse and recycling of by-products, foresting a more sustainable and resource-efficient approach to extraction.

1.4.1 The six principles of green extraction

Green chemistry, also known as sustainable and environmentally friendly chemistry is an approach that aims to minimize the use and the generation of hazardous substances. When applied to extraction processes, it gives rise to what is commonly referred to as green extraction (Anastas & Eghbali, 2010). To maintain a green extraction that follows standards and to guarantee a better process of biological molecule recovery, a list of six principles of green extraction should be followed (Chemat et al., 2012).

Principle 1: Innovation by select varieties and use of renewable plant resources

To satisfy the high demands for natural phytochemicals compounds in the pharmaceutical, cosmetic, and food industries, plant resources are being exploited excessively. Such excessive consumption may lead to the extinction of some plant species. The protection

and maintenance of biodiversity are crucial for future generations. Green extraction, plant selectivity, and utilization of renewable resources may be considered to protect biodiversity (Chemat et al., 2012).

Principle 2: Utilization of alternative solvents, bio-solvents (Agro-solvents)

In conventional extraction methods, organic solvents are consumed in huge quantities. The majority of those are qualified as volatile organic compounds (VOCs), known for their potential hazards to both human health and the environment (Kamal et al., 2016). They are flammable and promote global warming by contributing to air pollution (Li et al., 2021). To mitigate the risks associated with organic solvents, the adoption of agro-solvents and bio-solvents is encouraged. These alternative solvents, characterized by their non-toxic and non-flammable nature, offer a safer choice for various applications. Moreover, they demonstrate biodegradability, making them environmentally responsible options that align with sustainable practices. Some alternative solvents of green extraction are mentioned in Table 2.

Principle 3: Reduce of energy consumption via innovative technologies use and energy recovering

To reduce the high consumption of energy and to minimize waste, an optimization of the process is required, such as, the optimization of extraction time and the solvent used. It's recommended to use innovative methodologies with cleaning agents (Chemat et al., 2012).

Principle 4: Production of by-products instead of wastes to include the biorefining industry

To have added value, refining industries applied co-products to produce biofuels, energy, and more. An example of *Olea europaea* can be studied. There are different by-products of olive oil production generated including leaves, olive pomace, stones, and OMWs. Each one of them contains an abundance of bioactive compounds, particularly polyphenols, which can have adverse environmental consequences if they are disposed of improperly (Chemat et al., 2012).

Principle 5: Reduction of unit operations using technical innovations and safe, robust, and controlled processes

It consists of minimizing the number of steps of processes to reduce energy consumption and costs (Chemat et al., 2012).

Principle 6: The aim for biodegradable, green extract without contamination

Bioactive molecules are applied in foods, and pharmaceutical industries, so they must obey regulations of European directives (Chemat et al., 2012).

Table 2: Alternative solvents for green extraction (Chemat et al., 2012).

Solvent	Extraction technique (Application)	Polar	Weakly polar	Non-polar	Health and safety	Cost	Environment impact
Solvent-free	-Microwave Hydro-diffusion and gravity (antioxidants, essential oils);	+++	+		+++	+	+++
	-Pulse electric field (antioxidant, pigments).	+++	+		+++	+	+++
Water	-Steam distillation (essential oils);	++	+		+	++	+
	-Microwave-assisted distillation (essential oils);	+++	+++	+	+	+	++
	-Extraction by sub-critical water (Aromas)	+	++		+	+	+
CO_2	-Supercritical fluid extraction (decaffeination of tea and coffee.	-	+	+++	+	+	+
Ionic liquids	Ammonium salts (Artemisinin).	-	+	+++	-	-	++
Agrosolvents	-Ethanol (pigments and antioxidants);	+	+	-	-	++	+
	-Glycerol (polyphenols);	+	+	-	-	+	+
	-Terpenes such as -limonene (fats and oils).	-	-	+	-	+	+
Petrochemical solvents	n-hexane (fats and oils)	-	+	+++	---	++	---

1.4.2 Conventional and green extraction methodologies

Extraction is a technique that aims to recover chemicals and molecules from a solid in the case of solid-liquid extraction or from liquid if we have a liquid-liquid extraction. In solid-liquid extraction, the objective is to deplete the plant material of its natural compounds (Chanioti et al., 2014). Conventional extraction techniques, such as Soxhlet, maceration, decoction, and percolation, among others, are known for their efficacy in extracting different compounds. Nevertheless, these methods often necessitate extended extraction durations and a considerable volume of organic solvents. In light of this, the adoption of green or advanced extraction techniques presents a viable alternative to enhance the extraction process, reducing both time and resource requirements (Picot-Allain et al., 2021).

1.4.2.1 Dynamic maceration extraction

Maceration is a solid-liquid extraction technique that is based on the emergence of all the solids in a well-determined volume of solvent. This procedure entails the disintegration of plant material, enabling the release of target compounds into the extraction medium. For an effective extraction thorough agitation of the mixture is required. Despite its utility in recovering sensitive molecules, maceration is not lacking in limitations. One notable drawback is the substantial solvent consumption; however, it is not an expensive technique (Ishida & Chapman, 2009; Q.-W. Zhang et al., 2018).

1.4.2.2 Ultrasound-assisted extraction

Ultrasound-assisted extraction (UAE) is one of the important procedures to extract bioactive molecules. It is based on the use of mechanical waves. The sound waves generate energy, and their propagation is about 20 to 100 KHz. During this process, cavitations and bubbles are produced due to the production of cycles with negative pressure. This can induce the non-stability of the matrix, so the liquid can diffuse better into the solid by disrupting the cell wall and destroying them to release the different molecules (Luque-García & Luque De Castro, 2003). To conduct an effective extraction, some factors should be considered including the size of particles, the time of extraction, temperature, pressure, power, and humidity. The use of ultrasound extraction is justified by the fact that it presents high yields of extraction (Y.-C. Yang et al., 2013). In addition, it is a simple technique and very effective applied especially to extract sensitive molecules with a reduction of energy and time (Azmir et al., 2013).

1.4.2.3 Microwave-assisted extraction

Microwaves are electromagnetic radiation without ionization, infrared between 300 MHz and 300 GHz. They can penetrate the vegetal and induce fluctuations. Then, an elevation of cell temperature induces the vaporization of inside water (polar component) and breaks cells so that the different molecules can be recovered (Chan et al., 2011; Luque-García & Luque De Castro, 2003). The mass gradients and temperature are effective factors in obtaining a better yield of extraction. Also, the time of extraction, the solvent used, the power of microwaves, and the size of particles are the main parameters for microwave-assisted extraction (MAE). The process starts with the penetration of the solvent into a solid. After that, the power of waves facilitates the dissociation of intermolecular bonds and the solubilization of these molecules in the extraction solvent. Furthermore, MAE is one of the green extraction techniques used to extract components from *Olea europaea* (Da Rosa et al., 2019).

1.5 Experimental design

The integration of green extraction methodologies with experimental design is an imperative approach to optimize and enhance extraction processes while minimizing environmental impact. This synergistic approach allows for the development of extraction protocols that maximize yield, minimize waste, and reduce the use of harmful solvents, aligning with the principles of green extraction while contributing to the advancement of sustainable and eco-friendly practices in various industries.

1.5.1 Benefits and trade-offs

An experimental design is an approach established during a scientific study or in industrial research. The essential aim of this design is to plan experiences and work conditions to have significant results. It consists of measuring the value of outputs also known as response "*Y*", which is affected by inputs called factors "*x*". So, an experimental design is based on this equation: $Y= f(x)$. Codification is a common practice and usually, all factors vary between two values: -1 and +1 called levels. In a structured sequential approach, the process commences by identifying the variables to be used and defining the objectives (first step). Subsequently, the experimenter establishes an experimental plan and selects the specific methodology for the study (second step). Once all the parameters, conditions, and data outputs have been meticulously established, the net stage involves the in-depth analysis of the collected data (fourth step), followed by the interpretation and discussion of the obtained results. To ascertain

the model's reliability, it is imperative to undertake model verification (fifth step). Ultimately, the experimenter concludes by assessing the model's fitting capacities (sixth step). Additionally, such designs are used essentially to optimize resource utilization by reducing the number of experience repetitions, thereby minimizing resource consumption, exemplified by a notable reduction in solvent volume quantities. Also, it can focus on control parameters, factor behaviors, and factor interactions. Although it has plenty of advantages, an experimental design can also show some drawbacks, which will rely on experimental errors (Lundstedt et al., 1998).

1.5.2 Factorial design

To determine on which factors the response depends, experimenters establish a design called factorial design. The interesting point in such a type of design is that it can analyze two factors or more, at the same time. Two types of factorial design can be distinguished: full factorial design and fractional factorial design. In a factorial design, factors present as many levels as needed, but experimental runs will grow also according to the magnitude of levels and factors, it is characterized by 2^k with k corresponding to the number of factors used, this is a full factorial design. On the other hand, a fractional factorial design is described by 2^{k-p}. Such a design is used when the experimenter has an important number of factors to evaluate and wants to reduce experimental runs, although, sacrificing information (Mdege et al., 2014).

1.5.3 Central composite design

To understand interactions between variables and to estimate the response shape, a response surface methodology (RSM) is needed. Related to those objectives, different designs can be used such as Box-Behnken design using only three levels -1, 0, and +1, Taguchi design which is based on orthogonal arrays, predefined tables that select factors and their levels, and central composite design (Kayarogannam, 2023). Generally, central composite design or Box-Wilson central design is used to ensure working in optimal conditions and to determine the influence of factors on the response. It is composed of central points which are accompanied by a series of stars or axial points, so experimenters can predict curves (Bhattacharya, 2021). A central composite design (CCD) represents an origin point that corresponds to the center point and other points form a cube corresponding to axial -1 and +1, plus the star point which goes further than the axial and which is calculated according to the number of factors employed (Figure 12). CCD presents three types: circumscribed central composite design (CCC), inscribed central composite design (CCI), and face-centered composite design (FCCD). They differ in the distribution of axial points (Kayarogannam, 2023).

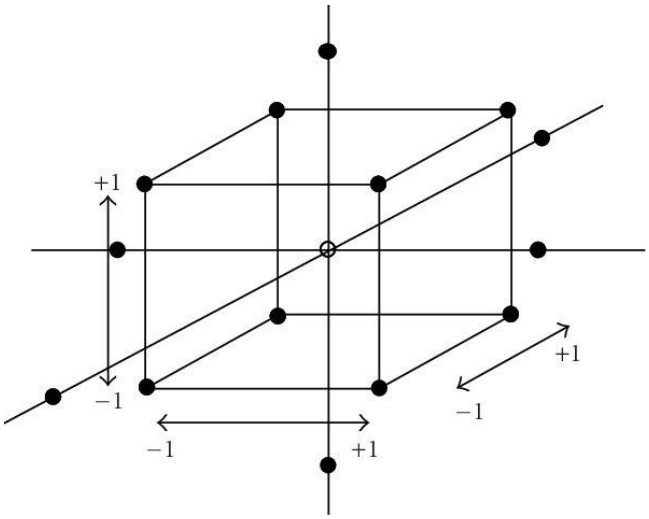


Figure 12: Example of central composite design using three factors (Kandananond, 2010).

1.5.4 Design modeling, optimization, and confirmation

The model and conditions that the experiment relies on must be selected to develop an experimental design. Linear, second-order interaction and quadratic models are the essential models that exist (Lundstedt et al., 1998). After selecting the different factors and choosing the response of interest, the next step consists of selecting the type of design and determining the different interactions. The model fitness must be evaluated by calculating two values: the predicted variation R^2_{pred} and the explained variation R^2. The acceptable values of these two are summarized in Table 3.

Table 3: Tips for evaluating R2 and R pred2 (Lundstedt et al., 1998).

Type of data	R^2	R^2_{pred}
Biological	Acceptable > 0.7	Acceptable > 0.4
Chemical	Acceptable ≥ 0.8	Acceptable ≥ 0.5 Excellent > 0.8

To ascertain if a relationship between the variables exists, the total variation of the response ANOVA must be evaluated by determining the significance of the model that corresponds to the *p*-value which should be ≤ 0.05. In addition, the objective of such designs is probably the optimization of data. This step depends on two methodologies: RSM and simplex optimization which corresponds to a shape with (k+1) angles and k is the number of factors (Lundstedt et al., 1998).

1.5.5 Exploratory data analysis as re-interpretation of data

Exploratory data analysis (EDA) is a crucial step in every research work. It is used to analyze data and to examine hypotheses. It also helps in verifying errors, trends, and mistakes. Furthermore, EDA can establish interactions between the different variables. EDA can present two essential types, univariate, and multivariate EDA. Some of the recurrent tools in EDA can be the use of histograms, heatmaps, scatterplots, etc, or numerical data (i.e., contingency tables) without graphics to define, summarize, and characterize data. EDA relies on graphical methods such as scatter plots, histograms, and Box plots, among many others (Morgenthaler, 2009).

1.5.6 Response surface methodology

Response surface methodology comprises a set of statistical and mathematical methods that are useful in enhancing and expanding processes where various variables can influence a specific response, which is the subject of optimization (Baş & Boyacı, 2007). Furthermore, it requires a lower investment of time and effort (Aydar, 2018). RSM has been applied in different studies to optimize the extraction of olive by-products (Giacometti et al., 2018; Madureira et al., 2021; Pedrosa et al., 2022; Vidal et al., 2022). An example of the optimization of olive leaves dynamic maceration extraction is illustrated in Figure 13.

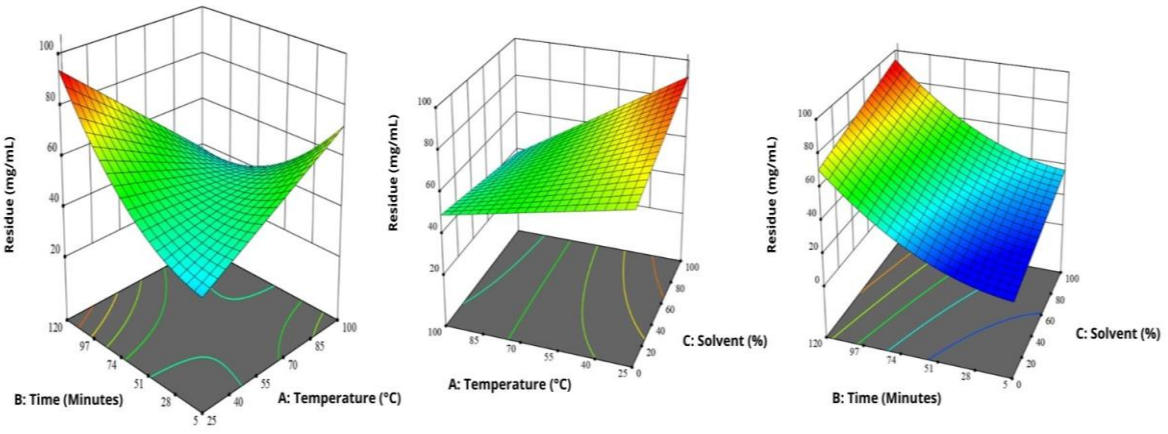


Figure 13: 3D plots of different applied factors (Pedrosa et al., 2022).

RSM can be visually represented using a contour plot, which is available in both two-dimensional and three-dimensional formats (Kayarogannam, 2023). Different steps in RSM should be followed to achieve the optimization in the process, starting by selecting the specific responses, then planning regarding the strategy or approach, followed by running the experiments. When the model is already obtained, it should be adjusted to align with the dataset

and then confirmed. After all these crucial steps, the optimization of the process might be performed (Kayarogannam, 2023).

In conclusion, experimental design offers a systematic and efficient way to study the multifaceted parameters involved in green extraction. By employing experimental design, researchers can methodically assess variables like solvent selection, temperature, pressure, and extraction time, ensuring that the extraction process is not only efficient but also environmentally, sustainable.

2 OBJECTIVES

The main objective of this research is to optimize extraction techniques for the efficient recovery of bioactive compounds from olive leaves. The study aims to determine the best extraction method in terms of total phenolic compounds (TPC), chlorophyll, and carotenoids extraction yield, as well as antioxidant capacity, thereby contributing to the development of sustainable and high-value applications for olive by-products.

Specific objectives:

- To review and analyze existing literature on extraction methods for bioactive compounds from olive leaves to establish a knowledge base;
- To compare three extraction procedures: dynamic maceration extraction (DE), ultrasound-assisted extraction (UAE), and microwave-assisted extraction (MAE);
- To determine the factors that influence the extraction method;
- To create a Model of prediction for solvent pH adjustments;
- To improve the DPPH EC_{50} value computing;
- To quantify the total phenolic content (TPC), chlorophyll, and carotenoid content in the extracted samples using appropriate analytical techniques;
- To evaluate the antioxidant capacity of the extracted bioactive molecules through various antioxidant assays, including DPPH, ABTS, FRAP, and reducing power;
- To establish statistical analysis and determine models, using one-way ANOVA and Tuckey test, central composite design (CCD), exploratory data analysis (EDA), and response surface methodology (RSM).

3 MATERIALS AND METHODS

3.1 Sampling

Olea europaea leaves were collected from the region of Bragança, Portugal, from different cultivars, in October 2022. Following collection, the olive leaves were freeze-dried (SCANVAC Coolsafe 110-4, Bjarkesvej, Denmark) for between 3 to 5 days. Subsequently, the dried olive leaves were subjected to mechanical milling (IKA, Model: M 20, 230V 50/60 Hz 620 W IP 21, Germany) (Figure 14). The olive leaves powder was further refined through sieving until the final diameter of 0.07 mm. The resultant samples were properly stored in a desiccator at room temperature in the dark, until further analysis.

Figure 14: Freeze-dried leaves placed in the milling machine.

3.2 Green extraction techniques

To optimize the antioxidant extraction from olive leaves, a comparative evaluation of three different green extraction techniques was performed, including dynamic maceration (DE), ultrasound-assisted extraction (UAE), and microwave-assisted extraction (MAE).

3.2.1 Dynamic maceration extraction

1.5 g of freeze-dried olive leaves powder was mixed with methanol, left under stirring in the dark and filtered (Figure 15), followed by methanol evaporation in a rotary evaporator at 35ºC (RE300DB, Stuart Stone, UK). The methanol volume used, and the time are described in Table 4. For the 180 min of extraction, the olive leaves powder was extracted with 50 mL of methanol, carefully filtered, and collected into an evaporating flask. This process was repeated

for a total of three cycles, with each cycle consisting of 1 hour of mixing and subsequent filtration.

Figure 15: Dynamic maceration extraction.

The collected filtrates from each cycle were then evaporated. Then, methanol was added to the dried sample obtaining a concentration of 0.5 mg/mL, filtrated with a Whatman Nylon (0,20 µm) filter, and stored in the dark, until further analysis. Each assay was repeated 2 times to achieve 2 independent trials.

Table 4: The dynamic maceration extraction conditions.

Condition	Methanol volume (mL)	Extraction time (min)
1	50	60
2	150	180

In parallel, 1.5 g of freeze-dried leaves powder was mixed with 50 mL of distilled water, left under a stirring in the dark and centrifuged at 15000 rpm for 15 minutes. The resulting extracts were freeze-dried (SCANVAC Coolsafe 110-4, Bjarkesvej, Denmark) for between 3 to 5 days. Then, distilled water was added to the dried sample obtaining a concentration of 0.5 mg/mL, filtrated with a Whatman Nylon (0,20 µm) filter, and stored in the dark, until further analysis. Each assay was repeated 2 times to achieve 2 independent trials.

3.2.2 Ultrasound-assisted extraction

1.5 g of the olive leaves powder and mixed with 50 mL of methanol and placed in the device of the UAE (Figure 16). The extraction was performed at 24°C, 250 W for 22 min, in

duplicate. At the end of the extraction, the methanol was evaporated in a rotary evaporator at 35ºC (RE300DB, Stuart Stone, UK). Then, methanol was added to the dried sample obtaining a concentration of 0.5 mg/mL and stored in the dark until further analysis.

Figure 16: Ultrasound-assisted extraction equipment.

3.2.3 Microwave-assisted extraction

1.5 g of olive leaves powder was mixed with 50 mL of methanol and placed in a vessel suitable for microwave irradiation. The extraction was performed at 113 ºC, 8 W for 26 min. This assay was performed in duplicate. At the end of the extraction, the methanol evaporated in a rotary evaporator at 35ºC (RE300DB, Stuart Stone, UK). Then methanol was added to the dried sample obtaining a concentration of 0.5 mg/mL and stored in the dark, until further analysis.

3.2.4 In vitro antioxidant tests

To evaluate the antioxidant activity of the biological compounds obtained through the three extraction techniques, different antioxidant methodologies were performed.

3.2.4.1 Ferric reducing antioxidant power

The ferric reducing antioxidant power (FRAP) assay is grounded in the swift reduction of ferric-tripyridiltriazine (Fe III-TPTZ) into ferrous-tripyridiltriazine (Fe II-TPTZ) by antioxidants found in the samples. This conversion results in the formation of blue-colored products as shown in Figure 17 (Benzie & Strain, 1996). A standard calibration curve should be created by introducing the FRAP reagent to a series of Fe^{2+} solutions with known

concentrations. This standard curve serves as a reference that enables the calculation of Fe^{2+} concentration in the samples to determine their antioxidant power.

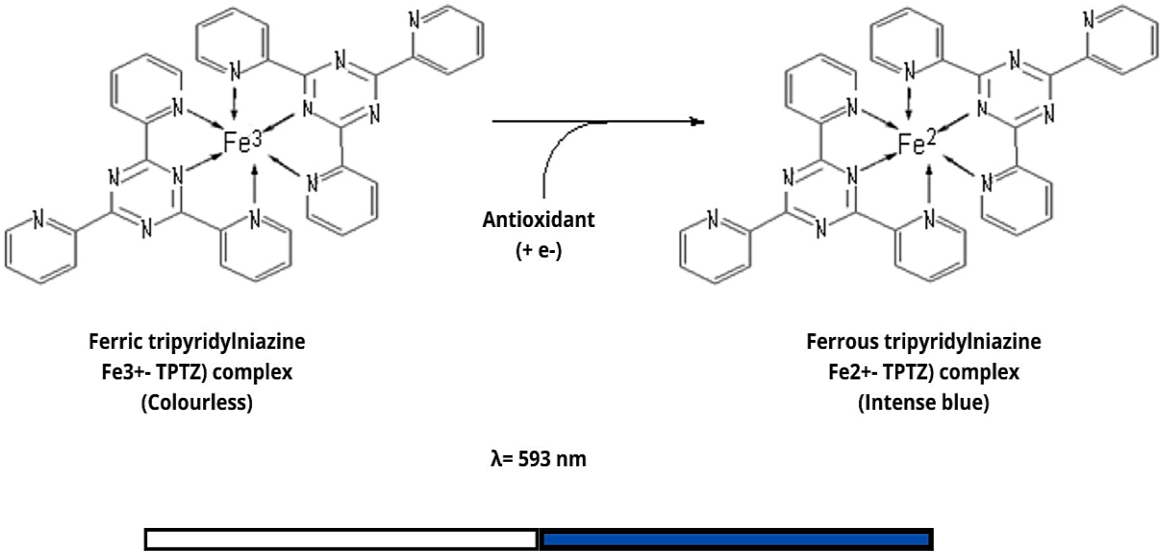


Figure 17: The ferric reducing antioxidant power (FRAP) reaction (Bhandari et al. 2015).

The FRAP assay is carried out following the method given by (Benzie and Strain 1996), with some modifications. In a flask, 1 mL of extract is blended with 2.9 mL of FRAP reagent and incubated at 37°C for 15 min. The FRAP reagent is prepared by mixing 10 mM 2,4,6-tri (2-pyridyl)-s-triazine (TPTZ) solution (made in 40 mM HCl) with a 20 mM $FeCl_3$ solution and 0.3 M acetate buffer (pH 3.6) in a proportion 1:1:10 (v/v/v). The absorbance is then measured at 593 nm (SPECTRO-star nano) using a methanol-prepared blank. An aqueous solution of ferrous sulfate $FeSO_4.7H_2O$ (20, 40, 60, 80, and 100 M) was used to create a calibration curve. FRAP readings are given in milligrams of ferrous sulfate equivalent per gram of dry extract (mg $_{FeSO4}$ /g $_{dry\ extract}$). Each sample was performed in triplicate.

3.2.4.2 Reducing power

The reducing power assessment technique relies on the concept that compounds with the ability to undergo reduction can interact with potassium ferricyanide (Fe^{3+}), leading to the creation of potassium ferrocyanide (Fe^{2+}). This resulting compound subsequently engages with ferric chloride, forming a ferric-ferrous complex with a peak absorption point at 700 nm.

The reducing power assay was performed according to the method described by (Malheiro et al., 2014). 1 mL of each concentration was mixed with 2.5 mL of 200 mmol/L sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. Following

vigorous agitation of the mixture, the tubes were, incubated at 50ºC for 20 min. After incubation, 2.5 mL of 10% trichloroacetic acid (w/v) was added, and the resulting mixture was subsequently centrifuged at 1000 rpm in a refrigerated centrifuge for 8 min. 2.5 mL of deionized water and 0.5 mL of 0.1% ferric chloride were added to the upper layer. The absorbance was read at 700 nm (SPECTRO-star nano). An aqueous solution of Trolox (6-hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic acid) (0,02 to 0,75 mg/mL) was used to create a calibration curve. The results of reducing power are given in grams of Trolox equivalent per gram of dry extract ($g_{\text{Trolox equivalent}}/g_{\text{dry extract}}$). Each sample was performed in triplicate.

3.2.4.3 Free radical scavenging activity DPPH

The free radical DPPH (2,2-diphenyl-1-picrylhydrazyl) includes an unpaired electron, making it highly reactive and capable of absorbing electrons or hydrogen atoms. The importance of antioxidants is represented by their capacity to deliver electrons or hydrogen atoms to free radicals. This may neutralize their reactivity and significantly reduce oxidative stress. Free radicals may interact with an antioxidant introduced to a methanol solution that contains DPPH. The reduction of DPPH is shown by a change in the color solution which becomes pale yellow from violet as illustrated in Figure 18 (Bibi Sadeer et al., 2020; Ioana et al., 2012).

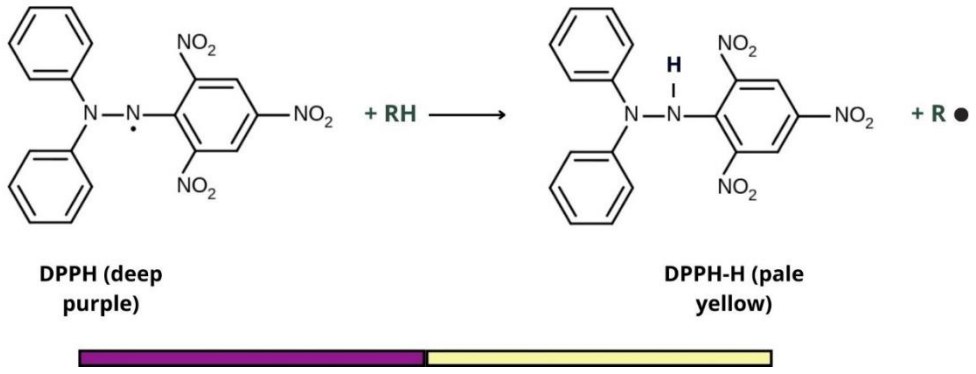


Figure 18: Scheme of a DPPH reaction (Ioana, Rugina, and Socaciu 2012; Bibi Sadeer et al. 2020).

For each sample, a series of dilutions (0.5 mg/mL to 0.1 mg/mL) were prepared, such that the percentage inhibition is between 20-80%. The reaction was carried out containing 30 µL of sample and 270 µL of 0.06 mM DPPH solution dissolved in methanol to an absorbance of 0.700 ± 0.01 at 515 nm in an ELISA (Model: SPL, Life Sciences Co., Ltd, Korea). The produced solutions are vortexed and allowed to stand for 30 min in the dark at room temperature. Then the absorbance is measured at 515 nm (SPECTRO-star nano) using methanol

as a blank. The control solution consists of using methanol instead of the sample. The radical scavenging activity is calculated by using the following equation:

$$\% \, inhibition = (1 - As/Ac) * 100$$

Where Ac and As represent, respectively, the absorbance of the control solution and the sample solutions. The calibration curve is prepared with a standard solution of Trolox diluted in methanol (0.5 to 0.004 mg/mL). DPPH percent inhibition data are plotted as a function of antioxidant concentrations to obtain DPPH inhibition concentrations at 50% (EC_{50}). The EC_{50} values are expressed as grams of Trolox equivalent per gram of dry extract (g $_{\text{Trolox equivalent}}$/ g $_{\text{dry extract}}$). Each sample was performed in triplicate.

3.2.4.4 ABTS

The 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) test or ABTS test is an assay used in biochemical and chemical analysis to evaluate the antioxidant capacity of substances. It starts by preparing the ABTS solution which represents a blue-green color, then it is mixed with a solution that contains the extracts. If the extract contains antioxidants, they will interact with the ABTS solution leading to a reduction of the solution intensity (Figure 19) (Re et al., 1999; Üstündaş et al., 2018).

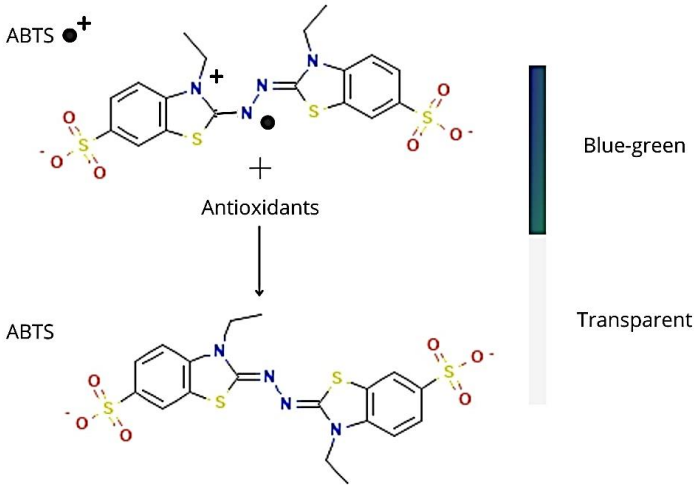


Figure 19: Representation of an ABTS reaction (Adapted, with some modifications, of Üstündaş, Yener, and Helvaci 2018).

The ABTS assay is performed according to the method described by Ballesteros et al. 2015, with some modifications. The ABTS cation is prepared by mixing 7 mM of 2,2' azino-bis-(3-ethylbenzothiazoline-6 sulphonic acid) diammonium salt (ABTS) dissolved in water with a 2.45 mM potassium persulfate solution. The resulting mixture was vortexed for 2 min

and left in the dark at 4 ºC between 12-16h for a stable oxidative state. Once this incubation period was over, ABTS radical cation solution was diluted in ethanol until it reached an absorbance of 0.700 ± 0.01 at a wavelength of 734 nm (SPECTRO-star nano). For each sample, a series of dilutions (0.5 mg/mL to 0.1 mg/mL) were prepared, such that the percentage inhibition is between 20-80%. Thus, assays are conducted by combining 10 µL of the sample with 200 µL of ABTS radical cation solution. The resulting solutions are maintained for 6 min in darkness at room temperature. The absorbance is measured at a wavelength of 734 nm, using distilled water as the control solution instead of the sample. The percent inhibition of ABTS radical cation is calculated by using the following equation:

$$\% \, inhibition = (1 - As/Ac) * 100$$

Where Ac is the absorbance of the control solution and As is the absorbance of the sample solutions. The calibration curve is constructed using a standard solution of Trolox diluted in methanol (0.5 to 0.004 mg/mL). The EC_{50} values are expressed as g of Trolox equivalent per g of dry extract ($g_{\text{Trolox equivalent}}$ /$g_{\text{dry extract}}$). Each sample was performed in triplicate.

3.2.5 Total phenolic content

The total phenolic compounds (TPC) test is a chemical analysis commonly used to determine the concentration of phenolic compounds present in a sample, such as plant extract or food products. Phenolic compounds are a diverse group of secondary metabolites found in plants that have various biological activities, including antioxidant properties.

For each sample, a concentration of 0.5 mg/mL was prepared. A 30 µL of the solution was mixed with 30 µL of Folin–Ciocalteu reagent (Aldrich Chemistry) and left for 3 minutes. Then, 30 µL of Na_2CO_3 was added, followed by 225 µL of distilled water. The reaction mixture is incubated in the dark at room temperature for 90 min. After that, the absorbance is determined at 725 nm using a spectrophotometer (SPECTRO-star nano) against a negative control (water) and a positive control (gallic acid at 0.5 mg/mL). A calibration curve was prepared using gallic acid dissolved in methanol at different concentrations (0.125 to 0.002 mg/mL). The TPC is expressed as grams of gallic acid equivalent per gram of dry extract ($g_{\text{gallic acid equivalent}}$/ $g_{\text{dry extract}}$). Each sample was performed in triplicate.

3.2.6 Carotenoids and chlorophylls content

The content of chlorophyll and carotenoids was estimated according to (Jeffrey & Humphrey, 1975) and (Wellburn, 1994) with a few modifications. 25 mg of extract were dissolved in 625 µL of acetone (100%), and subsequently homogenized for 1 min. The homogenate was centrifuged at 2500 rpm for 10 min. The absorbance was measured in the supernatant at 470 and 664 nm (SPECTRO-star nano), and chlorophylls and total carotenoids were determined according to the extract solvent following equations:

-For acetone extracts

$$Chl\ a = 10.82 \times Abs\ 664\ (\frac{\mu g}{mL})$$

$$Total\ carotenoids = \frac{1000 \times\ Abs\ 470\ - 1.90\ \times Clh\ a}{214}(\frac{\mu g}{mL})$$

-For methanol extracts

$$Chl\ a = 12.61 \times Abs\ 664\ (\frac{\mu g}{mL})$$

$$Total\ carotenoids = \frac{1000 \times\ Abs\ 470\ - 1.63\ \times Clh\ a}{221}(\frac{\mu g}{mL})$$

The results are expressed in µg of chlorophyll a, or total carotenoids per mL (µg/mL). Each sample was performed in triplicate.

3.2.7 Optimization of dynamic maceration extraction

In the next steps, all experimental design procedures were executed using the dynamic maceration extraction method as the primary method. This selection was based on the evaluation of the yields and antioxidant response. To optimize the dynamic maceration extraction two different solvents were used, methanol and acetone. In this study, multiple variables were systematically examined with the aim of optimizing the extraction of bioactive compounds from olive leaves utilizing the dynamic maceration technique. The primary goal in the subsequent phases of this research is to assess and discern the crucial parameters and factors that may exert an influence on the outcomes of the extraction process. These outcomes consider the determination of total phenolic content (TPC), the evaluation of DPPH radical scavenging potential, and the quantification of chlorophylls and carotenoids. Several key factors were considered, including extraction duration, plant material particle size, temperature, pH level, and the percentage of solvent employed. These variables were systematically varied and

investigated to ascertain their respective impacts on the efficiency and quality of the extraction process, while ensuring robust scientific rigor in our approach.

3.2.7.1 pH adjustments

The pH is one of the parameters which should be determined in the experimental designs. It must be maintained stable using a buffer solution such as the McIlvaine buffer. McIlvaine buffer is composed of two stock solutions, citric acid (0.1 M) and disodium hydrogen phosphate (0.2 M Na_2HPO_4) that were used to control the pH of solutions and prevent sudden changes in acidity or alkalinity from affecting experiment results. It can be prepared at pH 2.2 to 8 as mentioned in Table 5.

Table 5: Mixing table to obtain 20 mL of McIlvaine buffer.

pH	0,2 M Na_2HPO_4 (mL)	0,1 M citric acid (mL)
2.2	00.40	19.60
2.4	01.24	18.76
2.6	02.18	17.82
2.8	03.17	16.83
3.0	04.11	15.89
3.2	04.94	15.06
3.4	05.70	14.30
3.6	06.44	13.56
3.8	07.10	12.90
4.0	07.71	12.29
4.2	08.28	11.72
4.4	08.82	11.18
4.6	09.35	10.65
4.8	09.86	10.14
5.0	10.30	09.70
5.2	10.72	09.28
5.4	11.15	08.85
5.6	11.60	08.40
5.8	12.09	07.91
6.0	12.63	07.37
6.2	13.22	06.78
6.4	13.85	06.15
6.6	14.55	05.45
6.8	15.45	04.55
7.0	16.47	03.53
7.2	17.39	02.61
7.4	18.17	01.83
7.6	18.73	01.27
7.8	19.15	00.85
8.0	19.45	00.55

3.2.7.2 Extraction process

After adjusting the pH value for both methanol and acetone, following the guidelines of the McIlvaine buffer, the different extractions were performed. 1.5g of the dried olive leaves powder was vigorously mixed with 50 mL of the respective solvent for a specific duration. Subsequently, the extractions were centrifuged at 15000 rpm for 15 minutes, and the supernatant was recovered. After, an aliquot of 6 mL was extracted for yield determination, while the remaining volume was subjected to evaporation in a rotary evaporator at 35ºC (RE300DB, Stuart Stone, UK). The resulting extracts were resuspended in distilled water and freeze-dried (SCANVAC Coolsafe 110-4, Bjarkesvej, Denmark) for between 3 to 5 days. Then, the extracting solutions were added to the suitable dried samples obtaining stock solutions for each sample of a concentration of 0.5 mg/mL, then they were vortexed, and stored in the dark, until further analysis. The antioxidant activity/capacity and the total phenolic content analysis was performed according to the methodology described above. For the total carotenoids and chlorophyll analysis, the dried samples were directly dissolved in acetone 100% as previously detailed.

3.2.7.3 Determination of extraction yields

The percentage of yield was determined by evaporating the 3 mL of the different extract supernatant in the oven at 50ºC (DIGITRONIC-TFT, Model: J.P. SELECTA, s.a. Spain), until it was dried. Then, the samples were stored in a desiccator until a consistent weight was obtained.

3.2.8 Statistical analysis

All statistical analyses and graphical representations were developed using R Studio, version 2023.06.1+524 (Posit PBC, Boston, MA, USA) except for the experimental design and the response surface methodology (RSM) plots where Stat.Ease software, version 22.0 (Stat-Ease, Inc. Minneapolis, MN, USA) was used. For all tests, the necessary statistical assumptions were assessed, and a significance level of $\alpha = 0.05$ was consistently applied.

In cases where tests involved comparisons of more than two samples, appropriate post hoc tests were conducted and presented. All packages and scripts required for result replication are available in four separate scripts provided in the appendix of this document.

The main aim of this work was to optimize the different parameters of the hydrodynamic maceration extraction to achieve the most favorable outcomes. Therefore, an adapted central composite design CCD was applied to establish a model that captures the connections between the specific factors that need to be selected and to explore the impacts of these variables on the different responses.

3.2.8.1 CCD modeling

To delineate the relationships among various parameters and to fine-tune the operational settings for both methanol and acetone, employing Central Composite Designs (CCD) that encompass four key factors (3 quantitative and 1 qualitative): solvent percentage, pH level, extraction time, and solvent type was applied. This comprehensive experimental design involves 52 randomized trials which includes 2 True replicates in each factorial and axial points and eight central points (4 per solvent type), as visually illustrated in Figure 20. The CCD methodology allows for a systematic exploration of the parameter space, facilitating the optimization of extraction conditions for enhanced efficiency and accuracy. All the values consisted in triplicated measurements.

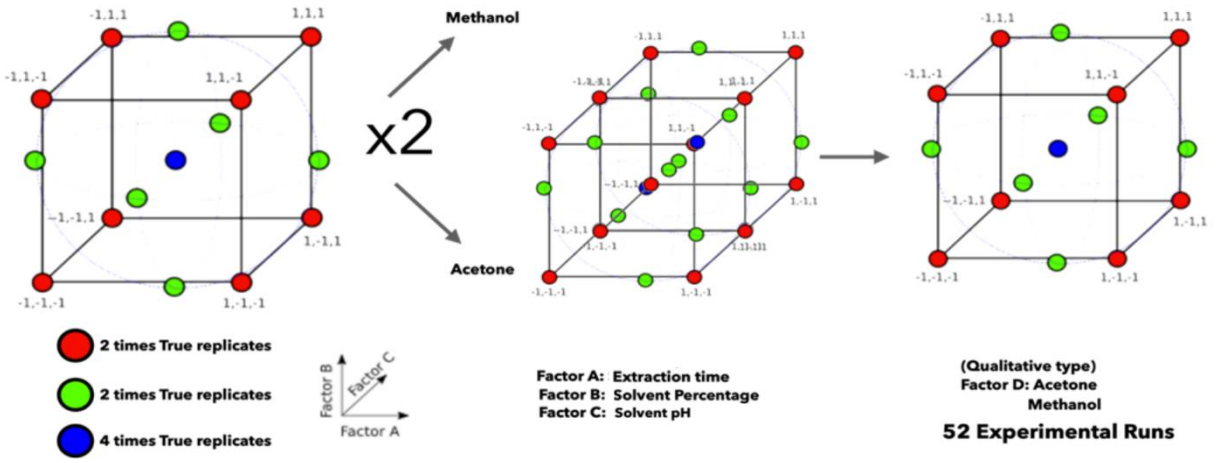


Figure 20: Adapted 52 experimental run Central Composite Designs for 4 factors.

The different factors and levels are summurized in the Following Table (Table 6).

Table 6: Summary of levels and factors used.

	LEVEL 1	LEVEL 2	LEVEL 3
FACTORS / CODED VALUES	-1	0	1
TIME	120	180	240
SOLVENT %	66	73	80
PH	5	6.5	8
SOLVENT TYPE	methanol		acetone

4 RESULTS and DISCUSSION

4.1 Dynamic maceration using water

The initial phase of the experimental development aimed at verifying the most suitable approach for the extraction, characterization, and utilization of olive tree leaf extract. Consisting of a rapid examination of the extraction process, the determination of total phenolic compound content (Folin-Ciocalteu), and the evaluation of its antioxidant activity using a quick, straightforward, and practical quantification method known as the DPPH assay.

Throughout the entire experimental development undertaken in this study, a series of extraction methods will be meticulously explored. The primary objectives will become the identification of the most efficient methodologies to achieve the results and to indicate the optimal extraction procedure for the specific matrix under investigation. With those objectives in mind, the initial extraction was conducted using a straightforward, rapid technique that avoided the use of organic solvents, relying on water and a dynamic extraction process of one hour long. The results obtained from these preliminary tests exhibited an extraction yield of 79.63 ± 3.03 mg of gallic acid equivalents per gram of extract (data provided in Table S1 of the supplementary material). As for the bioactivity of the extract, it revealed an inhibition percentage of 81.3 ± 3.35. These initial findings now serve as the foundational benchmark for subsequent experimental tests, which will contribute to forming a comprehensive and more precise understanding of the research objectives delineated in this experimental study.

4.2 Comparison of three extraction techniques: UAE, MAE, and dynamic maceration DE

4.2.1 Influence of the extraction techniques on antioxidant activities

For the whole work, *Olea europaea* leaves extracts were chosen to investigate their antioxidant capacity, rather than studying individual components. This approach was taken to explain the combined observation of the individual molecules, which may interact synergistically by modifying some of their characteristics, resulting in enhanced outcomes (Lee and Lee 2010). Choosing the appropriate extraction procedure is one of the most crucial factors in optimizing the recovery of bioactive compounds. Therefore, four extraction methods were tested, ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), dynamic maceration for 1 hour and 3 hours (DE-1h, DE-3h, respectively). To compare and evaluate the potential of antioxidant power, methanol was selected as extraction solvent due to its polarity

which tends to be better at extracting bioactive compounds (Altemimi et al. 2017). Cowan, 1999, suggested that methanol was one of the solvents that extracted a wider range of diverse compounds, justifying our use of this solvent accordingly (Cowan, 1999). The ABTS, DPPH, FRAP and RP tests were evaluated on the methanolic extracts obtained and the different results are shown in Table 7 which summarizes all the concentrations used for each antioxidant assay by displaying their average and standard deviations (SD) values from all the extractions. For both ABTS and DPPH the results are reported as percentage of inhibition (% inhibition), whereas FRAP and the reducing power assays were reported in compound equivalent (mg $_{FeSO4.H2O}$ or mg $_{Trolox}$ per g $_{dry\ extract}$). According to the results in Table 7, ABTS test exhibits a high coefficient of variation for DE-1h at 0.3 mg/mL (47.4%) and for DE-3h at 0.1 and 0.3 mg/mL (19% and 31.7%, respectively). This high CV suggests significant variability and dispersion in the data, indicating that the SD is noticeably greater when compared to the mean, and might be attributed to sub-optimal quality of the reagents used. However, in the case of DPPH test, there was found lower variability observations among the different treatments. As shown in Figure 21, the means are graphically represented in relation to the concentrations ranging from 0.1 to 0.5 mg/mL, showing an ascending trend that did not reached the totality (100%) of the inhibition, even though higher concentrations were tested. Another interesting highlight is that EC_{50} values might be around the 0.4 mg/mL concentration for the UAE and DE1h.

Table 7: Summary of the antioxidant activities evaluation of UAE, MAE, Dynamic maceration (1h) and Dynamic maceration (3h) presented with mean ±standard deviation

Concentrations	ABTS				DPPH			
(mg/mL)	UAE	MAE	DE1h	DE3h	UAE	MAE	DE1h	DE3h
0.1	54.84 ± 0.7	55.69 ± 0.5	2.98 ± 0.2	3.97 ± 0.8*	9.99 ± 0.7	8.54 ± 1.6*	5.98 ± 0.2	7.42 ± 0.6
0.2	61.41 ±0.9	60.45 ± 2.6	9.02 ± 0.1	10.32 ± 0.8	20.86 ± 1.0	16.89 ± 4.5*	10.92 ± 0.5	14.59 ± 0.4
0.3	66.63 ± 0.8	65.33 ± 3.2	12.2 ± 5.8*	13.31 ± 4.2*	29.69 ± 1.2	25.36 ± 8.1*	20.09 ± 3.2*	21.24 ± 0.0
0.4	73.09 ± 1.1	70.21 ± 3.0	21.84 ± 1.3	23.48 ± 0.4	40.24 ± 1.1	33.74 ± 4.8*	41.34 ± 1.7	27.89 ± 0.4
0.5	100.25 ± 1.6	96.78 ± 2.4	52.44 ± 0.8	52.67 ± 1.4	88.8 ± 0.2	82.94 ± 8.0	80.88 ± 2.0	81.6 ± 0.0
Concentrations	**FRAP**				**RP**			
(mg/mL)	UAE	MAE	DE1h	DE3h	UAE	MAE	DE1h	DE3h
0.1	39.73 ± 31.3*	3.78 ± 5.3*	47.71 ± 4.3	56.97 ± 9.6*	73.99 ± 3	84.49 ± 7	35.69 ± 3.6*	46.06 ± 2.9
0.2	58.46 ± 5.3	48.94 ± 7.8*	70.88 ± 2.7	92 ± 1.7	63.06 ± 4.5	65.21 ± 8.2*	56.79 ± 2.3	71.93 ± 4.3
0.3	47.85 ± 17.6*	57.55 ± 11.9*	75.49 ± 2.4	93.59 ± 3.1	57.96 ± 0.1	55.17 ± 1.5	62.76 ± 2.7	82.33 ± 5
0.4	61.88 ± 2.8	58.36 ± 12*	77.58 ± 7.1	95.59 ± 1.4	54.68 ± 4.8	83.47 ± 39.2*	67.87 ± 2.8	91.7 ± 0.1
0.5	68.97 ± 1.1	82.02 ± 8.7*	92.1 ± 2.8	115.8 ± 0.3	109.8 ± 4.5	117.95 ± 9	140.52 ± 6.7	178.18 ± 8.

*** Indicate a CV > 10**

The FRAP assay shows high CV values for all the concentrations using MAE, while the UAE showcases variations of 78.88% and 36.85 % at 0.1 and 0.3 mg/mL. Meanwhile, in the case of the reducing power (RP) test, MAE presented a high CV value in 0.2 and 0.4 mg/mL (12.6 % and 46.9%) and DE1h for concentration 0.1. At this point, it is worth noting that for sake of simplicity, all the numerical data is provided in the Table 7.

In almost every individual assay, dynamic maceration extraction shows better results as shown in Figure 21. Specifically, DE 3h outperformed the other extraction techniques in the FRAP and RP tests. To better describe the antioxidant effects display in the figure a clear separation can be made; in one hand, the FRAP and RP tests present their response on equivalents of a certain compound, therefore, higher equivalents response with lower extract concentration is desirable; whereas in the other hand, the DPPH and ABTS tests illustrate the percentage of inhibition achieved by the different concentrations tested, thus, higher or equal percentage of inhibition with lower concentrations corresponds to a stronger response.

DPPH test is known for its ease of use, time saving and specificity in reacting with hydrogen-donating antioxidants (Echegaray et al., 2021). Furthermore, the DPPH assay is used to measure the antioxidant's ability to scavenge radicals as the radical compound is stable and doesn't require generation (Gulcin and Alwasel 2023). Whereas the FRAP and RP tests lack specificity because they can be influenced by compounds other than antioxidants, for example, sugars might lead to an overestimation of antioxidant capacity. Additionally, FRAP test results can fluctuate based on the duration of the observed reaction between antioxidant and Fe^{+3}, which can range from a few minutes to several hours (Amorati and Valgimigli 2015; Pulido, Bravo, and Saura-Calixto 2000).

Given these considerations and the high variation coefficient achieved on some of the tests, the DPPH test was selected as the most suitable choice to perform in the next steps in analysis. As shown in Figure 21, low variation within extraction treatments seems a reasonable choice to extrapolate future results using any of the explored extraction techniques. All the screen analysis are provided in Table S2 (Supplement Material).

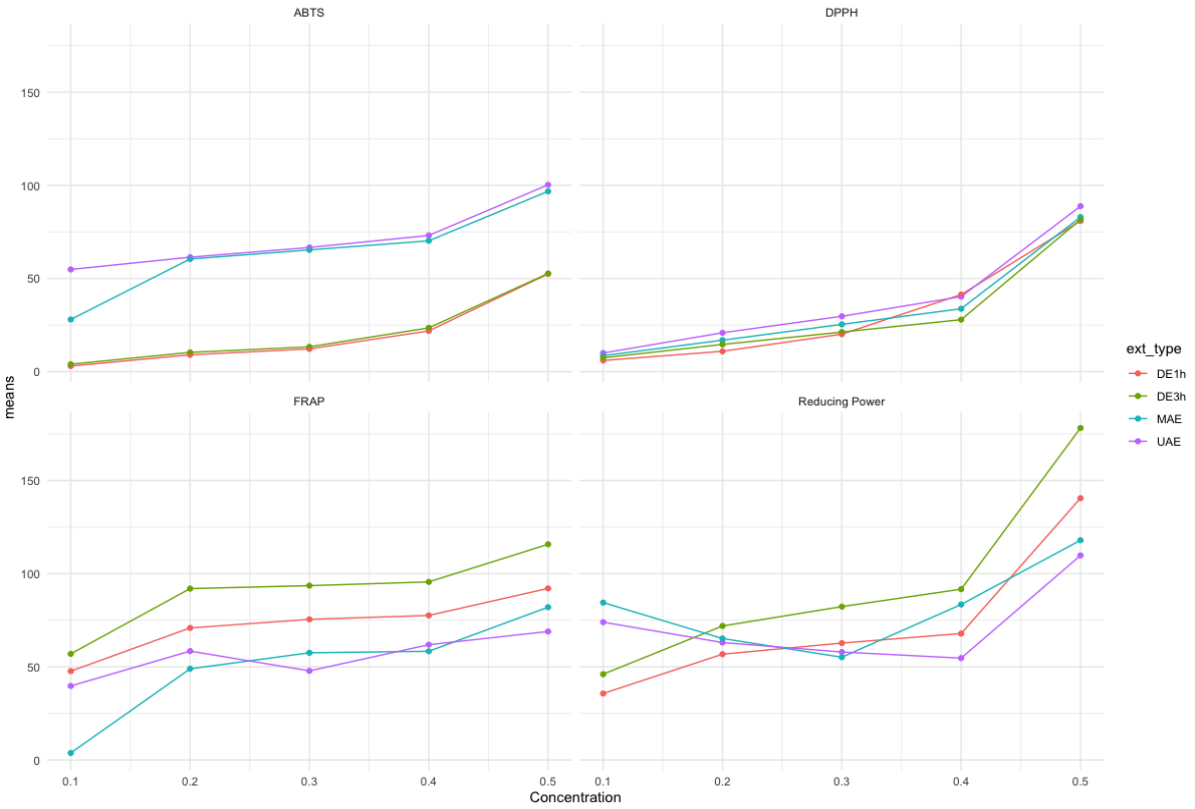


Figure 21: Line graphs representation of the ABTS, DPPH, FRAP and RP assays analyzed in the 4-extraction, at their different concentrations tested.

4.2.2 Total phenolic compounds estimation

The total phenolic compounds TPC test is a crucial assay to evaluate, indirectly, the antioxidant power of samples. As it is known, phenolic compounds are well known for their interesting antiradical activities (Balasundram et al., 2006). When the TPC value is high, this could suggest that the extract is more capable of combating the free radicals and oxidative stress (Noreen et al., 2017). The TPC of olive leaves extracts recover were assessed using 100% methanol as solvent and the 4 extraction procedures previously mentioned. Those results were expressed in mg equivalent of gallic acid per g of dried extract though the calibration curve of gallic acid (Figure 22a). Dynamic maceration extraction of 3h presents the highest TPC value 36.57 ± 0.58 $mg_{eq\ gallic\ acid}/g_{dry\ extract}$ as it is observed in both Table 8 and Figure 22b. After proper interpolation of the gallic acid calibration curve, the numerical values are presented in Table 8, where the UAE and MAE display 26.47 and 28.16 $mg_{eq\ gallic\ acid}/g_{dry\ extract}$, respectively. Both values from the advanced extracting techniques were lower than DE-1h (29.18 $mg_{eq\ gallic\ acid}/g_{dry\ extract}$), although these minimal variation (1.03 and 2.72 mg for MAE and UAE respectively), did not show significant statistical differences. Therefore, same outcomes could be achieved without incurring in the use of advanced equipment, which would end up producing higher

expenses on an industrial scale. Nevertheless, the DE-3h technique shown the maximal extractability of TPC displaying a difference of 7.38 $mg_{eq\ gallic\ acid}/g_{dried\ extract}$ when comparing with the second better result (DE-1h), which turned to show significant statistical differences as shown in Figure 22c.

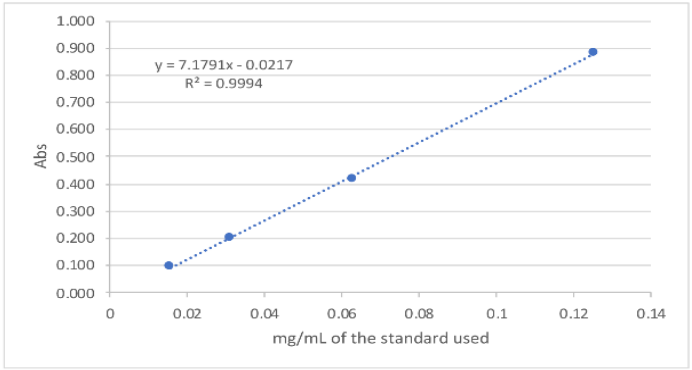
Table 8: TPC in olive leaves extracts estimation (expressed in mean ± standard deviation)

Extraction techniques	**TPC ($mg_{eq\ gallic\ acid}/g_{\ dry\ extract}$)**	**CV**
UAE	26.47 ± 2.37	8.96
MAE	28.16 ± 0.40	1.45
DE-1h	29.19 ± 0.69	2.35
DE-3h	36.57 ± 0.58	1.58

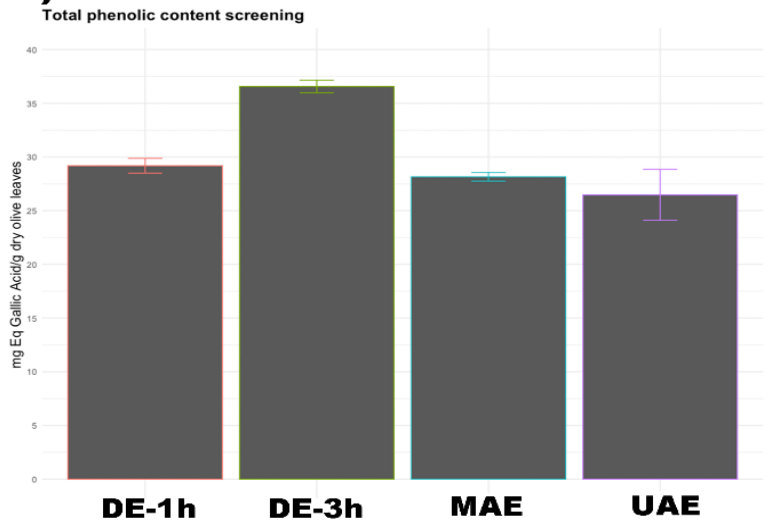
To understand the variation between the treatments and to determine their significance, the analysis of variance ANOVA test was computed. As it is represented in Figure 22c, the test shows significant effect (p <0.05). Therefore, to identify the specific pairs of group means that are significantly different from each other, Post Hoc Tukey test was applied. The statistical test showed significant difference when using DE-3h (Figure 22c), with p-adj of 0.016 for the couple DE-3h and DE-1h, 0.010 for DE-3h and MAE and, 0.005 for DE-3h and UAE. These results suggest that the conventional extraction technique (dynamic maceration extraction) is the best extracting procedure compared to the advanced techniques for this specific case and suggest that the extracts can present a higher scavenging activity. Additionally, it is less expensive technique with better solvent contact with the plant material (Ćujić et al., 2016). Even though DE-3h displays statistical differences, and DE-1h, UAE and MAE shown similar estimations of TPC in the olive leaves, the final decision of choice will be affected not only by the TPC extractability yield but by a sum of different responses. While DE-3h presented the better extractability value, DE-1h still showcasing a considerable high response compare with the more expensive advance technologies; therefore, to execute faster analysis considering those high extractability output, the selection of DE-1h seem reasonable to this study.

A)

mg/mL	Abs
0.5	Over Limit
0.25	Over Limit
0.125	0.881
0.0625	0.415
0.03125	0.202
0.015625	0.098
0.0078125	0.046
0.00390625	0.025
0.001953125	0.015



B)



C)

ANOVA

		Df	Sum Sq	Mean Sq	F value	Pr(>F)	
ext_type		3	119.2	39.72	24.06	0.00508	**
Residuals		4	6.6	1.65			

Signif.	codes:	0	'***'	0.05	'.'		
		0.001	'**'	0.1	' '		
		0.01	'*'	1			

Post hoc Tukey

	diff	lwr	upr	p adj
DE 3h-DE 1h	7.38	2.15	12.61	0.016
MAE-DE 1h	-1.03	-6.26	4.20	0.851
UAE-DE 1h	-2.71	-7.94	2.52	0.289
MAE-DE 3h	-8.41	-13.64	-3.18	0.010
UAE-DE 3h	-10.09	-15.32	-4.86	0.005
UAE-MAE	-1.68	-6.91	3.55	0.604

Figure 22: a) Gallic acid calibration curve, b) extractions behaviors plot, c) Statistical analysis including ANOVA test and Post Hoc Tukey test.

4.3 Factors selection

The first factor considered in this work is the pH level. This variable is critical for assessing how different changes in the concentrations of Hydrogen ions affect the acidity or alkalinity of the extract's solution (Chethan & Malleshi, 2007). Such fluctuations can influence the stability of polyphenols and bioactive compounds throughout the extraction procedure and can also impact specific responses. In addition, modifications in pH can have repercussions on the percentage of solvent, due to the miscibility of pH buffers with the extracting solvent. The pH of the extracting solution is important as it determines the solubility of bioactive soluble molecules and directly impacts their solubilization's degree. As an example, Rodriguèz-Juan and co-workers study the effect of extraction conditions on the phenolic compounds composition showed that a change of pH affect significantly results. In fact, their findings indicated that higher pH values have an adverse effect on the concentration of bioactive molecules obtained during the extraction procedure (Rodríguez-Juan et al., 2021). Hence, maintaining pH stability is essential to ensure consistent experimental conditions. To achieve this, McIlvaine buffer was used to carefully adjust the pH to the desired level for the solvent percentage using 2 solvents acetone and methanol. Several experiments were carried out, and the details are provided in Table 9. To accomplish this objective, McIlvaine buffer was used as a precise means of pH adjustment to achieve the desired pH level for both individual solvents, using acetone and testing methanol extractions. A series of systematic experiments were conducted, and the experimental data can be found in Table 9, presenting a detailed account of our methodological runs and results. This approach ensures both accuracy and consistency in the pH adjustment process and solvent composition, facilitating a robust and well-documented experimental framework for the future experimental design.

Table 9: Adjustments of pH using MLR and McIlvaine buffer.

Solvent	Buffer pH	Solvent %	Buffer %	pH (mean value)
Methanol	2.2	60	40	3.26
	2.2	80	20	3.79
	3	60	40	4.29
	2.2	97	3	4.39
	3	80	20	4.86
	4	60	40	5.24
	3	97	3	5.47
	4	80	20	5.80
	5	60	40	6.37
	4	97	3	6.61
	5	80	20	6.96
	6	60	40	7.44
	6	80	20	7.52
	5	97	3	7.95
	7	80	20	8.69
	6	80	20	8.17
	7	60	40	8.62
	8	60	40	9.58
	8	80	20	9.76
	6	97	3	9.37
	8	97	3	10.40
	7	97	3	10.21
Acetone	2.2	60	40	3.31
	2.2	80	20	3.87
	2.2	97	3	4.40
	3	60	40	4.49
	3	80	20	5.04
	3	97	3	5.05
	4	97	3	5.30
	4	60	40	5.48
	4	80	20	5.56
	5	97	3	5.81
	5	80	20	6.09
	5	60	40	6.38
	6	80	20	6.68
	6	97	3	6.72
	6	60	40	6.96
	7	60	40	8.32
	7	80	20	6.45
	7	97	3	9.52
	8	60	40	9.56
	8	80	20	10.10
	8	97	3	10.57

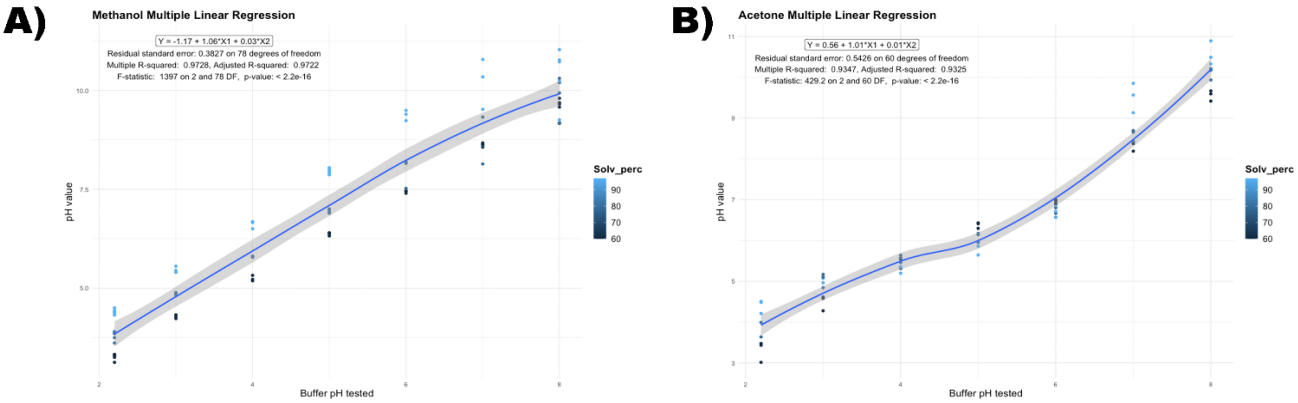
The main objective of these tests is to build a statistical and mathematical model capable of providing precise predictions for pH levels, particularly in relation to the desired solvent percentage. To achieve this goal, Multiple linear regression (MLR) analysis was employed on the experimental dataset, considering all pertinent variables and factors. The results of this analysis are presented in Figure 23, showcasing the relationships between pH and the solvent percentage for both methanol and acetone. In the case of methanol, the plotted curve closely

resembles a linear regression, with a highly R^2_{adj} = 0.97. Similarly, acetone exhibits a linear regression pattern with a coefficient of determination R^2_{adj} = 0.93. Using the adjusted R-squared (R^2_{adj}) instead of the plain R-squared (R^2) in a multiple linear regression (MLR) and higher order models is often preferred because it provides a more balanced and reliable measure of the model's goodness of fit when dealing with multiple predictor variables (Cohen, 2013). The MLR data are shown in Table S3 (Supplement Material).

Based on these curves, 2 mathematical equations were determined to calculate the predictable pH.

$$Buffer\ (met) = \frac{pH + 1.17 - 0.04met}{1.06} \qquad \text{Eq.1}$$

$$Buffer\ (ace) = \frac{pH - 0.56 - 0.01ace}{1.02} \qquad \text{Eq.2}$$



	Estimate	Std. Error	t value	Pr(> |t|)	
(Intercept)	-1.173	0.249	-4.711	1.05E-05	***
Buffer_pH	1.061	0.021	51.322	7.16E-62	***
Met_perc	0.036	0.003	12.676	1.24E-20	***

	Estimate	Std. Error	t value	Pr(> |t|)	
(Intercept)	0.562	0.404	1.391	0.16942334	
Buffer_pH	1.019	0.035	29.196	3.66E-37	***
Acet_perc	0.011	0.005	2.446	0.01739881	*

Figure 23: MLR results and mathematical models' establishment.

Considering the wide solvent percentages tested, these mathematical models have the capacity to forecast pH values accurately, while leaving the desired solvent concentration unaffected, which will provide a realistic solvent percentage and pH. Although methanol is widely used on bioactive extractions, acetone can also be used due to its higher effectiveness in extracting compounds from plant material, it is an environmentally friendly and biodegradable solvent (Metličar et al., 2021). The selection of the solvent percentage as the second factor in this study arises from its significant influence on the extraction yield, due to polarity change when creating the binary solvent using water. Therefore, the choice of solvents along with their concentrations, plays a pivotal role in this context. Furthermore, the solvent

percentage exerts a notable impact on the DPPH scavenging activity, as reported by (Turkmen et al., 2006).

The extraction time is the other critical factor that has been chosen for investigation. Several studies have exhibited interest in this variable and studied its impacts on extracting plant material (Abed et al., 2015; Kamal et al., 2023; Simões et al., 2022; Spigno et al., 2007). The duration over which solvents interact with the plant molecules plays a vital role in facilitating the transfer of chemical compounds into the extraction solution (Chakraborty et al., 2020).

In the upcoming sections, the investigation will encompass three key factors: pH level, percentage of solvents (specifically in acetone and methanol), and the duration of the extraction process. These factors have been thoughtfully selected based on their known significance in influencing the extraction process. However, before starting with the experimental design, it was essential to identify a room for improvement in the methodology employed for the DPPH assay, specifically concerning the interpolation of the EC_{50} (Effective Concentration 50) value. Up to this point, the calculation has relied solely on the "percentage of change" formula followed by Lagrange approximation adjustments. Nevertheless, as noted by De Menezes et al. (2021), a more accurate approach to this calculation can be achieved by incorporating the proper utilization of positive and negative controls within the percentage change formula (De Menezes et al., 2021). In addition, this adjustment has provided an opportunity to enhance the precision of the interpolated EC_{50} value through the implementation of a refined logistic regression model.

4.3.1 EC_{50} value improvement

The DPPH test proves to be a quick trustworthy approach for testing the antioxidant power of biological samples (Chen et al., 2013). In the DPPH assay, EC_{50} is an important measure for assessing the extract's antioxidant activity and it stands for "half maximum effective concentration" which means the concentration needed to achieve 50% antioxidant effect. EC_{50} provides crucial information about the effectiveness of samples, for instance a lower EC_{50} value reflects the greatest antioxidant capacity. Usually, in different studies, researchers use the EC_{50} values to compare and quantify the antioxidant potential of the extracts. In this work, an improvement of EC_{50} value was performed to achieve the optimal lower concentration with the potent scavenging effect. A series of experiments were conducted as shown in Table 10. The absorbance is measured at a wavelength of 515 nm (AlShaal et al., 2019; Hayes et al., 2020).

Table 10: Calibration curve for the improvement of EC_{50} value.

Sample	Standard Concentrations [Trolox mg/mL]	Absorbance (Mean ± S.D., n = 3)
Standard 1	0.5	0.054 ± 0.001
Standard 2	0.25	0.059 ± 0.002
Standard 3	0.125	0.093 ± 0.005
Standard 4	0.062	0.287 ± 0.026
Standard 5	0.031	0.436 ± 0.004
Standard 6	0.015	0.589 ± 0.030
Standard 7	0.008	0.665 ± 0.004
Standard 8	0.004	0.712 ± 0.004
Standard 11	0.0005	0.733 ± 0.004
Positive Control	0.5	0.054 ± 0.001
Negative Control	0	0.800 ± 0.027

The percentage of change for the DPPH was determined using the following equation (De Menezes et al., 2021).

$$\%\ DPPH\ sca = \left[\frac{(Abs\ sam - Abs\ NC)}{(Abs\ PC - Abs\ NC)}\right] \cdot 100\% \qquad \text{Eq.3}$$

Where:

- Abs $_{Sam}$ is the absorbance of the sample
- Abs $_{NC}$ is the absorbance of solvent (Negative control) and,
- Abs $_{PC}$ is the absorbance of the maximal concentration tested (Positive control).

The EC_{50} value is further determined through modeling with a four-parameter log-logistic regression model which is shown in Figure 24, displaying the anti-radical effectiveness against the concentrations of the evaluated extracts. From all the values in the Trolox dose-response graphed, three were indicated in the curve in red points, two of them from average experimental coordinates (0.03 mg/mL; 49.22 %), (0.05 mg/mL; 70.27 %), and the EC_{50} computed values presenting the half maximum effective concentrations of the Trolox antioxidant activity.

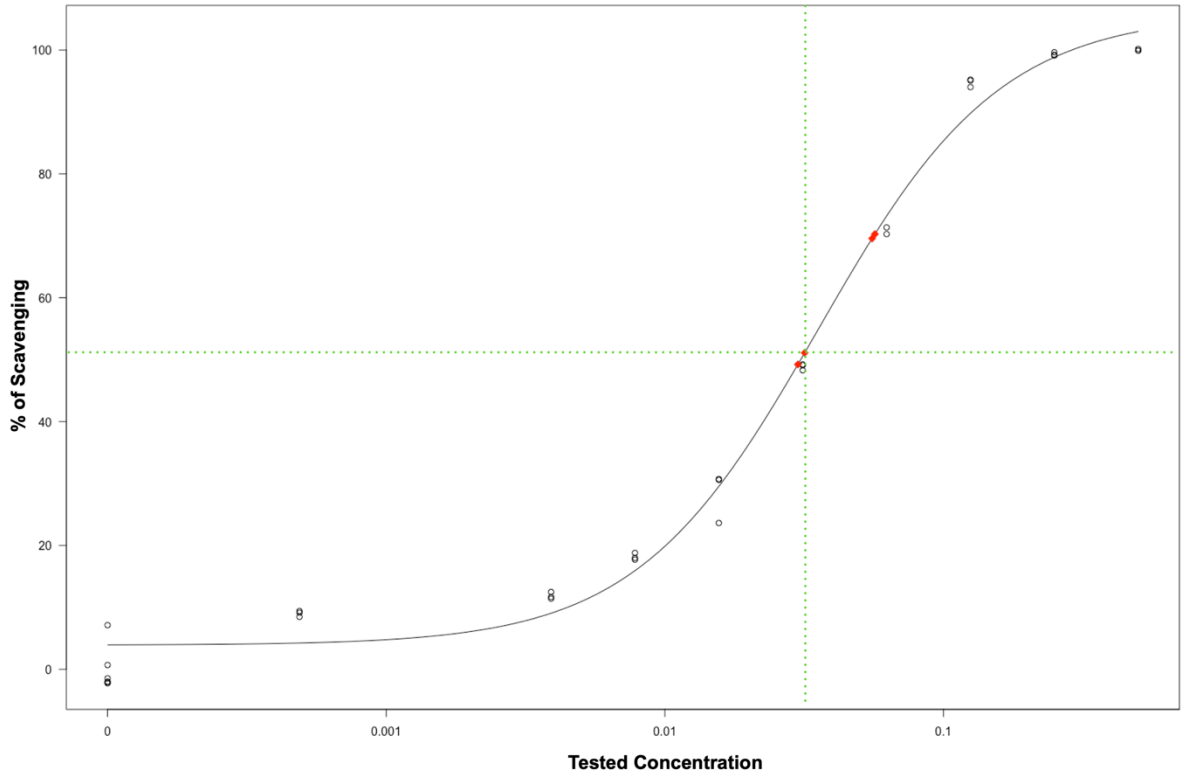


Figure 24: Trolox equivalents dose-response and EC50 four-parameter log-logistic regression model of DPPH test.

With the model derived from the Trolox dose-response using the four-parameter log-logistic regression model, experimental results will be interpolated within this experimental range, allowing an immediate comparison with Trolox equivalents. This interpolation provides a better fit in accordance with the generated dose-response model, eliminating the need for Lagrange approximation. This approach considers both the minimum and maximum values provided by the positive and negative controls. As a result, the obtained measurements are reliable representations of the actual values and should not be construed as theoretical values, particularly in the cases of 0% and 100% responses. The supplement data is in Table S4 (Supplement Material).

4.4 Adapted CCD for RSM modeling

4.4.1 CCD modeling

Based on the preceding experimental work, specific responses were designated for data collection. These responses encompassed DPPH, TPC, extraction yield, as well as two additional critical responses, namely Total Carotenoids Content (TCC) and Chlorophyll A content. The inclusion of TCC and Chlorophyll A content in our analysis is particularly

noteworthy due to their color-related properties, which are of relevance in assessing bioactivity. These chosen responses collectively provide a comprehensive overview of the chemical and color characteristics of the samples under investigation, enhancing the depth and breadth of the scientific analysis. The summary of the experimental design and results is concisely depicted in Tables 11 and 12 (for both methanol and acetone), exhibiting the mean average of responses per run (n = 3) along with their corresponding standard deviations. It is noteworthy that all measurements indicate coefficient of variation (CV) values below 1%, underscoring the precision and consistency of the data. To maintain clarity and brevity, a comprehensive listing of all complete cases is provided in the supplementary material, ensuring a streamlined presentation of the essential findings.

Table 11: Adapted Central Composite Design of 4 factors and 5 responses for methanol

Exp run	Block	Design point	X1: Time (min)	X2: Solvent (%)	X3: pH	X4: Solv	Y1: Carotenoids (µg/mL)	Y2: Chlorophyl A (µg/mL)	Y3: Trolox eq. (µg/mL)	Y4: Galic acid Eq. (mg/mL)	Y5: Extractions yield (%)
1	Block 1	Factorial	120	80	5	Methanol	1.69 ± 0.11	1.96 ± 0.1	16.71 ± 2.41	74.97 ± 30.38	52.5
2	Block 1	Factorial	120	66	5	Methanol	0.32 ± 0.02	0.63 ± 0.02	18.01 ± 5.13	91.62 ± 28.64	60.1
3	Block 1	Factorial	120	80	5	Methanol	1.79 ± 0.22	2.17 ± 0.23	36.07 ± 0.98	61.02 ± 2.35	60.4
4	Block 1	Factorial	120	80	8	Methanol	3.27 ± 0.02	4.34 ± 0.05	50.74 ± 4.41	54.39 ± 3.72	59.3
5	Block 1	Factorial	240	66	5	Methanol	0.51 ± 0.01	1.06 ± 0.02	17.45 ± 1.48	73.61 ± 11.06	55.4
6	Block 1	Factorial	240	66	5	Methanol	0.39 ± 0.02	0.79 ± 0.04	30.9 ± 7.71	126.56 ± 12.82	74.4
7	Block 1	Factorial	240	66	8	Methanol	0.63 ± 0.04	1.15 ± 0.06	26.56 ± 2.65	82.28 ± 3.38	70.9
8	Block 1	Factorial	120	66	8	Methanol	0.61 ± 0.02	1.28 ± 0.03	19.33 ± 1.49	73.21 ± 6.6	74.8
9	Block 1	Factorial	120	66	8	Methanol	0.69 ± 0.03	0.97 ± 0.04	28.63 ± 0.42	92.3 ± 9.09	74.8
10	Block 1	Factorial	240	80	5	Methanol	1.79 ± 0.13	2.57 ± 0.15	22.4 ± 4.79	106.25 ± 2.08	67.7
11	Block 1	Factorial	240	80	8	Methanol	1.80 ± 0.05	2.3 ± 0.05	48.01 ± 4.85	78.49 ± 16.58	65.9
12	Block 1	Factorial	120	80	8	Methanol	2.61 ± 0.13	2.61 ± 0.11	94.84 ± 9.65	129.67 ± 19.63	62.4
13	Block 1	Factorial	240	66	8	Methanol	0.48 ± 0.04	0.94 ± 0.07	15.64 ± 1.03	69.01 ± 1.86	73.6
14	Block 1	Factorial	240	80	5	Methanol	2.14 ± 0.65	3.01 ± 0.8	15.05 ± 4.31	70.37 ± 4.01	62.1
15	Block 1	Factorial	240	80	8	Methanol	1.84 ± 0.17	2.87 ± 0.27	20.14 ± 1.22	60.89 ± 7.75	56.6
16	Block 1	Factorial	120	66	5	Methanol	0.43 ± 0.04	0.78 ± 0.06	21.43 ± 1.12	71.85 ± 26.35	68.8
17	Block 1	Center	180	73	6.5	Methanol	0.48 ± 0.01	1.08 ± 0.02	61.39 ± 5.01	82.42 ± 42.36	67.3
18	Block 1	Center	180	73	6.5	Methanol	0.37 ± 0.04	0.8 ± 0.08	30.54 ± 7.6	83.63 ± 15.69	63.4
19	Block 2	Axial	180	73	9	Methanol	1.53 ± 0.15	2.34 ± 0.21	35.83 ± 2.94	113.15 ± 7.22	63.9
20	Block 2	Axial	79	73	6.5	Methanol	0.73 ± 0.02	1.19 ± 0.03	53.43 ± 9.78	111.8 ± 8.62	64.2
21	Block 2	Axial	281	73	6.5	Methanol	0.48 ± 0.04	1.08 ± 0.1	25.83 ± 0.95	69.55 ± 2.7	65.2
22	Block 2	Axial	180	85	6.5	Methanol	1.13 ± 0.36	1.56 ± 0.44	32.07 ± 7.75	91.35 ± 23.82	56.6
23	Block 2	Center	180	73	6.5	Methanol	0.48 ± 0.02	1.03 ± 0.05	48.62 ± 2.72	113.56 ± 12.68	65.6
24	Block 2	Center	180	73	6.5	Methanol	1.19 ± 0.01	2.21 ± 0.04	78.34 ± 9.23	103.95 ± 31.48	63.6
25	Block 2	Axial	180	61	6.5	Methanol	0.39 ± 0.05	0.69 ± 0.08	45.32 ± 7.31	86.88 ± 11.04	73.7
26	Block 2	Axial	180	73	4	Methanol	0.71 ± 0.04	1.21 ± 0.08	35.64 ± 7.57	159.46 ± 61.28	68.9

Table 12: Adapted Central Composite Design of 4 factors and 5 responses for acetone

Exp run	Block	Design point	X1: Time (min)	X2: Solvent (%)	X3: pH	X4: Solv	Y1: Carotenoids (µg/mL)	Y2: Chlorophyl A (µg/mL)	Y3: Trolox eq. (µg/mL)	Y4: Galic acid Eq. (mg/mL)	Y5: Extractions yield (%)
27	Block 3	Factorial	120	80	5	Acetone	2.63 ± 0.84	5.63 ± 2.16	14.14 ± 7.88	141.85 ± 23.42	52.3
28	Block 3	Factorial	240	66	5	Acetone	0.89 ± 0.07	1.9 ± 0.16	16.54 ± 11.08	94.44 ± 5.56	65.4
29	Block 3	Factorial	120	80	5	Acetone	2.78 ± 0.31	7.19 ± 0.81	22.58 ± 0.31	113.67 ± 4.18	47.5
30	Block 3	Factorial	240	80	8	Acetone	5.7 ± 0.12	11.57 ± 0.15	48.52 ± 9.32	134.35 ± 6.85	39.8
31	Block 3	Factorial	120	66	8	Acetone	2.89 ± 0.4	4.04 ± 0.69	16.76 ± 13.73	109.92 ± 6.03	47.4
32	Block 3	Center	180	73	6.5	Acetone	3.33 ± 0.04	7.23 ± 0.17	27.3 ± 0.32	108.13 ± 13.55	46.9
33	Block 3	Factorial	240	80	8	Acetone	5.85 ± 0.59	12.89 ± 1.23	22.76 ± 0.34	118.72 ± 40.92	41.5
34	Block 3	Factorial	240	66	5	Acetone	1.35 ± 0.28	2.66 ± 0.53	18.9 ± 0.69	70.5 ± 4.54	65.6
35	Block 3	Factorial	240	80	5	Acetone	1.31 ± 0.17	3 ± 0.54	2.32 ± 1.39	83.04 ± 1.85	50.0
36	Block 3	Factorial	120	80	8	Acetone	5.03 ± 0.03	10.32 ± 0.11	37.53 ± 15.78	127.35 ± 6.84	41.1
37	Block 3	Center	180	73	6.5	Acetone	2.79 ± 0.19	6.18 ± 0.39	21.19 ± 0.41	99.33 ± 8.18	46.2
38	Block 3	Factorial	120	66	5	Acetone	1.55 ± 0.1	2.85 ± 0.15	14.32 ± 1.48	52.42 ± 9.59	72.4
39	Block 3	Factorial	120	66	8	Acetone	2.4 ± 0.1)	3.6 ± 0.33	6.72 ± 3.2	64.96 ± 5.27	47.6
40	Block 3	Factorial	240	66	8	Acetone	5.05 ± 0.16	8.33 ± 0.24	17.52 ± 8.54	61.54 ± 13.02	49.2
41	Block 3	Factorial	240	80	5	Acetone	1.53 ± 0.12	3.65 ± 0.31	20.64 ± 0.7	76.36 ± 20.27	48.9
42	Block 3	Factorial	240	66	8	Acetone	3.53 ± 0.43	5.7 ± 0.66	30.24 ± 1	70.34 ± 5.73	47.1
43	Block 3	Factorial	120	80	8	Acetone	3.47 ± 0.38	7.51 ± 1.23	10.84 ± 6.64	58.77 ± 24.96	39.3
44	Block 3	Factorial	120	66	5	Acetone	1.73 ± 0.1	2.87 ± 0.16	13.67 ± 10.36	47.21 ± 33.07	65.3
45	Block 4	Axial	281	73	6.5	Acetone	2.93 ± 0.28	5.79 ± 0.53	23.27 ± 1.43	85 ± 3.67	50.9
46	Block 4	Axial	180	85	6.5	Acetone	3.22 ± 0.71	8.21 ± 1.74	24.22 ± 4.96	105.52 ± 3.67	42.2
47	Block 4	Center	180	73	6.5	Acetone	2.94 ± 0.25	6.06 ± 0.95	12.31 ± 0.16	119.69 ± 3.8	45.7
48	Block 4	Axial	79	73	6.5	Acetone	3.42 ± 0.16	7.47 ± 0.37	23.57 ± 11.71	112.69 ± 12.96	45.2
49	Block 4	Axial	180	61	6.5	Acetone	0.82 ± 0.08	1.42 ± 0.13	13.41 ± 0.57	53.56 ± 13.3	73.6
50	Block 4	Axial	180	73	9	Acetone	3.44 ± 0.57	5.79 ± 0.78	62.64 ± 15.92	100.8 ± 1.72	41.9
51	Block 4	Axial	180	73	4	Acetone	2.32 ± 0.13	5.49 ± 0.65	2.54 ± 1.6	84.83 ± 3.2	58.0
52	Block 4	Center	180	73	6.5	Acetone	4.62 ± 0.66	10.73 ± 1.69	14.5 ± 11.79	78.16 ± 9.15	45.7

4.4.2 Response surface methodology and exploratory data analysis

Due to the presence of two co-dependent factors in the experimental design, namely solvent percentage, and solvent pH, which cannot be separated, the results have been proceeded in a unified manner. However, it is essential to be aware of the potential impact this interdependence may have on result interpretation. To address this, the results analysis was augmented with specific statistical methods within an extensive Data Exploratory Analysis (EDA). Consequently, the discussion of each response factor considers both RSM and EDA, synergistically enhancing the resolution and comprehensiveness of our experimental design.

The aim of this study extends beyond the optimization of the extraction process; it encompasses the comprehensive interpretation of results to make well-founded decisions when approaching recommendations for optimal ranges. Due to the biological nature of the samples and the extensive range of experimentation, some of the models constructed may not consistently exhibit predictive capabilities. Nonetheless, they serve to provide valuable insights into general behavior and trends. For this reason, the subsequent sections present the complete process of analysis, integrating multiple specific statistical tests, along with the potential transformation of the dataset to view responses from the most advantageous perspectives. This approach ensures a thorough and informed assessment of the experimental outcomes.

4.4.2.1 Total carotenoid content

The total carotenoid content (TCC) of the 52 experimental runs was subjected to a "Square Root" transformation with λ (lambda) set to 0.5. This transformation involves a mathematical operation where each data point is replaced by the square root of that data point. It is a common technique used to align data with the assumptions of normality and is particularly beneficial when the data distribution exhibits right-skewness, where larger values exert a pronounced influence on the response, as observed in the data collected for acetone solvent. By implementing this transformation, the impact of extreme values was mitigated, thereby bringing the data closer to normal distribution. This not only ensures that the data conforms to statistical assumptions but also enhances the analytical and interpretive capabilities. The transformed dataset facilitates a more meaningful analysis of the underlying patterns and relationships, ultimately improving the reliability of the findings in scientific investigation.

Table 13: Response Surface Methodology statistics of TCC.

Fit summary					
Source	**Model *p-value***	**Lack of Fit *p-value***	**Adjusted R^2**	**Predicted R^2**	
Design Model	< 0.0001	0.007	0.8489	0.7103	Recommended
Linear	< 0.0001	0.0002	0.7408	0.7076	
2FI	**0.0541**	**0.0005**	**0.7773**	**0.7222**	**Suggested**
Quadratic	0.4628	0.0004	0.7752	0.6846	
Cubic	0.0139	0.0035	0.8489	0.5294	Aliased

ANOVA for Reduced 2FI model						
Source	**Sum of Squares**	**df**	**Mean Square**	**F-value**	***p-value***	
Model	11.0014927	5	2.200	36.085	<0.0001	Significant
A-Time	0.017039553	1	0.017	0.279	0.60	
B-Percentage	2.631537115	1	2.632	43.158	<0.0001	
C-pH	1.626353355	1	1.626	26.673	<0.0001	
D-Solvent	6.232952869	1	6.233	102.222	<0.0001	
CD	0.493609808	1	0.494	8.095	0.007	
Residual	2.804841964	46	0.061			
Lack of Fit	2.298184593	24	0.096	4.158	0.0007	Significant
Pure Error	0.50665737	22	0.023			
Cor Total	13.80633466	51				

Fit Statistics						
Std. Dev.	0.247		**R^2**	0.797		
Mean	1.332		**Adjusted R^2**	0.775		
C.V. %	18.545		**Predicted R^2**	0.741		
			Adeq Precision	19.96		

Final Equation in Terms of Coded Factors
Sqrt (TCC) = 1.33 - 0.02*A + 2.5*B + 0.19*C + 0.35*D + 0.1*CD

Table 13 provides a comprehensive summary of information starting from the transformation in the Fit summary section. It reveals that a model equal to or greater than the quadratic model would be unfavorable. Therefore, the selection of the previous model becomes a more favorable choice. As a result, the suggestion of a linear model with a two-factor interaction (2FI) stands out as a preferable option. It's worth noting, however, that the data presented in this summary consider all possible terms. By reducing terms and considering hierarchical terms with *p-values* less than 0.05, the model was further refined. Consequently, both the ANOVA for reduced 2FI and Fit statistics show statistical improvements. For instance, in the reduced model, a significant enhancement in the *p-value* for the "Model" term was observed, along with improved coefficients of determination (R-squared values). On the other

hand, the "Lack of Fit" remains significant, indicating that while the constructed model is generally valid and explains data trends well, it may not be perfect, and there are areas where it doesn't fit as well.

Like multiple linear regression, in Response Surface Methodology (RSM), it is crucial to focus on R-squared adjusted (R^2adj) and, additionally, on predictive R-squared (R^2pred). The difference between these two values should be less than 0.2 to ensure good predictive performance. This criterion is met for the TCC response.

Finally, it's essential to highlight that the *p-values* of the terms in the ANOVA section indicate their importance. Terms with *p*-values equal to or less than 0.05 are considered significant, and the smaller the *p*-value, the greater the importance of that term (factor or interaction) in the development of the final equation. This equation is presented in coded form at the end of the table, so the ranges and factors to be used should fall within the limits analyzed in this experimental design.

After presenting the numerical data, visual representations complement the information and to discern trends that may be more challenging to grasp through numerical analysis alone. In Figure 25, a dashboard comprising four graphs that display the behavior of the various experimental runs concerning Total Carotenoids Content (TCC) was provided. These graphs, resulting from a combination of RSM and EDA, are numerous, but the four most pertinent ones were selected to convey the discovered insights. In Figure 25-A), the normality plot of residuals is presented, a pivotal depiction following the data transformation via a square root with $\lambda = 0.5$. This plot allows to observe how most data aligns with the straight line that characterizes data normality. Following such transformation, a more pronounced alignment of the most extreme values with this line was achieved, which allows to keep forward the RSM.

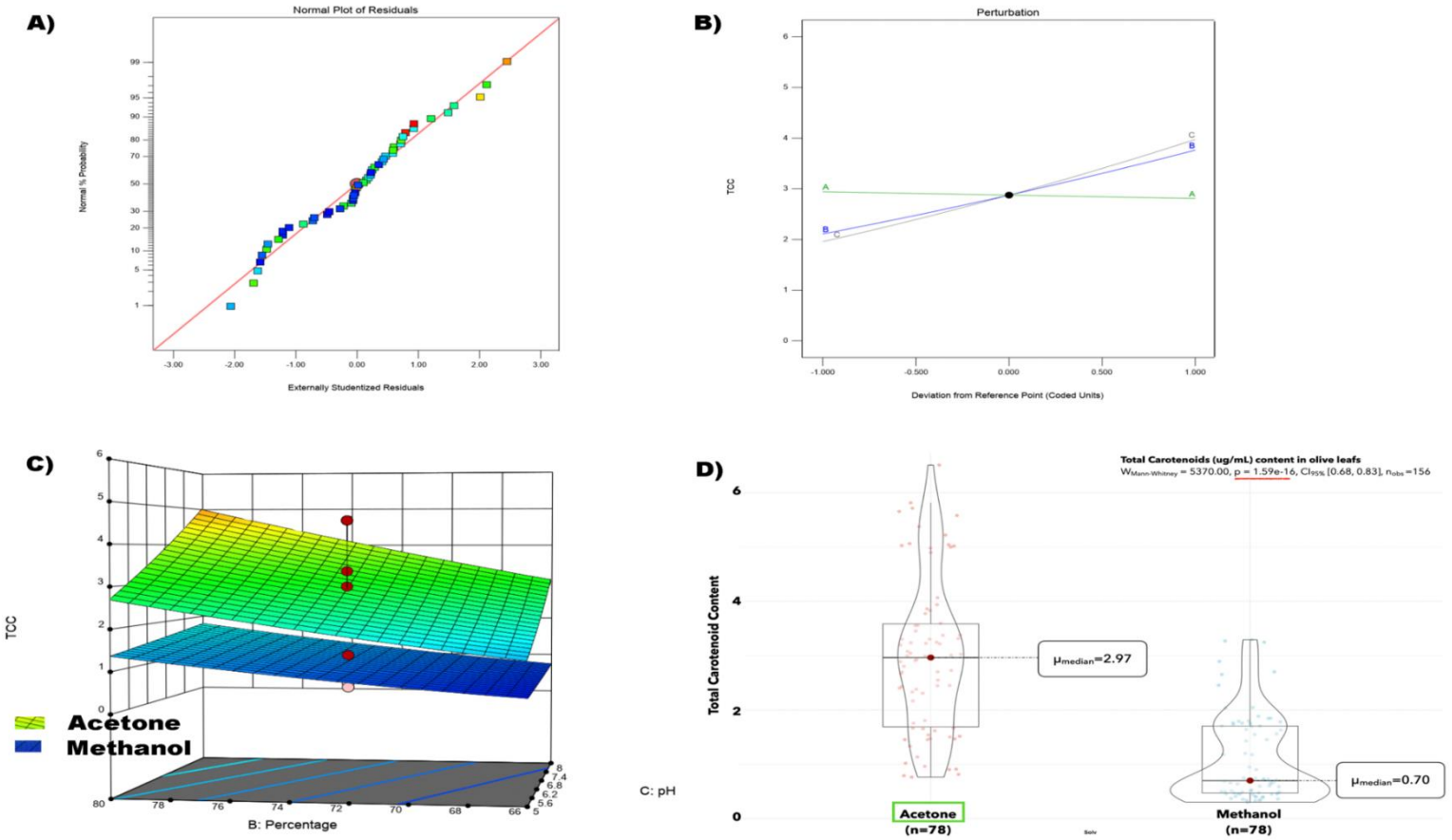


Figure 25: TCC Dashboard including a) Externally Studentized Residuals plot, b) Perturbation plot, c) RSM of methanol and acetone with fixed time and d) Violin, scatter and boxplot with statistical values and significance. .

Figure 25-B) introduces the perturbation plot, chosen for its ability to simplify the behavior of each factor, gathering three graphs into one. It showcases that Factor A (extraction time) exhibits an insignificant effect, with a constant behavior across the tested levels. In contrast, for Factors B and C (solvent percentage and solvent pH, respectively), it was observed that as the tested ranges increase, the TCC extraction levels also increase. This information mirrors the *p-values* in the ANOVA table, emphasizing that the most significant factor is solvent pH. Higher solvent percentages and pH values lead to greater TCC recovery. To delve into the individual behavior of each solvent, Figure 25-C) presents the response surface of both solvents used, with the extraction time fixed at 0 (180 minutes). The X and Y axes depict the different levels of solvent percentage and solvent pH, while the response surfaces for methanol and acetone have been superimposed. Methanol is represented in blue, and acetone in green. While the differences between the two solvents were observed, statistical significance is not apparent until Figure 25-D) was presented. Here, untransformed observations are overlaid with a violin plot, allowing to discern the non-parametric distribution in both cases. Additionally, a box plot is superimposed to easily visualize the mean of each solvent, as well as the skewness of each population. Essential statistical values are also provided numerically, along with the non-parametric Mann-Whitney test, revealing that the differences are highly significant, as evidenced by a *p-value* much lower than 0.05. This is evident because the median (n=78) of acetone, 2.97 µg/mL of TCC, is significantly higher than the median (n=78) of methanol, 0.70 µg/mL of TCC.

If TCC were the sole focus of this study, the choice of acetone as the extraction solvent would be straightforward. It is needed to navigate the design to identify the optimal conditions for maximizing compound recovery. However, this study considers other responses, so it is needed to describe the rest of the responses before providing specific recommendations. All the EDA for total carotenoids is provided in Table S6.

4.4.2.2 Chlorophyll a content

The criteria for analyzing various responses in RSM remain consistent, resulting in the same data order. However, the focus here is to present the key findings on each response, complementing the overall understanding of the extractions and the methodologies used. Regarding chlorophylls, it is noting that, according to Table 14, their behavior is relatively like the transformation of TCC. However, the chlorophyll data has undergone transformation using the mathematical function "Natural Log," which corresponds to the natural logarithm with base

"e" (Euler's number). This transformation is applied to stabilize the variance of the data, especially when data at higher values exhibit exponential growth.

The data from the fit summary reveals no apparent interaction between the factors, making a linear model sufficient. This straightforward model, as indicated by the final terms in the equation, highlights that factors D and B (solvent type and solvent percentage) are the most influential regarding the extractability of chlorophylls. The graphs in Figure 26 clearly illustrate the previously described observations from Table 14. In Figure 26-A), the plot demonstrates that the alignment of experimental data with respect to predicted values is notably precise at lower concentrations, while as values increase, they tend to exhibit greater dispersion. Figure 26-B) summarizes the trends of the two most influential factors (Factors D and B). Although no interaction is observed between these factors within the tested experimental range, which helps to visualize that the highest extraction of chlorophylls occurs when acetone is used. Furthermore, at higher concentrations of this solvent, the variance is considerably greater. Consequently, more polar solvents like acetone at higher purity levels aid in achieving higher chlorophyll values. This observation is once again corroborated in the statistical graph, Figure 26-C), where the median of acetone, 5.95 µg/mL, is supported by an extremely small *p*-value, which show greater statistically significancy than the median of methanol, 1.21 µg/mL. All the EDA for chlorophyll a is provided in Table S6.

Table 14: Response Surface Methodology statistics of Chlorophyll a content.

Fit summary					
Source	**Model *p-value***	**Lack of Fit *p-value***	**Adjusted R^2**	**Predicted R^2**	
Design Model	**< 0.0001**	**0.0584**	**0.8871**	**0.7957**	Recommended
Linear	**< 0.0001**	**0.0085**	**0.8393**	**0.8206**	**Suggested**
2FI	0.6897	0.0044	0.8318	0.7939	
Quadratic	0.2065	0.0052	0.8388	0.779	
Cubic	0.0326	0.0259	0.8824	0.6721	Aliased

ANOVA for Linear model						
Source	**Sum of Squares**	**Df**	**Mean Square**	**F-value**	***p-value***	
Model	31.77	4	7.94	67.58	< 0.0001	significant
A-Time	0.0002	1	0.0002	0.0016	0.9683	
B-Percentage	7.41	1	7.41	63.04	< 0.0001	
C-pH	1.99	1	1.99	16.91	0.0002	
D-Solvent	22.37	1	22.37	190.35	< 0.0001	
Residual	5.52	47	0.1175			
Lack of Fit	4.21	25	0.1682	2.81	0.0085	significant
Pure Error	1.32	22	0.0599			
Cor Total	37.3	51				

Fit Statistics						
Std. Dev.	0.3428		**R^2**	0.8519		
Mean	1.01		**Adjusted R^2**	0.8393		
C.V. %	33.88		**Predicted R^2**	0.8206		
			Adeq Precision	25.4263		

Final Equation in Terms of Coded Factors

Ln (Chlorophyll a) = 1.01 - 0.002*A + 0.41*B + 0.21*C + 0.66*D

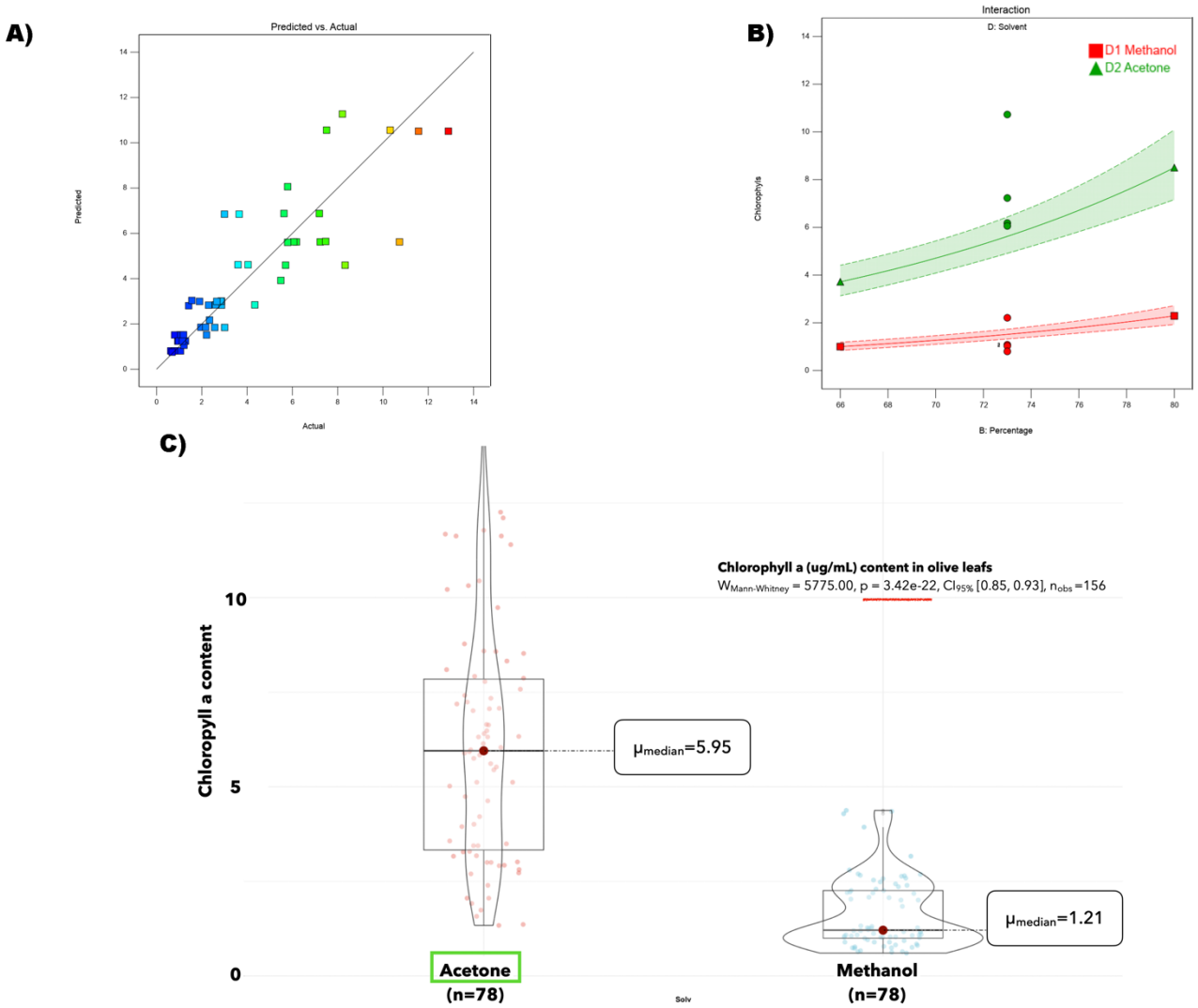


Figure 26: Chlorophyll a Dashboard including a) Predicted vs actual values plot, b) Interaction plot between solvent type and solvent porcentage, and c) Violin, scatter and boxplot with statistical values and significance.

4.4.2.3 Total phenolic content and DPPH responses

In this subsection, both responses have been presented together due to some similarities. For instance, the results of the models for DPPH (Table 15) and TPC (Table 16) are quite similar, and while a linear model is suggested for DPPH, and a quadratic model for TPC, in both cases, the *p*-values of the models are statistically significant. Additionally, the lack of fit is not statistically significant, indicating that the models fit the provided data. However, despite these seemingly contradictory combinations, both models reveal that the factors considered have an impact on the responses, implying some relationship between the factors and the responses. Nevertheless, the low correlation coefficients (R^2 values) indicate that the models are not capable of explaining the responses variability fully. This suggests that there may be other more relevant factors not considered in this experimental design. For this reason, these models can only be used for inferring results from the collected data, but they cannot be reliably used for prediction. Hence, final equations are not provided for either of the models.

Table 15: Response Surface Methodology statistics of DPPH Scavenging activity.

Fit summary						
Source	**Model *p-value***	**Lack of Fit *p-value***	**Adjusted R^2**	**Predicted R^2**		
Design Model	**0.0045**	**0.3184**	**0.406**	**-0.0515**	Recommended	
Linear	**0.0002**	**0.1885**	**0.3144**	**0.2297**	**Suggested**	
2FI	0.127	0.287	0.3766	0.1969		
Quadratic	0.3361	0.2928	0.384	0.1416		
Cubic	0.5641	0.1643	0.3635	-0.5726	Aliased	
ANOVA for Linear model						
Source	**Sum of Squares**	**df**	**Mean Square**	**F-value**	***p-value***	
Model	49.61	4	12.4	6.85	0.0002	significant
A-Time	1.04	1	1.04	0.5717	0.4534	
B-Percentage	4.09	1	4.09	2.26	0.1397	
C-pH	17.5	1	17.5	9.66	0.0032	
D-Solvent	26.99	1	26.99	14.9	0.0003	
Residual	85.13	47	1.81			
Lack of Fit	53.06	25	2.12	1.46	0.1885	Not significant
Pure Error	32.07	22	1.46			
Cor Total	134.74	51				
Fit Statistics						
Std. Dev.	1.35		**R^2**	0.3682		
Mean	5.06		**Adjusted R^2**	0.3144		
C.V. %	26.58		**Predicted R^2**	0.2297		
			Adeq Precision	8.7116		

Table 16: Response Surface Methodology statistics of TPC.

Fit summary						
Source	**Model *p-value***	**Lack of Fit *p-value***	**Adjusted R^2**	**Predicted R^2**		
Design Model	0.4013	0.1591	0.0334	-0.6313		
Linear	0.4171	0.1883	0	-0.1436		
2FI	0.1643	0.2614	0.0741	-0.2824		
Quadratic	**0.2181**	**0.3069**	**0.1097**	**-0.2787**	**Suggested**	
Cubic	0.5799	0.1694	0.0754	-0.8654	Aliased	
ANOVA for Quadratic model						
Source	**Sum of Squares**	**df**	**Mean Square**	**F-value**	***p-value***	
Model	0.2141	6	0.0357	2.82	0.0203	significant
A-Time	0.002	1	0.002	0.1616	0.6896	
B-Percentage	0.0592	1	0.0592	4.69	0.0357	
C-pH	7.95E-07	1	7.95E-07	0.0001	0.9937	
D-Solvent	0.0001	1	0.0001	0.0076	0.931	
BD	0.1065	1	0.1065	8.43	0.0057	
B^2	0.0462	1	0.0462	3.66	0.0623	
Residual	0.5683	45	0.0126			
Lack of Fit	0.2967	23	0.0129	1.04	0.4603	Not significant
Pure Error	0.2716	22	0.0123			
Cor Total	0.7824	51				
Fit Statistics						
Std. Dev.	0.1124		**R^2**	0.2736		
Mean	1.94		**Adjusted R^2**	0.1767		
C.V. %	5.79		**Predicted R^2**	0.026		
			Adeq Precision	7.6036		

On the other hand, the focus in these responses is primarily on the EDA, which is presented in Figure 27. Figure 27-A), exhibited that the median of methanol for the DPPH response (29.02 Trolox eq) is statistically greater than the median of acetone (19.96 Trolox eq), indicating that methanol is more effective for antioxidant capacity. Additionally, Figure 27-B)

reveals that there are no significant differences between the use of methanol and acetone for TPC extraction, suggesting that either solvent can be indiscriminately used for the recovery of these compounds. All the EDA for TPC and DPPH are provided in Table S7 and Table S8, respectively.

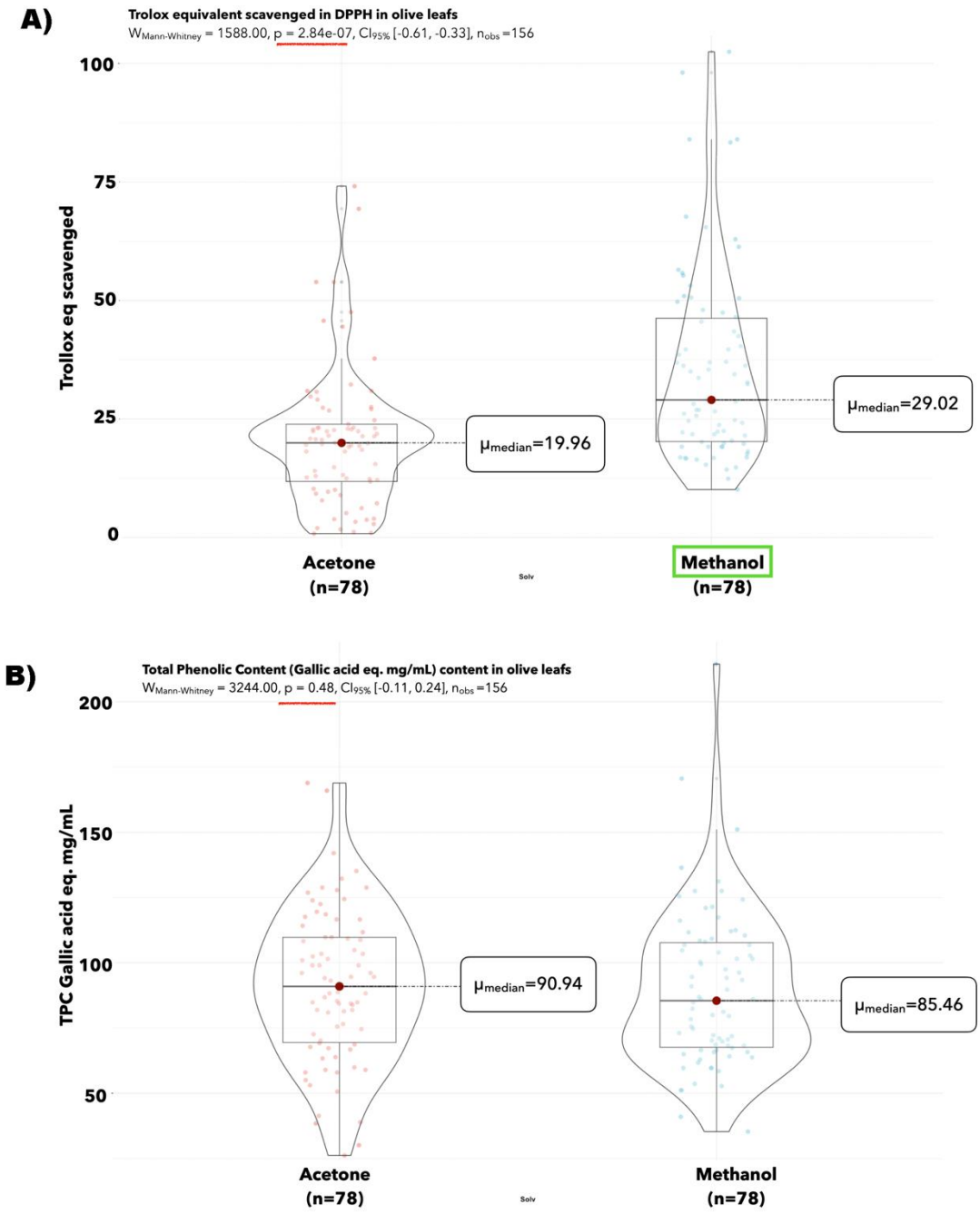


Figure 27: Violin, scatter and boxplot with statistical values and significance for A) DPPH and B) TPC.

4.4.2.4 Extraction yield

The extraction yield has been transformed using the "inverse" function, implying that there appears to be an opposing relationship between the factors and the extraction yield

response. The reciprocal transformation is one of the simplest transformations, in which the dataset numbers, denoted as "x," are reciprocally transformed as "1/x."

According to Table 17, the use of a two-factor interaction (2FI) model is suggested. Notably, in the ANOVA for the Reduced 2FI model, attention should be directed to the terms BD and CD. Furthermore, the *p*-value of the model is significant, while the lack of fit is not significant. In contrast to TPC and DPPH, the correlation values (R-squared) are considerably high. This indicates that not only does the model fit the data very well, but it also exhibits a high predictive capacity within the limits studied in this experiment.

Table 17: Response Surface Methodology statistics of Yield extraction.

Fit summary					
Source	**Model *p-value***	**Lack of Fit *p-value***	**Adjusted R^2**	**Predicted R^2**	
Design Model	**< 0.0001**	**0.4555**	**0.9089**	**0.8366**	Recommended
Linear	< 0.0001	0.0002	0.7215	0.6801	
2FI	**< 0.0001**	**0.1649**	**0.887**	**0.848**	**Suggested**
Quadratic	0.2983	0.1728	0.8892	0.8399	
Cubic	0.1275	0.3667	0.9067	0.7798	Aliased

ANOVA for Reduced 2FI model						
Source	**Sum of Squares**	**df**	**Mean Square**	**F-value**	***p-value***	
Model	0.0006	6	0.0001	72.45	< 0.0001	Significant
A-Time	1.37E-06	1	1.37E-06	0.994	0.3241	
B-Percentage	0.0001	1	0.0001	95.16	< 0.0001	
C-pH	0	1	0	36.2	< 0.0001	
D-Solvent	0.0003	1	0.0003	224.22	< 0.0001	
BD	0	1	0	16.52	0.0002	
CD	0.0001	1	0.0001	61.59	< 0.0001	
Residual	0.0001	45	1.38E-06			
Lack of Fit	0	23	1.57E-06	1.34	0.2454	Not significant
Pure Error	0	22	1.17E-06			
Cor Total	0.0007	51				

Fit Statistics						
Std. Dev.	0.0012		**R^2**	0.9062		
Mean	0.0179		**Adjusted R^2**	0.8937		
C.V. %	6.54		**Predicted R^2**	0.8722		
			Adeq Precision	27.1606		

Fit Statistics
1/(Yield) = 0.018 - 0.002*A + 0.001*B + 0.001*C + 0.002*D + 0.0007*BD + 0.001*CD

Figure 28 once again presents four graphs that will aid in the comprehension of the collected results. Beginning with Figure 28-A, which displays the perturbation plot of the three numerical factors. Factor A (extraction time) is again perpendicular to the X-axis, indicating its low statistical significance. Thus, if one is primarily interested in this response, shorter

extraction times could be chosen, extracting the same amount of compounds and resulting in significant energy and resource savings. Additionally, it is noteworthy that the magnitudes and negative directions of factors B and C are quite similar, suggesting the use of lower solvent percentages and lower pH values. Remembering that in the responses TCC and Chlorophyll a, the use of acetone led to higher compound extraction, it will be interesting to determine which compounds are primarily extracted by solvents at low percentages (high percentages of water). Typically, sugars are more akin to aqueous extractions. However, it's also worth mentioning that in DPPH section, methanol also performed well. Suggesting that some compounds with antioxidant properties are extracted by binary solvents like methanol-water, as observed in Figure 28-B. Figure 28-C presents the response surface of acetone with a fixed time value of 0 (180 minutes). This graph is display because it exbibits much greater variability with data close to 40% and nearly up to 80%. However, Figure 28-D highlights that despite this significant variability, most experimental values are skewed towards lower values, showing a median of 47.45%, which differs significantly from methanol's median of 64.70%, with much less dispersion. All the EDA for yield extract is provided in Table S9.

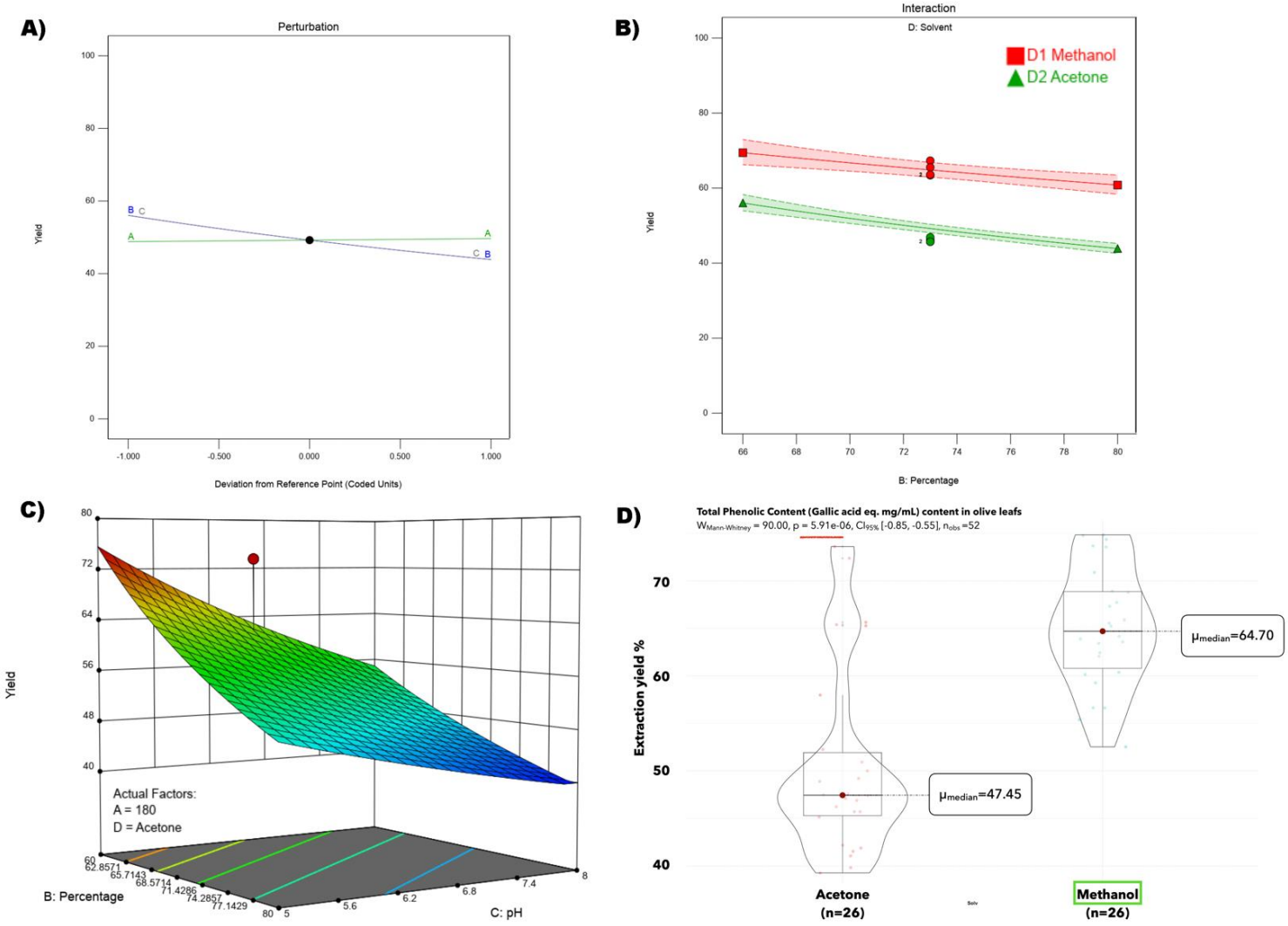


Figure 28: Extraction Yield Dashboard including a) Perturbation plot, b) 2 Factor interaction plot, c) RSM of acetone with fixed time and d) Violin, scatter and boxplot with statistical values and significance for both types of solvent extraction use

4.4.3 Total phenolic compounds estimation

As previously discussed in other sections, it's important to note that the goal of this study cannot be distilled into a single numeric value offering a one-size-fits-all solution to every olive leaf valorization problem. It was observed that, depending on the specific response variable under study, the extraction conditions studied tend to vary. Furthermore, considering the limited predictive power for the TPC and DPPH responses, there is a need for future work to delve into the factors that predominantly influence TPC and DPPH. Nonetheless, it is evident that to achieve higher concentrations of TCC and chlorophylls, extractions involving acetone are necessary. Lastly, the fact that the highest yield is obtained with methanol and low percentages of this same solvent leads to consider the possibility that the TPC values; in general, may not provide a clear explanation for why these extracts exhibit superior antioxidant activity, as reflected in the DPPH values.

Nevertheless, the selected parameters and values are presented in Table 18 to obtain a well-balanced extract, which could go to further studies. This extract prioritizes the reduction of extraction time since this factor was consistently found to be non-significant, while the other factors were not restricted, and all possible iterations between the studied values could be used. Regarding the response variables, the decision was made to maximize them, except in the cases of TPC and DPPH, for which our models demonstrated low predictive capacity. Therefore, these were allowed to vary within the experimentally obtained values.

Hence, the suggested optimization with a desirability value of 65.6% includes the following parameter values: Extraction time (A) = 120 minutes, Solvent percentage (B) = 78%, Solvent pH (C) = 5, and Solvent type = Acetone. This configuration results in an extraction that yields 2.51 μg/mL of TCC, 6.05 μg/mL of Chlorophyll A content, 18.4 μg $_{\text{Trolox eq.}}$/mL for the DPPH test, 108.72 μg$_{\text{gallic acid eq.}}$/mL for the TPC test, and an extraction yield of 50.7%.

Table 18: Goal setting and optimization values for olive leaf's by-products.

Name	Goal	Lower Limit	Upper Limit	Importance	Optimization values
A: Time	minimize	120	240	3	120
B: Percentage	is in range	66	80	3	77.832
C: pH	is in range	5	8	3	5
D: Solvent	is in range	Methanol	Acetone	3	Acetone
TCC	maximize	0.32	5.85	3	2.517
Chlorophylls	maximize	0.63	12.89	3	6.05
DPPH	is in range	2.32	94.84	3	18.409
TPC	is in range	47.21	159.46	3	108.726
Yield	maximize	39.27	74.84	3	50.715
Desirability					0.656

4.4.4 Future directions

The inability to create a fully predictive model for each response, coupled with the desire for a more specific attribution of bioactive compounds, necessitates the ongoing experimental development of this work. Therefore, the immediate next step is to perform the characterization, identification, and quantification of various phenolic compounds using high-performance liquid chromatography coupled with mass spectrometry. This approach aims to elucidate the compounds with the highest bioactivity. Subsequently, depending on the type of compounds identified, it will be essential to test the extracts with antioxidant methodologies that are more suited to the specific compound types. This will help provide a clearer profile that complements the information presented here.

Finally, the newly collected information should be leveraged to expand upon the existing experimental design. This expansion will aim to generate predictive models for all responses, incorporating higher coefficients and possible curvatures that can identify the inflection points of the studied responses more accurately.

5 Conclusions and future perspectives

The preliminary approach involved extracting olive tree leaf compounds through dynamic maceration with water displaying a TPC extraction yield of 79.6 mg of gallic acid equivalents per gram of extract and scavenging inhibition of 81.3% in the DPPH assay. Followed of a comparison of various extraction techniques, such as UAE, MAE, DE-1h and DE-3h using methanol as solvent and performing different test such as ABTS, DPPH, FRAP, and RP to evaluate antioxidant capacity and quantifying TPC as well. From where DE-3h extraction, excelled in antioxidant tests, and total phenolic compounds (TPC), although, DE-1h remains a reasonable choice for faster analysis providing higher values compare to de advance extraction technologies yielding on average 74.89 mg of gallic acid equivalents per gram of extract and scavenging inhibition of 80.8%.

As secondary objective the factor selection and creation of a predictive model for pH adjustment was performed using the McIlvaine buffer to adjust pH for Acetone and Methanol solvents, ensuring consistency and accuracy in our experiments. Creating a capable model through Multiple Linear Regression (MLR), achieving strong linear relationships, with R^2_{adj} values of 0.97 for Methanol and 0.93 for Acetone, and excellent accuracy of prediction.

The third broad objective consisted in the EC50 value determination improvement which was achieved through the implementation of positive and negative control, followed by Trolox dose-response modeling using a four-parameter log-logistic regression model. The final script is presented in the appendix-script 3, which will be useful for replication of this refined methodological improvement for other works, providing reliable measurements that accurately represent actual values, ensuring a more robust assessment of antioxidant activity in the DPPH assay.

And finally, better understanding of the factors employed on the experimental design, allows to create some predictive models for responses such as TCC, Chlorophyll A and extraction yield, as well as providing a global solution for optimization with a desirability value of 65.6% which includes the following parameter values: Extraction time (A) = 120 minutes, Solvent percentage (B) = 78%, Solvent pH (C) = 5, and Solvent type = Acetone. Such configuration resulted in an olive leaf extraction that yields 2.51 μg/mL of TCC, 6.05 μg/mL of Chlorophyll A content, 18.4 μg $_{\text{Trolox eq.}}$/mL for the DPPH test, 108.72 μg $_{\text{gallic acid eq.}}$/mL for the TPC test, and an extraction yield of 50.7%.

With the experimental design executed we were able to achieve the previously

mentioned optimization, but as well we can navigate through the design and point out towards better yielding of any of the responses collected, since higher values were achieved in the different experimental runs.

In terms of perspectives, for the understanding and improvement of various results, it is suggested the incorporation pf new factors into the model and studying their interactions.

After creating and confirming optimization models, identifying, characterizing, and quantifying phenolic compounds present in the different extracts using high performance liquid chromatography coupled with mass spectrometry (HPLC-MS) is proposed.

Additionally, other bioactive activities tests can be performed including ant-inflammatory activity, antimicrobial activity, antiproliferative activity and among others.

Finally, a SWOT analysis was suggested to assess the internal and external parameters in this project, as illustrated in the following Table (Table 19).

Table 19: SWOT analysis

INTERNAL FACTORS

STRENGTHS +	WEAKNESSES –
• Abundant Raw Material. • Sustainability. • Enhanced extraction efficiency. • Diverse compound analysis.	• Chemical solvent use. • Specificity of analysis.

EXTERNAL FACTORS

OPPORTUNITIES +	THREATS –
• Further optimization. • Exploration of alternative solvents. • Performance of HPLC-MS	• Competition with other companies (Animals feeding). • Higher cost after valorization.

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7 Supplement Material

R studio Script 1: Packages and theme call

```
rm(list = ls())     # clear objects
graphics.off()
#####################################
###### Sprectro data  #############
#####################################


# Packages ------------------------------------------------------------

inst <- function(pkg){
  new.pkg <- pkg[!(pkg %in% installed.packages()[, "Package"])]
  if (length(new.pkg))
    install.packages(new.pkg, dependencies = TRUE)
  sapply(pkg, require, character.only = TRUE)
}
packages <- c("tidyverse","cluster", "factoextra","NbClust","tidyr",
              "ggplot2", "ggpubr", "broom", "AICcmodavg", "ggcorrplot",
              "fpc","plot3D", "cluster", "readxl", "magrittr",
              "multipanelfigure","klaR","psych","MASS","ggord","devtools",
              "reshape2","RColorBrewer","SensoMineR","FactoMineR","stats","drc",
              "ggstatsplot", “dplyr”)
inst(packages)
theme_set(theme_minimal())
```

R studio Script 2: Screenign analysis

```
# Dataframe -----------------------------------------------------------
(df <- read_excel("Khaoula_20230509.xlsx",
                  sheet = "0 Summary"))


# Subsetting ----------------------------------------------------------
df_part1<-df[1:40,]
df_part2<-df[41:42,]


# Summary -------------------------------------------------------------
Antiox <- df_part1 %>%
  select(Concentration, Test, UAE, MAE, `DE 1h`, `DE 3h`) %>%
  group_by(Concentration, Test) %>%
  summarise(
    mean_UAE = mean(UAE),
    sd_UAE = sd(UAE),
    cv_UAE= (sd_UAE/mean_UAE)*100,
```

```
    mean_MAE = mean(MAE),
    sd_MAE = sd(MAE),
    cv_MAE = (sd_MAE/mean_MAE)*100,
    mean_DE1 = mean(`DE 1h`),
    sd_DE1 = sd(`DE 1h`),
    cv_DE1= (sd_DE1/mean_DE1)*100,
    mean_DE3 = mean(`DE 3h`),
    sd_DE3 = sd(`DE 3h`),
    cv_DE3= (sd_DE3/mean_DE3)*100
  )

## Printing ----------------------------------------------------------------
write.table(Antiox, "Antiox.csv", sep = ",")


# Plotting ----------------------------------------------------------------
## Subsetting ----------------------------------------------------------------
df1 <- Antiox[ , c(1:3,6,9,12)]
names(df1) <- c("Concentration","Test","UAE","MAE","DE1h","DE3h")


## Transforming and plot ----------------------------------------------------
### Faceting ----------------------------------------------------------------
(long <- df1 %>%
   pivot_longer(UAE:DE3h,names_to = "ext_type", values_to = "means") %>%
   ggplot(aes(x = Concentration, y = means, col = ext_type)) +
   facet_wrap(vars(Test), ncol = 2) +
   geom_point() +
   geom_line() +
   labs(
     title = "Antioxidant screening",
     x = "Tested concentrations",
     y = "Response mean values",
   ) +
   theme(
   plot.title = element_text(size = 16, face = "bold"),  # Title size and style
   axis.title.x = element_text(size = 14),              # Size X axis label
   axis.title.y = element_text(size = 14),              # Size Y axis label
   legend.title = element_text(size = 14, face = "bold"),            # Leyend title size
   strip.text = element_text(size = 14, face = "bold"), # Facet titles and sizes
   legend.text = element_text(size = 12)               # Legend text size
 ))



### TPC bars (means) ------------------------------------------------------
"""(bars<-df_part2 %>%
   pivot_longer(UAE:'DE 3h',names_to = "ext_type", values_to = "means") %>%
   ggplot(aes(ext_type, means, color = ext_type)) +
   geom_bar(stat = "summary") +
```

```
  theme(strip.text.x = element_text(size = 16, colour = "black", angle = 0)) +
  theme_minimal() +
  labs(
    title = "Total phenolic content screening",
    x = " ",
    y= "mg Eq Gallic Acid/g dry olive leaves"
  ) +
  scale_y_continuous(limits = c(0, 40), breaks = seq(0, 40, 5)) +
  theme(plot.title = element_text(size = 16, face = "bold"),  # Title size and style
        axis.title.y = element_text(size = 14),             # Size Y axis label
        legend.title = element_text(size = 14, face = "bold"),            # Leyend title size
        legend.text = element_text(size = 12)              # Legend text size
))
"""



# Calculate standard deviations for each ext_type
df_part2_summary <- df_part2 %>%
  pivot_longer(UAE:'DE 3h', names_to = "ext_type", values_to = "means") %>%
  group_by(ext_type) %>%
  summarize(mean_means = mean(means), sd_means = sd(means))

(bars <- ggplot(df_part2_summary, aes(ext_type, mean_means, col = ext_type)) +
    geom_bar(stat = "identity") +

    # Add error bars using geom_errorbar
    geom_errorbar(
      aes(ymin = mean_means - sd_means, ymax = mean_means + sd_means),
      width = 0.2,                    # Adjust the width of the error bars
      position = position_dodge(0.9)  # Adjust the dodge position
    ) +

    theme_minimal() +
    labs(
      title = "Total phenolic content screening",
      x = " ",
      y= "mg Eq Gallic Acid/g dry olive leaves"
    ) +
    scale_y_continuous(limits = c(0, 40), breaks = seq(0, 40, 5)) +
    theme(
      plot.title = element_text(size = 16, face = "bold"),  # Title size and style
      axis.title.y = element_text(size = 14),             # Size Y axis label
      legend.title = element_text(size = 14, face = "bold"), # Legend title size
      legend.text = element_text(size = 12),              # Legend text size
      legend.position="None"
    ))

# Significance ----------------------------------------------------------
## ANOVA -----------------------------------------------------------------
(long2<-df_part2 %>%
```

```
   pivot_longer(UAE:'DE 3h',names_to = "ext_type", values_to = "means"))
modelo_anova <- aov(means ~ ext_type, data = long2)
(t2<-summary(modelo_anova))
### Printing ------------------------------------------------------------
capture.output(t2, file = "t2.csv")

## Post hoc --------------------------------------------------------------
tukey <- TukeyHSD(modelo_anova)
summary(tukey)

### Dual comparison -----------------------------------------------------
(t1<-tukey[["ext_type"]])
### ploting -------------------------------------------------------------
plot(tukey)
### Printing ------------------------------------------------------------
write.table(t2, "t2.csv", sep = ",")
```

R studio Script 3: Multiple Linear Regression

```
# Dataframe ------------------------------------------------------------
(df <- read_excel("pH_solv.xlsx"))

# Subsetting -----------------------------------------------------------
(Met <- df %>%
  filter(Solvent == "Methanol") %>%
    dplyr::select(Buffer_pH:pH))
(Acet <- df %>%
    filter(Solvent == "Acetone") %>%
    dplyr::select(Buffer_pH:pH))

# First visualization --------------------------------------------------
(p1 <- Met %>%
   ggplot(aes(Buffer_pH, pH, col = Solv_perc)) +
   geom_point() +
   geom_smooth())
(p2 <- Acet %>%
   ggplot(aes(Buffer_pH, pH, col = Solv_perc)) +
   geom_point() +
   geom_smooth())

# Multiple regression --------------------------------------------------
model_m <- lm(pH ~ Buffer_pH + Solv_perc, data = Met)
summary(model_m)
model_a <- lm(pH ~ Buffer_pH + Solv_perc, data = Acet)
summary(model_a)

sm <- summary(model_m)$coefficient
sa <- summary(model_a)$coefficient

## Printing ------------------------------------------------------------
write.table(sm, "Estimates_m.csv", sep = ",")
write.table(sa, "Estimates_a.csv", sep = ",")

confint(model)

sigma(model)/mean(met$pH)
```

R studio Script 4: Experimental Design EDA

```
# Dataframes -----------------------------------------------------------
(TPC <- read_excel("Datos_Khaoula.xlsx", sheet = "TPC"))
(CnC <- read_excel("Datos_Khaoula.xlsx", sheet = "CnC"))
(Calib <- read_excel("Datos_Khaoula.xlsx", sheet = "DPPH_Calib"))
```

```
(DPPH <- read_excel("Datos_Khaoula.xlsx", sheet = "DPPH"))
(Yield <- read_excel("Datos_Khaoula.xlsx", sheet = "Yield"))


# ------------------------- #
#    1. Extraction Yield     #
#    1.1 Data glance         #
# ------------------------- #

Yield %>%
  ggplot(aes(Solv,Extractions_yield, fill = Solv)) +
  geom_boxplot(alpha = 0.8)

Yield %>%
  ggplot(aes(x=Extractions_yield, fill= Solv,col=Solv)) +
  geom_density(alpha=0.3)


# ------------------------- #
#    1.2 Statistics          #
# ------------------------- #

# Prueba de normalidad para el Solv "A"  ----------------------------------
shapiro.test(Yield$Extractions_yield[Yield$Solv == "Acetone"]) # Not Normal
distribution
# Prueba de normalidad para el Solv "M"  ----------------------------------
shapiro.test(Yield$Extractions_yield[Yield$Solv == "Methanol"]) # Normal distribution

# Prueba t de Student para comparar las medias (si los datos son normales) --
# t.test(interpolation ~ Solv, data = df)  #Both distributions are not normal distributions
# Prueba de Wilcoxon-Mann-Whitney para comparar las medianas (si los datos no son
normales) --
wilcox.test(Extractions_yield ~ Solv, data = Yield, paired = FALSE) #One distributions is
not a normal distributions and both are not with equal mean


# ------------------------- #
#    1.3 Visualization       #
# ------------------------- #

# Combine plot and statistical test with violin plots ----------------------
ggbetweenstats(
  Yield, Solv, Extractions_yield,
  type = "nonparametric",
  pairwise.comparisons = TRUE,
  pairwise.display = "all",
  p.adjust.method = "holm",
  effsize.type = "unbiased",
  title = "Extraction yield percentage of olive leafs with different solvents",
  package = "ggsci",
```

```
  palette = "nrc_npg"
)


# ---------------------------------- #
#    2. Total Phenolics              #
#    2.1 Subsetting and wrangling    #
# ---------------------------------- #

tpc.calib.met<-TPC[85:91,c(1:4,7)] %>%
  unite(ID,Solv:rep, sep = "_")
names(tpc.calib.met) <- c("ID","Concentration","Abs")

tpc.calib.acet<-TPC[176:181,c(1:4,7)] %>%
  unite(ID,Solv:rep, sep = "_")
names(tpc.calib.acet) <- c("ID","Concentration","Abs")

tpc.obs<-TPC[c(1:78,92:169),c(1:3,7)]
names(tpc.obs) <- c("Solv", "Run", "Rep", "Abs")

tpc.met<-TPC[1:78,c(1:3,7)]
names(tpc.met) <- c("Solv", "Run", "Rep", "Abs")

tpc.acet<-TPC[92:169,c(1:3,7)]
names(tpc.acet) <- c("Solv", "Run", "Rep", "Abs")


# ------------------------- #
#    2.2 Data glance        #
# ------------------------- #

tpc.obs %>%
ggplot(aes(Solv,Abs, fill = Solv)) +
  geom_boxplot(alpha = 0.8)

tpc.obs %>%
  ggplot(aes(x=Abs, fill= Solv,col=Solv)) +
  geom_density(alpha=0.3)


# ------------------------- #
#    2.3 Modeling           #
# ------------------------- #

# MetOH ---------------------------------------------------------------
lm.tpc.met<-lm(Abs ~ Concentration, data = tpc.calib.met)
lm.tpc.met
summary(lm.tpc.met)
plot(lm.tpc.met)
```

```
tpc.calib.met %>%
  ggplot(aes(Concentration,Abs)) +
  geom_point() +
  geom_smooth(method = 'lm', formula = y ~ x, size = 1)

# Acet ----------------------------------------------------------------
lm.tpc.acet<-lm(Abs ~ Concentration, data = tpc.calib.acet)
lm.tpc.acet
summary(lm.tpc.acet)
plot(lm.tpc.acet)

tpc.calib.acet %>%
  ggplot(aes(Concentration,Abs)) +
  geom_point() +
  geom_smooth(method = 'lm', formula = y ~ x, size = 1)


# ------------------------- #
#    2.4 Interpolation      #
# ------------------------- #

# MetOH ----------------------------------------------------------------
tpc.met$interpolation <- (tpc.met$Abs -
lm.tpc.met$coefficients[1])/lm.tpc.met$coefficients[2]

# Acet ----------------------------------------------------------------
tpc.acet$interpolation <- (tpc.acet$Abs -
lm.tpc.acet$coefficients[1])/lm.tpc.acet$coefficients[2]


# ---------------------------- #
#    2.5 Data Vizualization    #
# ---------------------------- #

# Stacking observations ------------------------------------------------
(tpc.obs.treated <- rbind(tpc.met, tpc.acet) %>%
  mutate(Galic_acid_Equiv_µg_mL = interpolation * 1000))


# Plotting -------------------------------------------------------------
tpc.obs.treated %>%
  ggplot(aes(x=Galic_acid_Equiv_µg_mL, fill= Solv,col=Solv)) +
  geom_density(alpha=0.3)


tpc.obs.treated %>%
  ggplot(aes(Solv,Galic_acid_Equiv_µg_mL, col=Solv)) +
  geom_violin(alpha=0.8) +
  geom_boxplot() +
  geom_jitter(alpha=0.3, width = 0.1, shape = 3) +
```

```
  labs(
    title = "Total phenolic content in olive leafs",
    subtitle = "From the solvents experimental designs",
    x= "Solvent used",
    y= "Galic acid equivalents ( µg/mL)") +
  scale_x_discrete(labels = c("Acetone", "Methanol"))


# ------------------------- #
#    2.6 Statistics         #
# ------------------------- #

# Subsetting  ------------------------------------------------------------
df <- tpc.obs.treated[,c(1,6)]

# Prueba de normalidad para el Solv "A"   --------------------------------
shapiro.test(df$Galic_acid_Equiv_µg_mL[df$Solv == "A"]) # Normal distribution
# Prueba de normalidad para el Solv "M"   --------------------------------
shapiro.test(df$Galic_acid_Equiv_µg_mL[df$Solv == "M"]) # Not Normal distribution

# Prueba t de Student para comparar las medias (si los datos son normales) ----
# t.test(interpolation ~ Solv, data = df)  #Both distributions are not normal distributions
# Prueba de Wilcoxon-Mann-Whitney para comparar las medianas (si los datos no son
normales) ----
wilcox.test(Galic_acid_Equiv_µg_mL ~ Solv, data = df, paired = FALSE)


# ------------------------- #
#    2.7 Statistical plot  #
# ------------------------- #

ggbetweenstats(
  df, Solv, Galic_acid_Equiv_µg_mL,
  type = "nonparametric",
  pairwise.comparisons = TRUE,
  pairwise.display = "all",
  p.adjust.method = "holm",
  effsize.type = "unbiased",
  title = "Total phenolic content in olive leafs",
  package = "ggsci",
  palette = "nrc_npg"
  )


# ------------------------------------ #
#    3. Chlorophyls and Carotenoids      #
#    3.1 Subsetting and wrangling        #
# ------------------------------------ #

# Data prep  ------------------------------------------------------------
```

```
cnc.obs<-CnC[ ,c(1:3,6:7)]
names(cnc.obs) <- c("Solv", "Run", "Rep", "Abs_470", "Abs_666")

# Calculations  ------------------------------------------------------------
cnc.obs$Chlorophyls <- cnc.obs$Abs_666*12.61 # (µg/mL)
cnc.obs$Carotenoids <- ((1000*cnc.obs$Abs_470)-(1.63*cnc.obs$Chlorophyls))/221 #
(µg/mL)

# Subsetting ---------------------------------------------------------------
chl.met<-cnc.obs[1:78,c(1:3,6)]
chl.acet<-cnc.obs[79:156,c(1:3,6)]
Carot.met<-cnc.obs[1:78,c(1:3,7)]
Carot.acet<-cnc.obs[79:156,c(1:3,7)]



# ------------------------- #
#    3.2 Data glance        #
# ------------------------- #

p1<- chl.met %>%
  ggplot(aes(x=Chlorophyls, fill= Solv, col=Solv)) +
  geom_density(alpha=0.3) +
  xlab("Chlorophyls (µg/mL)") +
  scale_colour_manual(values = "darkslategray4") +
  scale_fill_manual(values = "darkslategray4")

p2<- chl.acet %>%
  ggplot(aes(x=Chlorophyls, fill= Solv, col=Solv)) +
  geom_density(alpha=0.3) +
  xlab("Chlorophyls (µg/mL)")

p3<- Carot.met %>%
  ggplot(aes(x=Carotenoids, fill= Solv, col=Solv)) +
  geom_density(alpha=0.3) +
  xlab("Carotenoids (µg/mL)") +
  scale_colour_manual(values = "darkslategray4") +
  scale_fill_manual(values = "darkslategray4")

p4<- Carot.acet %>%
  ggplot(aes(x=Carotenoids, fill= Solv, col=Solv)) +
  geom_density(alpha=0.3) +
  xlab("Carotenoids (µg/mL)")

figboxs<-multi_panel_figure(columns = 2, rows = 2, panel_label_type = "upper-roman")
figboxs %<>%
  fill_panel(p1, column = 1, row = 1) %<>%
  fill_panel(p3, column = 2, row = 1) %<>%
  fill_panel(p2, column = 1, row = 2) %<>%
  fill_panel(p4, column = 2, row = 2)
figboxs
```

```
# ------------------------- #
#    3.3 Statistics         #
# ------------------------- #

# Prueba de normalidad para el Solv "A" en Chlorophyls ---------------------
shapiro.test(cnc.obs$Chlorophyls[cnc.obs$Solv == "A"]) # Not Normal distribution
# Prueba de normalidad para el Solv "M" en Chlorophyls ------------------
shapiro.test(cnc.obs$Chlorophyls[cnc.obs$Solv == "M"]) # Not Normal distribution
# Prueba de normalidad para el Solv "A" en Carotenoids ------------------
shapiro.test(cnc.obs$Carotenoids[cnc.obs$Solv == "A"]) # Not Normal distribution
# Prueba de normalidad para el Solv "M" en Carotenoids -------------------
shapiro.test(cnc.obs$Carotenoids[cnc.obs$Solv == "M"]) # Not Normal distribution

# Prueba t de Student para comparar las medias (si los datos son normales) ----
#t.test(Chlorophyls ~ Solv, data = cnc.obs)
#### Prueba de Wilcoxon-Mann-Whitney para comparar las medianas (si los datos no son
normales) ----
wilcox.test(Chlorophyls ~ Solv, data = cnc.obs, paired = FALSE) #Both distributions are
not normal distributions and are not with equal mean
wilcox.test(Carotenoids ~ Solv, data = cnc.obs, paired = FALSE) #Both distributions are
not normal distributions and are not with equal mean



# ------------------------- #
#    3.3 Stat plot          #
# ------------------------- #

# Combine plot and statistical test with violin plots for Chlorophyls -------
ggbetweenstats(
  cnc.obs, Solv, Chlorophyls,
  type = "nonparametric",
  pairwise.comparisons = TRUE,
  pairwise.display = "all",
  p.adjust.method = "holm",
  effsize.type = "unbiased",
  title = "Total Chlorophyl a (µg/mL) content in olive leafs",
  package = "ggsci",
  palette = "nrc_npg"
)

# Combine plot and statistical test with violin plots for Carotenoids  ------
ggbetweenstats(
  cnc.obs, Solv, Carotenoids,
  type = "nonparametric",
  pairwise.comparisons = TRUE,
  pairwise.display = "all",
  p.adjust.method = "holm",
  effsize.type = "unbiased",
```

```
  title = "Total Carotenoids (µg/mL)  content in olive leafs",
  package = "ggsci",
  palette = "nrc_npg"
)


# ------------------------- #
#    3.4 final DF prep       #
# ------------------------- #
# Stacking observations ---------------------------------------------------
chl.obs.treated <- rbind(chl.met, chl.acet)
carot.obs.treated <- rbind(Carot.met, Carot.acet)



# ------------------------------------- #
#    4. DPPH Calibration curve           #
#    4.1 Subsetting and wrangling         #
# ------------------------------------- #

# Controls prep -----------------------------------------------------------
PC.Calib <- Calib[grep("Positive", Calib$Content), c(1,4)] %>%
  summarise(Average = mean(`Raw Data (515)`))

NC.Calib <- Calib[grep("Negative", Calib$Content), c(1,4)] %>%
  summarise(Average = mean(`Raw Data (515)`))

names(Calib) <- c("ID", "Well", "Concent", "Abs_515")


# Scavenging calculation --------------------------------------------------
Calib <- Calib %>%
  mutate(Scav = (((Abs_515 - NC.Calib$Average)/(PC.Calib$Average -
NC.Calib$Average))*100))


# Model subsetting --------------------------------------------------------
(ss1 <-Calib %>%
   select(ID,Concent,Scav))

# Column format -----------------------------------------------------------
ss1$ID <- gsub(" ", "_", ss1$ID)
plot(ss1$Concent,ss1$Scav)


# ------------------------- #
#    4.2 Modeling  #
# ------------------------- #

mod1 <- drm(Scav ~ Concent,
```

```
          data = ss1, fct = LL.4(names = c("Slope", "Lower limit", "Upper limit", "EC50")))
plot(mod1)
plot(mod1, type = "all")
summary(mod1)


#  Model Selection ------------------------------------------------------------
mselect(mod1, fctList = list(W1.3(fixed=c(NA, 100, NA)),
                        W1.4(),
                        W2.3(fixed=c(NA, 100, NA)),
                        W2.4(),
                        LL.4()), linreg=TRUE)

#  Fit stats -----------------------------------------------------------------
coef(mod1)
plot(fitted(mod1), residuals(mod1))
hatvalues(mod1)
cooks.distance(mod1)
logLik(mod1)
plot(mod1, log="", main = "Logistic function")

# Subsetting for improved model ---------------------------------------------
ss2 <- ss1[c(1:9, 11:24, 31:33, 46:51),]
mod1.1 <- drm(Scav ~ Concent,
           data = ss2, fct = LL.4(names = c("Slope", "Lower limit", "Upper limit", "EC50")))
plot(mod1.1)
plot(mod1.1, type = "all")
summary(mod1.1)

#  Model Selection V.2 -------------------------------------------------------
mselect(mod1.1, fctList = list(W1.3(fixed=c(NA, 100, NA)),
                        W1.4(),
                        W2.3(fixed=c(NA, 100, NA)),
                        W2.4(),
                        LL.4()), linreg=TRUE)

####  Model comparison V.1 ----------------------------------------------------
mselect(mod1, fctList = list(W1.3(fixed=c(NA, 100, NA)),
                         W1.4(),
                         W2.3(fixed=c(NA, 100, NA)),
                         W2.4(),
                         LL.4()), linreg=TRUE)


# ---------------------------- #
#    4.3 Model interpolation    #
# ---------------------------- #

# Prep interpol terms mod1.1 ------------------------------------------------
S <- mod1.1$fit$par[1]
```

```
LL <- mod1.1$fit$par[2]
UL <- mod1.1$fit$par[3]
ED50 <- mod1.1$fit$par[4]

# Interpolation test 4 param log logistic ----------------------------------
# Calc conc from a given % of Scav  ----------------------------------------
Y <- c(70.27932961, 49.22905028)
Conc <- ED50/((UL-Y)/(Y-LL))^(-1/S)
Conc

#Calc % of Scav from a given conc  ----------------------------------------
X <- c(0.05545481, 0.03175231)
Scav <- LL + (UL - LL)/(1 + (ED50/X)^-S)
Scav

# Re-plot interpolations ---------------------------------------------------
plot(mod1.1, type="all")
points(c(X, Conc), c(Scav, Y), pch = 18, col = "red", cex=1.5)



# ------------------------------------ #
#    5. DPPH          #
#    5.1 Subsetting and wrangling        #
# ------------------------------------ #

dpph.obs<-DPPH[c(1:78,85:162),c(1:3,6)]
names(dpph.obs) <- c("Solv", "Run", "Rep", "Abs_515")

# Controls average computing for Scavenging formula ------------------------
PC.met <- DPPH[grep("Positive", DPPH$Exp), c(1,2,6)] %>%
  filter(Solv == "M") %>%
  summarise(Average = mean(`Raw Data (515) Rep 3`))

NC.met <- DPPH[grep("Negative", DPPH$Exp), c(1,2,6)] %>%
  filter(Solv == "M") %>%
  summarise(Average = mean(`Raw Data (515) Rep 3`))

PC.acet <- DPPH[grep("Positive", DPPH$Exp), c(1,2,6)] %>%
  filter(Solv == "A") %>%
  summarise(Average = mean(`Raw Data (515) Rep 3`))

NC.acet <- DPPH[grep("Negative", DPPH$Exp), c(1,2,6)] %>%
  filter(Solv == "A") %>%
  summarise(Average = mean(`Raw Data (515) Rep 3`))

# Scavenging Subsetting prep -----------------------------------------------
(dpph.acet<-dpph.obs[79:156, ])

(dpph.met<-dpph.obs[1:78, ])
```

```
# Scavenging calculation ----------------------------------------------
(dpph.met <- dpph.met %>%
  mutate(Scav = (((Abs_515 - NC.met$Average)/(PC.met$Average -
NC.met$Average))*100)))

(dpph.acet <- dpph.acet %>%
  mutate(Scav = (((Abs_515 - NC.acet$Average)/(PC.acet$Average -
NC.acet$Average))*100)))

# Working DPPH Dataset (Stacking observations) ---------------------------
(dpph.obs.treated <- rbind(dpph.met, dpph.acet))


# ------------------------- #
#    5.2 Data glance        #
# ------------------------- #

dpph.obs.treated %>%
  ggplot(aes(Solv,Scav, fill = Solv)) +
  geom_boxplot(alpha = 0.8)

dpph.obs.treated %>%
  ggplot(aes(x=Scav, fill= Solv,col=Solv)) +
  geom_density(alpha=0.3)


# ---------------------------------------- #
#    5.3 Interpolation                     #
#       of all observations in DPPH tests  #
# ---------------------------------------- #

dpph.obs.treated$Equiv_mg <- ED50/((UL -
dpph.obs.treated$Scav)/(dpph.obs.treated$Scav - LL))^(-1/S)

dpph.obs.treated <- dpph.obs.treated %>%
  mutate(Equiv_µg_mL = Equiv_mg * 1000)

# dpph.obs.treated <- dpph.obs.treated[complete.cases(dpph.obs.treated), ]


# --------------------------- #
#    5.4 Interpolation glance  #
# --------------------------- #
dpph.obs.treated %>%
  ggplot(aes(Solv,Equiv_µg_mL, fill = Solv)) +
  geom_boxplot(alpha = 0.8)


# ------------------------- #
```

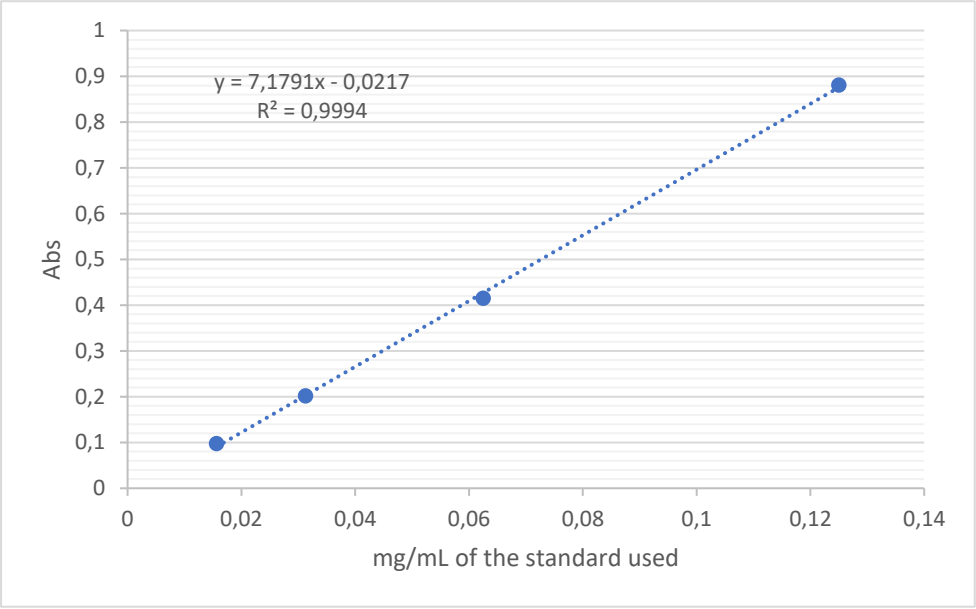
```
#    5.5. Statistics        #
# ------------------------- #


# Prueba de normalidad para el Solv "A" en DPPH --------------------------
shapiro.test(dpph.obs.treated$Equiv_µg_mL[dpph.obs.treated$Solv == "A"]) # Not
Normal distribution
#### Prueba de normalidad para el Solv "M" en DPPH ----------------------
shapiro.test(dpph.obs.treated$Equiv_µg_mL[dpph.obs.treated$Solv == "M"]) # Not
Normal distribution

# Prueba t de Student para comparar las medias (si los datos son normales)  ----
#t.test(Equiv_µg_mL ~ Solv, data = dpph.obs.treated)
#### Prueba de Wilcoxon-Mann-Whitney para comparar las medianas (si los datos no son
normales)  ------
wilcox.test(Equiv_µg_mL ~ Solv, data = dpph.obs.treated, paired = FALSE) #Both
distributions are not normal distributions and means are not equal



# ------------------------- #
#    5.5. Stat plot         #
# ------------------------- #

# Combine plot and statistical test with violin plots for DPPH -----------
ggbetweenstats(
  dpph.obs.treated, Solv, Equiv_µg_mL,
  type = "nonparametric",
  pairwise.comparisons = TRUE,
  pairwise.display = "all",
  p.adjust.method = "holm",
  effsize.type = "unbiased",
  title = "Trolox equivalent scavenged in DPPH in olive leafs",
  package = "ggsci",
  palette = "nrc_npg"
)



# ------------------------- #
#    6.1 final DF prep      #
# ------------------------- #

final <- cbind(carot.obs.treated,chl.obs.treated,dpph.obs.treated,tpc.obs.treated) %>%
  select( ,c(1:4,8,15,21)) %>%
  transform(Solv=factor(Solv, labels = c("Acetone","Methanol"))) #Double check correct
names in factors changed

names(final) <- c("Solvent",
                  "Run",
                  "Repetition",
                  "Carotenoids_µg_mL",
```

```
                "Chlorophyl_a_µg_mL",
                "Trolox_Equiv_µg_mL",
                "Galic_acid_Equiv")

final <- final %>%
  mutate(Exp_run = paste(Solvent, Run, sep = "_")) %>%
  select(-Solvent, -Run) %>%
  relocate(Exp_run, .before = 1)

View(final)


# ------------------------- #
#    6.2 Final DF summary    #
# ------------------------- #

final.summary <- final %>%
  group_by(Exp_run) %>%
  summarise(mean_Carotenoids_µg_mL = mean(Carotenoids_µg_mL),
            sd_Carotenoids_µg_mL = sd(Carotenoids_µg_mL),
            cv_Carotenoids_µg_mL = sd(Carotenoids_µg_mL) / mean(Carotenoids_µg_mL),
            mean_Chlorophyl_a_µg_mL = mean(Chlorophyl_a_µg_mL),
            sd_Chlorophyl_a_µg_mL = sd(Chlorophyl_a_µg_mL),
            cv_Chlorophyl_a_µg_mL = sd(Chlorophyl_a_µg_mL) /
mean(Chlorophyl_a_µg_mL),
            mean_Trolox_Equiv_µg_mL = mean(Trolox_Equiv_µg_mL, na.rm = TRUE),
            sd_Trolox_Equiv_µg_mL = sd(Trolox_Equiv_µg_mL, na.rm = TRUE),
            cv_Trolox_Equiv_µg_mL = sd(Trolox_Equiv_µg_mL, na.rm = TRUE) /
mean(Trolox_Equiv_µg_mL, na.rm = TRUE),
            mean_Galic_acid_Equiv = mean(Galic_acid_Equiv),
            sd_Galic_acid_Equiv = sd(Galic_acid_Equiv),
            cv_Galic_acid_Equiv = sd(Galic_acid_Equiv) / mean(Galic_acid_Equiv))


# ------------------------- #
#    6.3 Export Final DF     #
# ------------------------- #

write.table(final, "Spectro.csv", sep = ",")
write.table(final.summary, "Spectro_summary.csv", sep = ",")
```

Table S1: TPC interpolation, TPC calibration curve and DPPH % of inhibition of water extraction of olive leaves

Sample	Extraction type	m leaves (g)	mg of extract/mL	Sample2	Rep	Abs (725 nm)	[] ug eq gallic acid/mL	[] mg eq gallic acid/g extract	Average	SD	CV	Terms	
Olive dried leaves	Water (DE 1h)	3.0001	0.50	1	1	0.272	40.891	81.78	79.64	3.03322	3.8088	a	B
Olive dried leaves	Water (DE 1h)	3.0009	0.50	1	2	0.257	38.746	77.49				7.1801	0.0216

TPC Calib curve	
mg/mL	Abs
0.25	Acima do limite
0.125	0.881
0.0625	0.415
0.03125	0.202
0.015625	0.098
0.0078125	0.046
0.00390625	0.025
0.001953125	0.015



Sample	Blank average	Extraction type	Extraction rep	concentration (mg/mL)	Abs	% inhibition	Average	SD	CV
Olive dried leaves	0.760	Water (DE 1h)	1	0.5	0.124	83.684	**81.32**	3.35	4.12
Olive dried leaves	0.760	Water (DE 1h)	2	0.5	0.16	78.947			

Table S2: Screening analysis (File: Khaoula_20230509.xlsx, sheet "0 Summary"), R studio Script 2.

Sample	Replicate	Concentration	Result Report	Test	UAE	MAE	DE 1h	DE 3h
Olive dried leaves	1	0.1	% inh	DPPH	9.47	7.38	5.86	7.86
Olive dried leaves	2	0.1	% inh	DPPH	10.51	9.71	6.12	6.99
Olive dried leaves	1	0.2	% inh	DPPH	20.14	13.72	10.59	14.85
Olive dried leaves	2	0.2	% inh	DPPH	21.59	20.06	11.25	14.34
Olive dried leaves	1	0.3	% inh	DPPH	30.58	19.66	17.83	21.24
Olive dried leaves	2	0.3	% inh	DPPH	28.81	31.06	22.37	21.24
Olive dried leaves	1	0.4	% inh	DPPH	39.49	30.34	42.57	27.63
Olive dried leaves	2	0.4	% inh	DPPH	41.01	37.16	40.13	28.15
Olive dried leaves	1	0.5	% inh	DPPH	88.92	77.29	79.47	81.61
Olive dried leaves	2	0.5	% inh	DPPH	88.68	88.60	82.30	81.61

Olive dried leaves	1	0.1	% inh	ABTS	54.32	55.36	3.14	4.51
Olive dried leaves	2	0.1	% inh	ABTS	55.36	56.02	2.83	3.44
Olive dried leaves	1	0.2	% inh	ABTS	60.75	58.61	9.10	10.86
Olive dried leaves	2	0.2	% inh	ABTS	62.08	62.31	8.95	9.79
Olive dried leaves	1	0.3	% inh	ABTS	67.18	63.05	16.30	10.33
Olive dried leaves	2	0.3	% inh	ABTS	66.08	67.63	8.11	16.30
Olive dried leaves	1	0.4	% inh	ABTS	72.28	68.07	22.80	23.18
Olive dried leaves	2	0.4	% inh	ABTS	73.91	72.36	20.89	23.79
Olive dried leaves	1	0.5	% inh	ABTS	99.11	95.12	51.87	53.63
Olive dried leaves	2	0.5	% inh	ABTS	101.40	98.45	53.02	51.72
Olive dried leaves	1	0.1	Concent	Reducing Power	76.13	79.54	33.12	48.11
Olive dried leaves	2	0.1	Concent	Reducing Power	71.85	89.45	38.27	44.03
Olive dried leaves	1	0.2	Concent	Reducing Power	66.25	59.38	58.40	74.99
Olive dried leaves	2	0.2	Concent	Reducing Power	59.87	71.05	55.18	68.89
Olive dried leaves	1	0.3	Concent	Reducing Power	58.01	54.13	64.70	85.84

Olive dried leaves	2	0.3	Concent	Reducing Power	57.91	56.22	60.82	78.83
Olive dried leaves	1	0.4	Concent	Reducing Power	58.09	111.17	69.88	91.62
Olive dried leaves	2	0.4	Concent	Reducing Power	51.27	55.79	65.86	91.79
Olive dried leaves	1	0.5	Concent	Reducing Power	113.02	124.29	145.28	183.94
Olive dried leaves	2	0.5	Concent	Reducing Power	106.60	111.63	135.77	172.43
Olive dried leaves	1	0.1	Concent	FRAP	61.91	7.56	44.70	50.19
Olive dried leaves	2	0.1	Concent	FRAP	17.57	0.00	50.72	63.76
Olive dried leaves	1	0.2	Concent	FRAP	54.74	43.44	72.80	90.80
Olive dried leaves	2	0.2	Concent	FRAP	62.18	54.45	68.96	93.21
Olive dried leaves	1	0.3	Concent	FRAP	35.38	49.15	73.79	91.41
Olive dried leaves	2	0.3	Concent	FRAP	60.32	65.96	77.21	95.78
Olive dried leaves	1	0.4	Concent	FRAP	63.88	49.90	72.57	94.58
Olive dried leaves	2	0.4	Concent	FRAP	59.88	66.83	82.60	96.60
Olive dried leaves	1	0.5	Concent	FRAP	68.20	88.19	94.08	115.59
Olive dried leaves	2	0.5	Concent	FRAP	69.75	75.86	90.12	116.01

Olive dried leaves	1		mg Eq Gallic Acid/g dry olive leaves	TPC	28.15	28.44	28.70	36.16
Olive dried leaves	2		mg Eq Gallic Acid/g dry olive leaves	TPC	24.80	27.87	29.67	36.98

Table S3: Multiple Linear Regression Data (File: pH_solv.xlsx), R studio Script 3.

Assay	Exp_date	Solvent	Buffer_pH	Solv_perc	Buf_perc	pH
73	22-may	Methanol	2.2	60	40	3.124
52	22-may	Methanol	2.2	60	40	3.25
43	19-may	Methanol	2.2	60	40	3.299
1	19-may	Methanol	2.2	60	40	3.3
22	19-may	Methanol	2.2	60	40	3.325
74	22-may	Methanol	2.2	80	20	3.614
53	22-may	Methanol	2.2	80	20	3.749
29	19-may	Methanol	2.2	80	20	3.851
44	19-may	Methanol	2.2	80	20	3.856
8	19-may	Methanol	2.2	80	20	3.9
55	22-may	Methanol	3	60	40	4.23
2	19-may	Methanol	3	60	40	4.279
75	22-may	Methanol	2.2	97	3	4.32
23	19-may	Methanol	3	60	40	4.325
54	22-may	Methanol	2.2	97	3	4.378
36	19-may	Methanol	2.2	97	3	4.401
45	19-may	Methanol	2.2	97	3	4.435
15	19-may	Methanol	2.2	97	3	4.5
56	22-may	Methanol	3	80	20	4.826
9	19-may	Methanol	3	80	20	4.872
30	19-may	Methanol	3	80	20	4.888

3	19-may	Methanol	4	60	40	5.186
58	22-may	Methanol	4	60	40	5.222
24	19-may	Methanol	4	60	40	5.323
16	19-may	Methanol	3	97	3	5.4
57	22-may	Methanol	3	97	3	5.446
37	19-may	Methanol	3	97	3	5.554
59	22-may	Methanol	4	80	20	5.783
31	19-may	Methanol	4	80	20	5.805
10	19-may	Methanol	4	80	20	5.808
4	19-may	Methanol	5	60	40	6.319
76	22-may	Methanol	5	60	40	6.37
25	19-may	Methanol	5	60	40	6.38
61	22-may	Methanol	5	60	40	6.39
46	19-may	Methanol	5	60	40	6.401
60	22-may	Methanol	4	97	3	6.5
17	19-may	Methanol	4	97	3	6.66
38	19-may	Methanol	4	97	3	6.681
77	22-may	Methanol	5	80	20	6.897
62	22-may	Methanol	5	80	20	6.922
47	19-may	Methanol	5	80	20	6.973
11	19-may	Methanol	5	80	20	6.99
32	19-may	Methanol	5	80	20	7.001
64	22-may	Methanol	6	60	40	7.399
5	19-may	Methanol	6	60	40	7.45
26	19-may	Methanol	6	60	40	7.461
65	22-may	Methanol	6	80	20	7.522
78	22-may	Methanol	5	97	3	7.866
63	22-may	Methanol	5	97	3	7.921
18	19-may	Methanol	5	97	3	7.95

39	19-may	Methanol	5	97	3	7.99
48	19-may	Methanol	5	97	3	8.043
68	22-may	Methanol	7	80	20	8.14
12	19-may	Methanol	6	80	20	8.156
33	19-may	Methanol	6	80	20	8.19
67	22-may	Methanol	7	60	40	8.565
34	19-may	Methanol	7	80	20	8.607
27	19-may	Methanol	7	60	40	8.633
6	19-may	Methanol	7	60	40	8.671
70	22-may	Methanol	8	60	40	9.166
71	22-may	Methanol	8	80	20	9.168
80	22-may	Methanol	8	80	20	9.18
66	22-may	Methanol	6	97	3	9.235
81	22-may	Methanol	8	97	3	9.255
13	19-may	Methanol	7	80	20	9.324
40	19-may	Methanol	6	97	3	9.395
19	19-may	Methanol	6	97	3	9.494
69	22-may	Methanol	7	97	3	9.52
28	19-may	Methanol	8	60	40	9.579
49	19-may	Methanol	8	60	40	9.653
79	22-may	Methanol	8	60	40	9.688
7	19-may	Methanol	8	60	40	9.8
35	19-may	Methanol	8	80	20	9.936
14	19-may	Methanol	8	80	20	10.195
72	22-may	Methanol	8	97	3	10.233
50	19-may	Methanol	8	80	20	10.303
41	19-may	Methanol	7	97	3	10.34
42	19-may	Methanol	8	97	3	10.721
51	19-may	Methanol	8	97	3	10.77

20	19-may	Methanol	7	97	3	10.78
21	19-may	Methanol	8	97	3	11.03
124	23-may	Acetone	2.2	60	40	3.014
103	22-may	Acetone	2.2	60	40	3.433
82	22-may	Acetone	2.2	60	40	3.476
125	23-may	Acetone	2.2	80	20	3.636
104	22-may	Acetone	2.2	80	20	3.991
83	22-may	Acetone	2.2	80	20	3.992
126	23-may	Acetone	2.2	97	3	4.211
127	23-may	Acetone	3	60	40	4.277
84	22-may	Acetone	2.2	97	3	4.484
105	22-may	Acetone	2.2	97	3	4.506
106	22-may	Acetone	3	60	40	4.582
85	22-may	Acetone	3	60	40	4.611
128	23-may	Acetone	3	80	20	4.845
129	23-may	Acetone	3	97	3	4.963
108	22-may	Acetone	3	97	3	5.072
87	22-may	Acetone	3	97	3	5.101
107	22-may	Acetone	3	80	20	5.104
86	22-may	Acetone	3	80	20	5.17
132	23-may	Acetone	4	97	3	5.197
130	23-may	Acetone	4	60	40	5.316
111	22-may	Acetone	4	97	3	5.343
90	22-may	Acetone	4	97	3	5.356
131	23-may	Acetone	4	80	20	5.46
109	22-may	Acetone	4	60	40	5.542
88	22-may	Acetone	4	60	40	5.571

89	22-may	Acetone	4	80	20	5.578
110	22-may	Acetone	4	80	20	5.639
135	23-may	Acetone	5	97	3	5.645
93	22-may	Acetone	5	97	3	5.857
114	22-may	Acetone	5	97	3	5.942
134	23-may	Acetone	5	80	20	5.966
92	22-may	Acetone	5	80	20	6.143
113	22-may	Acetone	5	80	20	6.172
133	23-may	Acetone	5	60	40	6.297
91	22-may	Acetone	5	60	40	6.411
112	22-may	Acetone	5	60	40	6.431
137	23-may	Acetone	6	80	20	6.568
138	23-may	Acetone	6	97	3	6.573
95	22-may	Acetone	6	80	20	6.665
117	22-may	Acetone	6	97	3	6.713
116	22-may	Acetone	6	80	20	6.803
96	22-may	Acetone	6	97	3	6.861
94	22-may	Acetone	6	60	40	6.906
136	23-may	Acetone	6	60	40	6.966
115	22-may	Acetone	6	60	40	7.001
139	23-may	Acetone	7	60	40	8.189
118	22-may	Acetone	7	60	40	8.372
97	22-may	Acetone	7	60	40	8.403
140	23-may	Acetone	7	80	20	8.439
119	22-may	Acetone	7	80	20	8.655
98	22-may	Acetone	7	80	20	8.694
141	23-may	Acetone	7	97	3	9.132

142	23-may	Acetone	8	60	40	9.416
120	22-may	Acetone	7	97	3	9.565
121	22-may	Acetone	8	60	40	9.594
100	22-may	Acetone	8	60	40	9.666
99	22-may	Acetone	7	97	3	9.851
143	23-may	Acetone	8	80	20	9.933
101	22-may	Acetone	8	80	20	10.172
122	22-may	Acetone	8	80	20	10.209
144	23-may	Acetone	8	97	3	10.329
123	22-may	Acetone	8	97	3	10.49
102	22-may	Acetone	8	97	3	10.891

Table S4: DPPH Trolox dose response Modeling (File: datos_Khaoula.xlsx, sheet "DPPH_Calib"), R studio Script 4.

Content	Well	Standard Concentrations [mg/mL]	Raw Data (515)
Standard S1	A01	0.5	0.053
Standard S1	A02	0.5	0.055
Standard S1	A03	0.5	0.055
Standard S2	A04	0.25	0.057
Standard S2	A05	0.25	0.06
Standard S2	A06	0.25	0.061
Standard S3	A07	0.125	0.099
Standard S3	A08	0.125	0.09
Standard S3	A09	0.125	0.091

Standard S4	A10	0.0625	0.317
Standard S4	A11	0.0625	0.276
Standard S4	A12	0.0625	0.268
Standard S5	B01	0.03125	0.433
Standard S5	B02	0.03125	0.434
Standard S5	B03	0.03125	0.44
Standard S6	B04	0.015625	0.571
Standard S6	B05	0.015625	0.572
Standard S6	B06	0.015625	0.624
Standard S7	B07	0.0078125	0.666
Standard S7	B08	0.0078125	0.66
Standard S7	B09	0.0078125	0.668
Standard S8	B10	0.00390625	0.715
Standard S8	B11	0.00390625	0.707
Standard S8	B12	0.00390625	0.713
Standard S9	C01	0.001953125	0.612
Standard S9	C02	0.001953125	0.637
Standard S9	C03	0.001953125	0.641
Standard S10	C04	0.000976563	0.71
Standard S10	C05	0.000976563	0.692
Standard S10	C06	0.000976563	0.723
Standard S11	C07	0.000488281	0.737
Standard S11	C08	0.000488281	0.732
Standard S11	C09	0.000488281	0.73
Standard S12	C10	0.000244141	0.727
Standard S12	C11	0.000244141	0.728
Standard S12	C12	0.000244141	0.731

Standard S13	D01	0.00012207	0.662
Standard S13	D02	0.00012207	0.667
Standard S13	D03	0.00012207	0.664
Standard S14	D04	0.000061	0.717
Standard S14	D05	0.000061	0.718
Standard S14	D06	0.000061	0.75
Positive Control	A01	0.5	0.053
Positive Control	A02	0.5	0.055
Positive Control	A03	0.5	0.055
Negative control N	D07	0	0.795
Negative control N	D08	0	0.817
Negative control N	D09	0	0.816
Negative control N	D10	0	0.747
Negative control N	D11	0	0.811
Negative control N	D12	0	0.815

Table S5: CCD entire summary.

Exp	Block	Design point	time (min)	Solvent (%)	pH	Solv	mean_Carotenoids_µg_mL	sd_Carotenoids_µg_mL	cv_Carotenoids_µg_mL	mean_Chlorophyl_a_µg_mL	sd_Chlorophyl_a_µg_mL	cv_Chlorophyl_a_µg_mL	mean_Trolox_Equiv_µg_mL	sd_Trolox_Equiv_µg_mL	cv_Trolox_Equiv_µg_mL	mean_Galic_acid_Equiv	sd_Galic_acid_Equiv	cv_Galic_acid_Equiv	Extractions yield (%)
1	1	Fct	120	80	5	M	1.7	0.11	0.06	2.0	0.10	0.05	16.7	2.41	0.14	75.0	30.38	0.41	52.5
2	1	Fct	120	66	5	M	0.3	0.02	0.05	0.6	0.02	0.03	18.0	5.13	0.28	91.6	28.64	0.31	60.1
3	1	Fct	120	80	5	M	1.8	0.22	0.12	2.2	0.23	0.11	36.1	0.98	0.03	61.0	2.35	0.04	60.4
4	1	Fct	120	80	8	M	3.3	0.02	0.01	4.3	0.05	0.01	50.7	4.41	0.09	54.4	3.72	0.07	59.3
5	1	Fct	240	66	5	M	0.5	0.01	0.02	1.1	0.02	0.02	17.4	1.48	0.08	73.6	11.06	0.15	55.4
6	1	Fct	240	66	5	M	0.4	0.02	0.06	0.8	0.04	0.05	30.9	7.71	0.25	126.6	12.82	0.10	74.4
7	1	Fct	240	66	8	M	0.6	0.04	0.06	1.1	0.06	0.05	26.6	2.65	0.10	82.3	3.38	0.04	70.9
8	1	Fct	120	66	8	M	0.6	0.02	0.03	1.3	0.03	0.02	19.3	1.49	0.08	73.2	6.60	0.09	74.8
9	1	Fct	120	66	8	M	0.7	0.03	0.04	1.0	0.04	0.04	28.6	0.42	0.01	92.3	9.09	0.10	74.8
10	1	Fct	240	80	5	M	1.8	0.13	0.07	2.6	0.15	0.06	22.4	4.79	0.21	106.2	2.08	0.02	67.7
11	1	Fct	240	80	8	M	1.8	0.05	0.03	2.3	0.05	0.02	48.0	4.85	0.10	78.5	16.58	0.21	65.9
12	1	Fct	120	80	8	M	2.6	0.13	0.05	2.6	0.11	0.04	94.8	9.65	0.10	129.7	19.63	0.15	62.4
13	1	Fct	240	66	8	M	0.5	0.04	0.08	0.9	0.07	0.08	15.6	1.03	0.07	69.0	1.86	0.03	73.6
14	1	Fct	240	80	5	M	2.1	0.65	0.30	3.0	0.80	0.26	15.1	4.31	0.29	70.4	4.01	0.06	62.1
15	1	Fct	240	80	8	M	1.8	0.17	0.09	2.9	0.27	0.09	20.1	1.22	0.06	60.9	7.75	0.13	56.6
16	1	Fct	120	66	5	M	0.4	0.04	0.10	0.8	0.06	0.08	21.4	1.12	0.05	71.9	26.35	0.37	68.8
17	1	Cen	180	73	6.5	M	0.5	0.01	0.02	1.1	0.02	0.02	61.4	5.01	0.08	82.4	42.36	0.51	67.3
18	1	Cen	180	73	6.5	M	0.4	0.04	0.10	0.8	0.08	0.10	30.5	7.60	0.25	83.6	15.69	0.19	63.4
19	2	Ax	180	73	9	M	1.5	0.15	0.10	2.3	0.21	0.09	35.8	2.94	0.08	113.2	7.22	0.06	63.9
20	2	Ax	79	73	6.5	M	0.7	0.02	0.02	1.2	0.03	0.02	53.4	9.78	0.18	111.8	8.62	0.08	64.2
21	2	Ax	281	73	6.5	M	0.5	0.04	0.08	1.1	0.10	0.09	25.8	0.95	0.04	69.6	2.70	0.04	65.2
22	2	Ax	180	85	6.5	M	1.1	0.36	0.32	1.6	0.44	0.29	32.1	7.75	0.24	91.4	23.82	0.26	56.6

23	2	Cen	180	73	6.5	M	0.5	0.02	0.04	1.0	0.05	0.04	48.6	2.72	0.06	113.6	12.68	0.11	65.6
24	2	Cen	180	73	6.5	M	1.2	0.01	0.01	2.2	0.04	0.02	78.3	9.23	0.12	103.9	31.48	0.30	63.6
25	2	Ax	180	61	6.5	M	0.4	0.05	0.13	0.7	0.08	0.11	45.3	7.31	0.16	86.9	11.04	0.13	73.7
26	2	Ax	180	73	4	M	0.7	0.04	0.06	1.2	0.08	0.06	35.6	7.57	0.21	159.5	61.28	0.38	68.9
1	3	Fct	120	80	5	A	2.6	0.84	0.32	5.6	2.16	0.38	14.1	7.88	0.56	141.8	23.42	0.17	52.3
2	3	Fct	240	66	5	A	0.9	0.07	0.08	1.9	0.16	0.08	16.5	11.08	0.67	94.4	5.56	0.06	65.4
3	3	Fct	120	80	5	A	2.8	0.31	0.11	7.2	0.81	0.11	22.6	0.31	0.01	113.7	4.18	0.04	47.5
4	3	Fct	240	80	8	A	5.7	0.12	0.02	11.6	0.15	0.01	48.5	9.32	0.19	134.4	6.85	0.05	39.8
5	3	Fct	120	66	8	A	2.9	0.40	0.14	4.0	0.69	0.17	16.8	13.73	0.82	109.9	6.03	0.05	47.4
6	3	Cen	180	73	6.5	A	3.3	0.04	0.01	7.2	0.17	0.02	27.3	0.32	0.01	108.1	13.55	0.13	46.9
7	3	Fct	240	80	8	A	5.8	0.59	0.10	12.9	1.23	0.10	22.8	0.34	0.01	118.7	40.92	0.34	41.5
8	3	Fct	240	66	5	A	1.3	0.28	0.21	2.7	0.53	0.20	18.9	0.69	0.04	70.5	4.54	0.06	65.6
9	3	Fct	240	80	5	A	1.3	0.17	0.13	3.0	0.54	0.18	2.3	1.39	0.60	83.0	1.85	0.02	50.0
10	3	Fct	120	80	8	A	5.0	0.03	0.01	10.3	0.11	0.01	37.5	15.78	0.42	127.3	6.84	0.05	41.1
11	3	Cen	180	73	6.5	A	2.8	0.19	0.07	6.2	0.39	0.06	21.2	0.41	0.02	99.3	8.18	0.08	46.2
12	3	Fct	120	66	5	A	1.6	0.10	0.07	2.8	0.15	0.05	14.3	1.48	0.10	52.4	9.59	0.18	72.4
13	3	Fct	120	66	8	A	2.4	0.13	0.06	3.6	0.33	0.09	6.7	3.20	0.48	65.0	5.27	0.08	47.6
14	3	Fct	240	66	8	A	5.1	0.16	0.03	8.3	0.24	0.03	17.5	8.54	0.49	61.5	13.02	0.21	49.2
15	3	Fct	240	80	5	A	1.5	0.12	0.08	3.6	0.31	0.09	20.6	0.70	0.03	76.4	20.27	0.27	48.9
16	3	Fct	240	66	8	A	3.5	0.43	0.12	5.7	0.66	0.12	30.2	1.00	0.03	70.3	5.73	0.08	47.1
17	3	Fct	120	80	8	A	3.5	0.38	0.11	7.5	1.23	0.16	10.8	6.64	0.61	58.8	24.96	0.42	39.3
18	3	Fct	120	66	5	A	1.7	0.10	0.06	2.9	0.16	0.05	13.7	10.36	0.76	47.2	33.07	0.70	65.3
19	4	Ax	281	73	6.5	A	2.9	0.28	0.10	5.8	0.53	0.09	23.3	1.43	0.06	85.0	3.67	0.04	50.9
20	4	Ax	180	85	6.5	A	3.2	0.71	0.22	8.2	1.74	0.21	24.2	4.96	0.20	105.5	3.67	0.03	42.2
21	4	Cen	180	73	6.5	A	2.9	0.25	0.09	6.1	0.95	0.16	12.3	0.16	0.01	119.7	3.80	0.03	45.7
22	4	Ax	79	73	6.5	A	3.4	0.16	0.05	7.5	0.37	0.05	23.6	11.71	0.50	112.7	12.96	0.11	45.2

23	4	Ax	180	61	6.5	A	0.8	0.08	0.10	1.4	0.13	0.09	13.4	0.57	0.04	53.6	13.30	0.25	73.6
24	4	Ax	180	73	9	A	3.4	0.57	0.17	5.8	0.78	0.13	62.6	15.92	0.25	100.8	1.72	0.02	41.9
25	4	Ax	180	73	4	A	2.3	0.13	0.06	5.5	0.65	0.12	2.5	1.60	0.63	84.8	3.20	0.04	58.0
26	4	Cen	180	73	6.5	A	4.6	0.66	0.14	10.7	1.69	0.16	14.5	11.79	0.81	78.2	9.15	0.12	45.7

Table S6: Chlorophylls and Carotenoids EDA (File: datos_Khaoula.xlsx, sheet "CnC"), R studio Script 4.

Solv	Exp	rep	Standard Concentrations [mg/ml]	Well	Abs_470	Abs_666
M	1	1	20	A01	0.348	0.146
M	1	2	20	A02	0.386	0.159
M	1	3	20	A03	0.393	0.161
M	2	1	20	A04	0.074	0.051
M	2	2	20	A05	0.075	0.051
M	2	3	20	A06	0.068	0.048
M	3	1	20	A07	0.38	0.165
M	3	2	20	A08	0.364	0.159
M	3	3	20	A09	0.456	0.193
M	4	1	20	A10	0.725	0.34
M	4	2	20	A11	0.735	0.347
M	4	3	20	A12	0.73	0.345
M	5	1	20	B01	0.111	0.082
M	5	2	20	B02	0.114	0.084
M	5	3	20	B03	0.115	0.085
M	6	1	20	B04	0.081	0.059
M	6	2	20	B05	0.089	0.064
M	6	3	20	B06	0.09	0.065
M	7	1	20	B07	0.132	0.086

M	7	2	20	B08	0.143	0.092
M	7	3	20	B09	0.148	0.095
M	8	1	20	B10	0.134	0.1
M	8	2	20	B11	0.136	0.101
M	8	3	20	B12	0.143	0.104
M	9	1	20	C01	0.149	0.074
M	9	2	20	C02	0.154	0.076
M	9	3	20	C03	0.162	0.08
M	10	1	20	C04	0.367	0.19
M	10	2	20	C05	0.413	0.209
M	10	3	20	C06	0.421	0.213
M	11	1	20	C07	0.392	0.179
M	11	2	20	C08	0.4	0.182
M	11	3	20	C09	0.412	0.187
M	12	1	20	C10	0.547	0.197
M	12	2	20	C11	0.591	0.211
M	12	3	20	C12	0.602	0.214
M	13	1	20	D01	0.117	0.081
M	13	2	20	D02	0.103	0.071
M	13	3	20	D03	0.103	0.071
M	14	1	20	D04	0.646	0.312
M	14	2	20	D05	0.398	0.204
M	14	3	20	D06	0.391	0.201
M	15	1	20	D07	0.402	0.222
M	15	2	20	D08	0.379	0.21
M	15	3	20	D09	0.455	0.251
M	16	1	20	D10	0.106	0.067

M	16	2	20	D11	0.092	0.06
M	16	3	20	D12	0.089	0.058
M	17	1	20	E01	0.11	0.087
M	17	2	20	E02	0.106	0.084
M	17	3	20	E03	0.11	0.086
M	18	1	20	E04	0.092	0.071
M	18	2	20	E05	0.08	0.061
M	18	3	20	E06	0.076	0.059
M	19	1	20	E07	0.32	0.175
M	19	2	20	E08	0.325	0.176
M	19	3	20	E09	0.381	0.205
M	20	1	20	E10	0.166	0.097
M	20	2	20	E11	0.159	0.093
M	20	3	20	E12	0.162	0.093
M	21	1	20	F01	0.118	0.095
M	21	2	20	F02	0.102	0.081
M	21	3	20	F03	0.103	0.082
M	22	1	20	F04	0.346	0.164
M	22	2	20	F05	0.202	0.101
M	22	3	20	F06	0.212	0.105
M	23	1	20	F07	0.107	0.081
M	23	2	20	F08	0.103	0.079
M	23	3	20	F09	0.111	0.086
M	24	1	20	F10	0.269	0.179
M	24	2	20	F11	0.264	0.173
M	24	3	20	F12	0.264	0.174
M	25	1	20	G01	0.1	0.062

M	25	2	20	G02	0.083	0.053
M	25	3	20	G03	0.078	0.05
M	26	1	20	G04	0.148	0.089
M	26	2	20	G05	0.163	0.099
M	26	3	20	G06	0.167	0.1
A	1	1	20	A01	0.374	0.252
A	1	2	20	A02	0.672	0.514
A	1	3	20	A03	0.723	0.574
A	2	1	20	A04	0.183	0.138
A	2	2	20	A05	0.203	0.152
A	2	3	20	A06	0.216	0.163
A	3	1	20	A07	0.651	0.582
A	3	2	20	A08	0.682	0.628
A	3	3	20	A09	0.548	0.501
A	4	1	20	G10	1.252	0.904
A	4	2	20	G11	1.282	0.926
A	4	3	20	G12	1.304	0.922
A	5	1	20	B01	0.547	0.26
A	5	2	20	B02	0.668	0.334
A	5	3	20	B03	0.721	0.367
A	6	1	20	B04	0.742	0.561
A	6	2	20	B05	0.742	0.57
A	6	3	20	B06	0.758	0.588
A	7	1	20	Sample X19	1.27	0.972
A	7	2	20	Sample X20	1.462	1.134
A	7	3	20	Sample X21	1.208	0.96
A	8	1	20	B10	0.229	0.163

A	8	2	20	B11	0.34	0.238
A	8	3	20	B12	0.337	0.232
A	9	1	20	C01	0.255	0.19
A	9	2	20	C02	0.3	0.251
A	9	3	20	C03	0.331	0.273
A	10	1	20	H04	1.128	0.81
A	10	2	20	H05	1.136	0.818
A	10	3	20	H06	1.122	0.828
A	11	1	20	C07	0.607	0.474
A	11	2	20	C08	0.599	0.47
A	11	3	20	C09	0.675	0.526
A	12	1	20	C10	0.326	0.216
A	12	2	20	C11	0.371	0.239
A	12	3	20	C12	0.345	0.223
A	13	1	20	D01	0.521	0.261
A	13	2	20	D02	0.516	0.283
A	13	3	20	D03	0.57	0.313
A	14	1	20	H07	1.168	0.68
A	14	2	20	H08	1.128	0.66
A	14	3	20	H09	1.096	0.642
A	15	1	20	D07	0.33	0.277
A	15	2	20	D08	0.327	0.273
A	15	3	20	D09	0.375	0.318
A	16	1	20	D10	0.689	0.398
A	16	2	20	D11	0.881	0.502
A	16	3	20	D12	0.795	0.456
A	17	1	20	E01	0.683	0.487

A	17	2	20	E02	0.807	0.624
A	17	3	20	E03	0.848	0.676
A	18	1	20	E04	0.362	0.214
A	18	2	20	E05	0.393	0.231
A	18	3	20	E06	0.404	0.238
A	19	1	20	E07	0.629	0.438
A	19	2	20	E08	0.612	0.432
A	19	3	20	E09	0.728	0.508
A	20	1	20	E10	0.551	0.501
A	20	2	20	E11	0.863	0.772
A	20	3	20	E12	0.761	0.681
A	21	1	20	F01	0.605	0.406
A	21	2	20	F02	0.653	0.479
A	21	3	20	F03	0.718	0.556
A	22	1	20	F04	0.727	0.56
A	22	2	20	F05	0.776	0.601
A	22	3	20	F06	0.799	0.617
A	23	1	20	F07	0.177	0.108
A	23	2	20	F08	0.171	0.106
A	23	3	20	F09	0.204	0.125
A	24	1	20	F10	0.658	0.406
A	24	2	20	F11	0.742	0.445
A	24	3	20	F12	0.91	0.527
A	25	1	20	G01	0.487	0.376
A	25	2	20	G02	0.541	0.463
A	25	3	20	G03	0.536	0.467
A	26	1	20	H10	0.868	0.696

A	26	2	20	H11	1.12	0.934
A	26	3	20	H12	1.128	0.922

Table 7: Total Phenolic Content EDA (File: datos_Khaoula.xlsx, sheet "TPC"), R studio Script 4.

Solv	Exp	rep	Standard Concentrations [mg/ml]	Well	Raw Data (725)	Blank correction
M	1	1	0.5	A01	0.321	0.239
M	1	2	0.5	A02	0.284	0.202
M	1	3	0.5	A03	0.177	0.095
M	2	1	0.5	A04	0.273	0.191
M	2	2	0.5	A05	0.382	0.300
M	2	3	0.5	A06	0.25	0.168
M	3	1	0.5	G01	0.223	0.141
M	3	2	0.5	G02	0.233	0.151
M	3	3	0.5	G03	0.223	0.141
M	4	1	0.5	G04	0.202	0.120
M	4	2	0.5	G05	0.22	0.138
M	4	3	0.5	G06	0.208	0.126
M	5	1	0.5	B01	0.236	0.154
M	5	2	0.5	B02	0.248	0.166
M	5	3	0.5	B03	0.288	0.206
M	6	1	0.5	B04	0.412	0.330
M	6	2	0.5	B05	0.399	0.317
M	6	3	0.5	B06	0.352	0.270
M	7	1	0.5	B07	0.272	0.190
M	7	2	0.5	B08	0.288	0.206
M	7	3	0.5	B09	0.276	0.194
M	8	1	0.5	B10	0.238	0.156
M	8	2	0.5	B11	0.262	0.180

M	8	3	0.5	B12	0.269	0.187
M	9	1	0.5	C01	0.279	0.197
M	9	2	0.5	C02	0.308	0.226
M	9	3	0.5	C03	0.323	0.241
M	10	1	0.5	C04	0.342	0.260
M	10	2	0.5	C05	0.332	0.250
M	10	3	0.5	C06	0.339	0.257
M	11	1	0.5	C07	0.285	0.203
M	11	2	0.5	C08	0.3	0.218
M	11	3	0.5	C09	0.223	0.141
M	12	1	0.5	C10	0.385	0.303
M	12	2	0.5	C11	0.353	0.271
M	12	3	0.5	C12	0.448	0.366
M	13	1	0.5	D01	0.245	0.163
M	13	2	0.5	D02	0.251	0.169
M	13	3	0.5	D03	0.242	0.160
M	14	1	0.5	D04	0.238	0.156
M	14	2	0.5	D05	0.256	0.174
M	14	3	0.5	D06	0.254	0.172
M	15	1	0.5	D07	0.206	0.124
M	15	2	0.5	D08	0.244	0.162
M	15	3	0.5	D09	0.228	0.146
M	16	1	0.5	D10	0.202	0.120
M	16	2	0.5	D11	0.231	0.149
M	16	3	0.5	D12	0.326	0.244
M	17	1	0.5	E01	0.163	0.081
M	17	2	0.5	E02	0.309	0.227
M	17	3	0.5	E03	0.365	0.283
M	18	1	0.5	E04	0.29	0.208

M	18	2	0.5	E05	0.316	0.234
M	18	3	0.5	E06	0.24	0.158
M	19	1	0.5	E07	0.339	0.257
M	19	2	0.5	E08	0.351	0.269
M	19	3	0.5	E09	0.374	0.292
M	20	1	0.5	E10	0.334	0.252
M	20	2	0.5	E11	0.345	0.263
M	20	3	0.5	E12	0.375	0.293
M	21	1	0.5	F01	0.249	0.167
M	21	2	0.5	F02	0.24	0.158
M	21	3	0.5	F03	0.253	0.171
M	22	1	0.5	F04	0.234	0.152
M	22	2	0.5	F05	0.326	0.244
M	22	3	0.5	F06	0.343	0.261
M	23	1	0.5	F07	0.39	0.308
M	23	2	0.5	F08	0.348	0.266
M	23	3	0.5	F09	0.329	0.247
M	24	1	0.5	F10	0.362	0.280
M	24	2	0.5	F11	0.39	0.308
M	24	3	0.5	F12	0.244	0.162
M	25	1	0.5	G01	0.313	0.231
M	25	2	0.5	G02	0.26	0.178
M	25	3	0.5	G03	0.297	0.215
M	26	1	0.5	G04	0.306	0.224
M	26	2	0.5	G05	0.496	0.414
M	26	3	0.5	G06	0.604	0.522
M	Negative	Control	0	G07	0.079	-0.003
M	Negative	Control	0	G08	0.081	-0.001
M	Negative	Control	0	G09	0.086	0.004

M	Positive	Control	0.5	H01	1.348	1.266
M	Standard	S1	0.5	H01	1.348	1.266
M	Standard	S2	0.25	H02	0.62	0.538
M	Standard	S3	0.125	H03	0.375	0.293
M	Standard	S4	0.063	H04	0.206	0.124
M	Standard	S5	0.031	H05	0.148	0.066
M	Standard	S8	0.004	H08	0.125	0.043
M	Standard	S9	0.002	H09	0.111	0.029
A	1	1	0.5	A01	0.352	0.266
A	1	2	0.5	A02	0.434	0.348
A	1	3	0.5	A03	0.35	0.264
A	2	1	0.5	A04	0.269	0.183
A	2	2	0.5	A05	0.291	0.205
A	2	3	0.5	A06	0.285	0.199
A	3	1	0.5	A07	0.329	0.243
A	3	2	0.5	A08	0.322	0.236
A	3	3	0.5	A09	0.312	0.226
A	4	1	0.5	A10	0.379	0.293
A	4	2	0.5	A11	0.352	0.266
A	4	3	0.5	A12	0.359	0.273
A	5	1	0.5	B01	0.303	0.217
A	5	2	0.5	B02	0.327	0.241
A	5	3	0.5	B03	0.31	0.224
A	6	1	0.5	B04	0.279	0.193
A	6	2	0.5	B05	0.317	0.231
A	6	3	0.5	B06	0.333	0.247
A	7	1	0.5	B07	0.285	0.199

A	7	2	0.5	B08	0.428	0.342
A	7	3	0.5	B09	0.281	0.195
A	8	1	0.5	B10	0.225	0.139
A	8	2	0.5	B11	0.243	0.157
A	8	3	0.5	B12	0.23	0.144
A	9	1	0.5	C01	0.254	0.168
A	9	2	0.5	C02	0.261	0.175
A	9	3	0.5	C03	0.26	0.174
A	10	1	0.5	C04	0.339	0.253
A	10	2	0.5	C05	0.365	0.279
A	10	3	0.5	C06	0.343	0.257
A	11	1	0.5	C07	0.282	0.196
A	11	2	0.5	C08	0.282	0.196
A	11	3	0.5	C09	0.311	0.225
A	12	1	0.5	C10	0.173	0.087
A	12	2	0.5	C11	0.207	0.121
A	12	3	0.5	C12	0.207	0.121
A	13	1	0.5	D01	0.229	0.143
A	13	2	0.5	D02	0.209	0.123
A	13	3	0.5	D03	0.226	0.140
A	14	1	0.5	D04	0.201	0.115
A	14	2	0.5	D05	0.245	0.159
A	14	3	0.5	D06	0.197	0.111
A	15	1	0.5	D07	0.232	0.146
A	15	2	0.5	D08	0.211	0.125
A	15	3	0.5	D09	0.291	0.205
A	16	1	0.5	D10	0.219	0.133

A	16	2	0.5	D11	0.241	0.155
A	16	3	0.5	D12	0.237	0.151
A	17	1	0.5	E01	0.192	0.106
A	17	2	0.5	E02	0.168	0.082
A	17	3	0.5	E03	0.266	0.180
A	18	1	0.5	E04	0.142	0.056
A	18	2	0.5	E05	0.15	0.064
A	18	3	0.5	E06	0.263	0.177
A	19	1	0.5	E07	0.262	0.176
A	19	2	0.5	E08	0.255	0.169
A	19	3	0.5	E09	0.27	0.184
A	20	1	0.5	E10	0.313	0.227
A	20	2	0.5	E11	0.3	0.214
A	20	3	0.5	E12	0.3	0.214
A	21	1	0.5	F01	0.331	0.245
A	21	2	0.5	F02	0.342	0.256
A	21	3	0.5	F03	0.327	0.241
A	22	1	0.5	F04	0.296	0.210
A	22	2	0.5	F05	0.348	0.262
A	22	3	0.5	F06	0.313	0.227
A	23	1	0.5	F07	0.167	0.081
A	23	2	0.5	F08	0.209	0.123
A	23	3	0.5	F09	0.218	0.132
A	24	1	0.5	F10	0.291	0.205
A	24	2	0.5	F11	0.295	0.209
A	24	3	0.5	F12	0.298	0.212
A	25	1	0.5	G01	0.256	0.170

A	25	2	0.5	G02	0.261	0.175
A	25	3	0.5	G03	0.269	0.183
A	26	1	0.5	G04	0.262	0.176
A	26	2	0.5	G05	0.256	0.170
A	26	3	0.5	G06	0.227	0.141
A	Negative	Control	0	G07	0.087	0.000
A	Negative	Control	0	G08	0.085	0.000
A	Negative	Control	0	G09	0.085	0.000
A	Positive	Control	0.5	G10	1.132	1.046
A	Positive	Control	0.5	H01	1.113	1.027
A	Positive	Control	0.5	H10	1.115	1.029
A	Standard	S1	0.5	H01	1.113	1.027
A	Standard	S3	0.125	H03	0.342	0.256
A	Standard	S4	0.063	H04	0.205	0.119
A	Standard	S5	0.031	H05	0.162	0.076
A	Standard	S8	0.004	H08	0.102	0.016
A	Standard	S9	0.002	H09	0.09	0.004

Table S8: DPPH EDA (File: datos_Khaoula.xlsx, sheet "DPPH"), R studio Script 4.

Solv	Exp	rep	Concentration rep 3 (mg/mL)	Well	Raw Data (515) Rep 3
M	1	1	0.25	A01	0.397
M	1	2	0.25	A02	0.417
M	1	3	0.25	A03	0.437
M	2	1	0.25	A04	0.454
M	2	2	0.25	A05	0.397
M	2	3	0.25	A06	0.372

M	3	1	0.25	E07	0.296
M	3	2	0.25	E08	0.29
M	3	3	0.25	E09	0.2865
M	4	1	0.25	E10	0.2465
M	4	2	0.25	E11	0.2325
M	4	3	0.25	E12	0.2175
M	5	1	0.25	B01	0.397
M	5	2	0.25	B02	0.419
M	5	3	0.25	B03	0.416
M	6	1	0.25	B04	0.374
M	6	2	0.25	B05	0.303
M	6	3	0.25	B06	0.287
M	7	1	0.25	B07	0.363
M	7	2	0.25	B08	0.342
M	7	3	0.25	B09	0.329
M	8	1	0.25	B10	0.409
M	8	2	0.25	B11	0.391
M	8	3	0.25	B12	0.387
M	9	1	0.25	C01	0.331
M	9	2	0.25	C02	0.334
M	9	3	0.25	C03	0.329
M	10	1	0.25	C04	0.415
M	10	2	0.25	C05	0.357
M	10	3	0.25	C06	0.35
M	11	1	0.25	C07	0.258
M	11	2	0.25	C08	0.243
M	11	3	0.25	C09	0.224

M	12	1	0.25	C10	0.156
M	12	2	0.25	C11	0.137
M	12	3	0.25	C12	0.132
M	13	1	0.25	D01	0.416
M	13	2	0.25	D02	0.428
M	13	3	0.25	D03	0.433
M	14	1	0.25	D04	0.475
M	14	2	0.25	D05	0.415
M	14	3	0.25	D06	0.405
M	15	1	0.25	D07	0.397
M	15	2	0.25	D08	0.392
M	15	3	0.25	D09	0.379
M	16	1	0.25	D10	0.389
M	16	2	0.25	D11	0.373
M	16	3	0.25	D12	0.377
M	17	1	0.25	E01	0.197
M	17	2	0.25	E02	0.216
M	17	3	0.25	E03	0.191
M	18	1	0.25	E04	0.377
M	18	2	0.25	E05	0.299
M	18	3	0.25	E06	0.294
M	19	1	0.25	E07	0.308
M	19	2	0.25	E08	0.29
M	19	3	0.25	E09	0.279
M	20	1	0.25	E10	0.262
M	20	2	0.25	E11	0.201
M	20	3	0.25	E12	0.214

M	21	1	0.25	F01	0.344
M	21	2	0.25	F02	0.356
M	21	3	0.25	F03	0.347
M	22	1	0.25	F04	0.36
M	22	2	0.25	F05	0.31
M	22	3	0.25	F06	0.274
M	23	1	0.25	F07	0.25
M	23	2	0.25	F08	0.232
M	23	3	0.25	F09	0.235
M	24	1	0.25	F10	0.186
M	24	2	0.25	F11	0.156
M	24	3	0.25	F12	0.157
M	25	1	0.25	G01	0.231
M	25	2	0.25	G02	0.241
M	25	3	0.25	G03	0.286
M	26	1	0.25	G04	0.342
M	26	2	0.25	G05	0.274
M	26	3	0.25	G06	0.271
M	Negative	Control	0	G07	0.583
M	Negative	Control	0	G08	0.582
M	Negative	Control	0	G09	0.574
M	Positive	Control	0.5	G10	0.056
M	Positive	Control	0.5	H10	0.057
M	Positive	Control	0.5	H01	0.062
A	1	1	0.25	A01	0.51
A	1	2	0.25	A02	0.497
A	1	3	0.25	A03	0.385

A	2	1	0.25	A04	0.56
A	2	2	0.25	A05	0.386
A	2	3	0.25	A06	0.388
A	3	1	0.25	A07	0.387
A	3	2	0.25	A08	0.391
A	3	3	0.25	A09	0.391
A	4	1	0.25	A10	0.297
A	4	2	0.25	A11	0.233
A	4	3	0.25	A12	0.233
A	5	1	0.25	B01	0.537
A	5	2	0.25	B02	0.482
A	5	3	0.25	B03	0.326
A	6	1	0.25	B04	0.589
A	6	2	0.25	B05	0.355
A	6	3	0.25	B06	0.358
A	7	1	0.25	B07	0.391
A	7	2	0.25	B08	0.386
A	7	3	0.25	B09	0.388
A	8	1	0.25	B10	0.425
A	8	2	0.25	B11	0.414
A	8	3	0.25	B12	0.417
A	9	1	0.25	C01	0.581
A	9	2	0.25	C02	0.575
A	9	3	0.25	C03	0.559
A	10	1	0.25	C04	0.415
A	10	2	0.25	C05	0.262
A	10	3	0.25	C06	0.255

A	11	1	0.25	C07	0.398
A	11	2	0.25	C08	0.399
A	11	3	0.25	C09	0.404
A	12	1	0.25	C10	0.474
A	12	2	0.25	C11	0.454
A	12	3	0.25	C12	0.448
A	13	1	0.25	D01	0.564
A	13	2	0.25	D02	0.527
A	13	3	0.25	D03	0.503
A	14	1	0.25	D04	0.521
A	14	2	0.25	D05	0.403
A	14	3	0.25	D06	0.38
A	15	1	0.25	D07	0.41
A	15	2	0.25	D08	0.399
A	15	3	0.25	D09	0.405
A	16	1	0.25	D10	0.345
A	16	2	0.25	D11	0.334
A	16	3	0.25	D12	0.335
A	17	1	0.25	E01	0.547
A	17	2	0.25	E02	0.507
A	17	3	0.25	E03	0.425
A	18	1	0.25	E04	0.577
A	18	2	0.25	E05	0.412
A	18	3	0.25	E06	0.413
A	19	1	0.25	E07	0.385
A	19	2	0.25	E08	0.395
A	19	3	0.25	E09	0.374

A	20	1	0.25	E10	0.422
A	20	2	0.25	E11	0.36
A	20	3	0.25	E12	0.356
A	21	1	0.25	F01	0.479
A	21	2	0.25	F02	0.476
A	21	3	0.25	F03	0.477
A	22	1	0.25	F04	0.499
A	22	2	0.25	F05	0.334
A	22	3	0.25	F06	0.341
A	23	1	0.25	F07	0.471
A	23	2	0.25	F08	0.469
A	23	3	0.25	F09	0.461
A	24	1	0.25	F10	0.267
A	24	2	0.25	F11	0.192
A	24	3	0.25	F12	0.182
A	25	1	0.25	G01	0.583
A	25	2	0.25	G02	0.568
A	25	3	0.25	G03	0.558
A	26	1	0.25	G04	0.582
A	26	2	0.25	G05	0.389
A	26	3	0.25	G06	0.411
A	Negative	Control	0	G07	0.606
A	Negative	Control	0	G08	0.616
A	Negative	Control	0	G09	0.602
A	Positive	Control	0.5	G10	0.059
A	Positive	Control	0.5	H01	0.071
A	Positive	Control	0.5	H10	0.056

Table S9: Extraction Yield EDA (File: datos_Khaoula.xlsx, sheet "Yield"), R studio Script 4.

Solv	Extractions_yield
Methanol	52.53
Methanol	60.14
Methanol	60.36
Methanol	59.27
Methanol	55.38
Methanol	74.36
Methanol	70.89
Methanol	74.84
Methanol	74.76
Methanol	67.73
Methanol	65.87
Methanol	62.44
Methanol	73.56
Methanol	62.07
Methanol	56.64
Methanol	68.79
Methanol	67.33
Methanol	63.43
Methanol	63.88
Methanol	64.16
Methanol	65.24
Methanol	56.63
Methanol	65.55
Methanol	63.58
Methanol	73.68
Methanol	68.90

Acetone	52.25
Acetone	65.39
Acetone	47.48
Acetone	39.82
Acetone	47.41
Acetone	46.90
Acetone	41.53
Acetone	65.64
Acetone	50.01
Acetone	41.06
Acetone	46.23
Acetone	72.37
Acetone	47.57
Acetone	49.21
Acetone	48.90
Acetone	47.11
Acetone	39.27
Acetone	65.27
Acetone	50.93
Acetone	42.19
Acetone	45.70
Acetone	45.15
Acetone	73.61
Acetone	41.88
Acetone	57.99
Acetone	45.71